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Extracts of various species of *Epilobium* inhibit proliferation of human prostate cells

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Abstract

This study examined whether various species of *Epilobium*, a phytotherapeutic agent used in folk medicine as a treatment for benign prostatic hyperplasia, may have an antiproliferative effect in PZ-HPV-7 human prostatic epithelial cells in-vitro. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) test, [methyl-^{3}H]thymidine incorporation into DNA and flow cytometry analysis were used to evaluate cell proliferation. Ethanolic extracts of *E. spicatum, E. rosmarinifolium* and *E. tetragonum* inhibited DNA synthesis in PZ-HPV-7 cells. While at high concentrations all extracts were cytotoxic, DNA synthesis was also decreased at levels that caused no or little cytotoxicity. Treatment of cells with *Epilobium* extracts did not result in a formation of DNA fragments (evaluated by the TUNEL assay) or chromatin condensation (assessed by Hoechst staining). Flow cytometry analysis indicated that *Epilobium* extracts inhibit the progression of the cell cycle from the G_0/G_1 phase. These results suggest that extracts of *Epilobium* inhibit proliferation of human PZ-HPV-7 cells in-vitro by affecting progression of the cell cycle. This study provides some initial biological plausibility for the use of *Epilobium* extracts in benign prostatic hyperplasia.

Introduction

Benign prostatic hyperplasia (BPH) is the non-malignant enlargement of the prostate gland, usually accompanied by urinary symptoms and a peak flow rate of less than 15 mL s^{-1}. This condition commonly involves hyperplasia of both the prostatic epithelium and the stroma (Medina et al 1999). Autoptic studies have shown that BPH is present in 20% of men aged 40–50 years and has a prevalence of 50% by age 60, increasing to 70% by age 70 and to 90% by age 90 (Berry et al 1984). BPH might be even more prevalent, as it is believed that some cases go undiagnosed, because of embarrassment of discussing urinary problems and concern about surgical intervention and its complications, such as impotence, retrograde ejaculation and sterility (Garraway & Kirby 1994). BPH can have a substantial negative impact on the quality of life (QOL), as irritative symptoms, such as nocturia, frequency and urgency of urination, may lead to alterations in sleep patterns, anxiety, reduced mobility and a generally compromised sense of well-being (Garraway & Kirby 1994; Kirby 2000).

The cause of BPH is unknown, though androgens, oestrogens and growth factors seem to play important roles. BPH may result from an over-activity of cell proliferative processes induced by these hormones, or from a reduced rate of apoptosis (Kirby 1997). In particular, dihydrotestosterone, the metabolic product of testosterone reduction by 5α-reductase, appears to act indirectly, by influencing the expression of genes involved in cell proliferation and apoptosis, and directly, by inducing cell differentiation (Anderson et al 2001).

In addition to invasive or minimally invasive surgical treatments (Jonler et al 1994; Fitzpatrick 2000), various pharmacological approaches are currently utilized for the treatment of BPH, including α-adrenoceptor antagonists (Cooper et al 1999) and 5α-reductase inhibitors (Glassman et al 2001). These pharmacological treatments, though effective, have a few adverse effects, such as loss of potency and reduced libido. For these reasons, various plant extracts are being increasingly used in the treatment of lower urinary tract symptoms secondary to BPH. The most widely used are

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lipido-sterolic extracts of the fruit of *Serenoa repens*; root extracts of *Hypoxis rooperi*; chloroform extracts of *Pygeum africanum* bark; extracts of the roots of *Urtica dioica* and pollen extracts of *Secale cereale* (Buck 1996; Lowe & Fagelman 1999). Such extracts appear to be efficacious and their action has been ascribed to a variety of mechanisms, including inhibition of type-I and type-II 5α-reductase (Iehle et al 1995), increase of transforming growth factor-β1 levels, anti-androgenic and anti-oestrogenic activity (Ravenna et al 1996; Rosenberg Zand et al 2001), inhibition of production of 5-lipoxygenase metabolites (Paubert-Braquet et al 1994) and modulation of the binding of sex hormone-binding globulin to its receptor on human prostatic membranes (Hryb et al 1994). Moreover, inhibition of cell proliferation was also observed with some of these plant extracts. Lipido-sterolic extracts of *Serenoa repens* have been shown to affect the proliferative response induced by basic fibroblast growth factor in biopsies of human prostate (Paubert-Braquet et al 1998). Inhibition of proliferation of stromal and epithelial prostate cells was confirmed in other studies, and a specific and selective induction of apoptosis was also found (Vacherot et al 2000). Extracts from *Pygeum africanum* have also been shown to inhibit proliferation of rat prostatic stromal cells stimulated by different growth factors (Yablonsky et al 1997) and an antiproliferative action has also been demonstrated for *Urtica dioica* and pollen extracts in human stromal and epithelial prostatic cancer cell lines (Habib et al 1995; Konrad et al 2000).

