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Abstract

Background

Prostate cancer is an important public health problem. It is an excellent candidate disease for chemo-prevention because prostate cancer is typically slow growing and is usually diagnosed in elderly males. *Pygeum africanum (Prunus africana or Rosaceae)* is an African prune (plum) tree found in tropical Africa. An extract from the bark of *Pygeum africanum* has been used in Europe as a prevention and treatment of prostate disorders including benign prostatic hypertrophy (BPH). More recently in the USA, the phytotherapeutic preparations of *Pygeum africanum* and *Saw palmetto* have been marketed for prostate health including prostate cancer prevention and treatment.

Methods

The anti-cancer potential of *Pygeum africanum* has been tested both *in vitro* (PC-3 and LNCaP cells) and *in_vivo* (TRAMP mouse model).

Results

In tissue culture, ethanolic extracts (30%) of *Pygeum africanum* inhibited the growth of PC-3 and LNCaP cells; induced apoptosis and altered cell kinetics; down regulated ERα and PKC-α protein, and demonstrated good binding ability to both mouse uterine estrogen receptors and LNCaP human androgen receptors. TRAMP mice fed *Pygeum africanum* showed a significant reduction ($P = 0.034$) in prostate cancer incidence (35%) compared to casein fed mice (62.5%).

Conclusion

Pygeum africanum, which is widely used in Europe and USA for treatment of BPH, has a significant role in regulation of prostate cancer both *in_vitro* and *in_vivo* and therefore may be a useful supplement for people at high risk for developing prostate cancer.

Keywords

African herb, LNCaP, PC-3, TRAMP mice

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ORIGINAL PAPER

Phytosterol *Pygeum africanum* regulates prostate cancer *in vitro* and *in vivo*

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Keywords African herb · LNCaP · PC-3 · TRAMP mice

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Introduction

Prostate cancer is an important public health problem accounting for more than 232,000 new cases and 33,000 deaths in the USA in 2006 [1]. Prostate cancer is an excellent candidate disease for chemo-prevention because it is typically slow growing and is usually diagnosed in elderly males, therefore even a modest delay in the neoplastic development achieved through pharmacological or nutritional intervention could result in a substantial

reduction in the incidence of mortality [2]. The incidence of prostate cancer is lower in Asian populations and in vegetarians compared to populations consuming a more Western diet [3].

Components of the Western diet, most often cited as playing a role in increased prostate cancer risk are animal products [4]. The most consistent correlation for prostate cancer prevention is consumption of fruits, vegetables, and grains, which are potential sources of phytoestrogens [5]. Phytoestrogens are found in many plants which are commonly used in traditional medicine. Phytoestrogens may be either agonists or antagonists of estrogen receptors. Therefore, it has been proposed that phytoestrogens may influence prostate cancer cell growth and therefore herbal therapies have been developed which include these compounds [6]. *In vivo* and *in vitro* studies have shown that phytoestrogens may influence not only steroid hormone metabolism and biological activity, but also intracellular enzymes, growth factor action, protein synthesis, malignant cell proliferation, and angiogenesis, thus making them strong candidates for anticarcinogenic agents [7].

Pygeum africanum (Prunus africana or Rosaceae) is an African prune (plum) tree found in tropical Africa [8]. The bark has been used in Europe since 1969 as a prevention and treatment of prostate disorders including benign prostatic hypertrophy (BPH). Also in Asia, and Africa, phytotherapy is considered a first line treatment for prostate disorders and has been utilized effectively for centuries. In the USA, phytotherapeutic preparations containing *Pygeum africanum* and *Saw palmetto* are increasingly being marketed for the health of the prostate including prostate cancer prevention and treatment [9, 10]. Phytochemical investigations aimed at isolating the active ingredients of *Pygeum africanum* have identified many compounds including fatty acids, sterols (B-Sitosterol and Campesterol) and pentacyclic triterpenoids [11]. Multicenter clinical trials have been done to study the role of *Pygeum africanum* in prevention and treatment of BPH [12–22]; however one study on the mechanism of action of *Pygeum africanum* in prostate cancer has been described [23]. The major effects of *Pygeum africanum* have been reported to be caused by its main active ingredient, B-Sitosterol which is present in high concentrations in the *Pygeum africanum* extract [24, 25].

B-Sitosterol, a sterol related to cholesterol, is present in three commonly used prostate herbal supplements, *Pygeum africanum*, *Saw palmetto*, and *Hypoxis*. These three herbs are commonly used in commercial supplements for prostate health [26]. Epidemiological and experimental studies [2] suggest that dietary phytosterols may offer protection from the most common cancers in Western societies, such as colon, breast and prostate cancer. It has been proposed that phytosterols such as B-Sitosterol affect membrane structure and function of tumor and host tissues, signal transduction pathways that regulate tumor growth and apoptosis [27]. The role of B-Sitosterol in prevention and treatment of BPH has been studied both in tissue culture and in clinical trials [28–30] however there are few studies on the role of B-Sitosterol in prostate cancer. Von Holtz et al. [31] reported that B-Sitosterol inhibited the growth of LNCaP cells and induced apoptosis via activation of the sphingomyelin cycle. Also Awad et al. [32, 33] found B-Sitosterol inhibited the growth and dissemination of PC-3 cells both *in vitro* and *in vivo.*

The aim of this work was to explore the possible mechanisms by which *Pygeum africanum* regulates prostate cancer both *in vitro* (LNCaP and PC-3 human prostate cancer cell lines) and *in vivo* (in TRAMP Prostate Cancer Mouse model). These cell lines were selected to provide an androgen sensitive (LNCaP) and an androgen insensitive (PC-3) comparison. To our knowledge, this is the first report that extensively describes the anti-prostate cancer activity of *Pygeum africanum* both *in vitro* and *in vivo.*

Results

In vitro experiments

Pygeum africanum extract inhibited the growth of both PC-3 and LNCaP cells (Fig. 1). The IC_{50} (the concentration of the compound that is required to inhibit cell growth by 50%) was approximately 2.5 µl/ml (for both cell lines). B-Sitosterol inhibited the growth of LNCaP cells but had no effect on the growth of PC-3 cells (Fig. 2).

