A pharmacological study of the potential protective effect of Silymarin against testosterone-induced benign prostate hypertrophy in rats

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بسم الله الرحمن الرحيم

قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ

صدق الله العظيم

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Role of the phytoestrogenic, pro-apoptotic and anti-oxidative properties of silymarin in inhibiting experimental benign prostatic hyperplasia in rats

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HIGHLIGHTS

- Silymarin enhanced ER-β but decreased ER-α and androgen receptor expression.
- Silymarin showed powerful antioxidant and pro-apoptotic properties.
- Silymarin enhanced P21 expression, Bax/Bcl-x_l ratio and caspase-3 activity.
- Silymarin could be a promising candidate in the management of BPH.

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ABSTRACT

Androgen and estrogen play an important role in the pathogenesis of benign prostatic hyperplasia (BPH). Estrogen exerts its action through two distinct estrogen receptors (ERs) either ER-α or ER-β. The phytoestrogenic property of silymarin (SIL) has been previously characterized. Thus, this study examined the protective effect of SIL against testosterone-induced BPH in rats. In an initial dose–response study, SIL in a dose of 50 mg/kg was the most effective in preventing the rise in prostate weight, prostate weight/body weight ratio and histopathologic changes induced by testosterone. Testosterone significantly decreased ER-β and increased ER-α and AR expressions as compared to the control group and these effects were significantly ameliorated by SIL. Furthermore, SIL significantly protected against testosterone-provoked decline in mRNA expression of $P21^{WAF1/Cip1}$ and Bax/Bcl-x_l ratio as well as caspase-3 activity. SIL minimized the number of proliferating cell nuclear antigen (PCNA) positive cells as compared to testosterone-treated group. Moreover, SIL significantly blunted the inducible NF-κB expression and restored the oxidative status to within normal values in the prostatic tissues. Collectively these findings elucidate the effectiveness of SIL in preventing testosterone-induced BPH in rats. This could be attributed, at least partly, to its phytoestrogenic, pro-apoptotic and anti-oxidative properties.

1. Introduction

Benign prostate hyperplasia (BPH), a condition characterized by excessive and uncontrolled growth of the prostate gland which affects about 85% of men above 50 years of age (Lytton et al., 1968). Although prostate 5α-reductase inhibitors are widely used to manage BPH (Sandhu and Te, 2004), their uses are accompanied by undesirable side effects. Also, the magnitude of their therapeutic effect is relatively small and a clinically significant benefit is observed in less than 50% of the treated patients (Uygur et al., 1998).

Accumulating studies revealed that both androgen and estrogen play an important role in the prostate physiology and pathology (Harkonen and Makela, 2004; Weihua et al., 2002). Hence, targeting both hormones instead of only targeting androgen, has been suggested for effective management of prostatic disease (Johnson et al., 2010). Estrogen receptors (ERs) are responsible for the estrogen signaling; ER-α mediates proliferative response while ER-β facilitates pro-apoptotic and anti-proliferative effects (Ciocca et al., 1985). In human BPH, there has been shown an increased ER-α/ER-β expression ratio (Royuela et al., 2001). Also, ER-β agonists are effective in preventing prostatic hyperplasia (McPherson et al., 2007) which indicates the importance of ER-β signaling in the management of BPH. Some selective estrogen receptor modulators (SERMs) have demonstrated anti-proliferative action in human BPH-derived stromal cells (Glienke et al., 2004). In addition, some evidence suggested that oxidative stress may be involved in the pathogenesis of BPH (Pace et al., 2010).

Silymarin (SIL), a polyphenolic flavonoid extracted from *Silybum marianum*, is well tolerated and has been used clinically for its

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hepatoprotective activity (Wellington and Jarvis, 2001). It has been shown that SIL can be regarded as SERM with similar properties as raloxifene; it acts as a selective ER-β agonist (Seidlová-Wuttke et al., 2003). SIL also has emerged as a potent chemopreventive agent against various cancer models. Interestingly, SIL effectively induced apoptosis in skin cancer cell line mainly through inhibiting Bcl-x_l while enhancing Bax protein expressions and inducing caspase activation (Katiyar et al., 2005). In prostate cancer cell line, SIL induced the cyclin-dependent kinase inhibitors; p21 and p27 resulting in cell cycle arrest (Agarwal, 2000). SIL also possesses powerful antioxidant properties, acts as a reactive oxygen species scavenger, increasing the levels of antioxidant enzyme and decreasing the level of lipid peroxides (Muzes et al., 1990; Soto et al., 2003). The current study was designed to investigate the potential protective effect of SIL against testosterone-induced BPH in rats. In addition, the underlying mechanisms were explored by assessing different markers of apoptosis, proliferation and oxidative stress along with the effect of SIL on estrogen and androgen receptors expressions.

2. Materials and methods

2.1. Drugs and chemicals

Silymarin (SIL); (Indena S.P.A, Milano, Italy) was kindly supplied by (Medical Union Pharmaceuticals Co (MUP), Cairo, Egypt), Testosterone; (Steroid S.P.A, Cologno Monzese (MI), Italy) was kindly supplied by (Chemical Development Industries Co (CID), Cairo, Egypt).

Thiobarbituric acid (TBA) and 1,1′,3,3′-tetramethoxypropane were purchased from Sigma Aldrich Chemical Co. (St Louis, MO, USA). All other chemicals were of the highest available analytical grade.

2.2. Animals

The protocol for animal handling and treatment was approved by Bioethical and Research Committee of Ain Shams University, Cairo, Egypt. Male Sprague–Dawley rats (200–250 g) aged 10 weeks, were purchased from Nile Co. for Pharmaceutical and Chemical industries, Cairo, Egypt. Rats were housed in an air-conditioned atmosphere, at a temperature of 25 °C with alternatively 12 h light and dark cycles. Animals were acclimated for 1 week before experimentation. They were kept on a standard diet and water ad libitum.

2.3. Experimental design

A preliminary dose–response study was conducted in which animals were randomly divided into five groups, eight animals per group. A group of rats was administrated 3 mg/kg testosterone subcutaneously (S.C.) dissolved in olive oil, 5 days per week for 2 weeks; to induce BPH comparable to that occurred in patients as described by Maggi et al. (1989) and Thiyagarajan et al. (2002). Silymarin (SIL) was suspended in 0.5% w/v sodium carboxymethyl cellulose (Na CMC) and administrated orally by gastric lavage to three different groups of rats at three different doses (25 mg/kg, 50 mg/kg and 100 mg/kg) concomitantly with testosterone. The control group was administrated Na CMC (1 ml/kg) orally and olive oil (1 ml/kg) S.C. Rats were sacrificed 72 h after the last testosterone injection and the prostates were immediately removed and weighed then the ratio of the prostate weight to bodyweight was calculated. Sections of the ventral prostate lobe were fixed in 10% neutral buffered formalin and embedded in paraffin for histological examination. Based on the results obtained from the dose–response study, the optimal dose of SIL was chosen for further investigations.