In recent years, interest has emerged for the use of ethanolic extracts of the fresh aerial parts of *Epilobium* (Onagraceae). This plant, which is widely distributed all over the world, consists of over 200 species that have been used in folk medicine for the treatment of BPH and for inflammation of the prostate gland (Hiermann et al 1986; Van Hellemont 1986). The constituents of *Epilobium* are not well known, but the presence of sterols, triterpenes, fatty acids, macrocyclic tannins, flavonoid glycosides and a few other compounds has been reported (Hiermann 1983; Lesuisse et al 1996). Various species of *Epilobium* have been found to have anti-inflammatory (Juan et al 1988), analgesic (Tita et al 2001) and anti-androgenic (Hiermann & Bucar 1997) activity in-vivo. Though these properties may be useful for the treatment of BPH, no information exists on the effect of *Epilobium* on prostate cells. The aim of this study was, therefore, to investigate whether extracts of various *Epilobium* species could affect the proliferation of human epithelial prostate cells, as inhibition of proliferation is necessary to avoid hyperplasia and hypertrophy of the prostate gland. A preliminary account of this study has been published (Vitalone et al 2001).

Materials and Methods

Chemicals

Keratinocyte serum-free medium (K-SFM), Dulbecco's Modified Eagle Medium (DMEM), human recombinant epidermal growth factor (EGF human recombinant), bovine pituitary extract (BPE) and trypsin inhibitor-soybean were acquired from GIBCO (Grand Island, NY). [Methyl-^{3}H]thymidine (6.7 Ci mmol^{-1}) was obtained from New England Nuclear (Boston, MA). Hoechst 33258 was supplied by Molecular Probes (Eugene, OR). The TUNEL in-situ cell-death detection kit was purchased from Roche Diagnostics Corporation (Indianapolis, IN). Ethanolic extracts of fresh aerial parts of *Epilobium* species were kindly supplied by Boiron Laboratories (Riano, Rome). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO).

Cell cultures

PZ-HPV-7, a human prostate epithelial cell line derived from normal peripheral zone tissue and transfected with human papillomavirus type 18 (Weijerman et al 1994), was obtained from the American Type Culture Collection (ATCC). Cells were grown in K-SFM medium supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 5 ng mL^{-1} EGF human recombinant and 50 μg mL^{-1} BPE in 75-cm^2 flasks at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The culture medium was renewed every 2 days and cells were subcultured every 6 days. For the experiments, cells were seeded in 24-well plates at a density of 5×10^4 cells/well; after 48 h, when cells were 60–70% confluent, extracts of different *Epilobium* species were added. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), Trypan blue and thymidine incorporation assays were done 24 h after treatment, while flow cytometric analysis was carried out 48 h after treatment.

1321N1 astrocytoma cells were obtained and cultured as described by Guizzetti et al (1996). Briefly, cells were grown in DMEM medium, low glucose, supplemented with 5% of fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin in 75-cm^2 flasks at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The growth medium was renewed every 3 days and cells were subcultured every 6 days.

MTT Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay was used for measurement of cell proliferation. The test is based on the conversion of the tetrazolium salt MTT to an insoluble purple formazan by mitochondrial dehydrogenase enzymes (Romijn et al 1988). MTT was dissolved in distilled water (5 mg mL^{-1}) and 30 μL of this stock solution were added to each well, in a total volume of 1 mL. At the end of 80 min incubation period, the medium was carefully removed and 250 μL of dimethyl sulfoxide (DMSO) were added. The 24-well plate was shaken for 5 min to dissolve the formazan produced. Absorbance of converted dye was measured by a Molecular Device kinetic microplate reader at a wavelength of 590 nm with background subtraction at 650 nm.

