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Antiproliferative Activities of *Fagara xanthoxyloides* and *Pseudocedrela kotschyi* Against Prostate Cancer Cell Lines

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Abstract

Background/Aim—Roots of Fagara zanthoxyloides and Pseudocedrela kotchyii are used as chewing sticks and as medicinal remedies for diarrhea, cough and fever in West Africa. Extracts of the two plants also possess anti-bacterial, anti-fungal and anti-malarial activities. The aim of the present study was to determine the effects of such extracts on the growth, proliferation and induction of apoptosis in four prostate cancer cell lines.

Materials and Methods—Androgen-independent PC3 and DU-145 and androgen-dependent LNCaP and CWR-22 prostate cancer cell lines were cultured for five days with different concentrations of the extracts and examined for growth inhibition and evidence of apoptosis.

Results—Irrespective of their androgen dependence, all four cancer cell lines exhibited a dose-dependent decrease in cell proliferation and viability by the 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT) assay and in induction of apoptosis. The results also show that LNCap cells were the most sensitive to the two extracts, with highest inhibition at day 3 and exhibiting the highest rate of apoptosis.

Conclusion—These observations suggest that F. zanthoxyloides and P. kotchyii could serve as potential chemopreventive agents in the treatment of prostate cancer.

Keywords

Antiproliferative action; prostate cancer; *Fagara xanthoxyloides*; *Pseudocedrela kotschyi.*

Prostate cancer ranks as the second most commonly diagnosed cancer and the sixth in cancer mortality among men worldwide. It is also a leading cause of death among men in the United States. Over 241,000 new cases of prostate cancer will be diagnosed in 2014, along with about 28,000 deaths (1). About 903,500 new cases will also be diagnosed worldwide in 2015, with an expected number of 258,400 deaths (2). While the highest rates of prostate

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cancer are found in Europe and North America, it is one of the three most common types of cancer in sub-Saharan Africa (2). There are no known definitive causes of prostate cancer, but available evidence suggests that diet, genetic and hormonal factors may play a role in its pathogenesis. Hormonal involvement is suggested by published evidence that males who were castrated before puberty did not develop prostate cancer (3). Increased risk of prostate cancer among first-degree relatives of patients with the disease suggests the contribution of genetic factors (4). Other studies have also pointed to the possible role of diet as indicated by an elevated frequency of the disease in Scandinavian countries where the diet is rich in animal fat, compared to a low incidence in Japan and other Asian countries with low-fat diet (5).

Past studies have shown that some anticancer drugs inhibit prostate cell growth by inducing apoptosis and cell-cycle perturbations. Copeland *et al.* showed that a dichloro-naphthoquinone drug induced significant levels of apoptotic cell death in PC-3 and DU-145 prostate cell lines (6). Pourpak *et al.* reported that ethonafide inhibited DU-145 cell growth by inducing G_2 cell-cycle arrest and inhibiting topoisomerase II activity (7). Pidgeon *et al.* also reported that treatment of prostate cancer cell lines PC3 and DU-145 with two inhibitors, baicalein and BHPP, resulted in a dose-dependent decrease in cell proliferation (8). Yang *et al.* showed that while *Zanthoxyli fructus* inhibited the growth of various cancer cell lines, its highest inhibitory activity was against the androgen-dependent LNCaP prostate cancer cell line (9).

Roots of *Fagara zanthoxyloides* and *Pseudocedrela kotschyii* are widely used in West Africa as chewing sticks and as medicinal remedies for a variety of ailments (10, 11). Crude and purified root extracts of the two plants inhibited stage-specific *in vitro* growth of the human malaria parasite *Plasmodium falciparum* (12, 13). Other studies have separately demonstrated that exracts of *Fagara* and *Pseudocedrela* contain several natural compounds with different biological activities (10, 14). One of these compounds is fagaronine, which was found to inhibit DNA topoisomerases I and II and act as a DNA intercalating agent (15). On the other hand, *P. kotschyi* has hitherto not been shown to have any anticancer activity. We, therefore, designed experiments to investigate the antiproliferative and apoptosis-inducing activities of *Fagara* and *Pseudocedrela* extracts against androgen-independent prostate cancer PC3 and DU-145 cells and androgen-dependent LNCaP and CWR-22 prostate cancer cell lines.