Afterwards, a mechanistic study was conducted in which animals were randomly divided into four groups; eight animals per group and treated 5 days per week for 2 weeks as follows; the first group was kept as control received Na CMC orally and olive oil S.C. The second group was given 50 mg/kg SIL orally and olive oil S.C. The third group received Na CMC orally and 3 mg/kg testosterone, S.C. to induce benign prostatic hyperplasia (BPH). The fourth group received 50 mg/kg SIL orally concomitantly with 3 mg/kg testosterone, S.C. Rats were sacrificed 72 h after the last testosterone injection. Then, prostatic tissues were removed. Sections of the ventral lobes were fixed in 10% neutral buffered formalin and embedded in paraffin for immunohistochemical examinations. The remainder of each prostate was stored at −80 °C and used for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis as well as, further biochemical analyses.

2.4. Prostate weight and prostate weight/body weight ratio

Prostate tissues were collected and weighed immediately then the ratio of the prostate weight to the total body weight was calculated.

2.5. Histopathological examination

Ventral prostate tissues were embedded in 10% formalin and processed for paraffin sections of 4 μm thickness. After de-waxing and rehydration, sections were stained with haematoxylin and eosin (H&E) for routine histopathological examination using light microscopy and for quantitative determination of epithelial thickness and acini diameter in 10 non overlapping fields of vision from each slide using image analysis software (Image J, 1.46a, NIH, USA).

2.6. Immunohistochemical detection of ER-β, ER-α, AR, PCNA and NF-κB (p65)

Paraffin embedded tissue sections of 3 μm thickness, taken from three rats per group, were deparaffinized first with xylene then hydrated using an ethanol series and heated in citrate buffer (pH 6.0) for 5 min. After that, the sections were blocked with 5% bovine serum albumin (BSA) in tris buffered saline (TBS) for 2 h. The sections were then incubated overnight at 4 °C with one of the following specific primary antibodies; rabbit polyclonal antibody to NF-κB (p65), ER-β, AR, rabbit monoclonal antibody to ER-α, mouse monoclonal antibody to PCNA; all antibodies were purchased from (Thermo Fisher Scientific, Cheshire, UK). At a concentration of 1 μg/ml containing 5% BSA in TBS and incubated overnight at 4 °C. After washing the slides with TBS, the sections were incubated with the corresponding secondary antibody. Sections were then washed with TBS and incubated for 10 min in a solution of 0.02% diaminobenzidine containing 0.01% H_2O_2. Counter staining was performed using hematoxylin, and the slides were visualized under a light microscope. At least three sections per rat were investigated and immunohistochemical quantification was carried out using image analysis software (Image J, 1.46a, NIH, USA).

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of $p21^{WAF1/Cip1}$, Bax, Bcl-x_l gene expressions

RNeasy® Mini Kit (Qiagen, Valencia, CA, USA) was used for total RNA isolation according to the supplier protocol. Reverse transcription was undertaken to construct a cDNA library using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). PCR amplification reactions were then performed using a Taq PCR Master Mix Kit (Qiagen, Valencia, CA, USA) and the following primers were used: $P21^{WAF1/Cip1}$ sense primer, AGT-ATG-CCG-TCG-TCT-GTT-CG and the corresponding antisense primer, GAG-TGC-AAG-ACA-GCG-ACA-AG. Bax sense primer, GCA-GGG-AGG-ATG-GCT-GGG-GAG-A and the corresponding antisense primer TCC-AGA-CAA-GCA-GCC-GCT-CAC-G. Bcl-x_l sense primer, AAA-ATG-TCT-CAG-AGC-AAC-CGG and the corresponding antisense primer, TCA-CTT-CCG-ACT-GAA-GAG-TGA. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as reference housekeeping gene with sense primer, AAA-CCC-ATC-ACC-ATC-TTC-CA and antisense primer GTG-GTT-CAC-ACC-CAT-CAC-AA. All the primers were purchased from (Metabion international AG, Martinsried, Germany). Each PCR cycle consisted of a denaturation step for 1 min at 94 °C, an annealing step for 30 s at 61 °C (P21), 59 °C (Bax), 53 °C (Bcl-x_l), and 56 °C (GAPDH). All PCR products were resolved by 1.5% agarose gel electrophoresis and photomicrographs were taken of the ethidium bromide-stained gels. For quantification, gels were scanned, and the pixel intensity for each band was determined using the image analysis software (Image J, 1.46a, NIH, USA).

2.8. Biochemical analyses

2.8.1. Assessment of caspase-3 activity

Caspase-3 activity was examined using the colorimetric assay kit purchased from (Sigma Aldrich Chemical Co., St Louis, MO, USA). According to the manufacturer's protocol; the assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety. The absorbance of pNA is determined at 405 nm after incubation period of 90 min. The concentration of the pNA released from the substrate is calculated from a calibration curve prepared with a standard pNA solution (Nicholson et al., 1995). The results obtained were expressed as μmol/min/mg protein.

2.8.2. Assessment of oxidative stress markers

Prostatic tissues were homogenized in ice cooled phosphate-buffered saline (50 mM potassium phosphate, pH 7.5). Reduced glutathione (GSH) was assessed in the tissue homogenates using the commercially available kit (Biodiagnostic, Cairo, Egypt). Briefly, the procedure is based on the reduction of 2-nitrobenzoic acid by glutathione to produce a yellow compound measured spectrophotometrically at 405 nm (Beutler et al., 1963). Values were expressed as mmol/mg protein. Additionally, catalase activity was determined using the commercially available kit (Biodiagnostic, Cairo, Egypt). Briefly, the procedure is based on the addition of a known quantity of H_2O_2 then the reaction was stopped after exactly 1 min with a catalase inhibitor. In the presence of peroxidase, the remaining H_2O_2 reacts with 3,5-dichloro-2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore of pink color, detected at 510 nm, the intensity of which is inversely proportional to the amount of catalase in the original sample (Aebi, 1984). Specific catalase activity was expressed in terms of U/mg protein. Furthermore, lipid peroxidation was estimated spectrophotometrically using the thiobarbituric acid

Table 1
Effect of SIL treatment in three different doses (25 mg/kg, 50 mg/kg, 100 mg/kg, orally, for 2 weeks) on the prostate weight, prostate weight/body weight, epithelial thickness and acini diameter in testosterone-treated rats.

Groups	Prostate weight (mg)	Prostate weight/body weight ratio ($\times 10^{-3}$)	Epithelial thickness (μm)	Acini diameter (μm)
Control	423.40 ± 32.54	1.70 ± 0.16	11.11 ± 1.52	217.21 ± 19.9
Testosterone	679.36 ± 88.20^{a}	2.84 ± 0.50^{a}	36.95 ± 6.90^{a}	384.67 ± 3.94^{a}
Testosterone + 25 mg/kg SIL	659.40 ± 34.83^{a}	2.78 ± 0.30^{a}	31.80 ± 5.60^{a}	268.13 ± 30.97^{b}
Testosterone + 50 mg/kg SIL	$465.50 \pm 41.74^{b,c}$	$2.05 \pm 0.27^{b,c}$	$15.47 \pm 3.18^{b,c}$	270.25 ± 32.88^{b}
Testosterone + 100 mg/kg SIL	$518.58 \pm 39.80^{b,c}$	$2.2 \pm 0.12^{b,c}$	$14.07 \pm 2.00^{b,c}$	265.34 ± 14.68^{b}

Data are represented by mean ± SD ($n = 8$). a, b or c: statistically significant from control, testosterone or testosterone + 25 mg/kg SIL group, respectively at $P < 0.05$ using one-way ANOVA followed by Tukey–Kramer as a post hoc test.