Cell proliferation was measured using a thymidine incorporation assay in the two prostate cancer cell lines over 3 days incubated with and without the 2.5 µl/ml of *Pygeum africanum*. In PC-3 and LNCaP cells, *Pygeum africanum* inhibited growth by 50% from day 1 through day 3 (Fig. 3).

In both prostate cancer cell lines, a significant increase in the number of cells in the S phase of the cell cycle was induced by *Pygeum africanum* in PC-3 (Fig. 4B) and LNCaP (Fig. 4D) cells when compared to untreated cells (Fig. 4 A, C).

Pygeum africanum at 2.5 µl/ml induced significant apoptosis in both cell lines (PC-3 and LNCaP) when compared with untreated cells (Table 1).

Pygeum africanum and B-Sitosterol were tested for their potential to displace bound estradiol in mouse uterine cytosol using the competitive ^{3}H-estradiol ligand-binding assay. At 5 µl/ml *Pygeum africanum* displaced >70% of ^{3}H-estradiol; however, B-Sitosterol (5 µM) did not compete for ^{3}H-estradiol binding (Fig. 5).

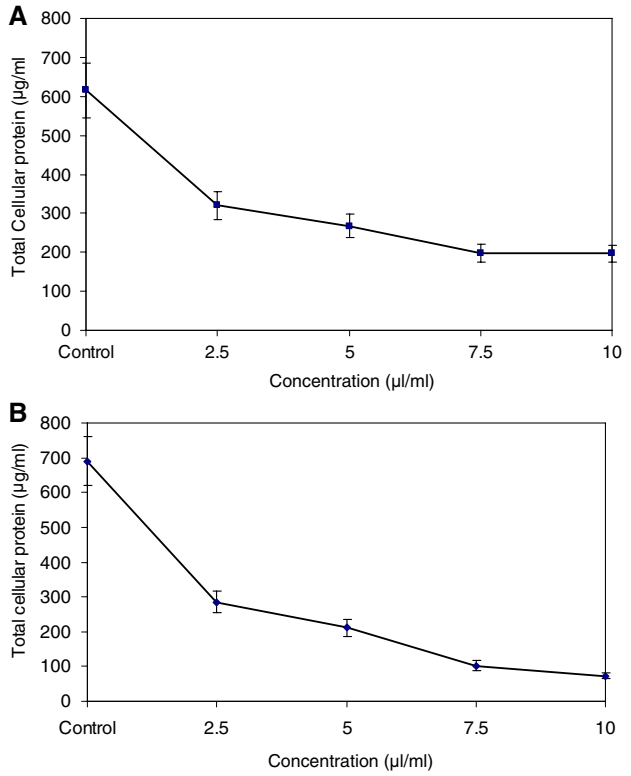

Fig. 1 PC-3 (**A**) and LNCaP (**B**) cells were plated at 1×10^4 cells per well in 24 well dishes and grown to 50% confluence. After 2 days the media was replaced with complete media containing 0–10 µl/ml of *Pygeum africanum* (30% ethanol extract) and incubated for 72 h. The total cellular protein concentration was determined by DC Bio-Rad assay. The IC_{50} for *Pygeum africanum* was calculated based on the growth inhibition after 72 h in comparison to the zero treatment control. Values are means ± SEM of three individual experiments (Each individual experiment was performed in duplicate)

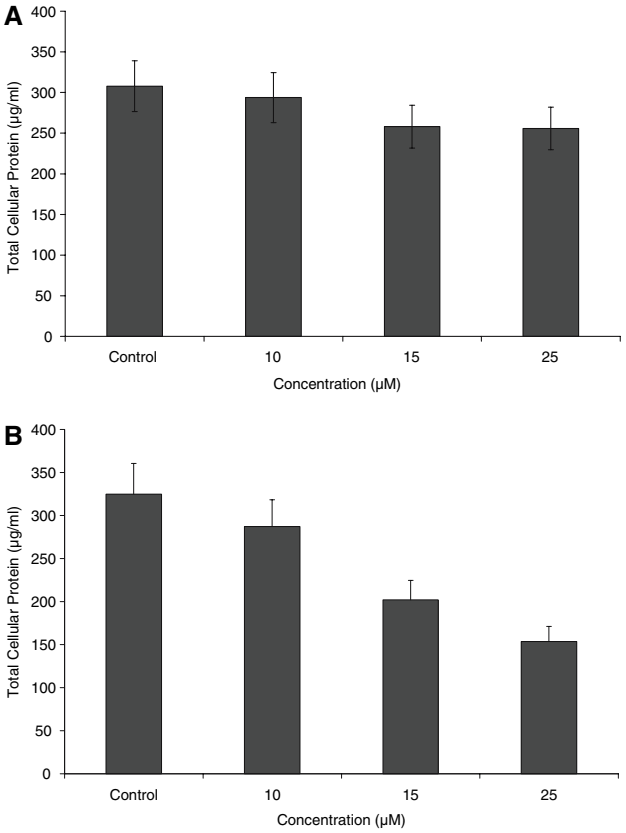

Fig. 2 PC-3 (**A**) and LNCaP (**B**) cells were plated at 1×10^4 cells per well in 24 well dishes and grown to 50% confluence. After 2 days the media was replaced with complete media containing 0–25 µM B-sitosterol loaded on cyclodextrin (CD) and incubated for 72 h. The total cellular protein concentration was determined by DC Bio-Rad assay. The IC_{50} for B-sitosterol was calculated based on the growth inhibition after 72 h in comparison to the zero treatment control. Values are means ± SEM of three individual experiments (Each individual experiment was performed in duplicate)

