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ORIGINAL ARTICLE

# Modulatory Effect of Silymarin on Apoptosis in Testosterone -Induced Benign Prostatic Hyperplasia in Rats

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## Abstract

Benign prostatic hyperplasia (BPH) is considered a normal part of the aging process in men, and is characterized by an imbalance between cell proliferation and apoptosis. Our study aimed to investigate the potential protective role of silymarin (SIL) against testosterone-induced BPH in rats and to elucidate the molecular mechanisms underlying SIL pro-apoptotic and anti-proliferative effects. Forty adult male Wistar rats were divided equally into four groups: control group, BPH group (3 mg/kg testosterone propionate, s.c. for 14 days, SIL group (50 mg/kg SIL, orally, once daily concomitantly with 3 mg/kg testosterone propionate s.c.) and inhibitor group (50 mg/kg SIL orally concomitantly with 3 mg/kg testosterone, s.c. and 0.5 mg/rat Z-VAD-FMK, i.p.). Silymarin induced caspase-dependent apoptosis in BPH as SIL significantly reduced prostatic Bcl-2 protein and increased Bax protein concentration. Also, SIL down-regulated survivin (Inhibitor of apoptosis protein (IAPs) gene expression in rat prostate assisting mainly caspase-dependent pathway. Silymarin significantly decreased cytochrome-*c* cytosolic concentration and increased caspase 3 activity compared to BPH group. Silymarin significantly increased the content of $p27^{kip1}$ (Cyclin dependent kinase inhibitor (CDKIs) promoting cell cycle arrest. The histological features of BPH such as hypertrophy, papillary projections formation, improved in SIL group. Silymarin showed a significant anti-proliferative and pro-apoptotic role in BPH and accordingly it could be effectively and safely used as a treatment tool in cases of BPH or prostatic disorders.

**Keywords** Silymarin · Benign prostatic hyperplasia · Survivin · Bax · Bcl-2 · $p27^{kip1}$

## Introduction

Benign prostatic hyperplasia is a noncancerous prostate disorder resulting from an imbalance between cell apoptosis and proliferation manifesting as increase in number and size of epithelial and stromal cells and enlargement of prostate [1].

Benign prostatic hyperplasia is linked to many complications such as urinary tract infections, acute urine retention, hematuria, and urinary stones with 33% of BPH patients mostly go through medical or surgical procedures [2].

Androgens, estrogens, growth factors are ones of different factors that are associated with BPH pathogenesis [3]. These factors are thought to cause a gradual growth involving both fibromuscular stroma and glandular epithelium, resulting in enlargement of the prostate [4]. Altered expression of genes related to apoptosis and proliferation leading to decreased cell death and increased proliferation are thought to be a potential mechanism in BPH pathogenesis [5].

Programmed cell death (apoptosis) includes many molecular steps that lead to the clearance of damaged and altered cells while avoiding the leaking of damaging substances into the nearby tissues [6].

The genes expressing B cell lymphoma (Bcl-2) and Bcl-2 associated X protein (Bax) play an essential role in BPH, with the expression of Bax in epithelial cells decreased, while the expression of Bcl-2 increased [7].

Inhibitors of apoptosis (IAPs) are a family of proteins that can control programmed cell death (PCD). Without inhibition, IAPs can be suppressors of apoptosis and stimulators to cell

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cycle [8]. Subsequently, enhancing cell apoptosis and cell cycle arrest have been proposed as a promising strategy for the evolution of anti-BPH agents.

Herbal products, with limited side effects, have become increasingly crucial in BPH management and have the benefit of being relatively non-toxic agents [9].

Milk thistle (*Silybum marianum* L.), a flowering plant growing in Mediterranean Europe, has been increasingly used as a treatment for various chronic liver diseases over many centuries. Milk thistle seeds extract, known as silymarin, is a complex mixture of seven significant flavonolignans and one flavonoid. [10].

Silymarin is broadly considered to be a phytoestrogen and is associated with numerous biological properties including antioxidant, anti-inflammatory, neuroprotective, and anti-carcinogenic [11–13].

Silymarin showed potential effectiveness through pro-apoptotic and anti-proliferative properties affecting the cascade of apoptosis and cell cycle regulators in many previous studies [12, 14].

Hence, our study aimed to investigate the possible protective effects of SIL in male Wistar rats with induced BPH and to elucidate the anti-proliferative and pro-apoptotic mechanisms underlying these effects. Z-VAD-FMK was used as a pan-caspase inhibitor to selectively inhibit the caspase-dependent apoptotic pathway.

## Materials and Methods

### Animals and Experimental Design

Forty adult male Wistar rats (200-250 g), aged 10-12 weeks old and were purchased from the National Research Center (Giza, Egypt). Rats were kept in a controlled room temperature of 25 °C with alternate 12 h light /12 h dark cycles. Animals were accustomed for 1 week before experimentation. The animals were fed standard rodents diet and allowed free access to tap water.