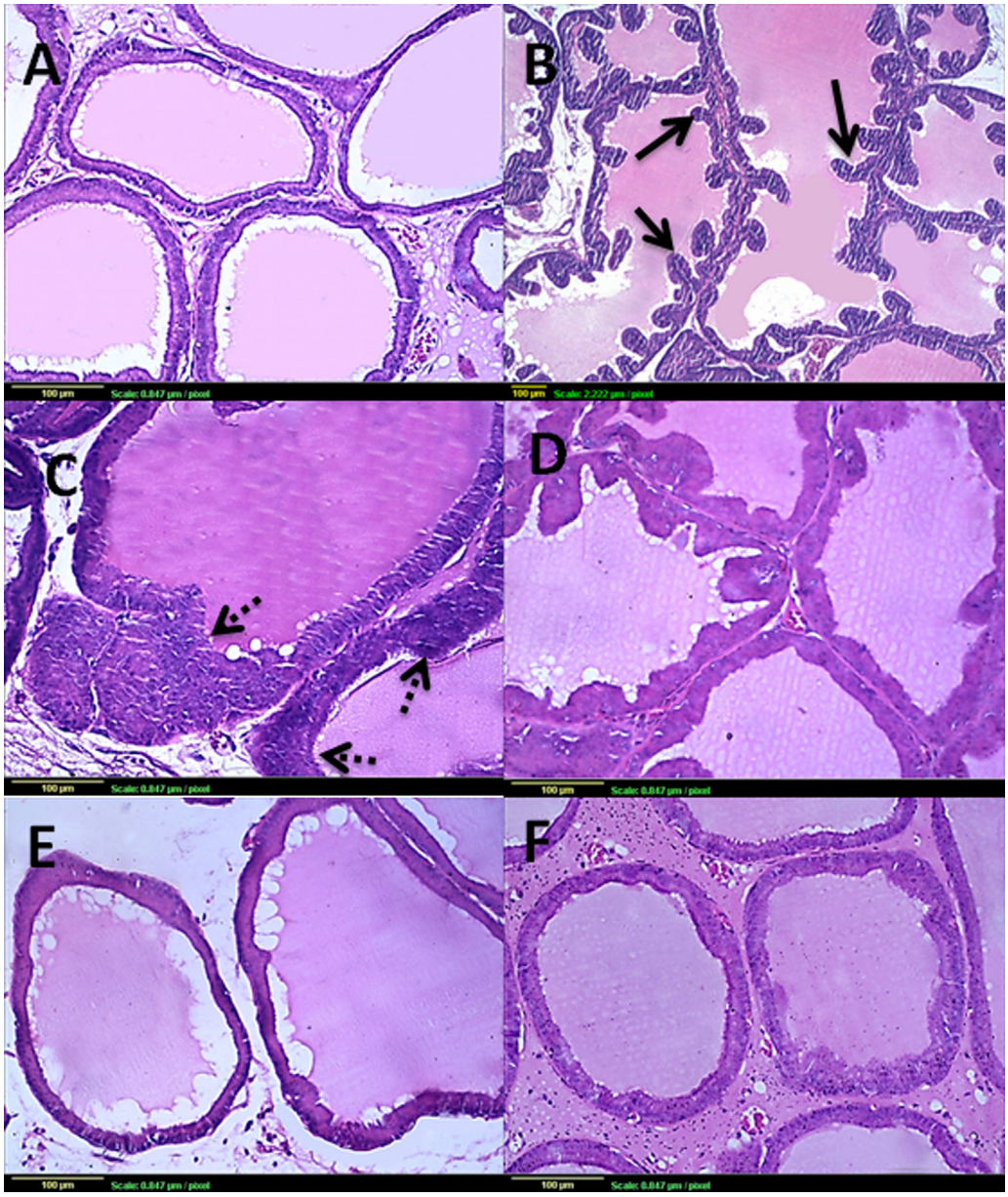

Fig. 1. Histological examination of hematoxylin-eosin sections of rat ventral prostates. A: Section taken from the prostate of the control group shows normal morphological structure of the lining epithelial cells. B and C: Sections taken from the prostate of testosterone only treated group exhibit hypertrophy with increased epithelial thickness (dotted arrows) and polyps formations (solid arrows). D: Section taken from the prostate of the group treated with testosterone and 25 mg/kg SIL shows mild reduction in epithelial thickness. E and F: Sections taken from the prostate of testosterone groups co-treated with 50 mg/kg (E) or 100 mg/kg (F) SIL show marked reduction in prostate hypertrophy and hyperplasia induced by testosterone. Scale bar, 100 μm.

reactive substance (TBARS) method by measuring malondialdehyde (MDA) level, as described by Mihara and Uchiyama (1978). Results were expressed in terms of nmol MDA/mg protein. Calibration curve was done using 1,1′,3,3′-tetraethoxypropane as a standard. Briefly, the homogenate was supplemented with 20% trichloroacetic acid (TCA) and 0.6% TBA. The mixture was heated in boiling water bath then cooled in ice. After cooling, n-butanol was added, shaken vigorously and centrifuged to separate the n-butanol layer. The absorbance of the pink colored product was read at 535 nm. Protein content was determined according to Biuret method using the commercially available kit (Biodiagnostic, Cairo, Egypt).

2.9. Statistical analysis

Data are presented as mean ± SD. Multiple comparisons were performed using one-way ANOVA followed by Tukey–Kramer as a post hoc test. The 0.05 level of probability was used as the criterion for significance. All statistical analyses were performed using GraphPad Instat software version 3. Graphs were sketched using GraphPad Prism software version 4 (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. Dose–response study

3.1.1. Prostate weight and prostate weight/body weight ratio

Animals treated with testosterone showed a significant increase in prostate weight and prostate weight/body weight ratio by 60.45% and 67% respectively compared to the control group. In comparison with the testosterone-treated group, SIL (50 mg/kg and 100 mg/kg) significantly decreased the prostate weight gain induced with testosterone by 29.5% and 23.67% respectively, and decreased the prostate weight/body weight ratio by 27.8% and 22.5% respectively. However, SIL (25 mg/kg) insignificantly reduced the prostate weight and prostate weight/body weight ratio by 2.9% and 2.1% respectively. In addition, prostate weights of animals treated with SIL (50 mg/kg or 100 mg/kg) were significantly lower than those in the group of SIL (25 mg/kg) (Table 1).