Pygeum africanum and B-Sitosterol were tested for their potential to displace bound DHT in LNCaP cell cytosol using the competitive ^{3}H-DHT ligand-binding assay. At 5 µl/ml *Pygeum africanum* displaced >60% of ^{3}H-DHT; however, B-Sitosterol (5 µM) was not able to compete for ^{3}H-DHT binding (Fig. 6).

Pygeum africanum showed a dose dependent inhibition of ERα protein expression in PC-3 cells (Fig. 7). Also *Pygeum africanum* at 2.5 µl/ml inhibited PKC-α expression in LNCaP but not in PC-3 cells (Fig. 8).

In vivo experiments

Among TRAMP mice fed *Pygeum africanum* extract for 5 months, fewer animals developed prostate cancer (35%) than mice fed the casein diet (62.5%). The largest effect was on WDC incidence ($P = 0.034$; Table 2). There was a non-significant effect on PDC ''neuro-endocrine like carcinoma incidence'' ($P = 0.78$).

There was no significant effect of diet on total body weight, reproductive tract, testes or prostate weights (Table 3). The large variation in the prostate weight in the *in vivo* treatment group was because one of the tumors in PDC stage of the *Pygeum africanum* fed group was huge. The difference in PDC incidence was still not statistically significant ($P > 0.05$).

Discussion

In the present study, an extract of *Pygeum africanum* induced 50% growth inhibition of both PC-3 and LNCaP cells. This was in agreement with Margalef et al. [8] who reported growth inhibition of prostate cancer-derived cells (LNCaP and CA-HPV-10). The inhibition of the growth of PC-3 and LNCaP cells was present from day 1 through day 3 of incubation with the *Pygeum africanum* extract. The effect of *Pygeum* on the prostate cancer cell lines was

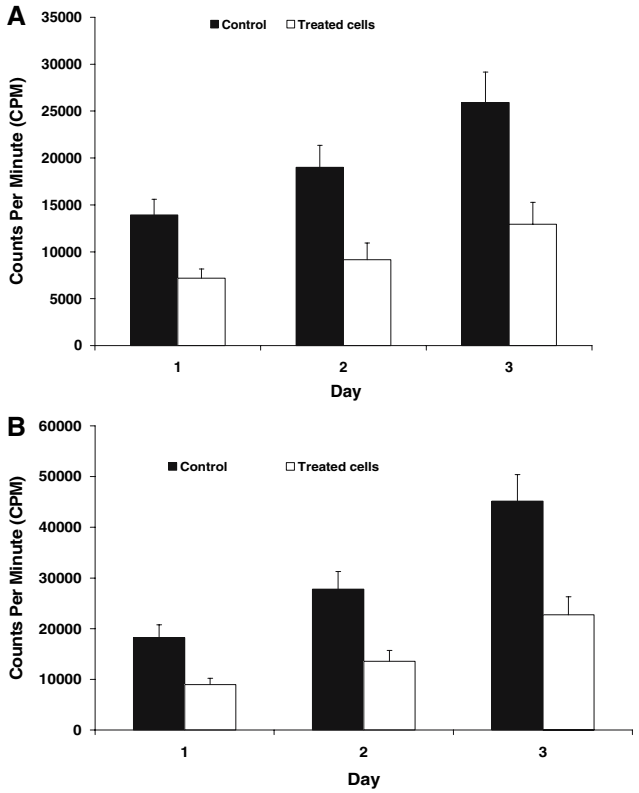

Fig. 3 PC-3 (**A**) and LNCaP (**B**) cells were plated at 1×10^4 cells per well in 24 well dishes and grown to 50% confluence. After 2 days the media was replaced with complete media containing 2.5 µl/ml of *Pygeum africanum* (30% ethanol extract) and incubated for 72 h. The total DNA concentration was determined at day 1, 2 and 3 by thymidine incorporation assay. Values are means ± SEM of three individual experiments (Each individual experiment was performed in duplicate)

not a toxic effect as we saw no effect of *Pygeum africanum* on the $CaCO_2$ (Colon Cancer) cell line that has similar metabolic activity and cell cycle time, at concentrations up to 20 µl/ml of the ethanolic extract of *Pygeum africanum* (Data not shown).

