

Anti-proliferative effect on a prostatic epithelial cell line (PZ-HPV-7) by *Epilobium angustifolium* L.[☆]

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Abstract

Symptomatic benign prostatic hyperplasia (BPH) is a common condition in elderly men and has a significant impact on their daily lives. The drugs prescribed for treatment include α_1 -blockers, 5- α -reductase inhibitors and plant preparations. *Epilobium angustifolium* L. is deemed to be helpful in BPH therapy, although there is less information regarding the mechanism of its biological activity. The present study evaluated the effect of *E. angustifolium* extract on human prostatic epithelial cells (PZ-HPV-7). The exposure to *E. angustifolium* extract induced a marked inhibition of cell growth in all tested conditions. The anti-proliferative effect observed in in vitro systems clearly indicates a biologically relevant effect of compounds present in the extract. Considering these results, the use in traditional medicine of *E. angustifolium* extract against BPH seems to be justified. However, further experimental studies are needed to determine the biochemical mechanism of the action and the clinical value of the *E. angustifolium* extract. © 2001 Éditions scientifiques et médicales Elsevier SAS

Keywords: *Epilobium angustifolium* L.; Benign prostatic hyperplasia; Prostatic epithelial cell; Proliferation

1. Introduction

Benign prostatic hyperplasia (BPH) is the most important urologic disorder affecting the ageing male population, due to excessive cellular proliferation of both the glandular (epithelial) and the stromal elements of the prostate. Epidemiological data have given the prevalence of histological BPH as high as approximately 8% in the fourth decade of life, rising to approximately 90% in the ninth decade.

The natural history of BPH involves a pathological phase and a clinical phase. During the early phase, hyperplastic nodules develop in the transition zone of the prostate, progressively compress normal prostatic tissue and cause increase of the prostate size with predominance of stromal element, narrowing of the urethra and voiding difficulties. Nevertheless, enlargement of the prostate alone may be insufficient for development of the clinical phase and other factors,

such as the compliance of the prostatic capsule and the presence of prostatitis, have a role in the onset of clinical BPH.

The clinical symptoms of BPH have a major impact on the daily life of sufferers. These symptoms can be classified as being obstructive or irritative in nature. Obstructive symptoms are associated with the narrowing of prostatic urethra and include hesitancy in initiating the urinary stream, weak or intermittent stream, and dribbling of urine. Most of the patients also have irritative symptoms such as pollakiuria, urgency, nocturia and a sensation of incomplete bladder emptying, linked to the presence of detrusor instability.

Although the aetiology of BPH is not fully known, BPH is regarded as an endocrine disorder caused by age-related hormone imbalance [1]. Most etiological theories of this disorder have focused on the role of hormones, especially androgens. In particular, accumulation of 5- α -dihydrotestosterone following shift in prostatic androgen metabolism has been implicated. In addition, age-related increase in the estrogen/androgen ratio may contribute to the maintenance of BPH. Pharmacological approaches based on the effect of male hormones on the growth of the prostate are androgen

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ablation and blockade, which have not been widely used because of adverse effects such as loss of potency and reduced libido. Inhibition of the intracellular prostatic enzymes 5- α -reductase (which converts testosterone to dihydrotestosterone) and aromatase (which converts testosterone to estrogens) has introduced a hormonal therapy with few side effects. In fact, aromatase inhibitors block the estrogen biosynthesis stemming from the aromatization of androstenedione and testosterone, and the 5- α -reductase inhibitors block the conversion of testosterone to the more potent tissue-specific androgen 5- α -dihydrotestosterone. Moreover, changes in the binding capacity of sex-hormone-binding globulin have also been implicated in the pathogenesis of BPH [2]. Finally, another hypothesis regards the possibility that elevated levels of prostaglandins and leukotrienes may be partially involved in the maintenance of BPH. This hypothesis suggests that substances with anti-inflammatory action would be effective in BPH patients.