Materials and Methods

Plant extract preparation

The identities of the plants used in the study, *P. kotschyi* and *F. zanthoxyloides*, were confirmed by comparison with authentic specimens at the University of Lagos Herbarium. The roots of the plants were washed, air-dried, pulverized into powder form and extracted in cold sterile deionized water (20 g/l00 ml H_2O) for 24 h by mechanical stirring. The aqueous extracts were centrifuged twice at 800 ×*g* for 15 min to remove particulate materials. The supernatants were then filter-sterilized (0.45 μm; Millipore Corp., Bedford, MA, USA) and freeze-dried in a Labconco lyophilizer (Marshall Scientific, Brentwood, NJ, USA). The dried powder extracts were used in all experiments.

Cell cultures

Stock cultures of two androgen-independent PC-3 and DU-145 and two androgen-dependent LNCaP and CWR-22 prostate cancer cell lines (Manassas, VA, USA) were propagated in complete RPMI-1640, supplemented with 10% fetal bovine serum, 2 mM L-glutamine and penicillin/streptomycin (100 ug /ml,100 units). The cultures were maintained at 37° C in a humidified atmosphere of 5% CO_2 and 95% air until reaching about 80% confluency. The cells were trypsinized with trypsin-EDTA solution, stained with 0.2% trypan blue and enumerated in a hemacytometer for assessment of viability, with cell density adjusted for each experiment.

Cell proliferation

Cells (2×10^4 cells/well), in 200 ul of RPMI-1640 medium, were plated in triplicate in 96-well plates and allowed to grow for 24 h. The cells were then incubated with different concentrations (12, 25, 50 and 200 μg/ml) of *Fagara* and *Pseudocedrela* extracts. Control cells were similarly incubated without the extracts. For determination of cell proliferation and viability at 1, 3 and 5 days of incubation, the culture medium was aspirated and cells were treated for 3 h with 30 μl MTT colorimetric reagent (0.5 mg/ml) at 37° C. After aspiration, 100 μl of 0.04 N HCl in isopropanol was added to each well. Spectrophotometric measurements of the blue-colored product, formazan, at 570 nm with a background reading at 630 nm were used to determine cell proliferation. The amount of formazan dye is a direct measurement of the number of metabolically active cells in the culture.

Determination of apoptosis

Cells were harvested by centrifugation at 200 ×*g* for 5 min and washed twice with PBS to remove residual inhibitors. The cells were resuspended in 75% ethanol overnight at 4° C. The fixed cells were centrifuged, washed twice with PBS, readjusted to a volume of 200 μl with PBS and stained for 3 h with a 40-μl mixture of acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml). The cells were examined under fluorescence microscopy for morphological evidence of membrane blebbing, nuclear disintegration and chromatin condensation. The proportion of apoptotic cells was calculated from multiple readings of 1,000 cells each.

Statistical analysis

All results are expressed as the cell growth as a percentage that of control cultures and each data point on graphs represents the mean of three independent experiments that were run in triplicate. The differences within and between different groups of treatments were analyzed using one-way analysis of variance (ANOVA), with $p<0.05$ considered statistically significant.

Results

Increasing concentrations of *F. zanthoxyloides* and *P. kotchyii* extracts were tested for their anti-proliferative and apoptosis-inducing activities on four prostate cancer cell lines over a five-day incubation period. The extract concentrations used in the experiments ranged from 12 to 200 μg/ml. The results of their effects on the proliferation of the four cell lines are

presented in Figures 1–4. The Figures show that the *Fagara* and *Pseudocedrela* extracts at all concentrations had little or no effect on the growth of PC-3, DU-145 and LNCaP cells at day 1 but reduced the proliferation of the CWR-22 cells to 69% and 57% of the control only at 200 μg/ml. While the *Fagara* extract also did not have any appreciable effect on the PC-3 cells at day 3, the *Pseudocedrela* extract reduced its growth to about 61% of the control (Figure 1B). The growth of DU145 cells was reduced to 48% by *Fagara* extract and to 36% by the *Pseudocedrela* extract (Figures 2A, B). The results also show that the LNCap cells were the most sensitive to the two extracts, with their highest inhibition being found at day 3 instead of day 5 as was observed with the other three cell lines (Figures 3A, B). Irrespective of their androgen dependence, all four cell lines were reduced to their lowest levels of proliferation in a concentration-dependent manner by day 3 or 5 (Figures 1–4).