Phytochemical investigations aimed at isolating the active ingredients of *Pygeum africanum* have been extensively undertaken. Many compounds have been identified including fatty acids, sterols (B-Sitosterol and Campesterol) and pentcyclic triterpenoids [11]. B-Sitosterol, as one of the marker compounds for *Pygeum africanum*, has been tested for its ability to inhibit the growth of both PC-3 and LNCaP cells [32, 33]. Few studies on the role of B-Sitosterol in prostate cancer have been described. We found that B-Sitosterol inhibited the growth of LNCaP cells. This agrees with Von Holtz et al. [31] who reported that B-Sitosterol inhibited the growth of LNCaP cells and induced apoptosis via activation of the sphingomyelin cycle or by regulation of the activity of PKC-alpha [34]. Also Awad et al. [27] reported that B-Sitosterol might affect membrane structure and function of tumor and host tissues, signal transduction pathways that regulate tumor growth, and apoptosis However, we found no effect of B-Sitosterol on PC-3 cell growth but we did see an effect similar to Awad et al. [32] who showed that B-sitosterol inhibited the growth of LNCaP cells *in vitro*. However, in contrast to our results Awad et al. found an effect of B-Sitosterol on PC-3 cell growth in 5% non-stripped serum [33].

The present study showed that *Pygeum africanum* at 2.5 µl/ml induced an accumulation of the cells in the S phase of the cell cycle for both PC-3 and LNCaP cells, as well as significant apoptosis compared to untreated cells. These results differed from Margalef et al. [18] who reported a block in the transition from G1 to S phase in the cell cycle of PZ-HPV7 cells with an ethanolic extract of *Pygeum africanum.*

Interest in the physiological roles of bioactive compounds present in plants has increased dramatically over the last decade. Of particular interest because of their reported roles in human health is the class of compounds known as phytoestrogens. Phytoestrogens embody several groups of non-steroidal estrogens that are widely distributed within the plant kingdom [35]. The phytoestrogen genistein for example binds to ER with a preference for the more recently described ER β [36]. In the present study, *Pygeum africanum* extract displaced ^{3}H-estradiol in a competitive ligand-binding assay using mouse uterine cytosol. This was in agreement with Mathe [21, 22] who reported that *Pygeum africanum* had a phytoestrogenic effect. Also, *Pygeum africanum* at the same concentration displaced ^{3}H-DHT in a competitive ligand-binding assay using LNCaP cell cytosol. The androgen receptor binding affinity of *Pygeum africanum* might help to explain the inhibitory effect on 5 alpha reductase reported by Rhodes et al. [23]. Recently some phytoestrogens (genistein and daidzein) have been found to induce apoptosis and inhibition of the PC-3 and LNCaP cell growth through their binding and regulation of both ER and AR. Cao et al [37] found that the expression of the ER-alpha and ER-beta genes decreased, while AR gene was not expressed, after incubation with genistein and daidzein in PC-3 cells. Also, they reported that the apoptosis percentage of LNCaP cells was elevated significantly by daidzein.

The effects of estrogens on prostate cancer have been reviewed by Ho [38] and by Steiner and Raghow [39]. Estrogen therapy, principally the use of diethylstilbestrol (DES), is a suggested practice in prostate tumor treatment. Its primary mode of action is through feedback on the anterior pituitary with suppression of gonadotropin secretion and subsequent decrease in testosterone production by Leydig cells of the testis. This in turn causes a decrease in androgens required for hormone dependent cancer. However, direct effects of DES in the prostate are also possible. Whether DES works directly in the prostate through a

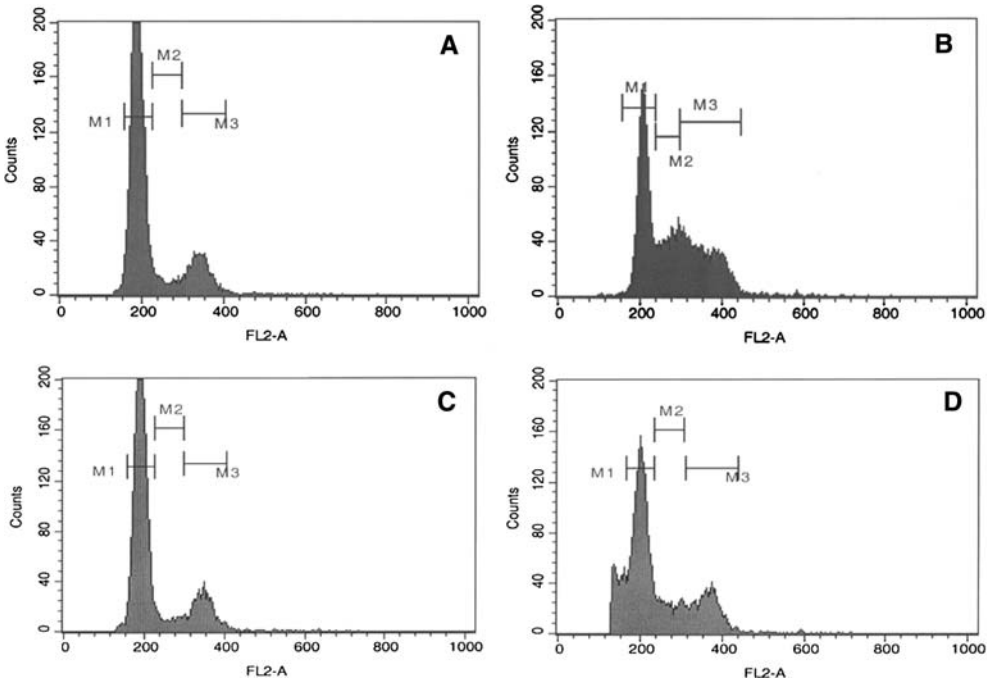

Fig. 4 Cell cycle changes in untreated PC-3 and LNCaP cells (**A** & **C**) and PC-3 and LNCaP cells treated with 2.5 µl/ml of *Pygeum africanum* (**B** & **D**) were detected by flow cytometry. The untreated PC-3 and LNCaP cells showed normal cell cycle events (**A** & **C**) while the cells treated with 2.5 µl/ml of *Pygeum africanum* showed accumulation of cells in S phase of the cell cycle (**B** & **D**). M1 = G1 phase; M2 = S phase; M3 = G2/M phase

