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SAW PALMETTO BERRY EXTRACT INHIBITS CELL GROWTH AND COX-2 EXPRESSION IN PROSTATIC CANCER CELLS

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The cytotoxicity of a commonly used material to alleviate the symptoms of benign prostatic hyperplasia (BPH), Saw Palmetto Berry Extract (SPBE), was examined as neat oil using a set of prostatic cell lines; 267B-1, BRFF-41T and LNCaP. Proliferation of these prostatic derived cell lines is inhibited to different degrees when dosed for 3 days with SPBE. The amount of SPBE required to inhibit 50% growth (IC_{50}) of these cell lines was 20–30 nl equivalents of SPBE per ml of medium for cell lines 267B-1 and BRFF-41T and approximately 10-fold more for the LNCaP cell line. The effect of SPBE dosing on these cell lines is not irreversible, since a 30 min treatment with SPBE at an IC_{50} concentration does not inhibit their growth. Normal prostate cells were inhibited by 20–25% when grown in the presence of 200 nl SPBE equivalent per ml media. Growth of other non-prostatic cancer cell lines, i.e. Jurkat and HT-29, was affected by approx. 50% and 40%, respectively. When LNCaP cells were grown in the presence of dihydrotestosterone and SPBE, the IC_{50} concentration decreased significantly compared to LNCaP cells grown in the presence of serum and SPBE. Reduced cellular growth after SPBE treatment of these cell lines may relate to decreased expression of Cox-2 and may be due to changes observed in the expression of Bcl-2. Expression of Cox-1 under similar conditions is not affected because of its constitutive expression. Since increased Cox-2 expression is associated with an increased incidence of prostate cancer, and decrease in its expression by SPBE would provide a basis for further investigation of its use against BPH and in prostatic cancer chemoprevention.

KEYWORDS: Saw Palmetto berry extract; cell growth inhibition; MTT and SRB assays; Cox-2; Cox-1; Bcl-2.

INTRODUCTION

Phytotherapeutic formulations based on Saw Palmetto berry extract (*Serenoa repens*) (SPBE) have traditionally been used for treating prostate-related problems, and clinical research has supported the application of SPBE in the fight against benign prostatic hyperplasia BPH (Wilt *et al.*, 1998; Plosker and Brogden, 1996; DiSilverio *et al.*, 1998; McKinney, 1999; Lowe and Ku, 1996). The combination of this extract with other herbs has been shown to lower not only testosterone concentrations but also the concentration of prostate-specific antigens (DiPaola *et al.*, 1998). Researchers have further demonstrated in animal studies that SPBE inhibits the binding of dihydrotestosterone DHT to its receptor (Carilla *et al.*, 1984; Sultan *et al.*, 1984) and blocks the conversion of testosterone to DHT by inhibiting the activity of 5-α-reductase. So far the mechanism of action is not known for this phytotherapy but it may include an anti-oestrogenic, anti-androgenic, anti-spasmolytic,

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or anti-inflammatory effect, or the inhibition of 5-α-reductase, since all of these activities have been documented for it in *in vitro* experiments (Stoner, 1996; Carraro *et al.*, 1996).

In the present study, we investigate the role of SPBE in prostate cancer by comparing the growth of prostatic cancer cell lines in the presence and absence of SPBE. The data presented here demonstrate that SPBE inhibits the growth of a normal prostatic derived cell line and two prostatic carcinoma cell lines. The results may suggest an 'operating mechanism' involving growth inhibition via alterations in the expression of Bcl-2 and prevention of prostate carcinoma development through the inhibition of expression of Cox-2.