All experiments were performed under the ethical guidelines for animal care approved by Research Ethical Committee of Faculty of Pharmacy, Tanta University, Egypt (FPTU-REC,141\2013\970) and the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). Animals were grouped randomly into four equal groups ($n = 10$) being 1 control group and 3 BPH groups with each receiving different treatment as follows: (A) Control group; rats were given 5 mL/kg 0.5% *w/v* sodium carboxymethyl cellulose (Na CMC) (Oxford Co., India) orally and 100 μL corn oil (Iso Chem Co., Egypt) subcutaneously (s.c.) as vehicles once daily for 14 days. (B) BPH group; rats were given 3 mg/kg testosterone (CID-Chemical industries development Co., Giza, Egypt) dissolved in 100 μL corn oil, s.c. daily for 14 days to induce benign prostatic hyperplasia (BPH) [15].

(C) Silymarin (SIL) group; rats were given 50 mg/kg SIL (SEDICO-South Egypt Drug Industries Co., Giza, Egypt) suspended in 0.5% *w/v* (Na CMC) orally [16] concomitantly with 3 mg/kg testosterone dissolved in 100 μL corn oil, s.c. (D) Inhibitor group; rats were given 0.5 mg/rat Z-valinyl-alanyl-DL-aspartyl-fluoromethylketone (Z-VAD-FMK) (AdooQ BioScience., Irvine, CA, USA) dissolved in 2 mL 5% DMSO in sterile saline intraperitoneally (i.p.) [17] divided on days 1,7,13 along with 50 mg/kg SIL orally concomitantly with 3 mg/kg testosterone, s.c.

### Specimen Collection

After the final treatment, all rats were denied food and water overnight then they were weighed and anesthetized using pentobarbital 100 mg/kg body weight [18] injected intraperitoneally. Rats were sacrificed by cervical dislocation then dissected and the prostates were rapidly removed, cleaned of the adhering connective tissues and bladders, washed in saline and weighed, then prostate index (PI) was calculated for each rat as the ratio of prostate weight in (mg) to body weight in (g). Sections of the ventral prostate lobes were fixed in 10% neutral buffered formalin and embedded in paraffin for histological examinations. The remaining part 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.

### Enzyme Linked Immunosorbent Assay (ELISA) for Bax, Bcl-2, p27, and Cytochrome-*c*

For Bax, Bcl-2, and p27, 0.1 g of prostatic tissue was homogenized in cold PBS (1:10 *w/v*). All biomarkers levels in prostatic tissue homogenate were measured by ELISA kits obtained from Shanghai Sunred Biological Technology Co., Ltd. (China) according to the manufacturer's protocol and were expressed as ng/g tissue. Cytochrome c cytoplasmic fraction was measured in the cytosolic supernatant that was prepared using the mitochondria isolation kit purchased from (Biovision Incorporated, Milpitas, CA, USA). Then the supernatant from the 2nd centrifugation step after homogenization was used as the soluble cytosolic fraction.

### Gene Expression of Survivin by Quantitative Real-Time PCR

Total RNA was extracted from prostatic tissues using total RNA extraction Kit (Bioer Technology, China). The purity of obtained RNA was verified spectrophotometrically at 260/280 nm by nanodrop. 1-5 μg total RNA was converted to cDNA using HiSenScript RH (−) cDNA synthesis kit

(iNtRON Biotechnology, Korea). 1.6 μL of the cDNA was used for quantitative PCR using SensiFAST ™ SYBR No-ROX kit (Bioline, USA) as described by the manufacturer. Primer sequences were designed by Primer 3 plus Program (version 2.0). Primer sequences (Biosearch Technologies Co., California, USA) used in RT-PCR were as follows in Table 1:

The PCR conditions were initial polymerase activation for 2 min at 94 °C, followed by 45 cycles (94 °C for 5 s for denaturation, 62 °C for 10 s for annealing and 72 °C for 20 s for extension). After that, the reaction mix was placed in a qRT- PCR system (Thermo Fisher scientific, Pikoreal5100, Finland) for consistent quantification of targets from all regions of mRNA transcripts. All samples were analyzed and standardized to the level of reference gene (GAPDH). Threshold cycle (Ct) values were calculated, and the transcript levels were calculated and expressed as relative quantification (RQ) or the relative expression of target genes using the $2^{-\Delta\Delta CT}$ formula. ΔCt value = target gene Ct value − reference gene Ct value. ΔΔCt = experimental group ΔCt − normal control group ΔCt as described previously by **[19].**

### Determination of Caspase-3 Activity

Caspase-3 activity was examined using the colorimetric assay kit purchased from (Biovision Incorporated, Milpitas, CA, USA). According to the manufacturer's protocol; the assay is based on the spectrophotometric detection of the chromophore *p*-nitroaniline (*p*NA) after being cleaved from the labeled substrate (DEVD-*p*NA) by caspase-3. The absorbance of *p*NA is determined at 405 nm using a microtiter plate reader after an incubation period of 1.5 h. 200 μg protein (20 to 50 μL volume) was added to 10 μL of the substrate (final concentration 50 μmol/L). The volume was made up to 200 μL with the caspase assay buffer. The results obtained were expressed as absorbance (OD) fold difference related to control as described in [20].