Table 1 Induction of apoptosis by the *Pygeum africanum* extract in PC-3 and LNCaP cell lines as determined by the TUNEL assay

	LNCaP (% apoptotic events)	PC-3 (% apoptotic events)
Negative control	0.94 ± 0.08[a*]	1.01 ± 0.10[a]
Positive control	22.90 ± 1.41[b]	20.75 ± 1.02[b]
Pygeum africanum	15.20 ± 1.19[c]	17.85 ± 0.86[b]

Mean ± SEM. Values are percent of total events counted. Values within each column with different letter superscripts are significantly different, $P < 0.05$ or less. $N = 3$ individual experiments and each experiment was performed in duplicate. Negative control is the untreated cells. Positive control is camphotechin treated cells

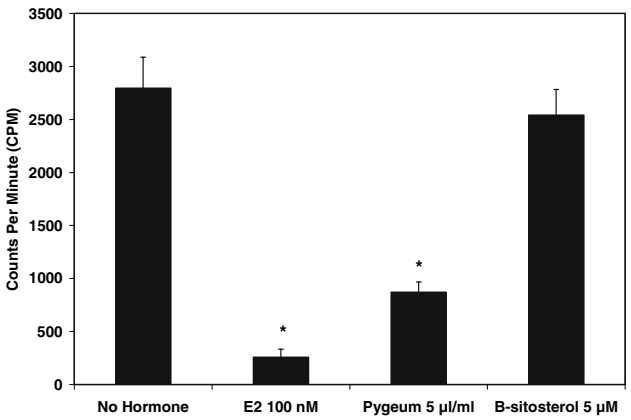

Fig. 5 *ER Saturation Binding Analysis*—WT uterine cytosol was incubated overnight at 4°C with ^{3}H-estradiol (5 nM final concentration) and the aliquot of *Pygeum africanum* (5µl/ml of 30% ethanol extract) and B-sitosterol (5 µM). Bound and free ligands were separated by dextran-coated charcoal and an aliquot of bound radioactivity was measured by scintillation counting. Values are means ± SEM of three individual experiments (Each individual experiment was performed in duplicate). Bars designated with asterisks are significantly different from no hormone, $P < 0\ 05$ or less

classical ERα pathway, through ERβ or another estrogen-dependent mechanism has not been fully determined. The present study showed a dose dependent inhibition of ERα protein expression in PC-3 cells which express a significant amount of ERα protein [2]. This was in agreement with Mathe [21, 22] who reported that *Pygeum africanum* had a phytoestrogenic effect on the prostate resulting in a significant effect on the morphology of the glandular epithelium of the mouse prostate presumably due to the effect of weak anti-estrogens.

A recent review identified protein kinase C-α (PKC- α), a serine–threonine kinase involved in cellular signaling pathways, as a target to modulate prostate cancer. PKC-α has been found in both hormone insensitive (PC-3) and hormone sensitive (LNCaP) prostate cancer cells [40]. The present study found that *Pygeum africanum* at 2.5 µl/ml inhibited the expression of PKC-α in LNCaP but not in PC-3 cells.

The present study showed that TRAMP mice fed *Pygeum africanum* in a dose of 0.128 gm/kg of the diet showed a significant and marked reduction of WDC tumor incidence (35%) compared to mice fed casein diet (62.5%). This could be explained by *Pygeum africanum* potentially regulating prostate cancer *in vivo* through several mechanisms including ERα, AR, PKC-α and apoptosis-related mechanisms. The mice fed *Pygeum africanum* did not show any significant difference from the mice fed casein in

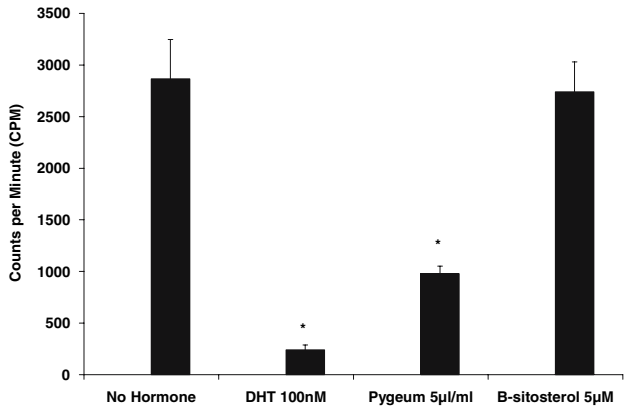

Fig. 6 *AR Saturation Binding Analysis*—LNCaP cell cytosol was incubated overnight at 4°C with ^{3}H-DHT (5 nM final concentration) and the aliquot of *Pygeum africanum* (5µl/ml of 30% ethanol extract) and B-sitosterol (5 µM). Bound and free ligands were separated by dextran-coated charcoal and an aliquot of bound radioactivity was measured by scintillation counting. Values are means ± SEM of three individual experiments (Each individual experiment was performed in duplicate). Bars designated with asterisks are significantly different from no hormone, $P < 0\ 05$ or less