MATERIALS AND METHODS

Cell lines and cell culture

Human prostate cancer cell lines, 267B-1 and BRFF-41T, were obtained from BRFF (Ijamsville, MD, U.S.A.), and LNCaP, Jurkat (lymphoma) and HT-29 cells (human colon adenocarcinoma) were from ATCC (Rockville, MD, U.S.A.). Normal human prostate epithelial cells were purchased as growing cultures and maintained as described by the supplier (BioWhittaker, Walkersville, MD, U.S.A.). Cell lines were grown in media as recommended by the suppliers i.e., 267B-1 cells in P4/8F media, BRFF-41T cells in basal media BRFF-HPC1, LNCaP and Jurkat cells were maintained in RPMI and HT-29 in DMEM supplemented with 10% serum. All cell lines were maintained at 37°C in humidified atmosphere of 5% CO_2 in air. The cells were maintained as sub-confluent monolayers in T-25 flasks and subcultured once or twice per week. Cell passage levels were between 2–8 after receipt of cell lines from the supplier. 267B-1 and BRFF-41T cell lines were developed from prostate carcinoma (Type P). Antibodies against Cox-1 and Cox-2 were obtained from Cayman Chemical Company (Ann Arbor, MI, U.S.A.) and Bcl-2 from Transduction Laboratory (San Diego, CA, U.S.A.). All other reagents were purchased from Sigma Chemicals.

Preparation of SPBE

The neat SPBE oil was originally prepared as an ethanolic extract of Saw Palmetto berries obtained from PharmaPrint (Irvine, CA, U.S.A.). A homogeneous stock solution of the SPBE for test was prepared by diluting the SPBE with ethanol (20% solution, w/v) at room temperature and shaking with constant agitation for at least 10 h. This preparation was passed through 3-mm filters and finally centrifuged at 1000 × *g* for 10 min to remove herbal debris. This preparation was then concentrated by passing a stream of nitrogen over it. The concentrated material was stored in the dark at room temperature until use. Fresh dilutions were prepared for each experiment to minimize any non-specific degradation of SPBE constituents.

MTT assay

MTT assays were performed as described by Mosmann (1983) with minor modifications. Briefly, exponential growing cells were harvested by trypsinization, counted, and then plated on flat-bottomed 96-well culture dishes. Optimal seeding densities for each cell line were determined and used to ensure exponential growth during 4-day assays. Cells (1×10^4) in 100 µl media per well were seeded and allowed to attach overnight. Initially, a 10% stock solution of SPBE was prepared in ethanol and all further dilutions were made in cell growth media. To each well, 100 µl of media containing different concentrations of SPBE were added. After 3 days, 20 µl of MTT solution (5 mg/ml) were added to each well. Following 4 h of incubation at 37°C, the media was removed and farmazan crystals, which result from the reduction of MTT by active cells, were dissolved in 50 µl of 0.04 N HCl-isopropanol. The optical density at 590 nm was determined by a microplate reader. All experiments were performed in triplicate.

Sulfo rhodaminesulfate-B (SRB) assay

SRB assays were performed as described by Skehan *et al.* (1990). Cell plating and incubation were carried out in the same way as described for the MTT assay. The cells were then fixed with 50 µl 50% trichloroacetic acid at 37°C for 1 h. The cells were washed five times with deionized water, dried, and stained by the addition of 50 µl SRB solution (0.4% SRB in 1% acetic acid). Following staining, cells were first washed five times with 1% acetic acid to remove unbound dye and then air-dried. Subsequently, bound dye was solubilized with 100 µl of 10 mM Tris base (pH 10.5) and detected at 560 mm by a microplate reader.

Cell survival assay, counting, and data analysis

Cell plating was performed in the same way as in MTT assays. After cell growth, fresh media was used (0.2 ml) containing various concentrations of SPBE and cells were then incubated at 37°C. After

30 min of incubation, the cells were washed again with fresh medium and grown for 3 d in the absence of SPBE. MTT assays were performed as described above.

Trypsinized cells were seeded in 96-well plates as described for the MTT assay. After the cells had been cultured for three days, the medium was removed and the cells were incubated with 50 µl of trypsin-EDTA solution for 5 min at room temperature. Cells were suspended in trypsin-EDTA solution and counted with a hemocytometer. Cell counting was carried out in duplicate, and for each sample, the mean of all fields was calculated.

Microplate absorbance data were exported to Microsoft Excel, and the average as well as the standard deviation were calculated. The final data were analysed in Cricket Graph, which was used to prepare the figures.