[Methyl-^{3}H]thymidine incorporation

To assess DNA synthesis, incorporation of [methyl-^{3}H]thymidine into cell DNA was measured as described by Guizzetti et al (1996), with some modifications. After 48 h in K-SFM, the *Epilobium* extracts were added for 24 h. [Methyl-^{3}H]thymidine (1 μCi/well) was added for the last 6 h of the incubation at 37 °C under an atmosphere of 5% CO_2 and 95% air. Cells were washed twice with phosphate-buffered saline (PBS) and fixed in methanol. The DNA was precipitated by two washes with 10% and 0.5% trichloroacetic acid. The monolayer was dissolved in 250 μL of 1 M NaOH, and 125 μL of every sample were transferred to scintillation fluid and counted by Beckmann LS 5000 CE scintillation counter.

Trypan blue assay

The Trypan blue exclusion assay was performed to determine the real proportion of living and dead cells (Patterson 1979). After 24 h of treatment with *Epilobium* extracts, attached cells were collected by trypsinization, followed by the addition of trypsin inhibitor-soybean. Cells were counted in a hemocytometer using equal volumes of cell suspension and trypan blue solution (0.2% in PBS).

Flow cytometry analysis

Flow cytometric analysis of the cell cycle was carried out using the 5-bromodeoxyuridine-Hoechst 33258 method, as described by Rabinovitch et al (1988) and Guizzetti & Costa (1996), with slight modifications. Briefly, after addition of *Epilobium* species and 150 μM 5-bromodeoxyuridine, cells were incubated for 48 h at 37 °C in a humidified 5% CO_2 atmosphere. Cells were harvested by adding 200 μL of a 0.25% trypsin-EDTA solution, which was neutralized by adding the same amount of trypsin inhibitor-soybean. The cells were transferred in Eppendorf tubes and collected by centrifugation at 1000 rev min^{-1} for 10 min at 4 °C. The supernatant was removed by aspiration and the pellet was resuspended in 500 μL Hoechst buffer with 5% DMSO, and the samples were frozen until the flow cytometric analysis. Fifteen minutes before the analysis, 1.5 μg mL^{-1} of ethidium bromide was added. Ultraviolet excitation for analysis of Hoechst 33258 and ethidium bromide fluorescence was used. Hoechst 33258 fluorescence emission at wavelength of 450 nm and ethidium bromide fluorescence emission at 590 nm were measured with a Coulter EPICS Elite ESP flow cytometer and the results were analysed using MPLUS AV program.

TUNEL assay

DNA fragmentation was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL). PZ-HPV-7 cells were seeded in 8-well plates at a density of 2.5×10^4 cells/well; after 48 h, extracts of *Epilobium* were added. The in-situ cell death detection protocol provided by Roche was followed with minor modifications. Briefly, after 6 or 24 h of exposure, cells were fixed with cold acetone for 10 min, then washed three times with phosphate-buffered saline (PBS) and incubated for 10 min at room temperature with 3% H_2O_2 in methanol. After rinsing the samples three times with PBS, 0.1% triton X-100 in 0.1% sodium citrate was added for 2 min on ice. The TUNEL reaction mixture, properly diluted, was added for 1 h in a humidified chamber at 37 °C. Following this treatment, cells were washed three times and 40 μL of converter-POD were added. At the end of the incubation time, 100 μL of diaminobenzidine solution were added for 10 min at room temperature. The slides were washed twice with PBS and cover-slips were fixed with glycerol 50% in water. The samples were analysed and photographed under a light microscope by objective magnification × 20, using a Nikon SPOT camera (Diagnostic Instruments Inc., Michigan, MI), and quantified using MetaMorph software (Universal Imaging Corporation, Downingtown, PA).