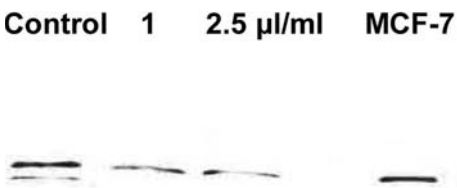

Fig. 7 Western blot analysis of ERα protein expression in PC-3 cells treated with different concentrations of *Pygeum africanum* using human reactive polyclonal antibody. Extracts of PC-3 cell line were resolved by gel electrophoresis, electro-blotted onto nitrocellulose membrane, and immuno-detected as described in Materials and Methods. Lane 1, 2 and 3 extracts of PC-3 cells treated with *Pygeum africanum* (zero treatment control, 1 and 2.5 µl/ml of 30% ethanol extract); lane 4, protein standard; lane 5, MCF-7 cells as a positive control

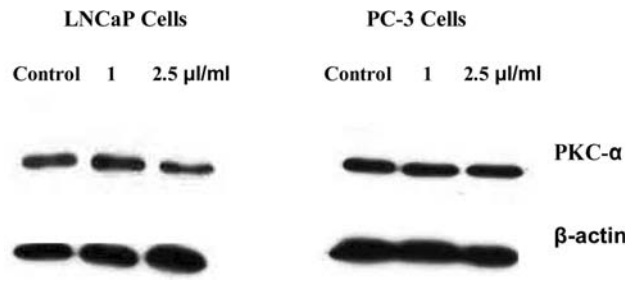

Fig. 8 Western blot analysis of PKC-α protein expression in PC-3 and LNCaP cells treated with different concentrations of *Pygeum africanum* using human reactive polyclonal antibody. Extracts of PC-3 and LNCaP cell lines were resolved by gel electrophoresis, electro-blotted onto nitrocellulose membrane, and immuno-detected as described in Materials and Methods. (**A**) Lane 1, 2 and 3 extracts of PC-3 cells treated with *Pygeum africanum* (zero treatment control, 1 and 2.5 µl/ml of 30% ethanol extract). (**B**) Lane 1, 2, and 3 extracts of LNCaP cells treated with *Pygeum africanum* (zero treatment control, 1 and 2.5 µl/ml of 30% ethanol extract)

terms of their total body weight, reproductive tract, testes and prostate weights. This finding confirms the absence of any obvious toxic effects among the mice fed *Pygeum africanum* at this dose.

In conclusion, this study showed that *Pygeum africanum* which is widely used in Europe and the USA for treatment of BPH might be able to play a significant role in regulation of prostate cancer both *in vitro* and *in vivo.* Thus, *Pygeum africanum* may be of dual benefit to men who are at high risk of developing either BPH or prostate cancer.

Materials and methods

Pygeum africanum bark was obtained from Nutraceutical Corp./Solaray (Park City, UT) and was by weight 13% total sterols. Ethanolic extracts (30% W/V) were made for tissue culture experiments and stocks were kept at –20°C. Human reactive polyclonal antibody for ERα (PA1–308) and PKC-α (PA1–405) were purchased from Affinity Bio Reagents (Golden, CO). B-Sitosterol (98% pure) was obtained from Sigma Chemical Co. (St. Louis, MO) and used in tissue culture after being loaded on Cyclodextrin (CD) vehicle to be accessible by the cultured cells [41]. Unlabeled 17β estradiol and DHT were obtained from Sigma Chemical Co. (St. Louis, MO). ^{3}H-17β estradiol and ^{3}H-DHT were obtained from Amersham Pharmacia Biotech (Piscataway, NJ).

In vitro experiments

Cell culture experiments

Human prostate carcinoma (PC3 and LNCaP) cells were obtained from the ATCC and cultured in complete RPMI 1640 medium (Gibco Rockville, MD) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) streptomycin (100 µg/ml)), L-glutamine (5 mM)), NEAA (0.1 mM Non Essential Amino Acids), HEPES buffer (10 mM), glucose (2.5 gm/l) in 5% CO_2 at 37°C.

Growth inhibition curve and IC_{50}: PC3 and LNCaP cells were plated at 1×10^4 cells per well in 24 well dishes with RPMI 1640 complete culture medium and cells were grown to 50% confluence. After 2 days the media were replaced with complete media with charcoal stripped serum [2] containing 0–10 µl/ml of the *Pygeum africanum* extract or 0–25 µM of B-Sitosterol and incubated for 72 h. The media were aspirated, cells washed twice with phosphate buffered saline (PBS) (10 mM, pH 7.4), and 1N NaOH (250 µl) added to lyse the cells. The total cellular protein concentration was determined by DC Bio-Rad assay using the manufacturer's protocol (Bio-Rad laboratories, Hercules, CA). The IC_{50} is the concentration where 50% of the maximum

Table 2 Effect of *Pygeum africanum* on the incidence of prostate tumorigenesis in ERαWT/TRAMP mice

Diet	*n*	Phenotype					
		Non-cancer			Cancer		
		Normal	HYP	PIN	WDC	MDC	PDC
Casein	16	0	0	6(37.5%)	8(50%)	0	2(12.5%)
Pygeum africanum[a]	20	0	1(5%)	12(60%)	4(20%)	0	3(15%)

There was a statistically significant difference ($P = 0.034$) between the *Pygeum africanum* and casein diet regarding prostate tumorigenesis

Table 3 Body and organ weights of mice from different dietary groups

Weights[a] (gm)				
Diet	Testes	Body	Repro. tract	Prostate
Casein	0.19 ± 0.006	30.37 ± 0.45	1.55 ± 0.18	0.30 ± 0.18
Pygeum africanum	0.18 ± 0.008	29.46 ± 0.37	1.80 ± 0.47	0.40 ± 0.49

[a] Means ± SEM. No significant differences were seen between treatment groups

inhibition is observed. To calculate IC_{50}, we first take the difference between the protein value of the maximum inhibition (where the growth curve levels out) and the protein value of no inhibition at zero concentration. This difference is multiplied by 0.5 and then the resulting protein value is subtracted from the no inhibition protein value to obtain the protein value that corresponds to the IC_{50} concentration of the extract being used. 100% ethanol was used in the treatment control group up to 1% final concentration without any observed cell toxicity.