There is controversy regarding the indications and efficacy of the various treatments for BPH and while surgical option is preferred in USA, medical treatment is considered an effective option in Europe. Synthetic drugs, e.g. α -receptor blockers and 5- α -reductase inhibitors, are available, but most of them possess serious adverse effects.

The wide use in Europe of preparations made from *Serenoa repens* for the treatment of stage I–II BPH traces back to the early 1900s. The results of animal studies have demonstrated that *S. repens* extracts possess anti-androgenic actions due to inhibitory effects on 5- α -reductase. The enzymatic inhibition is partly due to its content of free fatty acids, and *S. repens* extracts have other effects, such as anti-inflammatory and antioxidant effect, which can be useful in BPH patients [3].

Recently, preparations from *Urtica dioica* have been employed for the treatment of BPH [4] and many studies have shown that *U. dioica* extracts inhibit aromatase, interact with sex-hormone-binding globulin and possess anti-inflammatory effect.

Another plant, *Hypoxis rooperi*, is used as a natural remedy for bladder and prostate diseases. This herb contains β -sitosterol, which acts on prostaglandin metabolism, reducing interstitial oedema [5].

Lipophilic extracts of *Pygeum africanum*, long used in Africa for micturition problems, contain at least three classes of active constituents, free and conjugated phytosterols, pentacyclic terpenes and ferulic acid esters of fatty alcohols, which can improve several symptoms of BPH. However, the precise mechanism of action of plant extracts is not completely understood.

Various members of the genus *Epilobium* (Family: Onagraceae) have been used in folk medicine for the

treatment of prostatic diseases, mainly benign prostatic hypertrophy and prostatitis [6]. Teas of dried aerial parts of *Epilobium* are very commonly used because of the high prevalence of these diseases in the population. *E. angustifolium* L. (from Latin, 'narrow leaved') or Fireweed because it is one of the first plants to spring up after fire, is a perennial herb, which grows in temperate climates worldwide, in disturbed ground such as cut-over or burned forest and waste places.

Experimental studies on intact and testosterone-stimulated castrated male rats demonstrated that the oral administration of an aqueous extract of *E. angustifolium* affects the growth of accessory sexual organs [7]. The constituents of *Epilobium* are not well known, but the presence of sterols [8], triperpenes and flavonoids [9] has been reported. Flavonoid content of the aerial parts of various members of the genus *Epilobium* can be used as a chemotaxonomic marker. Chemotaxonomy based on flavonoids permits identification of the different species, even if botanically very similar.

The evaluation of the constitutive polyphenolics of the whole plant extract of *Epilobium hirsutum*, carried out by reverse-phase HPLC, revealed that the aqueous ethanolic extract contains a complicated polyphenolic mixture of ellagitannins, gallotannins and flavonoids [10].

Lesuisse et al. [11] have demonstrated that a decoction of *E. parviflorum* displayed inhibition of the enzyme 5- α -reductase, the current target in the search for medical treatment of BPH. The active principle responsible for this inhibition has been identified and characterised as a macrocyclic tannin, oenothien B. This molecule possess antiviral [12] and anti-tumour [13a,b] activities.

Ducrey et al. [14], during a screening of different *Epilobium* extracts from Europe and Africa have demonstrated that an aqueous methanol extract of *E. capense* Buch. showed the highest inhibition of aromatase and 5- α -reductase activity and the bioactivity-guided fractionation by gel filtration on Sephadex LH-20, followed by semipreparative HPLC led to the isolation of two active compounds identified from NMR experiments as the ellagitannins oenothien A and oenothien B. In the case of aromatase, oenothien A induced 70% inhibition at 50 μ M and oenothien B 33% inhibition at 50 μ M. On the contrary, against 5- α -reductase oenothien B showed IC_{50} 0.44 μ M and oenothien A IC_{50} 1.24 μ M.

However, the beneficial effect of *Epilobium* extracts on BPH is still controversially discussed, since the mechanisms of action remain unclear. In the present study, we examined the in vitro effect of an extract of *E. angustifolium* on proliferative activity of human prostatic epithelial cells.