Hoechst staining

Chromatin condensation was measured by Hoechst staining, as described by Xia et al (1995), with slight modifications. In brief, the cells were plated in dishes of 100 mm at a density of 2.5×10^6 cells/dish, and cultured for 48 h in 10 mL of medium. Cells were exposed for 24 h to 0.1 mg mL^{-1} of *E. rosmarinifolium* and were rinsed twice with PBS. Each sample was fixed with 4% of paraformaldehyde for 15 min in a cold room and then washed twice with 0.1% triton X-100 in PBS. The staining was carried out using Hoechst 33258 green dye (5 μg mL^{-1}) in 0.1% of triton X-100/PBS solution, for 10 min at room temperature. The samples were visualized under a fluorescence microscope by magnification × 40, and nuclei that appeared condensed or fragmented were considered as apoptotic.

Statistical analysis

All values are expressed as means ± standard error of the mean (s.e.m.). Statistical analysis was performed by one-way analysis of variance followed by the Bonferroni *t*-test. A value of $P < 0.05$ was considered to denote statistical significance.

Results and Discussion

Extracts from five different species of *Epilobium* (*hirsutum, palustre, rosmarinifolium, spicatum, tetragonum*) were initially tested for their ability to inhibit proliferation of PZ-HPV-7 cells using the MTT reduction assay and the incorporation of [methyl-^{3}H]thymidine into DNA. As shown in Figure 1A, all ethanolic extracts caused a statistically significant decrease of MTT reduction at the highest concentration tested (1 mg mL^{-1}). Similar results were also found when incorporation of [methyl-^{3}H]thymidine was measured (Figure 1B). At this concentration,

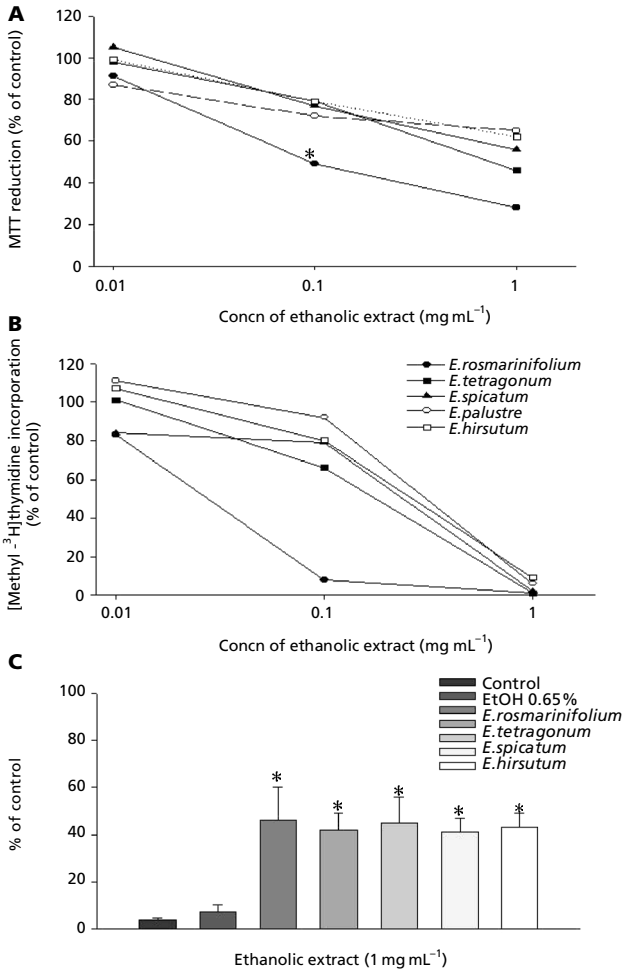

Figure 1 Effect of *Epilobium* extracts on proliferation of PZ-HPV-7 human prostatic epithelial cells measured by the MTT assay (A) or by incorporation of [methyl-^{3}H]thymidine into cellular DNA (B). All data at 1 mg mL^{-1} are statistically different from control ($P < 0.05$). Panel C shows the cytotoxic effect of *Epilobium* extracts (1 mg mL^{-1}) assayed by the Trypan blue exclusion assay. Data represent the mean (± s.e.m.) of six replicates. *$P < 0.05$ vs control.