3.1.2. Histopathological examination

Section from the ventral prostates of the control group stained with hematoxylin-eosin showed no histological alteration in the acini or the lining epithelium. Epithelial cells were cuboidal in shape and of regular size (Fig. 1A). Testosterone administration induced disrupted morphology in the prostate; marked thickening and hypertrophy as well as hyperplasia with polyps formation in the lining epithelium of the acini (Fig. 1B). Widening of the lumen diameter was also observed for this group (Fig. 1C). Treatment with 25 mg/kg SIL showed mild hypertrophy and hyperplasia in the lining epithelium. The decrease in the epithelial thickness was insignificant compared to the testosterone-treated group while the lumen diameter was normalized to the control base line (Fig. 1D) (Table 1). Interestingly, SIL in the dose of either 50 mg/kg or 100 mg/kg was effective in reducing the hypertrophy and hyperplasia seen in the testosterone-treated group preserving the normal histological structure. Epithelial thickness and acini diameter were reduced to levels comparable to that of the control ones (Fig. 1E and F) (Table 1). In the light of these

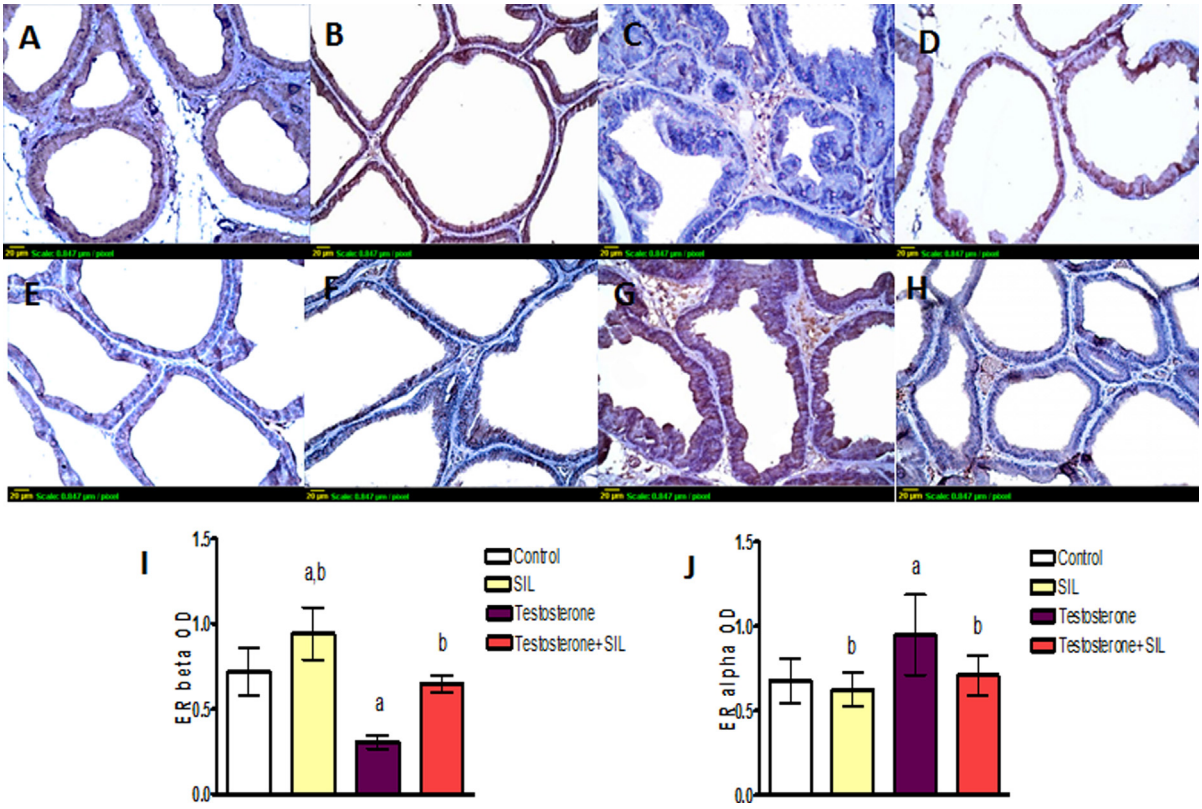

Fig. 2. Immunohistochemical localization of estrogen receptor-β (ER-β) (A–D) and estrogen receptor-α (ER-α) (E–H) in the prostate tissues. A: Expression of ER-β in the prostate epithelial cells of the control group. B: Expression of ER-β in prostate epithelial cells of SIL only treated group shows an extensive degree (brown stain). C: Expression of ER-β in the prostate epithelial cells of testosterone only treated group shows a minimal degree. D: Expression of ER-β in the prostate epithelial cells of the testosterone group co-treated with SIL shows a high degree (brown stain). E: Expression of ER-α in the prostate epithelial cells of the control group. F: Expression of ER-α in the prostate epithelial cells of SIL only treated group shows a minimal degree (faint stain). G: Expression of ER-α in the prostate epithelial cells of testosterone only treated group shows an extensive degree (brown stain). H: Expression of ER-α in the prostate epithelial cells of the testosterone group co-treated with SIL shows an expression level similar to that of the control group. Scale bar, 20 μm. I and J: Quantitative image analysis for immunohistochemical staining of ER-β and ER-α respectively expressed as optical densities (OD) across 10 different fields for each rat section. Each column represents mean ± SD. a or b: Statistically significant from control or testosterone group, respectively at $P < 0.05$ using one-way ANOVA followed by Tukey–Kramer as a post hoc test.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