### Histopathological Examination

Ventral prostate tissues were fixed in 10% neutral buffered formalin solution for 24 h, washed under water then dehydrated using serial dilutions of alcohol. Specimens were cleared in xylene, embedded in paraffin wax and then tissue blocks were sectioned at 5 μm thickness using slide microtome (Leica RM2155, Germany). The acquired tissue sections were fixed on glass slides, de-waxed then rehydrated, stained by hematoxylin and eosin (H&E) then studied using an Olympus light microscope (Olympus, Tokyo, Japan) and photographed [21].

**Table 1** Primer sequences (Biosearch Technologies Co., California, USA) used in RT-PCR were as follows

| | |
|---|---|
| Survivin (BIRC5) gene | Forward primer: (5-CCCTACCGAGAATGAGCCTG-3) Reverse primer: (5-ACGGTCAGTTCTTCCACCTG-3) |
| GAPDH gene | Forward primer: (5- TGATGGGTGTGAACCACGAG −3) Reverse primer: (5-AGTGATGGCATGGACTGTGG −3) |

### Statistical Analysis

Analysis of data was executed with statistical package for social science (SPSS) software version 20 [22]. Data are presented as mean ± SD and % change. Statistical comparison among groups was performed by one-way analysis of variance (ANOVA) using Fisher's least-significant differences (LSD) method for comparison between two groups. Statistical significance was fixed at $p > 0.05$. Graphs were sketched using GraphPad Prism software version 5 (GraphPad Software, Inc., La Jolla, CA, USA).

## Results

### Effect on Prostate Weight and Prostate Index

Figure 1 and Table 2 show that testosterone propionate injection significantly elevated the prostate weight and prostate index by 102% and 92%, respectively compared to the control group. Prostate index for each rat was calculated as the ratio of prostate weight in (mg) to body weight in (g) **[**23**]**. Silymarin significantly reduced the prostate weight by 32% and the prostate index by 34% of the testosterone group. The Z-VAD-FMK + SIL treated group showed a non-significant change in prostate weight and prostate index in comparison to the testosterone-treated group and showed a significant elevation in the prostate weight and prostate index by 87% and 81%, compared to the SIL treated group.

### Effect on Bax and Bcl-2 Concentrations in Prostatic Tissue

Figure 2 shows that BPH induction with testosterone significantly increased the Bcl-2 concentration in prostatic tissue (49.83 ± 5.41 ng/g tissue) by 172% while it significantly decreased Bax concentration (80.39 ± 15.69 ng/g tissue) by 63% & Bax /Bcl-2 ratio (1.62 ± 0.33) by 86% of the control group (18.25 ± 3.36, 220.39 ± 26.6 ng/g tissue &12.44 ± 2.89) respectively. Comparatively to the BPH group, Silymarin significantly decreased Bcl-2 concentration (30.48 ± 4.38 ng/ g tissue) by 40% while it increased Bax concentration (163.43 ± 19.6 ng/g tissue) by 103% & Bax /Bcl-2 ratio (5.46 ± 1.11) by 237%. The Z-VAD-FMK + SIL treated group showed

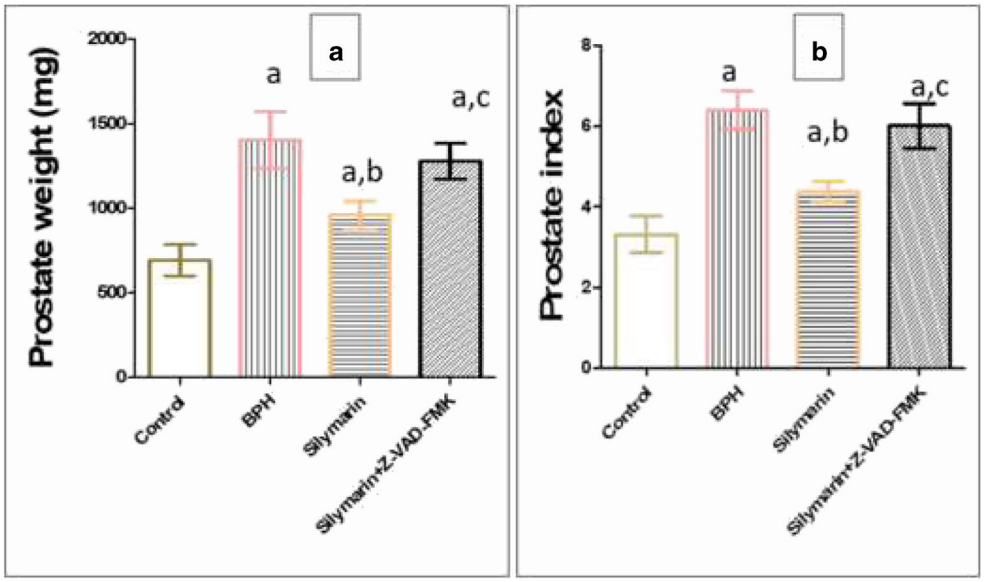


**Fig. 1** Effect of SIL and SIL + Z-VAD in BPH induced groups on **a** Prostate weight (mg). **b** Prostate index of studied groups. Data are stated as mean ± SD, significance was set at $p < 0.05$; a: significant vs control group, b: significant vs BPH group, c: significant vs Silymarin group using one-way ANOVA followed by LSD as a post hoc test