Table I shows the IC_{50} values for the two extracts that were derived from the proliferation graphs, using the plots for the 5-day cultures. *Pseudocedrela* extract had lower IC_{50} values of 12–20 μg/ml for PC-3, DU145 and LNCap cells, indicating that these cells were more sensitive to the extract than to the *Fagara* extract, which had significantly higher IC_{50} values of 25–44 μg/ml.

Table II shows the mean proportions of each cell line that were determined as viable by the MTT assay and as apoptotic at day 5 of incubation with 50 μg/ml of each of the two extracts. The LNCap cells entitled the highest proportions (42–45%) of apoptotic cells induced by the two extracts, compared to the low proportions of 24–26% for CWR-22 cells. Figure 2 shows fluorescence microscopy images of apoptosis of DU-145 cells, as indicated by membrane blebbing, chromatin condensation and nuclear disintegration after treatment with *Fagara* and *Pseudocedrela* extracts and staining with a mixture of acridine orange and ethidium bromide. Similar apoptotic images were also obtained with the three other extract-treated cell lines.

Discussion

This study examined the effects of *Fagara* and *Pseudocedrela* root extracts on the *in vitro* growth and proliferation of four prostate tumor cell lines. The cells used included androgen-independent PC-3 and DU145 cell lines and androgen-dependent LNCaP and CWR-22 cells. We chose these four cell lines because of their reported differential responses to chemopreventive agents. The extract-treated cultures were monitored for their growth and proliferation during five days of incubation. While the extracts did not inhibit the growth and multiplication of three of the cell lines on day 1, significant dose-dependent growth inhibition of all four cell lines was evident at day 3. In particular, maximum growth inhibition of LNCaP cells was achieved by the two extracts at day 3 (Figures 3A, C). All four cell lines, irrespective of their androgen sensitivity, exhibited a dose-dependent proliferation inhibition by the two plant extracts (Figures 1–4). The results also showed that LNCap cells had the highest proportions (42–45%) of apoptotic cells after treatment with the two extracts, compared to the low proportions of 24–26% for CWR-22 cells. These observations of dose-dependent decrease of cell proliferation and induction of apoptosis are similar to the findings reported by other investigators. Kumar *et al.* reported a dose-dependent decrease in viability, along with an increase of DNA fragmentation of PC3

prostate cancer cell line after treatment with *Azadirachta indica* neem leaf extract (20). In another study, Yang *et al.* reported that an extract of *Zanthoxyli fructus*, used as seasoning in Asian countries, exhibited significant *in vitro* growth-inhibitory activity against leukemia, breast, lung and prostate cancer cell lines (9). They showed that the extract also inhibited androgen receptor signaling and induced apoptosis of these cell lines. In a series of reports, Singh and Agarwal showed that silibinin, derived from milk thistle, inhibited cell growth and proliferation and arrested G_1G_2 /M cell progression (16–18). It also induced apoptosis and suppressed tumor xenograft development of PC-3, DU145 and LNCaP prostate cancer cell lines.

All the observations from the aforementioned reports suggest that growth inhibition of the prostate cancer cell lines may be mediated by a blockage of multiple pathways, including androgen receptor (AR) signaling. Prostate cancer is a slow developing tumor and generally progresses from androgen dependence in its early stage to androgen independence in the late stage. The growth and progression of prostate cancer also involves the interaction of AR and dihydrotestosterone (DHT), which is derived from the conversion of testosterone by 5-α-reductase. The ratio of testosterone to DHT in serum is about 10:1, but the ratio is reversed in the prostate. In the Guevedoce population of the Dominican Republic, male adults are known to develop relatively small-sized prostates (19). Their testosterone levels are normal, but their DHT levels are deficient. Prostate cancer is absent in this population, suggesting that DHT may play an important role in the development of the disease. Since our study used four cell lines that were derived from prostate tumors, it is possible that the anti-growth and antiproliferative properties of our *Fagara* and *Pseudocedrela* extracts may partly be associated with the inhibition of DHT.

