

EXPERIMENTAL PAPER

SRC kinase mRNA transcription changes in testosterone-induced rat ventral prostate lobes under the influence of *Epilobium angustifolium* extract

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## Summary

The aim of this study was to investigate the influence of standardized crude aqueous *Epilobium angustifolium* L. extract [100 mg/kg/day, p.o.] on the expression level of SRC kinase mRNA - a representatives of non-genomics xenobiotics signaling pathway in prostate ventral lobes of testosterone-induced, castrated rats. We have shown that in all analyzed groups induced by testosterone an elevation of SRC kinase mRNA transcription was observed, in comparison to control animals (not receiving the testosterone), ( $p < 0.05$ ). Finasteride in rats induced by testosterone caused the strongest inhibition of SRC mRNA transcription ( $p < 0.05$ ). In rats receiving testosterone and the plant extract a ca. 90% decrease of mRNA level was observed vs. testosterone-induced animals ( $p < 0.05$ ), while in testosterone-induced animals receiving concomitantly *E. angustifolium* extract and finasteride the observed reduction reached 87.3% ( $p < 0.05$ ).

We did not observed, however, any positive feedback between studied plant extract and finasteride in the inhibitory activity ( $p < 0.05$ ). Further experimental studies should be performed in order to the understanding the molecular basis of interactions, the efficacy and safety of tested plant extract.

**Key words:** *hormonally induced prostate hyperplasia, rats, BPH, Epilobium angustifolium, SRC kinase, androgen receptor non-genomics signaling pathway*

## INTRODUCTION

One of the examples of herbal materials, with potential antiproliferative, anti-inflammatory, antioxidant properties [1-6], being usefull in the prevention and complementary treatment in the early stages of benign prostatic hyperplasia (BPH) are *Epilobium* sp. representatives [7-11], especially following species: *E. angustifolium* L., *E. parviflorum* Schreb. and *E. hirsutum* L. [1, 4, 6]. Individual members of the *Epilobium* genus and extracts obtained from these plants are very well phytochemically characterized [1, 6, 7, 11, 12]. Although, according to current knowledge, the influence of *Epilobium* sp. various extracts, fractions and their bio-active metabolites on androgens/estrogens related enzymes and apoptosis pathways proteins i.e. 5- $\alpha$ -steroid reductase and aromatase [13, 14], capsase 3 [6], estrogen receptors [15] and several metalloproteinases activities [16] have been previously noticed, the exact molecular mechanism of action of *Epilobium* sp. preparations, especially in a prostate gland, still remains not fully known.

Research results obtained in past two decades, predominantly carried out in cell cultures [17-19], indicate that androgens (progesterone) and estrogens as well, in prostate cells, apart from androgen receptor-related a classical – so-called genomic signaling pathway, can cause some cellular and phenotypic effects indicating the existence of other, so-called nongenomic pathway of steroid hormones action in the prostate [19-22]. This signaling pathway is characterized by speed indicating a lack of transcription and translation from androgen-responsive genes [20-22]. In this signaling cascade a number of protein kinases are involved,

including *Ras/Raf/MAPK (ERK1/2)* kinases [18, 19, 23, 24], as well as non-repceptor tyrosine *SRC* kinases [25].

In this study we have tried to assess the impact of a crude aqueous extract from *Epilobium angustifolium* leaves on the *SRC* kinase mRNA (a representative of one of the non-genomic cellular signal transduction protein) in prostate ventral lobes of testosterone-induced, castrated rats. Subsequently, the observed expression changes were compared with changes in rat prostate weights. Understanding the molecular basis of interactions caused by tested plant extracts in a rat model of hormone-induced prostatic hyperplasia may, in longer period, significantly contribute to the establishment of their effective and safe doses in the prevention and symptomatic treatment of BPH.

## MATERIAL AND METHODS

### Plant extract preparation

Standardized dried water extract from a herb of *Epilobium angustifolium* (0.91% m/m flavonoglycoside compounds expressed as quercetin, 24.36% m/m phenolic compounds expressed as gallic acid, 0.09% m/m sterol compounds expressed as  $\beta$ -sitosterol and 0.01% m/m tannin compounds expressed as pyrogallol) was obtained in the Institute of Natural Fibers and Medicinal Plants (Poland).

### Animals treatment

Adult male Wistar rats weighing between 180 and 250 g, four-week-old, were housed in plastic cages at the Department of Pharmacology, Poznan University of Medical Sciences. The animals were kept in a climate-controlled room with 12-h light/dark cycle and allowed access to a commercial rat chow and tap water ad libitum. They were acclimatized for at least a few days prior to experiment. All rats were castrated before the testosterone induction, divided by randomization into 7 groups (each group consisted of 10 animals). All substances were administered for 21 consecutive days.