significant difference in Bcl-2, Bax concentrations & Bax/Bcl-2 ratio from the SIL treated group (36 ± 3.15, 124.31 ± 22.08 ng/g tissue & 3.47 ± 0.68) respectively. In comparison to the BPH group, the Z-VAD-FMK + SIL treated group significantly decreased Bcl-2 concentration by 27%, while it increased Bax concentration by 55% and significantly elevated Bax/Bcl-2 ratio by 114%.

### Effect on p27/$^{kip1}$ Concentration in Prostatic Tissue

Figure 3a reveals that testosterone injection significantly decreased the p27/$^{kip1}$ concentration (218.65 ± 32.43 ng/ g tissue) in prostatic tissue by 50% of the control group (439.8 ± 25 ng/ g tissue). Silymarin significantly increased p27/$^{kip1}$ concentration (398.46 ± 27.59 ng/g tissue) by 82% of the testosterone-induced BPH group and it retained 90% of the control group p27/$^{kip1}$ concentration. The Z-VAD-FMK + SIL treated group showed a significant increase by 20% in p27/$^{kip1}$ concentration (262.88 ± 41.49 ng/ g tissue) in comparison to the testosterone-treated group.

**Table 2** Effect of SIL and SIL + Z-VAD-FMK in BPH induced rats on prostate weight and prostate index

| | Prostate weight (mg) | Prostate index |
|---|---|---|
| Control | 694.2 ± 91.8 | 3.32 ± 0.45 |
| Testosterone | 1404.2$^{a}$ ± 168.66 | 6.39$^{a}$ ± 0.47 |
| Testosterone + SIL | 960.7$^{a,b}$ ± 84 | 4.37$^{a,b}$ ± 0.24 |
| Testosterone + SIL+ Z-VAD | 1297.5$^{a,c}$ ± 104.76 | 6$^{a,c}$ ± 0.55 |

### Effect on Cytochrome-*c* Concentration in Prostatic Tissue: Cytosolic Fraction

BPH group showed a significant reduction in cyt-c concentration (46.5 ± 4.8 ng/ g tissue) in prostate by 44% of the control group (81.92 ± 6.87 ng/ g tissue). Silymarin showed a significant decrease in cyt-c concentration (75.93 ± 6.33 ng/ g tissue,) compared to the control group and it increased significantly cyt-c concentration by 65% of the testosterone-induced BPH group. The Z-VAD-FMK + SIL treated group showed a non-significant difference in cyt-c concentration (47.61 ± 7.21 ng/ g tissue) compared to the testosterone-treated group as illustrated in Fig. 3b.

### Effect on Caspase-3 Activity

Figure 4a shows that testosterone-induced BPH significantly decreased the caspase-3 activity (0.47 ± 0.045) in prostatic tissue by 53% of the control group (1 ± 0.08). Silymarin retained caspase-3 activity (0.79 ± 0.06) by 80% of the control group showing a significant difference and it significantly increased caspase-3 activity by 68% of the testosterone-induced BPH group. The Z-VAD-FMK + SIL treated group expectedly showed diminished caspase-3 activity (0.17 ± 0.05).

### Effect on Prostatic Survivin Gene Expression

The results, as shown in Fig. 4b demonstrate that there was 2.9 times up-regulation in the prostatic survivin gene expression of the testosterone-treated group (2.92 ± 0.37 RQ) compared to the control group. Silymarin concomitant treatment significantly downregulated survivin gene expression (1.7 ± 0.14