Western blotting

Cells were seeded at a density of 500,000 cells/well in 6-well plates. Once the cells reached 80–90% confluency, the medium was replaced by serum-free medium for 24 h. The cells were induced at different concentrations of FCS for 24 h in the presence and absence of various concentrations of SPBE. The cells were then washed in PBS, removed, centrifuged and, lyzed for 15 min on ice using 50 mM Tris–HCl, 110 mM NaCl, 1% Triton-X100, 5 mM EDTA; 10 µg/ml each of leupeptin, chymostatin, antipain, pepstatin, and 2 mM phenylmethylsulfonyl-fluoride (PMSF). The lysates were centrifuged in an Eppendorf minifuge for 10 min at 4°C, and aliquots of 5 µl from each sample were used to quantify the protein using Bradford dye. Cell lysates were denatured in Laemmli sample buffer, and 50–100 µg of protein were analysed by 12% SDS–Page under reducing conditions. Following SDS–Page, proteins were transferred electrophoretically onto sheets of Immobilon-P membrane (Millipore, Bradford, MA, U.S.A.) in a buffer containing 50 mM Tris–glycine, pH 8.3, and 20% methanol for 5–6 h or overnight at a constant current of 60 mA. Transferred proteins were visualized with Coomassie blue and identified separately with polyclonal antibodies against Cox-1, Cox-2, and Bcl-2.

RESULTS

Effect of SPBE on cell growth

Prior to measuring the effect of SPBE on the proliferation or cytotoxicity of different prostate-derived cell lines, we performed a series of MTT assays to optimize cell plating assay conditions. For BRFF-41T and LNCaP cells, we determined the range to allow for a linear relationship between cell number and the MTT assay readout, and decided to plate 20,000 cells per well in a 96-well plate. In all subsequent experiments using other cells, 10,000 cells were added per well.

Initial analyses determined the effects of SPBE on the growth of prostate-derived cell lines. SPBE was prepared as described in Materials and Methods. The cells were either grown in the presence of SPBE or incubated for 30 min and then grown in the absence of SPBE. BRFF-41T and 467B-1 cells in the presence of SPBE were completely inhibited at concentrations ranging between 40–50 nl equivalents per ml of media, resulting in an IC_{50} between 20–25 nl equivalents per ml of media (Figs 1A and 1B, cytotoxicity assay, open circles; Table 1). In contrast, the IC_{50} for LNCaP cells was in the range of 200 nl equivalents per ml of media (Fig. 2A, closed squares; Table 1). No inhibition of cell-growth was observed when calls were only grown in the presence of vehicles (Fig. 2A, closed circles). Normal prostate cells—when grown in the presence of 200 nl SPBE equivalent per ml media—were partially inhibited (20–25%), suggesting a specificity of SPBE for prostate cancer cells (Fig. 2B). To further demonstrate the specificity of SPBE, we also assessed the cytotoxicity of SPBE on HT-29 and Jurkat cells. When these cells were grown in the presence of 200 nl of SPBE, their growth decreased by about 40–50% (Fig. 2C), thus suggesting a more specific effect of SPBE on prostate cancer cell lines.

A 30-min pulse of the cell lines with SPBE at its IC_{50} concentration was not effective in inhibiting the growth of any of these cell lines (Figs 1A, B, cell survival assay, closed circles). Since prostate cell growth can be modulated by male hormones (Sonnenschein *et al.*, 1989; Lee *et al.*, 1995), we determined what impact the male hormone DHT has on the cytotoxicity/proliferation of the more SPBE-resistant LNCaP cell line. Usually, this hormone acts as a mitogen enhancing cellular growth. When these cells were grown in the presence of different concentrations of DHT, they responded favourably up to 1 nM. At higher concentrations, the growth of LNCaP cells decreased slightly in comparison to cells grown in 1 nM DHT (data not shown and Lee *et al.*, 1995). Under these conditions, it was found that there was a significant decrease in the IC_{50} concentration for SPBE when LNCaP cells were co-cultured in 1 nM DHT and