While we have not yet isolated and identified the active antitumor components of our two extracts; some studies have isolated biologically-active components from these two plants, including fagaronine and kotschyins A-C (10, 14, 15, 16, 21). Fagaronine has been shown to be a DNA-inter-calating agent and an inhibitor of topoisomerases I and II (16, 21). Prostate cancers produce high levels of topoisomerase II enzymes in proportion to their histological grading (22). These enzymes play a role in DNA replication and chromosome segregation through breaking and rejoining of DNA strands (23, 24). The mode of action of some anticancer agents involves the inhibition of topoisomerases. These agents include topotecan and irinotecan, which are derivatives of the natural alkaloid camptothecin (25). Others are daunorubicin and doxorubicin, which are anthracycline derivates and are topoisomerase II inhibitors (26, 27). In a previous study, we showed that a purified constituent of F. zanthoxylloides, identified as fagaronine, inhibited *in vitro* multiplication and stage-specific development of the malaria parasite *Plasmodium falciparum* (12), possibly by DNA intercalation and topoisomerase II inhibition. The inhibition of replication of the four prostate cell lines by our plant extracts may be due in part to their fagaronine content and/-or to other constituents. It is, therefore, imperative to isolate, purify and identify the anti-proliferative constituents of the two plant extracts, and determine their mode(s) of action.

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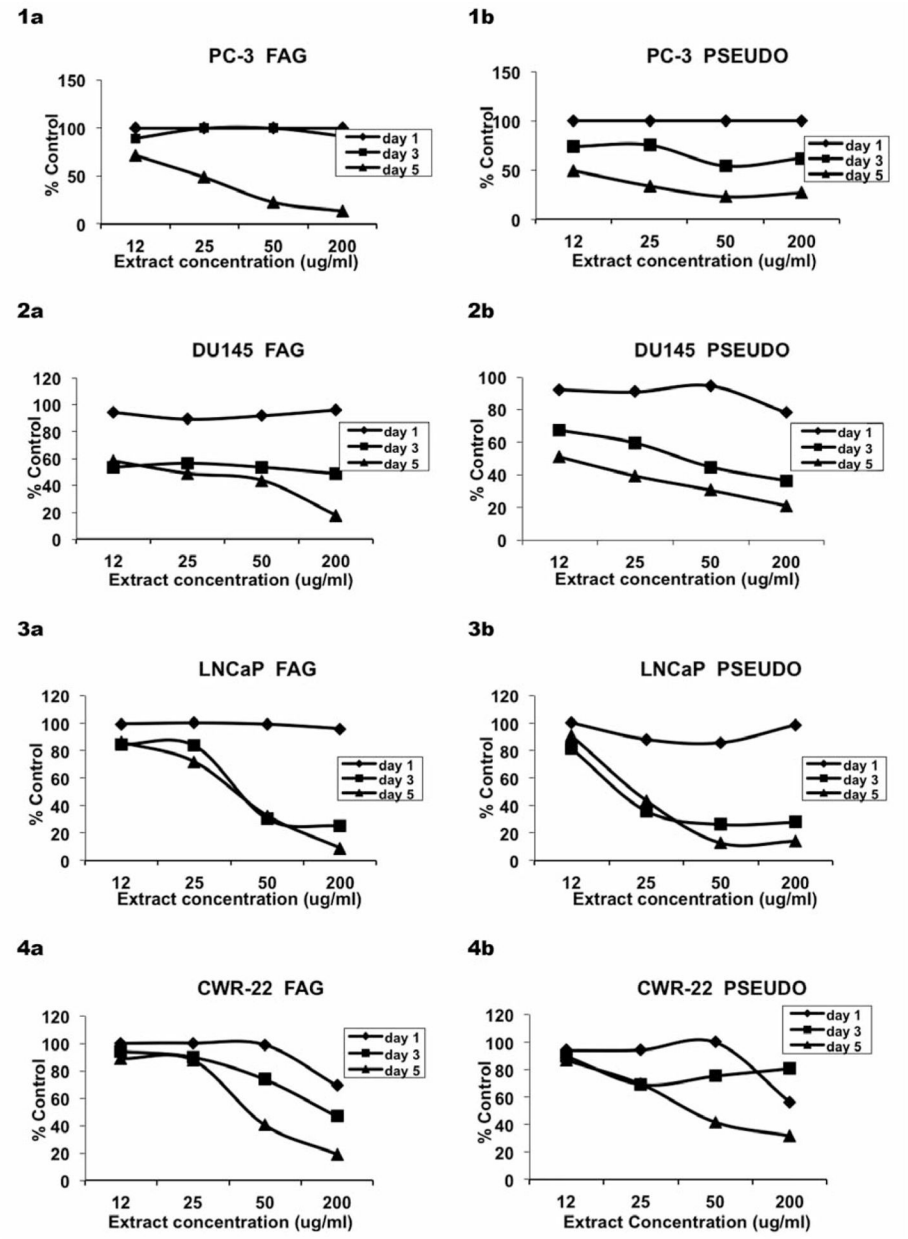