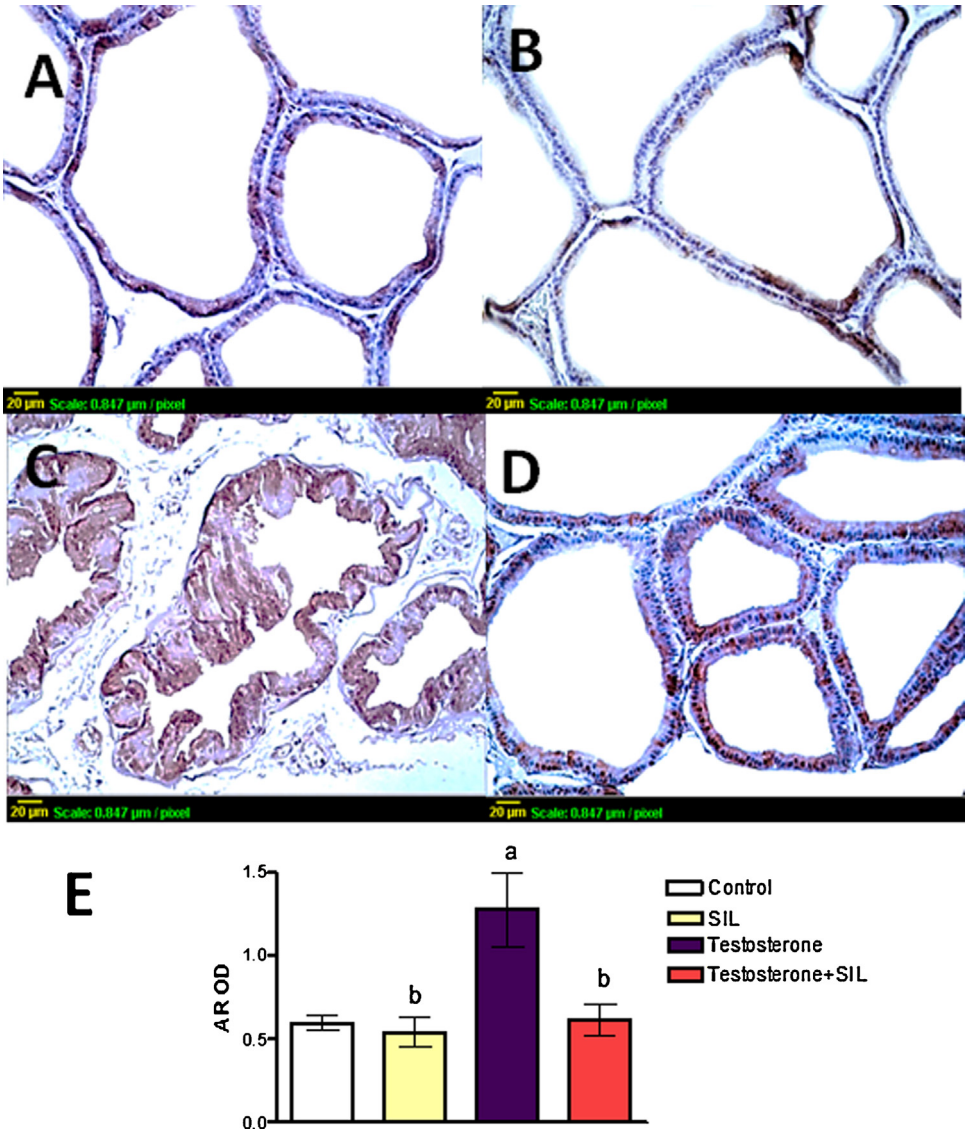

Fig. 3. Immunohistochemical staining of androgen receptor (AR) in the prostate tissues. A: Expression of AR in the prostate epithelial cells of the control group. B: Expression of AR in prostate epithelial cells of SIL only treated group shows a limited degree. C: Expression of AR in prostate epithelial cells of testosterone only treated group shows an extensive degree (brown stain). D: Expression of AR in prostate epithelial cells of the testosterone group co-treated with SIL shows an expression level similar to that of the control group. Scale bar, 20 µm. E: Quantitative image analysis for immunohistochemical staining expressed as optical densities (OD) across 10 different fields for each rat section. Each column represents mean ± SD. a or b: Statistically significant from control or testosterone group, respectively at $P < 0.05$ using one-way ANOVA followed by Tukey–Kramer as a post hoc test.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

findings, a dose of 50 mg/kg SIL was chosen for further investigations.

3.2. Mechanistic study

3.2.1. Estrogen and androgen receptor expression

The expression of ER-β, ER-α and AR in the acinar epithelia was assessed immunohistochemically. SIL only treated group showed a significant elevation in the expression of ER-β as evidenced by intense brown staining compared to the control group (Fig. 2B) while that of ER-α and AR was not altered as only minimal staining was observed (Figs. 2F and 3B). Testosterone significantly diminished ER-β expression as manifested by faint brown staining (Fig. 2C) and elevated the expressions of both ER-α and AR as shown by the intense brown staining of prostate sections (Figs. 2G and 3C). Administration of SIL significantly restored expression of ER-β, ER-α and AR to almost the control level (Figs. 2D, 2H and 3D). The immunohistochemical staining was quantified as optical density (OD) of the stained regions using the image analysis software, and the results are represented in (Figs. 2I, 2J and 3E).

3.2.2. $P21^{WAF1/Cip1}$ (P21), Bax and Bcl-x_l mRNA expressions

SIL only treated group showed no significant change in the gene expression of P21, Bax, Bcl-x_l in comparison to the control group. Treatment with testosterone triggered a significant decrease in mRNA level of P21 (Fig. 4A) and Bax (Fig. 4B) while significantly increased that of Bcl-x_l (Fig. 4C) as compared to the control group. Concomitant treatment with SIL afforded significant protection against the testosterone-driven decline in mRNA expression of P21 (Fig. 4A) and Bax (Fig. 4B) and maintained them at near basal levels as seen in the control group. SIL showed minor decrease in Bcl-x_l (Fig. 4C) but still insignificant from both the control group and the group treated with testosterone. Interestingly, treatment of animals with SIL along with testosterone afforded significant protection against testosterone-induced decline in Bax/Bcl-x_l ratio (Fig. 4D).