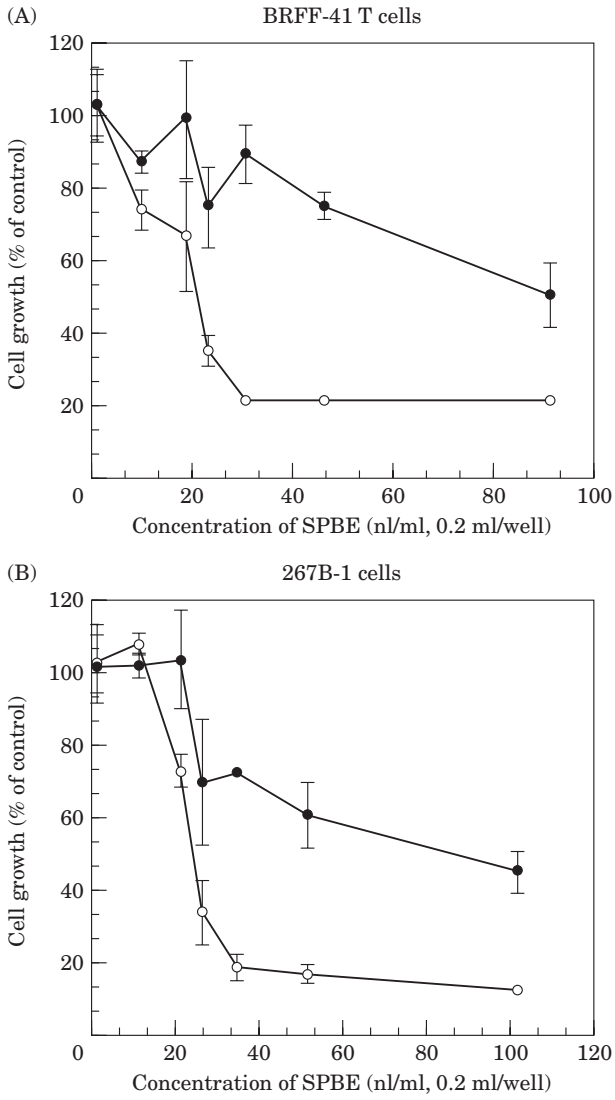


Fig. 1. Effect of SPBE on the growth of BRFF-41T and 267B-1 cells. Trypsinized cells suspended in HPC-1 media were seeded on fibronectin-coated, flat-bottomed 96-well culture dishes. After incubation for 24 h the medium was replaced with medium prepared in the presence/absence of different concentrations of SPBE. The MTT assay was performed after three days of cell culturing. The cell survival assay was carried out as follows: the cells were trypsinized and grown as mentioned above. The media containing different concentrations of SPBE were placed in wells for 30 min, and the cells were washed and further incubated in fresh media containing no SPBE. After three days of cell growth, the MTT assay was performed. —●—, Cell survival; —○—, cytotoxicity.

with different equivalents of SPBE (Fig. 2A, closed triangles, and Table 1).

Since the MTT assay is dependent on the reduction of a tetrazolium salt to formazan by mitochondria of viable cells, its reaction can correlate with both cell number and incubation time. It is known that this reaction is also sensitive to the concentration of glucose, hydrogen ions, NADH, and NADPH (Jabbar *et al.*, 1989; Vistca *et al.*, 1991). Since we do not know how SPBE affects those molecules that can influence the results of the MTT assay, they were verified using SRB assay. In this assay SRB stains proteins under weakly acidic conditions, a property that provides a quantitative measurement of the cell number. Thus, it gives no measure of cell viability. The previous MTT results were confirmed in this assay. As before, the SPBE treatment resulted in a concentration-dependent reduction in cell number for all three cell lines (Fig. 3A and 3B). Again, the inhibition of cell proliferation was dose-dependent for the SRB assay and no different to results from MTT assays. Comparing the two assays, the only difference was that the SRB assay was 2–4 times more sensitive in detecting cell numbers than the MTT assay. The reduction in cell numbers after 72 h of culture in the presence of SPBE was also verified by counting the actual number of cells after culturing in the presence of different concentrations of SPBE (data not shown).