however, all extracts caused cytotoxicity, as evidenced by the results of the Trypan blue assay (Figure 1C). At the highest concentration tested (0.65%), ethanol had no effect on Trypan blue exclusion (Figure 1C), nor caused any inhibition of MTT reduction (92 ± 5% of control, $P > 0.05$) or [methyl-^{3}H]thymidine incorporation (89 ± 7% of control, $P > 0.05$). An analysis of the concentration–response effect (Figure 1A, B) indicated that *E. tetragonum* and *E. rosmarinifolium* were the most potent species tested, and these were chosen for further study. In addition, *E. spicatum* (also called *E. angustifolium*) was also chosen, as several reports in the literature focus on this species (Juan et al 1988; Hiermann & Bucar 1997; Tita et al 2001; Vitalone et al 2001).

In a second series of experiments, extracts of these three *Epilobium* species were tested in a wider range of concentrations, with the purpose of identifying concentrations that would inhibit DNA synthesis but causing no or

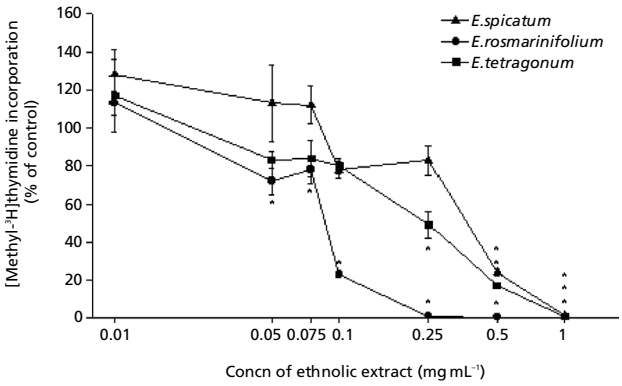

Figure 2 Concentration–response effect of *Epilobium* extracts on [methyl-^{3}H]thymidine incorporation in PZ-HPV-7 cells. Data represent the mean (± s.e.m.) of nine replicates. *$P < 0.05$ vs control.

minimal cytotoxicity. As shown in Figure 2 and Table 1, such concentrations were 0.05–0.1 mg mL^{-1} for *E. rosmarinifolium*, 0.25 mg mL^{-1} for *E. tetragonum* and 0.5 mg mL^{-1} for *E. spicatum*.

Flow cytometric analysis was then carried out to assess the effects of non-cytotoxic concentrations of *Epilobium* extracts on the progression of the cell cycle. Figure 3 and Table 2 show that control PZ-HPV-7 cells are actively proliferating, with only 2% of cells in the G_0/G_1 phase. All *Epilobium* extracts, at concentrations that were only minimally cytotoxic, significantly increased the percent of cells in the G_0/G_1 phase to about 40% (Table 2). As several plant extracts are sold as a combination of extracts from several plants (Lowe & Fagelman 2002), the effect of low concentrations of different *Epilobium* extracts was also investigated. Of interest is that the combination of

Table 1 Effects of *Epilobium* extracts on viability of PZ-HPV-7 human prostatic epithelial cells.

Treatment (mg mL^{-1})		% viable cells	% dead cells
Control		95 ± 1	5 ± 1
Ethanol	0.65%	94 ± 6	6 ± 6
	0.325%	94 ± 2	6 ± 2
E. rosmarinifolium	0.05	89 ± 3	11 ± 3
	0.075	82 ± 3*	18 ± 3*
	0.1	66 ± 5*	34 ± 5*
E. spicatum	0.25	74 ± 3*	26 ± 3*
	0.5	78 ± 2*	22 ± 2*
	1.0	67 ± 4*	33 ± 4*
E. tetragonum	0.25	85 ± 3	15 ± 3
	0.5	54 ± 5*	46 ± 5*
E. rosmarinifolium + *E. tetragonum*	0.05 + 0.25	79 ± 5*	21 ± 5*

Percent of viable and dead cells after exposure to ethanol and various concentrations of *Epilobium* extracts assessed by the Trypan blue exclusion test. Data are the mean (± s.e.m.) of six replicates. *$P < 0.05$ vs control.