Figures 1–4.

Effects of Fagara and Pseudocedrela extracts on the proliferation of androgen-independent PC-3 and DU-145 and androgen-dependent LNCaP and CWR-22 prostate cell lines. The cells were incubated with 12, 25, 100 and 200 μg/ml for 1, 3 and 5 days and their viability was quantitatively assessed by the MTT colorimetric assay. The results are expressed as percentages of control cultures without extract incubation.

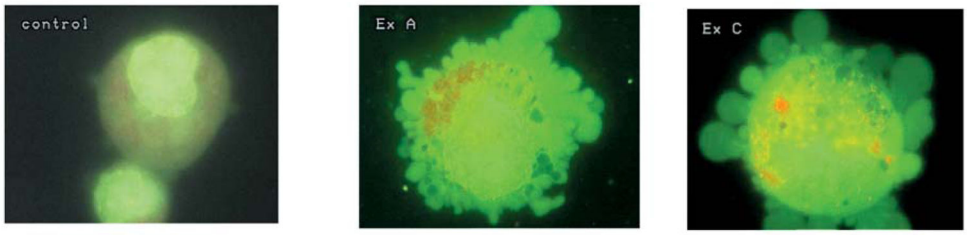

Figure 5.

Representative DU145 control and cells treated with 50 μg/ml of Fagara (A) and Pseudocedrela (C) extracts on day 5. Apoptosis in treated cells is indicated by the appearance of membrane blebs and nuclear disintegration.

Table I

IC_{50} values of Fagara and Pseudocedrela root extracts obtained from the dose response curves of 5-day cultures of the four prostate cancer cell lines.

Prostate cell line	*Fagara extract*	*Pseudocedrela extract*
PC-3	25±2.8 μg/ml	12±0.7μg /ml
DU-145	25±2.6 μg /ml	12±0.8μg /ml
LNCaP	39±3.5 μg /ml	20±1.6μg /ml
CWR-22	44±3.8 μg /ml	42±3.6μg /ml

Table II

Percent viable and apoptotic prostate cancer cell lines at 5 days of treatment with 50 μg/ml of Fagara and Pseudocedrela root extracts. Viability was determined by the MTT assay and percent apoptosis was calculated from cells stained with acridine orange and ethidium bromide.

Prostate cell line	Percent viable cells by MTT		Percent apoptotic cells	
	Fagara	*Pseudocedrela*	*Fagara*	*Pseudocedrela*
PC-3	22±2.5%	22±1.8%	38±3.4%	35±3.8%
DU-145	43±3.8%	30±2.5%	28±1.9%	33±2.7%
LNCaP	0±2.7%	12±0.8%	45±3.4%	42±3.5%
CWR-22	40±3.4%	42±3.2%	26±1.7%	24±1.5%