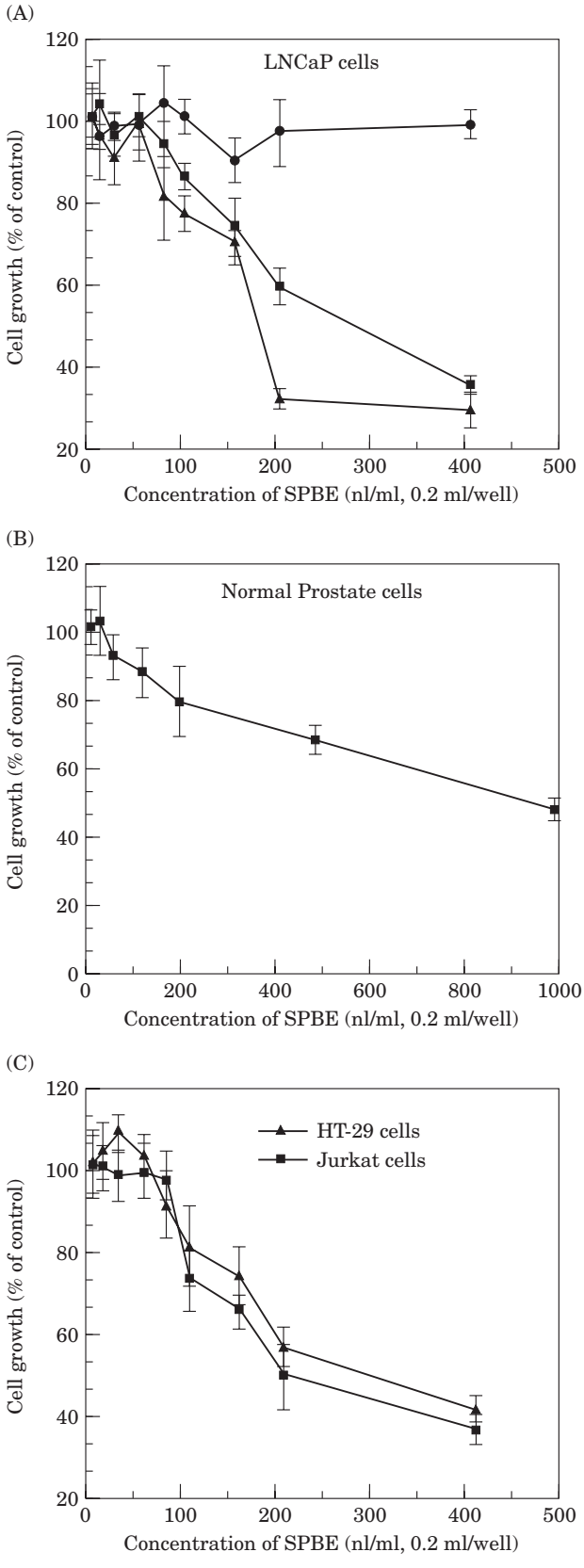
Table 1.
Effect of SPBE on different cell lines' growth as measured by the MTT assay

Cell lines	*SPBE; IC_{50} (nl/ml)*
Normal prostate cells	>400
BRFF-41T	~20
267B-1	~20
LNCaP	~200
LNCaP+DHT	<200*
Jurkat	~200
HT-29	>200

*LNCaP cells were grown in the presence of DHT instead of serum.

Effect of SPBE on Cox-1 and Cox-2 expression in the LNCaP cell line

Having shown that SPBE inhibits the growth of prostate derived cell lines, we investigated its effect on the expression of Cox-1 and Cox-2. We chose the LNCaP cell line as a model for this investigation. LNCaP cells constitutively express high levels of the inducible Cox-2 mRNA and its protein (Tjandrawinata *et al.*, 1997; Liu *et al.*, 1998). It has been previously shown that specific Cox-2 inhibitors induce apoptosis in cell lines known to express high levels of Cox-2 (Kyprianou *et al.*, 1996). To determine the effect of SPBE on Cox-2 expression,