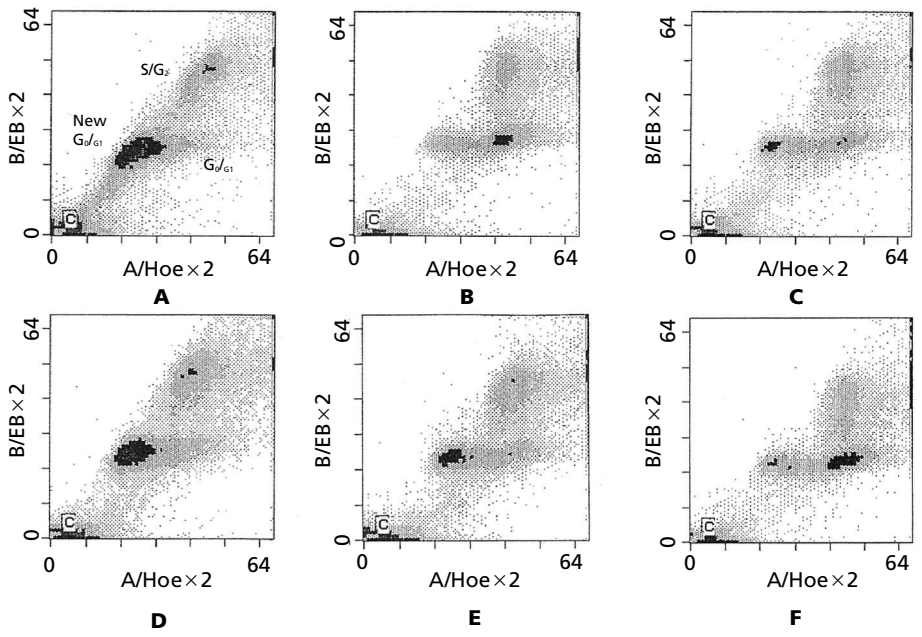

Figure 3 Effect of different concentrations of *Epilobium* extracts on PZ-HPV-7 cell cycle distribution. Hoechst 33258 (A/Hoe) and ethidium bromide (B/EB) fluorescence of PZ-HPV-7 cells were analysed after 48 h incubation with 5-bromodeoxyuridine in serum-free medium (control, A), *E. rosmarinifolium* 0.1 mg mL^{-1} (B), *E. spicatum* 0.5 mg mL^{-1} (C), *E. rosmarinifolium* 0.05 mg mL^{-1} (D), *E. tetragonum* 0.25 mg mL^{-1} (E) and *E. rosmarinifolium* 0.05 mg mL^{-1} plus *E. tetragonum* 0.25 mg mL^{-1} (F).

Table 2 Effects of *Epilobium* extracts on cell cycle distribution of PZ-HPV-7 human prostatic epithelial cells.

Treatment (mg mL^{-1})		Cell phase (% of cells in phase)		
		G_0/G_1	S/G_2	new G_0/G_1
Control		2 ± 0.1	37 ± 0.7	61 ± 0.8
E. spicatum	0.5	31 ± 1.7*	47 ± 1.3	22 ± 0.4*
E. rosmarinifolium	0.05	3 ± 0.1	44 ± 1.3	52 ± 1.4*
	0.1	42 ± 1.1*	41 ± 1.3	16 ± 0.2*
E. tetragonum	0.25	23 ± 0.9*	50 ± 0.6	27 ± 0.7*
E. rosmarinifolium	0.05			
+ *E. tetragonum*	0.25	48 ± 1.1*#	37 ± 0.6	14 ± 0.8*#

5-Bromodeoxyuridine-Hoechst 33258 flow cytometry of human prostate epithelial cells. Data are the mean (± s.e.m.) of six replicates. *$P < 0.01$ vs control; #$P < 0.01$ vs *E. rosmarinifolium* 0.05 mg mL^{-1} or *E. tetragonum* 0.25 mg mL^{-1}.

low concentrations (0.05 mg mL^{-1}) of *E. rosmarinifolium*, which had no effect by itself on the G_0/G_1 phase, and *E. tetragonum* yielded an apparent synergistic inhibition of the cell cycle (Figure 3F and Table 2).