2. Materials and methods

2.1. Materials

The *E. angustifolium* ethanolic extract was purchased from Boiron Laboratories. For biological assays the extracts were diluted in order to obtain an alcohol concentration that did not interfere with proliferation tests. The concentrations assayed were expressed as dry extract, preliminarily determined by evaporating the extract under vacuum at 45°C and weighing the dry residue.

Keratinocyte-serum-free medium (K-SFM), epidermal growth factor 1-53 (EGF-1-53), bovine pituitary extract, penicillin, streptomycin, trypsin and trypsin inhibitor were obtained from Life Technologies Italia (S. Giuliano milanese, Milan); sodium phosphate monobasic monohydrate, sodium azide, sodium chloride, dimethyl sulfoxide were obtained from Sigma (Milan, Italy).

2.2. Cell culture

PZ-HPV-7 cells (American Type Culture Collection 2221; derived from human prostatic cells transformed by transfection with HPV18 DNA) were employed in the experiments. The human prostatic epithelial cell line was maintained in K-SFM supplemented with bovine pituitary extract (50 µg/ml), epidermal growth factor (5 ng/ml), penicillin G (100 U/ml) and streptomycin sulphate (100 µg/ml) in 25-cm² flasks under a humidified atmosphere of 5% CO₂/95% air at 37°C. Cells were subcultured every seven days, and the growth medium was changed every two to three days.

2.3. Assays of cell proliferation and cell cytotoxicity

For proliferation experiments, cells were plated in 24-well plates at a concentration of 5×10^4 cells per well, and cultured undisturbed for 72 h after seeding to maximize cell attachment to the growth surface. After three days, media were replaced with media containing *E. angustifolium* extract. Each plate was treated only with one concentration of *E. angustifolium* extract (1900, 190 and 19 µg of dry extract/ml of medium) (4 wells/*E. angustifolium* extract/incubation time), while control wells were treated with the same amount of ethanol present in the extract or with the medium. The plates were incubated for three days. At different times of incubation (0, 24, 48 h), cells were harvested by adding 200 µl of 0.25% trypsin. The trypsin was neutralized with 200 µl of 0.1% soybean trypsin inhibitor, cells were gently dislodged by tapping the plate and counted with a microscope. The whole experiment was repeated at least twice.

Cell cytotoxicity was measured using commercially available techniques. More particularly, cell proliferation of PZ-HPV-7 cells was measured with the MTT colorimetric assay [15], that is a rapid and convenient non-radioactive alternative to [³H]thymidine for determining viable cell number. This assay is based on the cellular conversion by the mitochondrial dehydrogenase of viable cells of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), a tetrazolium salt, into a blue formazan product that is easily detected and quantified using a 96-well plate reader at 590 nm: the intensity of the colour is a measure of cell viability. Each experiment was performed in triplicate, testing the above-mentioned concentrations of *E. angustifolium* extract.

Release of lactate dehydrogenase (LDH) in the medium was measured as indicator of cytotoxicity. We used the SIGMA diagnostic kit for the quantitative kinetic determination of LDH activity. Lactate dehydrogenase catalyses the interconversion of lactate and pyruvate. During the reduction of pyruvate, an equimolar amount of NADH is oxidised into NAD and this reaction is measured by a decrease in absorbance at 340 nm. The rate of decrease is directly proportional to LDH activity in the sample; this activity is measured in U/l, where one unit of LDH activity is defined as the amount of enzyme which catalyses the formation of 1 µmol/l of NAD per minute, under the condition of the assay. LDH activity in the medium was assayed 24 h after treatment of cells with *E. angustifolium* extract. Data were related to the maximal leakage obtained by treating cells with 0.1% Triton X-100. Each assay was done in triplicate.

2.4. Statistical analysis

Data were reported as mean \pm SEM or as percent variation from control group.