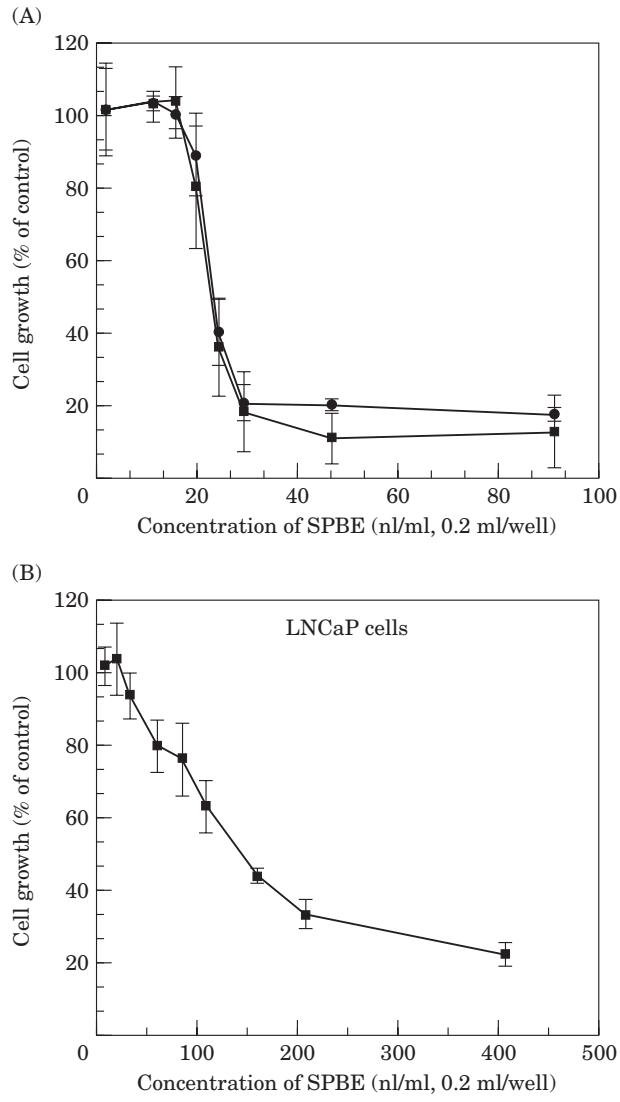


Fig. 3. Cytotoxicity of SPBE in different cell lines using SRB assay. (A) BRFF-41T (■) and 267B-1 (●) cells, (B) LNCaP (■) cells were each plated in wells and SRB assays were performed.

LNCaP cells were cultured in the presence or absence of both FCS and SPBE. Cell lysates were prepared after culturing the cells for 24 h and the proteins were determined using SDS–Page. The separated proteins were transferred onto an Immobilon-P membrane and subjected to Western blot, using polyclonal antibodies against Cox-1 or

Fig. 2. Effect of SPBE on cell growth of LNCaP (A), normal prostate (B) and HT-29 and Jurkat cells (C). Trypsinized cells suspended in RPMI medium were plated on flat-bottomed 96-well culture dish and cytotoxicity assays were performed as described in Materials and Methods. Cytotoxicity: ●, vehicle; ■, serum+SPBE; ▲, 1 nM DHT+SPBE.

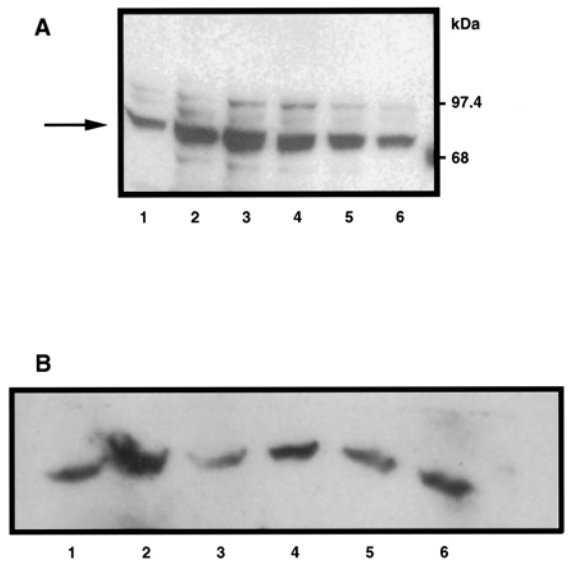


Fig. 4. Effect of SPBE on the regulation of Cox-2 (upper panel) and Cox-1 (lower panel) in LNCaP cells. Cells were grown in six-well plates as described in Materials and Methods in the absence (lane 1) or presence of 2% FCS (lane 2) or 10% FCS (lanes 3–6). Some cells were treated with increasing concentrations of SPBE (50, 100 and 200 nl per ml media, lane 4–6, respectively). Cells were harvested after 24 h and analysed by SDS–Page and Western blotting, using antibodies against Cox-1 and Cox-2.