Time course study using thymidine incorporation assay: PC3 and LNCaP cells were plated at 1×10^4 cells per well in 24 well dishes with RPMI 1640 complete culture medium and cells grown to 50% confluence. After 2 days the media was replaced with complete media containing 2.5 µl/ml of *Pygeum africanum* extract and incubated for 24, 48 or 72 h. The cellular proliferation was determined by thymidine incorporation assay [42].

Cell cycle kinetics: PC3 and LNCaP cells were plated at 1×10^4 cells per well in 24 well dishes with RPMI 1640 complete culture medium and cells grown to 50% confluence. After 2 days the media was replaced with complete media containing 2.5 µl/ml of *Pygeum africanum* extract and incubated for 72 h. The adherent cells were released with trypsin, washed twice with phosphate buffered saline (PBS) and collected by centrifugation. The cell pellet was resuspended in 2.5 ml of cold ethanol (95%) for 1 h at 4°C, washed with PBS, and incubated with 5 µl RNase (20 µg/ml final concentration) at 37°C for 30 min. The cells were chilled on ice for 10 min then stained with propidium iodide (50 µg/ml final concentration) for 1 h and analyzed by flow cytometry (CyAn Flow, Cytomation, Inc, Somerville, NJ).

Detection of apoptosis using TUNEL assay: PC3 and LNCaP cells were plated at 1×10^6 cells in 100-mm culture dishes in RPMI 1640 complete media and grown to 50% confluence. After 2 days the media was replaced with complete media containing 2.5 µl/ml of *Pygeum africanum* extract and incubated for 72 h. The adherent cells were released with trypsin, washed with PBS and processed for labeling with fluorescein-tagged deoxyuridine triphosphate nucleotide and propidium iodide by the use of an APO-Direct Apoptosis Kit obtained from Phoenix Flow Systems (San Diego, CA). The labeled cells were then analyzed by flow cytometry.

Binding assays

^{3}H-estradiol binding assay: Tissue cytosol preparation (WT mouse uterine cytosol) and estradiol competition binding analysis of *Pygeum africanum* extract were performed as previously described [2].

^{3}H-DHT binding assay: The capacity of *pygeum africanum* extract to bind androgen receptor was assessed by DHT competition binding assay using LNCaP cell cytosol as previously described [43].

Western blotting for ER detection

Expression levels of both ERα and PKC-α in PC-3 and LNCaP cells treated with IC50 of *Pygeum africanum* were quantified by western immunoblot as previously described [2]. We have controlled for the decrease in cell number through cell cycle inhibition and apoptosis by loading the same amount of protein in each lane (20 µg).

In vivo experiments

Prostate cancer model

Animal protocols followed were approved by the University of Missouri Animal Care and Use Committee and followed NRC guidelines. All breeder pairs were maintained on a casein-based diet (AIN 93G) as previously described [44]. This model, known as TRAMP [45] for TRansgenic Adenocarcinoma of the Mouse Prostate, was developed by placing the SV40 early genes encoding T and t antigens under the control of the rat probasin promoter, which has been shown to be highly and specifically expressed in the mouse prostate [46]. The probasin (PB) promoter is androgen and zinc regulated, with two androgen response elements located in this region. PB is localized in the ducts and nuclei of prostate epithelial cells, yet PB's function has not been identified. The transgene, known as PBTag, is highly expressed in the dorsal and ventral prostate lobes. This oncoprotein is known to abrogate tumor suppressor functions of p53 and Retinoblastoma (Rb) and thereby drive the cell through the normal cell cycle checkpoints. Mutations in p53 are important in the prostate and occur in one third of early stage prostate cancers [47, 48]. In our hands ~60–85% of TRAMP mice develop prostate cancer spontaneously within 5 months.

The C57BL6/J TRAMP mice were housed in pairs in breeder boxes with micro-isolator lids and given free access to food and water. A daily light: dark cycle of 12:12 h was used, with ambient temperature and humidity set at 21°C, and 50%, respectively. Animals were monitored weekly for body weight and tumor burden and were euthanized after 5 months.

Dietary Pygeum africanum treatments

All breeder pairs were maintained on the casein-based diet through pregnancy and lactation. At 6 weeks of age, male TRAMP mice were randomly assigned to either the casein-based diet (AIN 93G) or to the same diet to which *Pygeum africanum* was added. The concentration of *Pygeum africanum* was chosen in reference to the human consumption dose in BPH [9, 10]. The mouse diet formulation contained 0.128 grams *Pygeum africanum*/kg diet, which was higher than the recommended human dose in BPH but well within the range attainable through supplements. The bark of *Pygeum Africanum* was grounded and added as a dry powder, the diet mixed in a Hobart mixer and pelleted by dry, low heat extrusion into pellets. Animals were given continual access to the diet and body weights were recorded weekly.