As other phytotherapeutic agents have been shown to induce apoptosis of prostate cells in addition to inhibiting cell proliferation (Vacherot et al 2000), an extract of *E. rosmarinifolium*, the most potent among the species, was tested to determine whether it could induce apoptosis of PZ-HPV-7 cells. The TUNEL assay indicated that no apoptotic response was present 6 h (not shown) or 24 h (Figure 4) after exposure to *E. rosmarinifolium*. However, significant necrosis was evident at the high concentration tested (1 mg mL^{-1}), as indicated by altered cell morphology (Figure 4E). Further experiments were done using the Hoechst 33258 staining method. Figure 5 confirms that *E. rosmarinifolium* (0.1 mg mL^{-1}) caused no apoptosis in PZ-HPV-7 cells (Figure 4C). Interestingly, neither staurosporine (Figure 4B) nor chelerythrine (Figure 5B), two known apoptotic agents chosen as positive controls, caused apoptosis in PZ-HPV-7 cells, confirming previous observations of a strong resistance of these cells to apoptosis-inducing chemicals (Gupta et al 2001). On the other hand, chelerythrine chloride induced apoptosis in 1321N1

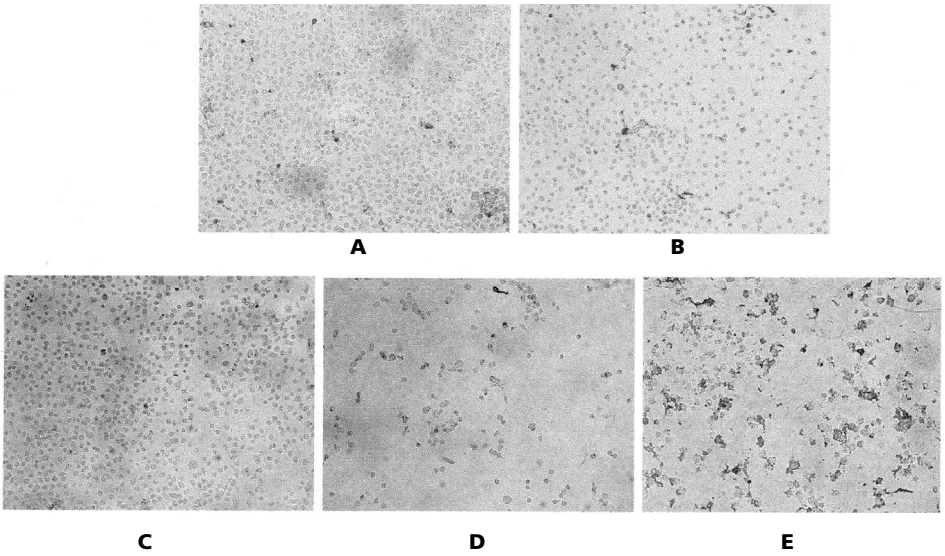

Figure 4 TUNEL assay in PZ-HPV-7 prostate cells (A), after 24 h exposure to staurosporine 5 μM (B), *E. rosmarinifolium* extract 0.05 mg mL^{-1} (C), 0.1 mg mL^{-1} (D) or 1 mg mL^{-1} (E). No apoptotic morphology is present. At the highest concentration, necrotic cells are evident (E). Pictures shown are representative of six replicates. Original magnification: × 10.