Cox-2. Results from this experiment are shown in Figure 4. In lane 1 of Figure 4A, the basal Cox-2 expression is shown for LNCaP cells grown in culture medium not supplemented with FCS. However, when LNCaP cells were stimulated for 24 h with culture medium containing either 2% or 10% FCS, respectively, there was 3.7 and 4.2 fold increase in Cox-2 protein expression as measured by densitometry (Fig. 4A; lanes 2 and 3, respectively). In experiments where LNCaP cells were stimulated with culture medium supplemented with 10% FCS and 50, 100, or 200 nl equivalents of SPBE, we detected a significant decrease in Cox-2 expression, i.e., 51, 64 and 86%, respectively (Fig. 4A; lanes 4–6). Not unexpectedly, under the same experimental conditions, no change in Cox-1 protein expression was detected after stimulation with FCS or in co-culture with SPBE, except an inter-sample variation (Fig. 4B). It was assumed from the Coomassie stain of total proteins, that equal amounts of protein were loaded on SDS–Page gels (data not shown).

Effect of SPBE on Bcl-2 expression

To clarify why the cell number does not increase for the prostate cell lines when cultured with SPBE,

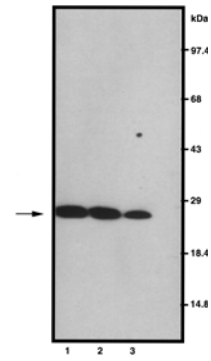


Fig. 5. Effect of SPBE on Bcl-2 expression. Jurkat cells were grown in the presence of media (lane 1), in the presence of media and 10% FCS (lane 2), and in the presence of media plus 10% FCS and 200 µl equivalents of SPBE (lane 3). Western blotting was performed against Bcl-2 using specific antibodies.

we determined the level of Bcl-2 expression in the LNCaP prostatic carcinoma cell line (Fig. 5). There was insignificant expression of Bcl-2 in LNCaP cells. Therefore, we used a Jurkat cell line to demonstrate the effect of SPBE on the expression of Bcl-2, since this cell line is known to express higher levels of the Bcl-2 protein in comparison to LNCaP. Unlike the expression of the Cox-2 protein, FCS does not stimulate Bcl-2 protein expression in Jurkat cells. However, basal expression of the Bcl-2 protein is decreased by 39% when Jurkat cells are co-cultured in the presence of 200 nl equivalents of SPBE (Fig. 5, lane 3). A decrease in expression of Bcl-2 caused by SPBE suggests that these cells are susceptible to apoptosis, resulting in an inability to increase in cell number when cultured over a 72-h time period.

DISCUSSION

We have demonstrated a concentration-dependent growth inhibition of three prostatic cancer cell lines, BRFF-41T, 267B-1, and LNCaP, when co-cultured with SPBE. The response of two of these cell lines was similar whereas the LNCaP cell line was inherently more resistant to the inhibitory activity of SPBE (Figs 1–3 and Table 1). Growth inhibition of all three cell lines' is not irreversible since pulsing the cell lines for 30 min with SPBE at its IC_{50} concentration—as determined by co-culturing the cells with the extract for a period of 72 h—is not inhibitory (Fig. 1 and data not shown). It is interesting to note that in LNCaP cells co-cultured with both SPBE and 1 nM of the usually mitogenic androgen DHT, the IC_{50} value

decreases significantly in comparison to cells that were grown in the presence of serum alone. Several possible mechanisms of action might explain this observation. It has been demonstrated in animal studies that SPBE inhibits the binding of DHT to its receptor (Carilla *et al.*, 1984; Sultan *et al.*, 1984) and blocks the conversation of testosterone to DHT by inhibiting the activity of 5-α-reductase (Bayne *et al.*, 1999; Iehle *et al.*, 1995; Delos *et al.*, 1994). The lack of interaction of DHT with its receptor or inhibition of 5-α-reductase may be responsible for the further decrease in the IC_{50} value in comparison to cells grown in the presence of serum. Another possible reason may be the presence of large numbers of other grown factors or hormones in serum that are affected less severely by SPBE compared to DHT.