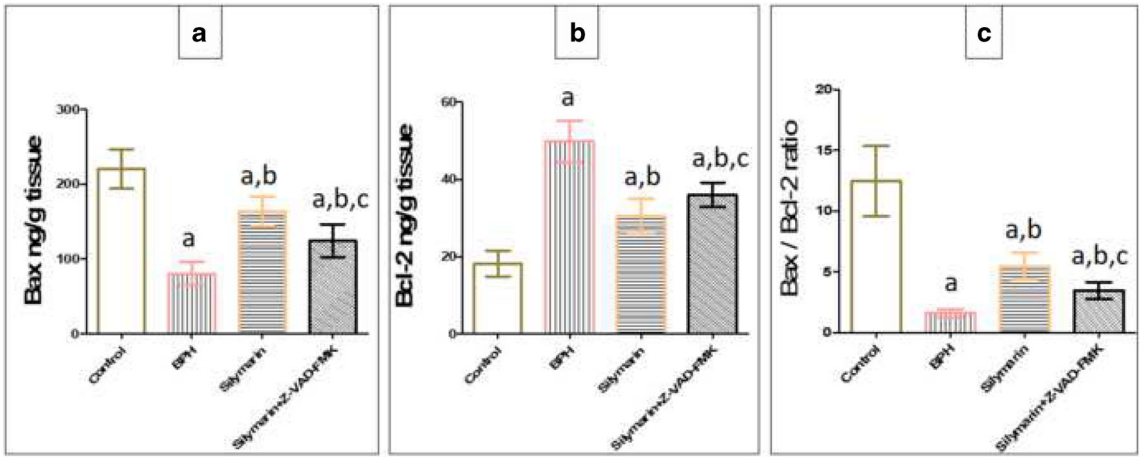


**Fig. 2** Effect of SIL and SIL + Z-VAD in BPH induced groups on **a** Bax concentration in prostates of studied groups expressed in ng/g tissue. **b** Bcl-2 concentration in prostates of studied groups expressed in ng/g tissue. **c** Bax/Bcl-2 ratio in prostates of studied groups. Data are stated as mean ± SD, significance was set at $p < 0.05$; a: significant vs control group, b: significant vs BPH group, c: significant vs Silymarin group using one-way ANOVA followed by LSD as a post hoc test

RQ) to 58% of the respective testosterone-treated group. The Z-VAD-FMK + SIL treated group down regulated prostatic survivin gene expression (1.6 ± 0.2 RQ) by 55% compared to the BPH group and it showed no significant difference respectively of the SIL treated group.

## Histopathological Examination

Figures 5 and 6 show representative photomicrographs of histological examination of hematoxylin-eosin stained sections of rat ventral prostates. (A): Section taken from the prostates of the control group show normal morphological structure of the lining epithelial cells, no histological changes in the acini (solid arrows show normal epithelial lining). Epithelial cells were cuboidal in shape and of regular size. (B, C, D, E): Sections taken from the prostates of the BPH group show disfigured morphology in the prostate epithelia indicated by notable thickening and hypertrophy with increased epithelial thickness (double arrows), papillary projections formation (solid arrows) and thick fibromuscular stroma (f) with widening of prostatic acini. (F): Section taken from the prostates of the BPH group co-treated with 50 mg/kg SIL shows notable reduction in prostate hypertrophy and hyperplasia induced by testosterone maintaining the normal histological structure and regular shape of acini with decreased epithelial thickness and papillary projections formation.

(G, H, I): Sections taken from the testosterone+SIL + Z-VAD-FMK group show abnormal prostatic morphology, increased epithelial thickness (arrow head) and irregular shape of acini with papillary projections (arrows) and thick fibromuscular stroma (f).

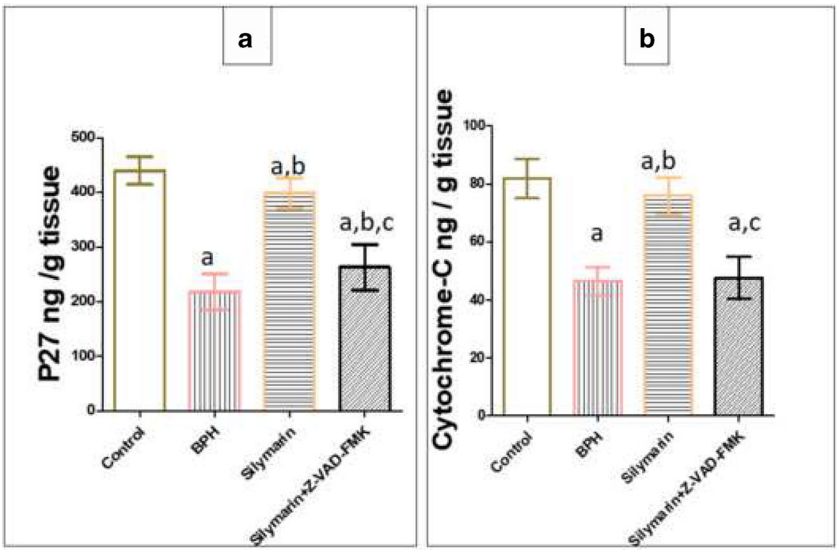


**Fig. 3** Effect of SIL and SIL + Z-VAD in BPH induced groups on **a** P27 concentration in prostates of studied groups expressed in ng/g tissue. **b** Cytochrome-C concentration in prostates of studied groups expressed in ng/g tissue. Data are stated as mean ± SD, significance was set at $p < 0.05$; a: significant vs control group, b: significant vs BPH group, c: significant vs Silymarin group using one-way ANOVA followed by LSD as a post hoc test