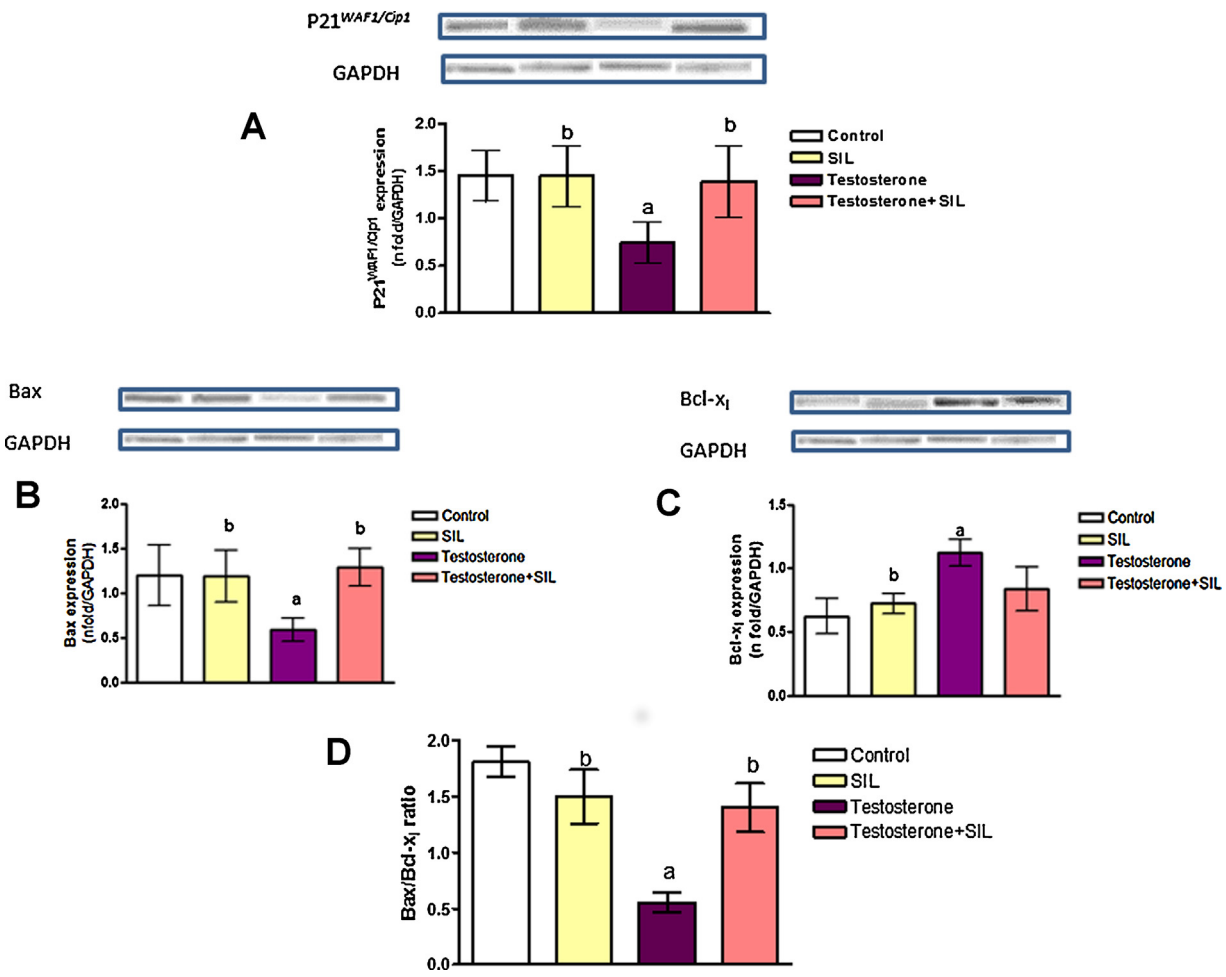

Fig. 4. RT-PCR analysis of p21$^{WAF1/Cip1}$ (P21), Bax and Bcl-x_l. Effect of testosterone and/or SIL on (A) p21, (B) Bax and (C) Bcl-x_l mRNA expressions of *n*-fold difference relative to the endogenous control gene GAPDH (relative expression levels). D: The ratio of Bax to Bcl-x_l after SIL administration in testosterone-treated/untreated groups. Data are expressed as mean ± SD ($n = 5$). a or b: Statistically significant from control or testosterone group, respectively at $P < 0.05$ using one-way ANOVA followed by Tukey–Kramer as a post hoc test.

3.2.3. Proliferating cell nuclear antigen (PCNA)

The immunohistochemistry of the proliferation marker (PCNA) revealed that there was a boost in the number of stained cells in the testosterone-treated group, indicating an increase of the proliferation rate within this group (Fig. 5C). Concomitant treatment with SIL was able to significantly minimize the number of PCNA stained cells compared to the testosterone-treated group (Fig. 5D). The immunohistochemical staining was quantified as percentage of PCNA positive cells and the results are represented in Fig. 5E.

3.2.4. Caspase-3 activity

As shown in Fig. 5F, caspase-3 activity was significantly decreased in testosterone-treated rats as compared with control rats by 39.2% while SIL significantly ameliorated testosterone-stimulated reduction in its activity by 66% which was evident in the group co-treated with both drugs.

3.2.5. NF-κB (p65)

The activated p65 subunit of NF-κB was detected immunohistochemically. The group treated with SIL showed no significant change in the expression of NF-κB as compared to the control group, both groups were just minimally stained (Fig. 6B). However, testosterone only treated group showed significant increase in NF-κB expression compared to that of the control group manifested by intense brown staining in prostate tissues (Fig. 6C). Treatment of rats with SIL conferred significant protection against testosterone exaggerated expression of NF-κB to a level similar to that of the control group (Fig. 6D). The immunohistochemical staining was quantified as optical density (OD) of the stained regions using the image analysis software, and the results are represented in (Fig. 6E).

3.2.6. Oxidative stress markers

Oxidative status was assessed by determining the level of GSH, lipid peroxides estimated as MDA and catalase activity. SIL only treated group showed no significant variation in the oxidative stress markers as compared to the control group. However, testosterone-treated group exhibited a significant decrease in the GSH level and catalase activity by 0.33 and 0.52 folds respectively, lipid peroxides were significantly elevated in the testosterone-treated group by 1.38 folds as compared to the control group. On the other side, the group treated with testosterone and SIL was effective in correcting testosterone-induced depletion of GSH and catalase activity as well as decreasing the elevated level of the lipid peroxides showed in the testosterone-treated group (Table 2).

4. Discussion

Benign prostatic hyperplasia is considered the most common benign tumor in men (Donnell, 2011). Many factors have been involved in the pathogenesis of BPH; impairment of the balance