On similar lines, Hsieh *et al.* (1997) have shown that a complex herbal mixture containing Saw Palmetto, PC-SPES, inhibits the proliferation of LNCaP cells and that this decrease is accompanied by a 60–70% down-regulation in proliferating cell nuclear antigens (PCNA) and levels of secreted PSA. PC-SPES is believed to decrease androgen-receptor expression, as shown by Western blot analysis, and this may explain the lower expression of PSA. Recent work by Tiwari *et al.* (1999) also supports the *in vivo* anti-tumor effects of PC-SPES.

The induction of apoptosis is associated with decreased expression of Bcl-2 proteins. A potential role for the anti-apoptotic gene Bcl-2 as a survival mechanism for androgen-resistant prostate cancers is supported by the observation that Bcl-2 is not expressed in normal secretory prostatic epithelial cells. Yet, it is expressed in a variety of prostate hyperplasia and cancer specimens. It is known that over-expression of Bcl-2 protects cancer cells from apoptosis *in vitro* and confers resistance to androgen-deprivation therapy *in vivo* (Raffo *et al.*, 1995). A role for Bcl-2 in survival of the androgen-resistant cells is experimentally supported when analysing androgen-dependent and androgen-independent LNCaP cell-lines. Androgen-independent LNCaP-AI cells express much higher levels of Bcl-2 than the androgen-dependent cells. Expression of Bcl-2 was also not modulated by the addition of the androgen agonist R1881 (Lu *et al.*, 1999), but was shown in Figure 5 to be down-regulated in Jurkat cells when co-cultured with SPBE. Reduced expression of Bcl-2 may render the androgen-resistant prostate cancer cells more susceptible to apoptosis, resulting in a reduction in tumour burden in the cancer patients. Our inability to detect Bcl-2 in LNCaP cells may be due to the variation in their growth conditions and/or differentiation.

Further, we investigated the effects of FCS and SPBE on the expression of either Cox-1 or Cox-2 in LNCaP cells. Exposing these cells to increasing concentrations of FCS induced the expression of Cox-2, but not Cox-1 (Fig. 4). However, this induction of Cox-2 was reversed in a dose-response manner when the LNCaP cells were co-cultured with SPBE (Fig. 4). The lack of induction of Cox-1 expression by FCS is not surprising since its expression is usually not amenable to induction by a variety of growth factors and cytokines. The importance of reducing the expression of Cox-2 is emphasized by the fact that NS398, a specific inhibitor of Cox-2, induces apoptosis in LNCaP cells but not in human fetal prostate fibroblasts (Guinan *et al.*, 1997). Studies have also revealed that treatment with highly selective Cox-2 inhibitors results in both decreased size and number of premalignant colonic lesions in several animal model systems and that decreased expression of Cox-2 potentially protects against colon cancer (Tsulii and DuBois, 1995). Comparing observations from this study with previously published results, we hypothesize that Cox-2 inhibition induced by SPBE may provide an important basis for potential chemopreventative action. Further experimental work will address this important issue.

[*Note:* The 'mechanism of action' of SPBE is reported to be its ability to inhibit the conversion of testosterone to DHT (Bayne *et al.*, 1999; Iehle *et al.*, 1995; Delos *et al.*, 1994). SPBE also inhibits the binding of DHT to its receptors (Sultan *et al.*, 1984; Carilla *et al.*, 1984). Further, it has been suggested that too much DHT could cause prostate cells to divide at an abnormal rate leading to enlargement of the prostate gland. Other effects of SPBE are anti-inflammatory (Di Silverio *et al.*, 1992) and anti-estrogenic (Di Silverio *et al.*, 1992; Briley *et al.*, 1983). In conclusion, SPBE is a well-tolerated drug that has an efficacy similar to Finasteride in improving symptoms in men with BPH (Plosker and Brogden, 1996; Gerber *et al.*, 1998). An additional effect of SPBE is the inhibition of growth factor-induced proliferation of human prostate cell-cultures (Paubert-Braquet, 1998). This drug also induces a significant reduction in DHT and epidermal growth factor concentrations in human BPH following three months of treatment (Di Silverio, 1998). Amongst other effects suggested for how SPBE functions, is the inhibition of signal transduction through the prolectin receptor (Vacher *et al.*, 1995)].

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