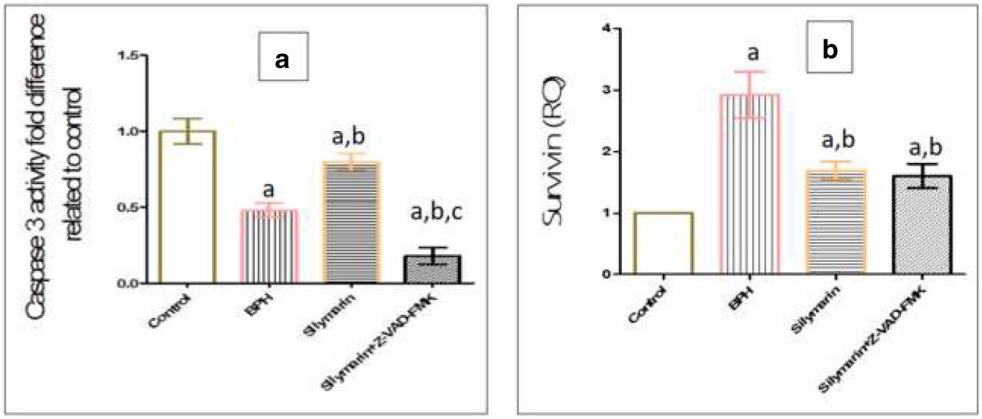


**Fig. 4** Effect of SIL + Z-VAD in BPH induced groups on **a** Caspase-3 activity (OD) in prostates of studied groups expressed as fold difference related to control **b** survivin mRNA expressions of n-fold difference relative to the reference gene GAPDH (relative expression levels), RQ expresses relative quantity. Data are stated as mean ± SD, significance was set at $p < 0.05$; a: significant vs control group, b: significant vs BPH group, c: significant vs Silymarin group using one-way ANOVA followed by LSD as a post hoc test

## Discussion

BPH is one of the most common benign tumors in males, with an old age-related incidence. BPH constitutes an enlargement of the prostate gland consequent of over proliferation of stromal and glandular cells. Oxidative stress and androgens are important factors that increase the imbalance between cell proliferation and cell apoptosis, which consequently enhance the pathogenesis of BPH [23].

The present study aimed to investigate the pro-apoptotic and the anti-proliferative role of silymarin in inhibiting the progression of BPH using a rat model of testosterone-induced BPH. Z-VAD-FMK was used as a pancaspase inhibitor to selectively inhibit the caspase dependent apoptotic pathway.

In the present study BPH was induced in rats by testosterone propionate injection and was evidenced by the histopathological changes in rats' prostates. The prostates showed severe hyperplasia, desquamation of lining epithelial cells, inflammatory cell infiltration, and papillary projections. In addition, prostate weight and prostate index were elevated in BPH group. These observations were in agreement with previous reports [24].

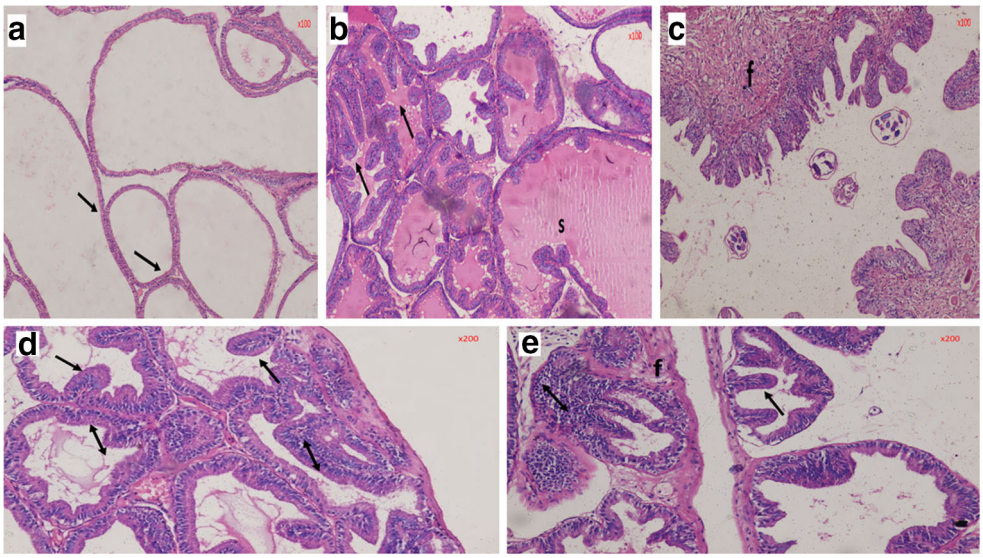


**Fig. 5 and 6** Show representative photomicrographs of histological examination of hematoxylin-eosin stained sections of rat ventral prostates. **a** Section taken from the prostates of the control group show normal morphological structure of the lining epithelial cells. **b**, **c**, **d**, and **e** Sections taken from the prostates of the BPH group show disrupted morphology in the prostate epithelia. **f** Section taken from the prostates of the BPH group co-treated with 50 mg/kg SIL shows marked reduction in prostate hypertrophy and hyperplasia induced by testosterone. **g**, **h**, **i** Sections taken from the testosterone+SIL + Z-VAD-FMK group show disrupted prostatic morphology, increased epithelial thickness