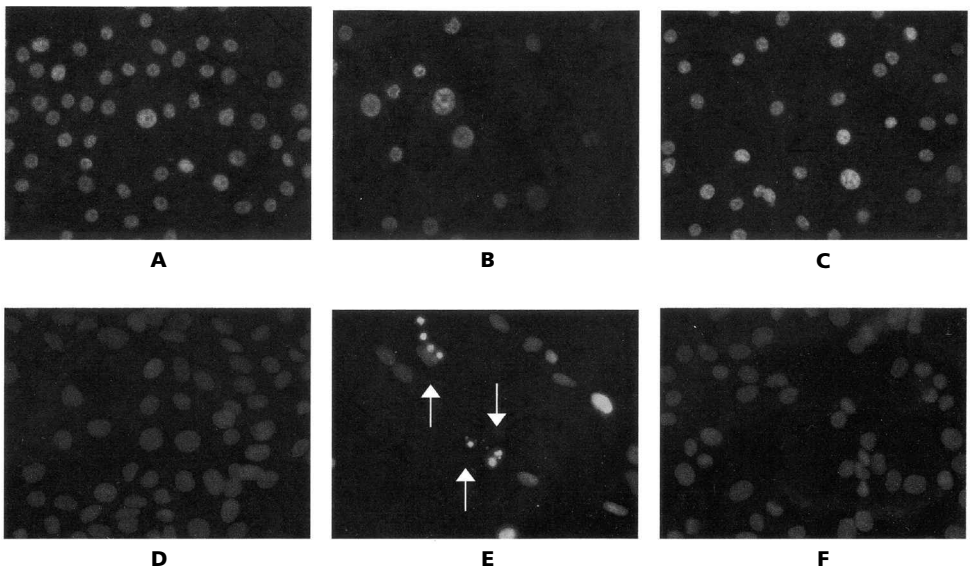

Figure 5 Hoechst 33258 staining in PZ-HPV-7 prostate cells (A–C) and 1321N1 astrocytoma cells (D–F) 24 h after treatment with *E. rosmarinifolium* extract (0.1 mg mL^{-1}) (C and F) or chelerythrine chloride (10 μM) (B and E). A and D show control cultures. The arrows indicate condensed chromatin, evidence of an apoptotic effect. Pictures shown are representative of six replicates. Original magnification: × 40.

human astrocytoma cells (Figure 5E), whereas *E. rosmarinifolium* did not (Figure 5F).

Results of this study indicate that various species of *Epilobium*, used in folk medicine for the treatment of BPH, inhibit the proliferation of human PH-HPV-7 prostatic epithelial cells in-vitro. These cells, derived from normal peripheral zone tissue and transfected with human papillomavirus type 18 (Weijerman et al 1994), have been previously used as a model of non-malignant human prostate cells (Gupta et al 2001). High concentrations of extracts (1 mg mL^{-1}) were cytotoxic; however, at lower concentrations, extracts inhibited DNA synthesis with minimal or no cytotoxicity. The antiproliferative effect of *Epilobium* extracts was further confirmed by flow cytometric analysis, which indicated their ability to interfere with the cell cycle and to increase the percentage of cells in the G_0/G_1 phase. Experiments with *E. rosmarinifolium* also indicated that this extract did not induce

apoptosis at any concentration tested. Though PZ-HPV-7 cells appear to be particularly resistant to apoptotic agents, as previously suggested (Gupta et al 2001), the *E. rosmarinifolium* extract also did not induce apoptosis in another human cell line.

The ability of *Epilobium* extracts to inhibit proliferation of human prostate cells may be ascribed to one or more of the compounds identified in these extracts, which include flavonoids, macrocyclic ellagitannins and sterols (Hiermann 1983; Lesuisse et al 1996). A potentially active compound is oenothein (A and B), an ellagitannin with antitumour activity (Miyamoto et al 1993), which has been shown to inhibit proliferation of several cell lines (Miyamoto et al 1993; Wang et al 1999). Oenothein B was identified and quantitated in different *Epilobium* species (Ducrey et al 1997), and was also shown to inhibit 5α-reductase and aromatase. The aerial parts of *Epilobium* are also rich in other polyphenols, such as kaempferol, quercetin, miricetin and their glycosides (Slacanin et al 1991), that may be responsible for the antiproliferative effect. Some studies (Kampa et al 2000; Knowles et al 2000) have reported an inhibitory effect of these compounds on the growth of both androgen-dependent and independent human prostate cancer cell lines. Inhibition of inducible nitric oxide synthase and interferences in the cell cycle progression were proposed as possible mechanisms for their action.

Conclusions

In summary, this study provides evidence that ethanolic extracts of various *Epilobium* species can inhibit the proliferation of PZ-HPV-7 human prostate cells in-vitro. This effect may be useful for the prevention of epithelial hyperplasia, and offers some initial evidence of biological plausibility for the use of these extracts as a remedy against BPH in folk medicine. Further studies should elucidate the specificity of this action and identify the compound(s) responsible for such effect.

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