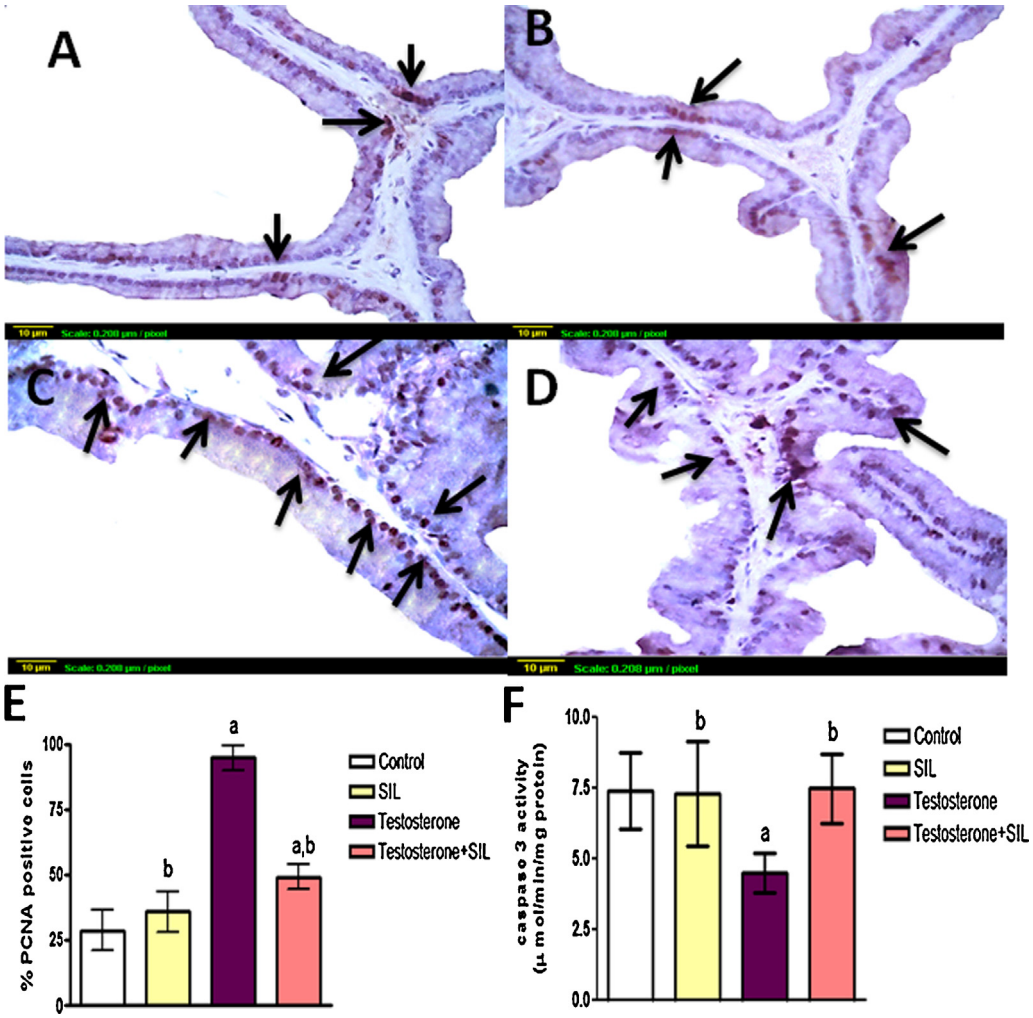

Fig. 5. Expression of PCNA in the rat prostate tissues by immunohistochemical staining. A: Section obtained from the control group shows limited PCNA expression in the prostate epithelial cells. B: Section obtained from SIL only treated group shows limited PCNA expression in the prostate epithelial cells. C: Section obtained from testosterone only treated group shows a high degree of PCNA expression in the prostate epithelial cells (brown stain). D: Section obtained from the group treated with both testosterone and SIL shows an expression level comparable to that in the control group. Scale bar, 10 µm. The arrows point to PCNA stained cells. E: Quantitative image analysis for immunohistochemical staining expressed as a percentage of PCNA positive cells against the total number of cells across seven higher power fields (×40) for each rat section. F: Effect of SIL on the activity of caspase-3 measured as µmol pNA/min/mg protein in testosterone-treated and untreated rats. Data are expressed as mean ± SD ($n = 8$). a or b: Statistically significant from control or testosterone group, respectively at $P < 0.05$ using one-way ANOVA followed by Tukey–Kramer as a post hoc test.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

between cell proliferation and cell death is of particular concern (Pawlicki et al., 2004). In the current study; treatment of rats with 50 mg/kg SIL orally for 14 days significantly inhibited testosterone-mediated increase in the prostate weight, prostate weight/body weight as compared to the lower dose (25 mg/kg). While the highest dose (100 mg/kg) showed no significant benefit over the middle dose (50 mg/kg). In addition, histopathological examination as well as assessments of epithelial thickness confirmed the previous results while SIL at all doses studied was effective in restoring the acini diameter to almost the normal values. Based on these data, SIL in the dose 50 mg/kg has proven efficacy in protecting against testosterone-induced BPH and was used for further investigations. This was in accordance with a preliminary data which supported the beneficial effect of SIL in the management of prostate cancer and hypertrophy (Young and Xing, 2004).

Two estrogen receptors subtypes (ER-α and ER-β) have been identified in the prostate gland. They play an important role in the pathogenesis of BPH and prostate cancer where ER-α is responsible for mediating the effect of estradiol in promoting cellular proliferation. Meanwhile, ER-β is responsible for preventing

Table 2
Effect of SIL (50 mg/kg, orally, for 2 weeks) on oxidative stress markers in prostatic tissues of testosterone-treated rats.

Groups	GSH (mmol/mg protein)	Catalase (U/mg protein)	TBARS (nmol/mg protein)
Control	0.12 ± 0.03	1.77 ± 0.33	0.81 ± 0.12
SIL	0.13 ± 0.02[b]	1.96 ± 0.41[b]	0.73 ± 0.16[b]
Testosterone	0.04 ± 0.01[a]	0.92 ± 0.18[a]	1.12 ± 0.14[a]
Testosterone + SIL	0.09 ± 0.02[b]	1.50 ± 0.34[b]	0.83 ± 0.17[b]

Data are represented by mean ± SD ($n = 8$). a or b: Statistically significant from control or testosterone group, respectively at $P < 0.05$ using one-way ANOVA followed by Tukey–Kramer as a post hoc test.

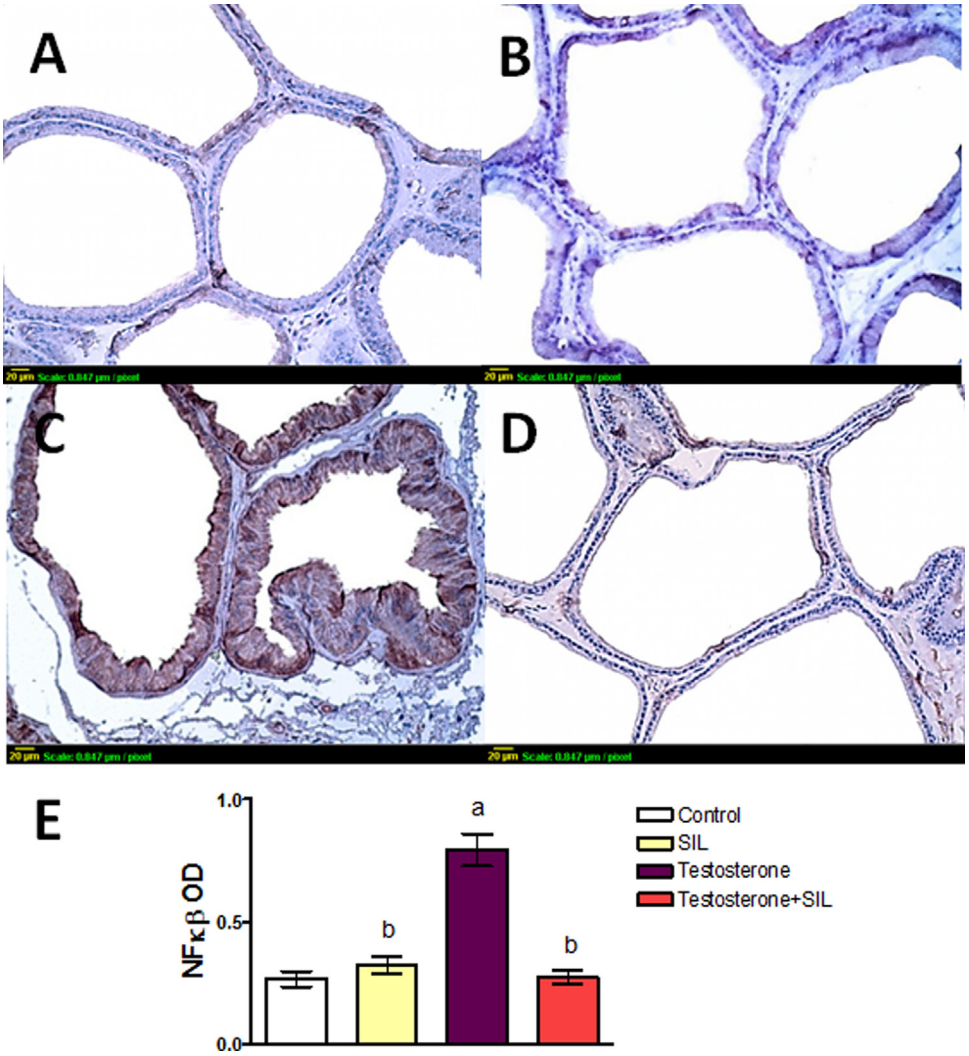

Fig. 6. Expression of nuclear factor-κB (NF-κB) by immunohistochemical staining. A: Section of a prostate taken from the control group reveals a minimal degree of NF-κB expression. B: Section of a prostate taken from SIL only treated group reveals a minimal degree of NF-κB expression. C: Section of a prostate taken from testosterone only treated group reveals an intensive degree of NF-κB expression (brown stain). D: Section of a prostate taken from the group co-treated with both testosterone and SIL reveals a minimal degree of NF-κB expression (brown stain). Scale bar, 20 μm. E: Quantitative image analysis for immunohistochemical staining expressed as optical densities (OD) across 10 different fields for each rat section. Each column represents mean ± SD. a or b: Statistically significant from control or testosterone group, respectively at $P < 0.05$ using one-way ANOVA followed by Tukey–Kramer as a post hoc test.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