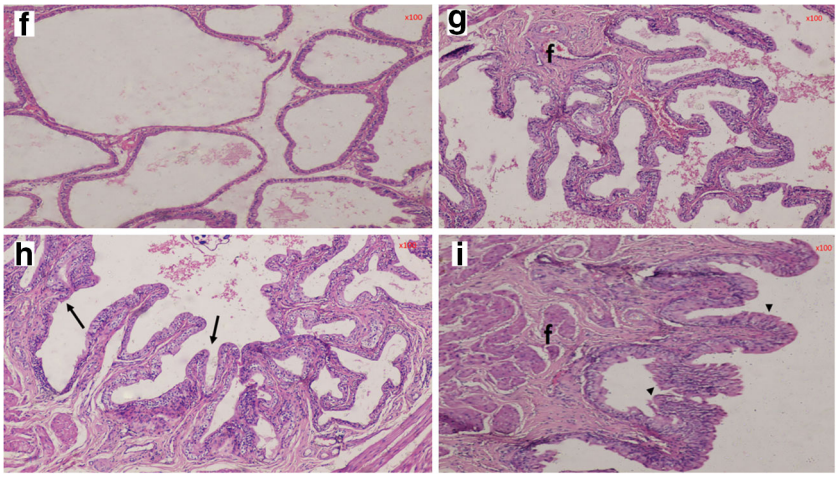


**Fig. 5 and 6** continued.

In our study, histopathological investigations of prostate sections of rats group treated with SIL concomitantly with BPH induction showed improvement in prostatic hyperplasia, decreased papillary projections and a significant decrease in the prostates weight and prostates index compared with the BPH group. These findings were consistent with those of [25], who reported that the treatment with SIL in Sprague–Dawley rats with induced BPH significantly reduced prostates weight, prostates index and improved prostatic hyperplasia. These results were explained as the modulatory effect of SIL on inflammatory mediators such as PTEN, HIF-1$\alpha$, and NF-$\kappa$B.

Histopathological alterations observed in the BPH group treated with Z-VAD-FMK concomitant with SIL ingestion were similar to those observed in the BPH group showing severe hyperplasia, disrupted prostate epithelial morphology and papillary projections also it showed non-significant difference in prostates weight and prostate index from the BPH group suggesting that inhibiting the caspase cascade of apoptosis abolished the SIL induced therapeutic pro- apoptotic effect.

Apoptosis refers to the cell's inherent ability to self-destruct through a process of programmed cell death and considered a tumor suppressor mechanism [26]. The B cell lymphoma/leukemia-2 (Bcl-2) family has been speculated as one of the fundamental elements determining apoptosis. Bcl-2 family members are divided into proteins that protect cells from apoptosis (e.g., Bcl-2), and those that induce apoptosis (e.g., Bax) [27]. The Bax/Bcl-2 ratio works as a measure to determine a cell's proneness or resistance to apoptosis [28]. They trigger opposing actions on the mitochondria; Bcl-2 inhibits while Bax induces the release of apoptogenic cytochrome c which binds to Apaf-1 leading to the formation of apoptosome and activating caspase-9; activated caspase 9 that in turn cleaves and activates the effector caspases such as caspase-3 and results in apoptosis [29].

With this apoptosis plays a critical role in the preservation of tissue homeostasis, and rising evidence has shown that reduction of cell apoptosis is associated with the pathogenesis of BPH [30]. Due to the inhibition of apoptosis, the total number of prostatic stromal and epithelial cells is increased and eventually results in prostate hyperplasia.

Our study showed that BPH rats group exhibited a significant reduction in the pro-apoptotic Bax and a significant increase in the anti-apoptotic Bcl-2 levels in rat prostate tissues compared to normal control group in agreement with the results shown in [16].

Whereas, SIL treated rats showed a significant increase in prostate content of Bax compared to BPH group. These results were in accordance with the results reported by [31]. Silymarin significantly decreased the concentration of the anti-apoptotic protein Bcl-2, as compared with the BPH group which goes in accordance with reports shown in [12]. BPH rats group treated with SIL showed a significant increase in Bax / Bcl-2 ratio, as compared with the BPH group.

The Z-VAD-FMK group showed a significant decrease in Bax level compared to the SIL group while it showed a significant increase in Bcl-2 level compared to the SIL group. Also, Z-VAD-FMK group showed a significant decrease in Bax / Bcl-2 ratio in relation to SIL group. The results shown in the Z-VAD-FMK group suggested that inhibiting caspase activity doesn't abolish the effect of SIL on Bax and Bcl-2 levels.

Our results suggested that, the therapeutic effect of SIL on BPH was mainly dependent on induction of apoptosis through regulation of the levels of anti- and pro-apoptotic Bcl-2 family proteins (Bax and Bcl-2, respectively). In harmony with our results, the pro-apoptotic properties of SIL were previously reported in [14].