All data were analysed by one-way ANOVA followed by Tukey test to determine significant differences between treatments. Data with *P* values < 0.05 were considered to be statistically significant.

3. Results and discussion

Cell treatment with ethanol at the concentration of 0.65% modestly affected cell growth. In fact, ethanol treatment resulted in 13.2% variation of cell number after 24 h of exposure and 15.3% after 48 h (Table 1, Fig. 1).

Cell exposure to alcoholic extract of *E. angustifolium* induced an inhibition of cell proliferation and the effect was statistically significant (*P* < 0.05) at the concentration of 1900 µg/ml of culture medium (Table 1, Fig. 1). Compared with untreated control, *E. angustifolium*

Table 1

Effect of *Epilobium angustifolium* extract and of EtOH after 24 and 48 h of exposure on proliferation of PZ-HPV-7 cells counted with a microscope. Data are reported as mean value \pm SEM of four wells for every dose level

	Number of viable cells	
	24 h	48 h
Control	53 000 \pm 1914.85	65 000 \pm 3696.85
EtOH 0.65%	46 000 \pm 7615.77	75 000 \pm 2516.61
<i>E. angustifolium</i> 1900 μ g/ml	17 000 \pm 1914.85 *	21 500 \pm 1258.31 *
<i>E. angustifolium</i> 190 μ g/ml	58 000 \pm 3741.66	73 000 \pm 7852.81
<i>E. angustifolium</i> 19 μ g/ml	60 500 \pm 6652.07	90 500 \pm 3774.92

* $P < 0.05$, Tukey test.

treatment (1900 μ g/ml) resulted in 68% growth inhibition after 24 h of exposure and in 67% growth inhibition after 48 h.

As reported in Table 2 and Fig. 2, *E. angustifolium* induced similar results when the MTT cell viability assay was employed. Compared with untreated control, maximum and statistically significant inhibitory effect (43.6 and 76.6% of viable cells) was reached 24 and 48 h following cell exposure to 1900 μ g/ml of *Epilobium*. The concentration of 190 μ g/ml of *E. angustifolium* extract induced an inhibition of 22.5 and 20.5% after 24 and 48 h exposure, respectively.

No unspecific cytotoxic effect of the *Epilobium* extract (1900 μ g/ml) on PZ-HPV-7 cell proliferation was

Table 2

Effect of *Epilobium angustifolium* extract after 24 and 48 h of exposure on proliferation of PZ-HPV-7 cells as measured by MTT viability assay. Data are reported as mean value \pm SEM of six wells for every dose level

	Absorbance ($A_{590}-A_{650}$)	
	24 h	48 h
Control	0.66 \pm 0.04	0.77 \pm 0.05
<i>E. angustifolium</i> 1900 μ g/ml	0.37 \pm 0.02 *	0.26 \pm 0.05 *
<i>E. angustifolium</i> 190 μ g/ml	0.51 \pm 0.05	0.61 \pm 0.05
<i>E. angustifolium</i> 19 μ g/ml	0.7 \pm 0.03	0.58 \pm 0.07

* $P < 0.05$, Tukey test.

detected, as shown by measurements of LDH released in the culture medium (Table 3, Fig. 3). In fact, LDH levels evaluated in culture medium of *Epilobium* treated cells were not statistically different from those determined for control group.

The present preliminary findings demonstrate for the first time a specific and significant anti-proliferative effect of *E. angustifolium* extract on human epithelial prostatic cells and may justify, at least partially, the employment of *Epilobium* extracts in the traditional medicine for the prevention and treatment of BPH.

Recently a similar approach with prostate cell lines was used to demonstrate the anti-proliferative effect of an *Urtica dioica* extract [16].