proliferation when being activated by estradiol (Bodker et al., 1999; Royuela et al., 2001). Since, there exists a rationale for using ER-β agonists for the management of BPH, it has been reported that SIL exhibits a selective ER-β agonistic activity (Seidlová-Wuttke et al., 2003). This study showed that testosterone significantly decreased ER-β expression while enhanced that of ER-α thus promoting the prostatic hyperplasia. Interestingly, SIL significantly enhanced ER-β and reduced ER-α expressions in the prostate epithelia and this was in line with a previous study which indicated that SIL increased the expression of ER-β in the colon cancer model in vivo (Barone et al., 2010). The inverse relationship between ER-β and ER-α expression has been studied in endometrial stromal cells. The data revealed that overexpression of ER-β suppressed ER-α expression and knockdown of ER-β increased ER-α mRNA and protein levels significantly (Trukhacheva et al., 2009).

Binding of testosterone or dihydrotestosterone (DHT) to AR leads to activation of numerous genes that promotes prostatic growth (Chatterjee, 2003). It has been shown that overexpression of epithelial AR increased the prostate proliferation which supports the idea that AR is a major driver of prostate cell growth and survival (Saha et al., 2011; Stanbrough et al., 2001). Overexpression of AR was demonstrated in testosterone-treated animals. In this context, it was evident that castration significantly decreased, while androgens increased the AR mRNA levels in the prostate (Takeda et al., 1991). In the current study, co-treatment with SIL significantly precluded testosterone-stimulated expression of AR in the prostate tissues. Our data was in agreement with previous investigations, which showed that SIL decreased the level of AR in human prostate cancer cell lines (Deep et al., 2010, 2008).

The ability of SIL to regulate cell cycle progression and thus enhance the apoptotic machinery was evidenced by its induction of cyclin-dependent kinase inhibitor 1A; $P21^{WAF1/Cip1}$ (P21) mRNA level as shown in the results of the current study. It has been demonstrated that ER-β inhibits prostate cell growth and proliferation through induction of P21 gene expression (Yu et al., 2008). In addition, P21 is believed to act as an up-stream signal to provide activation of caspases and induction of apoptosis (Huo et al., 2004). It is well characterized that P21 can directly inhibit DNA replication by blocking the proliferating cell nuclear antigen (PCNA)-dependent DNA polymerase-δ activity (Waga et al.,

1994). Moreover, it has been shown that P21 is involved in the proteasome-dependent degradation of PCNA in adult cardiomyocytes thus inhibiting DNA replication (Engel et al., 2003). A previous study showed that selective ER-β agonist caused down-regulation while ER-α agonist caused up-regulation of PCNA in normal murine mammary epithelial cell line (Helguero et al., 2005). In the present study, the immunohistochemical detection of PCNA has shown a marked increase in its expression in the group treated with testosterone, which was minimized in the group treated with both testosterone and SIL.

The B-cell lymphoma/leukemia-2 (Bcl-2) family is the best-characterized group of apoptosis mediating factors. Bcl-x_l is regarded as the key anti-apoptotic protein, whereas, Bax is considered as the critical pro-apoptotic Bcl-2 family member (Papaliagkas et al., 2007). They elicit opposing effects on the mitochondria; Bcl-x_l blocks while Bax induces the release of cytochrome c which in turn, activates the executioner caspase-3 which is crucial for apoptosis (Donovan and Cotter, 2004). The results obtained in the current study demonstrated that SIL possess a pro-apoptotic activity as it significantly protected against testosterone-mediated down expression of Bax. Since the ratio of Bax/Bcl-x_l is an important determining factor for the induction of apoptosis (Lee et al., 2008). SIL has been shown to preserve this ratio at a level comparable to that of the control group.

Nuclear factor kappa B (NF-κB) is a heterodimer, composed of p50 and p65 subunits of the Rel protein family (Baeuerle and Baltimore, 1996). Several physiological and pathological conditions such as infection, inflammation and particularly, oxidative stress contributes to the activation of NF-κB (Pahl, 1999). The activated NF-κB (p65) subunit is involved in several stages of the inflammatory-proliferative process by regulating a number of genes, including cell adhesion molecules, growth factors, inflammatory cytokines and chemokines (Baeuerle and Henkel, 1994). In particular, the gene expression of Bax and Bcl-x_l is regulated at least partly by NF-κB; inhibiting the expression of Bax while enhancing that of Bcl-x_l (Bentires-Alj et al., 2001; Chen et al., 2000). This may contribute to the pro-apoptotic effects of SIL through inhibition of NF-κB expression. Increased expression of NF-κB was observed in experimentally induced BPH (Rick et al., 2011). Moreover, it has been demonstrated that constitutive activation of NF-κB can hinder TNF-α induced apoptotic pathway via blocking the activation of caspase-8 and caspase-3 in prostate cancer cell line; DU145 (Wang et al., 1998). SIL effectively down-regulated the expression of NF-κB subunits. Thus, DU145 cell line became sensitive to TNFα-induced apoptosis resulting in activation of the downstream caspases such as caspase-3 (Dhanalakshmi et al., 2002). In the current study, SIL significantly protected against testosterone-induced NF-κB expression and alleviated caspase-3 activity to almost the normal levels. Inhibition of apoptosis could be a major contributing factor to the pathogenesis of BPH (Lee and Peehl, 2004). Our results support the pro-apoptotic activity of SIL and thus its efficacy in combating BPH.

Some evidence suggested the implication of oxidative stress in BPH and prostate cancer pathogenesis. Androgens have been shown to increase prostatic cellular metabolism resulting in extensive production of free radicals along with depletion of the antioxidant enzymes. As mentioned before; oxidative stress can contribute to the induction of NF-κB. This could be another target for free radicals induced by testosterone promoting cellular proliferation. On the other hand, several antioxidant plant extracts and phytochemicals exert an inhibitory effect on BPH (Ali et al., 2013; Pace et al., 2010; van Breemen et al., 2011). This was in coherence with our results which revealed that SIL was effective in countering testosterone-induced reactive oxygen species as evidenced by significantly decreasing the level of MDA, and enhancing that of GSH as well as catalase enzymatic activity to almost the control level.

In conclusion, the current study affords an evidence for the protective effects of SIL against testosterone-induced BPH in rats. This can be attributed, at least partly, to the augmentation of ER-β and decreasing ER-α and AR expressions; the pro-apoptotic and anti-proliferative activities of SIL. Additionally, SIL can guard against the oxidative damage and NF-κB expression induced in BPH.

Conflict of interest

The authors declare that there are no conflicts of interest.

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