In the present study, induction of BPH exhibited a significant reduction in prostatic cytosolic cyt-c concentration and caspase-3 activity compared to normal control group. These

results provided an evidence between release of cyt-c into cytosol and activation of pro-caspase-3 in BPH supporting results reported in [32].

Furthermore, BPH rats treated with SIL showed a significant elevation in prostatic cytosolic cyt-c and caspase-3 activity compared to BPH group. Our data were in agreement with the results of [33] who stated that SIL and its components promote apoptosis via caspase pathway. In our study, BPH rats treated with Z-VAD-FMK concomitant with SIL showed a significant decrease in caspase-3 activity and a significant reduction in cyt-c levels compared with SIL group while showing a non-significant change compared to BPH group suggesting that caspase inhibition and cancelling the effect of SIL on caspase activity may hinder its effect on the cyt-c release from mitochondria into cytosol. Thus, our results indicate that SIL-induced apoptosis is also associated with the activation of caspases via a mitochondrial pathway. Our results resonated with reports shown in [34].

Inhibitors of apoptosis proteins impact apoptosis by direct negative regulation of caspases impeding the intrinsic apoptosis pathway and alteration of the transcription factor Nuclear Factor-κB (NF-κB) impeding the extrinsic death receptor pathway. Eight mammalian IAPs are known at present: Survivin, X chromosome-linked IAP (XIAP), cellular IAP-1 and IAP-2 (c-IAP-1 and c-IAP-2), neuronal apoptosis inhibitory protein (NAIP), Bir-ubiquitin conjugating enzyme (BRUCE), ML-IAP (LIVIN) and testis-specific IAP (Ts-IAP) [35]. Increased IAPs expression has been shown in human pathological prostate including benign hyperplasia, prostatic intraepithelial neoplasia, and cancer [36]. Survivin is one of the IAPs that is directly involved in the intrinsic apoptosis pathway. Previous studies revealed that survivin inhibits the signaling cascades including caspases 3, 8, and 9 [37]. Also, antagonism of survivin function elicits the apoptosis of HCC cells. It makes cancer cells sensitive to the pro-apoptotic effects of TNF-α, suggesting that survivin blocks the extrinsic pathway of apoptosis [38].

In our study, the BPH rat group showed up-regulation of survivin gene expression compared to control group. The increase in survivin gene expression in the BPH group goes in agreement with [39]. Moreover, The BPH group that received SIL exhibited down-regulation of survivin gene expression compared to BPH group which was in line with [40] regarding the effect of silbinin (a component of silymarin) on survivin expression in pancreatic cancer cells.

Interestingly, in the present study, the BPH group that received SIL and Z-VAD-FMK concomitantly exhibited down-regulation of survivin gene expression compared to the BPH group. These results suggest inhibiting the caspase pathway doesn't alter the SIL effect on survivin gene expression.

$P27^{/kip1}$ is one of the CIP1/KIP1 family which are cyclin-dependent kinase (CDK) inhibitors implicated in mediating a wide range of cell growth control signals. $P27^{/kip1}$ is functioning as a negative regulator of cyclins and protein kinase, which lead to cell cycle arrest at the G0/G1 resting phase [41].

Our results indicated that, the BPH group was associated with a significant decrease in $p27^{/kip1}$ concentration in rat prostate compared to control group. The SIL group showed a significant increase in $p27^{/kip1}$ concentration compared to the BPH group which goes in agreement with results reported by [42] about the effect of Silbinin on human breast cancer cell lines. The Z-VAD-FMK group showed a significant decrease in $p27^{/kip1}$ level almost comparable to the BPH group which indicated that inhibiting the SIL effect on caspase cascade hindered its effect on cell cycle regulation and aggravated cell proliferation in BPH.

The previous results suggested that the use of Z-VAD-FMK as an inhibitor to caspase mediated apoptosis has abolished the SIL induced therapeutic pro-apoptotic effect. Therefore, the present study provided an evidence that inhibition of cell apoptosis is associated with the pathogenesis and development of BPH.

## Conclusion

Our study demonstrated that concomitant administration of SIL along with testosterone injection significantly attenuated morphological, biochemical and histological features of BPH. The pro-apoptotic and anti-proliferative action of SIL could presumably be attributed to the reduction of the anti-apoptotic Bcl-2 and elevation of the pro-apoptotic Bax, cytosolic cyt-c, $p27^{/kip1}$ concentration, and caspase-3 activity. Silymarin also down-regulated the IAP survivin gene exprsession. These results were further assisted by inhibiting the caspase-dependent apoptotic effect of SIL with Z-VAD-FMK. As a result, enhancing cell apoptosis and cell cycle arrest is suggested as a promising strategy for the evolution of anti-BPH agents. As SIL is a relatively safe plant-based product, it could be useful as a prophylactic measure or adjuvant therapy in controlling BPH. More studies are needed to investigate the potential therapeutic efficacy of SIL in BPH and its clinical application.

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**Author Contribution** NE and EG conceived and designed the research study. NN conducted experiments and analyzed data. EG and HA wrote the manuscript. All authors read and approved the manuscript.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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