At termination the reproductive tract (testes, vas deferens, empty urinary bladder, seminal vesicles and prostate lobes), testes and prostates were removed and weighed. At the time of collection (5 months), a portion of each prostate was fixed in neutral buffered formalin for histological analysis and the remainder snap-frozen in liquid nitrogen and stored at –80°C.

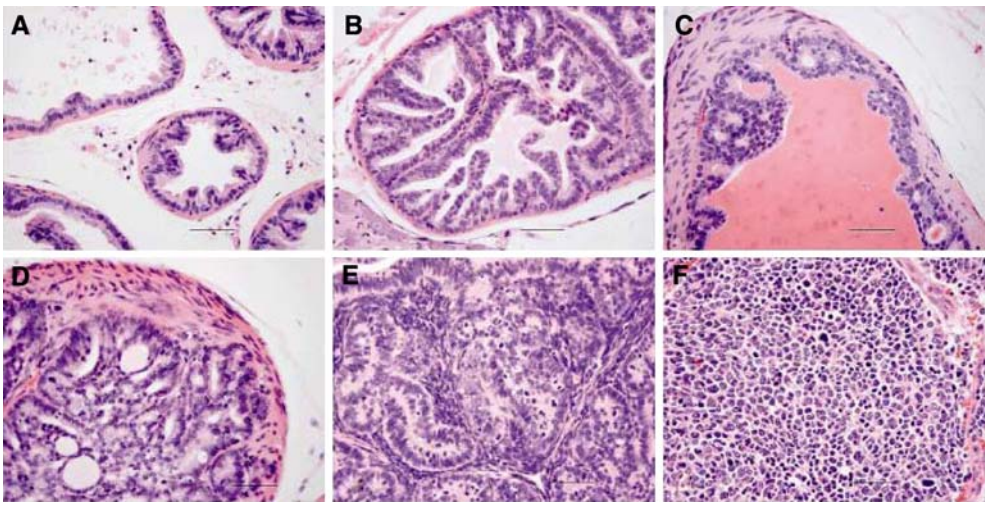

Fig. 9 Stages of cancer in the TRAMP mouse prostate (H&E stained sections, bar = 50 mm). (**A**) Normal prostate tubule (dorsal prostate) with single epithelial layer and thin muscle wall. (**B**) Hyperplastic prostate tubule with more numerous epithelial cells but retention of simple layer and thin muscle wall. (**C**) Prostate tubule with prostate intraepithelial neoplasia (PIN) and cribriform hyperplastic lesions and thickened muscle wall. (**D**) Well-differentiated adenocarcinoma with increased cribriform lesions, invasion of tubule wall and increased mitotic rate in epithelium. (**E**) Moderately well-differentiated adenocarcinoma with stratification or layering of epithelium and loss of formation of tubules. (**F**) Poorly differentiated carcinoma with ''neuroendocrine-like'' pattern

Tumor analysis

All lobes of the prostate glands were examined for nodular expansion, excised, and weighed. If their appearance was normal, then one dorsal lobe and all other lobes of the prostate were snap-frozen in liquid nitrogen and subsequently stored at –80°C. If nodules or abnormal findings were observed in regions of the prostate other than the dorsal lobe, the lesions were bisected, with half of the sample fixed for histological evaluation and the other half snap-frozen. The other dorsal prostate lobe and any abnormal prostate lesions were immersed in 10% neutral buffered formalin for 12–18 h, transferred to 70% ethanol and then processed for paraffin embedment. Paraffin-embedded tissues were cut in 4 micron sections and stained with hematoxylin and eosin.

One or more sections of the dorsal prostate lobe were assigned random numbers so as to blind the pathologist. Prostate sections were examined and graded according to morphologic criteria established for mouse models of prostate cancer [49, 50]. The proliferative changes of the secretory epithelia of the prostate tubules were recorded, scored by independent veterinary pathologist who were unaware of the treatment groups and assigned to one of the following designations: (A) normal, (B) hyperplastic, (C) prostatic intraepithelial neoplasia (PIN), (D) well-differentiated carcinoma (WDC), (E) moderately well-differentiated carcinoma (MDC), (F) poorly differentiated carcinoma (PDC), invasive carcinoma with features suggestive of neuroendocrine differentiation (Fig. 9) as described previously [49, 50]. Proliferative and neoplastic changes occur simultaneously throughout regions within the prostate lobe(s) [49]. For example, a lobe of the dorsal prostate may have several tubular sections with hyperplastic changes, and half the tubules with well-differentiated adenocarcinoma. So the percentage of prostate epithelium with hyperplastic or neoplastic changes is recorded, and a morphologic diagnosis is assigned to the alteration observed in the greatest portion of the prostate sample.

Statistical analysis

In vitro experiments

Data in Tables 1–3 were analyzed by one-way ANOVA using the GLM procedure of SAS (Version 8, SAS Institute Cary, NC). Post-hoc mean comparisons were made using the Duncan's Multiple Range component. Means were considered significantly different at $P < 0.05$ or less. Data in Figs. 5 and 6 were analyzed by student *t* test. Means were considered significantly different at $P < 0.05$ or less.

In vivo experiments

Prostate histology was classified as non-cancer stages (Normal, Hyperplasia and PIN) or cancer stages (WDC, MDC, and PDC or neuro-endocrine like carcinoma). Tumor incidence data was analyzed using X^2 test. Values that achieved $P < 0.05$ were considered to be significantly different. Comparison within the same tumor stage between the two dietary groups has also been done. Body weight, reproductive tract weight, testicular weight, and prostate weight were analyzed using a two-sample *t*-test, assuming unequal variance.

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