The anti-proliferative effect of the *E. angustifolium* extract tested against PZ-HPV-7 cells may be likely attributed to various (and perhaps yet unidentified) compounds acting through different biochemical mech-

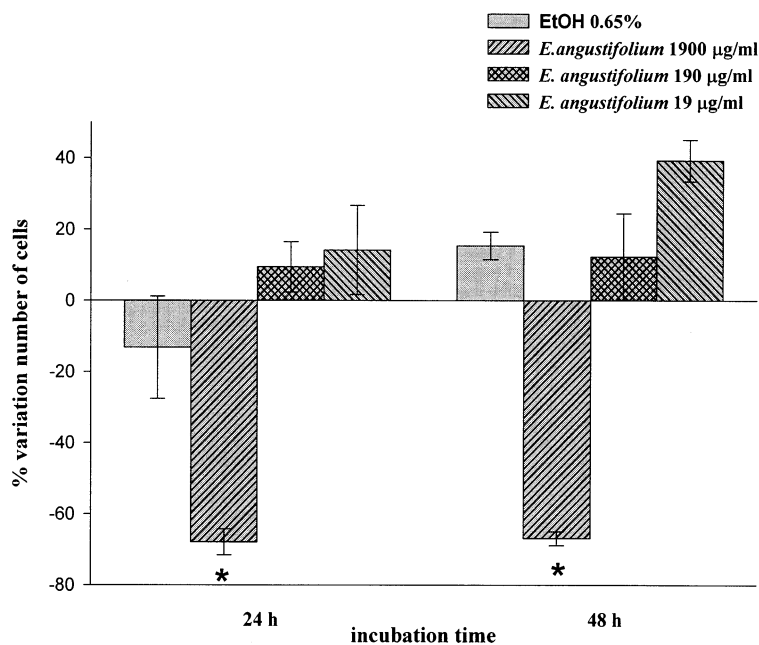


Fig. 1. Effect of *Epilobium angustifolium* extract on proliferation of PZ-HPV-7 cells after 24 and 48 h incubation, counted with a microscope. * $P < 0.05$, Tukey test.

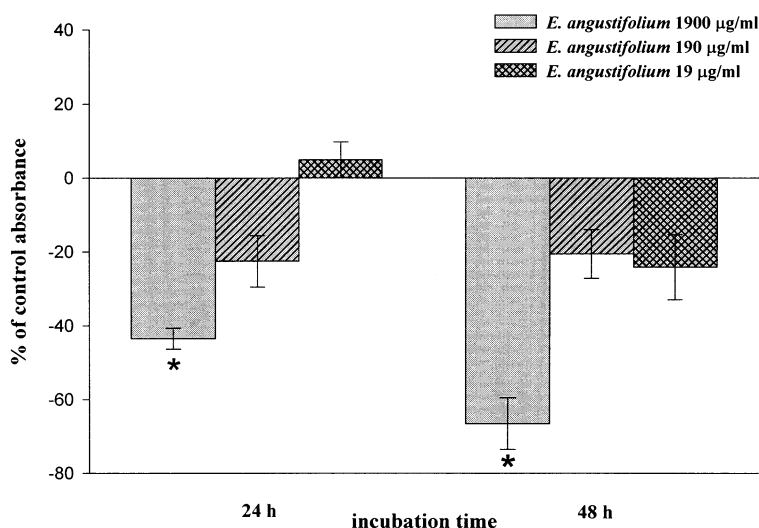


Fig. 2. Effect of *Epilobium angustifolium* extract on proliferation of PZ-HPV-7 cells after 24 and 48 h incubation, as measured by MTT viability assay. * $P < 0.05$, Tukey test.

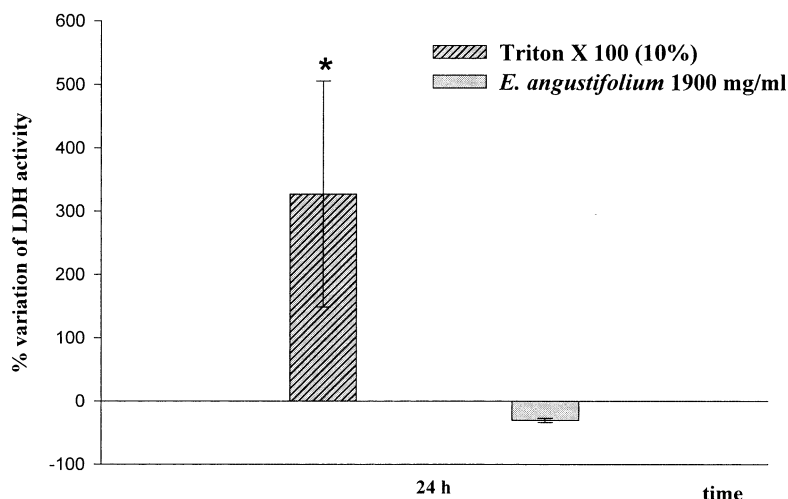


Fig. 3. Effect of *Epilobium angustifolium* extract on proliferation of PZ-HPV-7 cells after 24 h incubation, as measured by LDH assay. * $P < 0.05$, Tukey test.

anisms. Several hypotheses may be suggested to explain this inhibition of prostatic cell growth.

Firstly, the *Epilobium* extract could interfere in vitro with 5- α -reductase and aromatase, which are known to have a part in the development of prostatic hyperplasia. In fact, *Epilobium* has been shown to contain two macrocyclic ellagitannins, oenothin A and oenothin B, endowed with strong inhibitory activity against 5- α -reductase and aromatase, and several flavonoids are able to significantly inhibit aromatase (although inactive against 5- α -reductase) [14]. Consistently with this hypothesis, finasteride, a 5- α -reductase inhibitor, was shown to reduce proliferation of LNCaP cells [17].

Secondly, several flavonoids proved to be potent growth inhibitors when tested on various hormone-sen-

sitive and non-sensitive cell lines [18,19], although there are very few data in literature concerning a possible effect of these phenols on prostatic cells. Agarwal et al.

Table 3

Effect of *Epilobium angustifolium* extract after 24 h of exposure on proliferation of PZ-HPV-7 cells as measured by LDH assay. Data are reported as mean value \pm SEM

	LDH activity (U/l) 24 h
Control	38.5 \pm 5.2
<i>E. angustifolium</i> 1900 µg/ml	26.8 \pm 1.3
Triton \times 100 (10%)	164.6 \pm 68.7 *

* $P < 0.05$, Tukey test.

[20] have reported that an extract from grape seeds, rich in procyanidins, induces a growth-inhibitory and cell-death effect on human prostate carcinoma cells. Thus one could speculate that flavonoids contained in the *Epilobium* extract tested might directly inhibit proliferation of PZ-HPV-7 cells.

Third, some flavonoids exhibit significant steroid activity [21]. The phytoestrogen genistein (an isoflavone) proved to inhibit in vitro the growth of prostatic cells [22,23]. Thus, the observed anti-proliferative effect might be related to the estrogenic activity of the flavonoids contained in the *Epilobium* extract under investigation.

Finally, vitamin E and other antioxidants have been found to modulate human prostate cancer cell proliferation by altering apoptosis in proliferating cells [24]. The well-recognized antioxidant effectiveness of the polyphenolic compounds present in the *Epilobium* extract tested might contribute to explain the anti-proliferative effect. Similarly, Jonas et al. [25] attributed the ability of cactus flower extracts to exert an effect on BPH to their antioxidant components.

In conclusion, in this preliminary study we have demonstrated that ethanolic extract of *E. angustifolium* is active in suppressing the growth of human epithelial prostate cells; this anti-proliferative activity could be responsible for a beneficial effect in BPH treatment. Considering these results, the use in traditional medicine of *E. angustifolium* extract as a remedy against BPH seems to be justified. Moreover, it may possess a more favourable therapeutic profile over hormonal and non-hormonal treatment in patients with BPH. However, further experimental studies are needed to determine the biochemical mechanism of action and the clinical value of *E. angustifolium*.

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