The control group (control for finasteride treated animals) was treated with an arachidonic oil with dimethyl sulfoxide (DMSO) [40 mg/kg, each 7th day, s.c.] and PEG300 (2:8) [50 mg/kg, each 3th day, s.c.]. Another control group (K1) – was treated with an arachidonic oil with H<sub>2</sub>O and PEG400 [50 mg/kg/day; p.o.]. One group was administered with a dried aqueous extract of *Epilobium angustifolium* [100 mg/kg/day, p.o.]. Next four groups of rats were testosterone-induced in order to increase a prostate mass. Induced control rats received testosterone (*Testosteronum prolongatum*, 100 mg/ml, Jelfa; 40mg/kg/day, 3 times, each 7th day; s.c.) dissolved in arachidonic oil [25]. A “mixed” group represented a combined treatment of

rats with testosterone (*Testosteronum prolongatum* 100 mg/ml, Jelfa; 40mg/kg/day, 3 times, each 7th day; s.c.) dissolved in arachidonic oil and dried water extract of *Epilobium angustifolium* [100 mg/kg/day, p.o.]). Rats were also administered with testosterone (in above mentioned dose) and finasteride (Proscar, 5 mg tabl., Merck Sharp&Dohme, 0,2% water solution + PEG300 (2:8) [50 mg/kg/3 days; p.o.]. In animals from the last group – testosterone, finasteride (in abovementioned dosages) and *Epilobium angustifolium* extract [100 mg/kg/day, p.o.] were administered. Sixteen hours after the last administration, rats were decapitated and prostate ventral lobes were immediately weighted, frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until used. The experiment with rats was performed in accordance with Polish governmental regulations (01.21.2005, Dz.U. No.33;289) and in an agreement with Local Ethic Committee of the Use Laboratory Animals in Poznan (No. 54/2007).

## RNA isolation and reverse transcription reaction

Total RNA isolation was carried out using TriPure Isolation Reagent (Roche Applied Science, Germany) according to the manufacturer's protocol. The RNA pellet was washed with 70% ethanol and dissolved in DEPC water. The integrity of RNA was visually assessed by conventional agarose gel electrophoresis and the concentration was assessed by measuring the absorbance at 260 and 280 nm in a spectrophotometer (BioPhotometer, Eppendorf). RNA samples were stored at  $-80^{\circ}\text{C}$  until used. One  $\mu\text{g}$  of total RNA from all samples was reverse-transcribed into cDNA using Transcriptor cDNA First Strand Synthesis Kit (Roche, Germany) and oligo(dT)<sub>20</sub> primer (Roche, Germany) according to manufacturer's protocol. Obtained cDNA samples were stored at  $-20^{\circ}\text{C}$  or used directly for the quantitative real-time PCR (qRT-PCR) reaction.

## Real-time PCR assay

The genes expression level was analyzed by real-time quantitative PCR reaction using a LightCycler TM Instrument (Roche Applied Science, Germany) and a LightCycler Fast Start DNA Master SYBR Green I kit (Roche Applied Science, Germany) according to the instructions of the manufacturer. All primers sequences were designed using the Oligo 6.0 software (National Biosciences, USA), based on the sequence entries in the Genbank and synthesized from TIB Molbiol (TIB Molbiol Sp. z o.o., Poland). Primers sequences and specific PCR reaction conditions used for 5ar2, MAPK3, RafA and a reference gene – GAPDH expression measurement are described in table 1. Primer specificity was verified by the assessment of a single PCR product on agarose gel and single temperature dissociation peak (melting curve analysis) of GAPDH cDNA amplification product was used as a housekeeping

gene (endogenous internal standard) for normalization. The relative quantification for any given gene was expressed as a signal relative to the average signal value for the internal standard. A qRT-PCR reaction was carried out using a SYBR Green I in a reaction volume of 10  $\mu$ l reaction mixture. Standard curves were prepared from dilution of cDNA and generated from a minimum of four data points. Complementary DNA was quantified by comparison of the number of cycles required for amplification of unknown samples with those of series of cDNA standard dilutions. All quantitative PCR reactions were repeated twice. The data were evaluated using LightCycler Run 5.32 software (Roche Applied Science, Germany).

Table 1.

Sequences of oligonucleotide primers and reaction conditions used for the qRT-PCR analysis. All primers were designed using the Oligo 6.0 software (National Biosciences)

Genes/Primers	Primer sequence (5' → 3')	Product size (bp)	qRT reaction conditions			
			Cycle	Denaturation	Annealing	Elongation
GAPDHF	GATGGTGAAGGTCGGTGTG	108	30	95°C, 4 s.	56°C, 8 s.	72°C, 8 s.
GAPDHR	ATGAAGGGGTCGTTGATGG					
SRC-F	AGAACTGGGAATGGAGGAA	210	45	95°C, 4 s.	58°C, 6 s.	72°C, 8 s.
SRC-R	AGAATTATCTAAACCGTCGAG					

## Statistical analysis

The results were expressed as means  $\pm$  SEM. Statistical significance of the difference between the control and experimental group was assessed by SPSS 17.0 software (IBM Corporation, USA) using one-way ANOVA test and Fischer LSD post-hoc test. The values of  $p < 0.05$  were considered as a statistically significant difference.

## RESULTS

### Influence of *Epilobium angustifolium* extract on prostates weights in rats

It was shown that the testosterone induction caused the largest increase of rat prostate weights (induction over 100%) ( $p < 0.05$ ), while the administration with the *E. angustifolium* extract caused a statistically insignificant (19.5%) decrease of prostate weights ( $p > 0.05$ ), in comparison to control group, respectively. In practice, testosterone-induced rats the presence of the studied extract did not affect the changes of prostate weights (only ca. 4% reduction was observed) ( $p > 0.05$ ), in comparison to control testosterone-induced rats. In animals administered with testosterone and

finasteride; there was also no significant reduction in prostates weight (a decrease by 8% vs. testosterone administered rats) ( $p < 0.05$ ). The administration of finasteride and tested plant extract in animals injected with testosterone-induced caused a reduction in weights by ca. 25%, in comparison to testosterone alone induced rats ( $p < 0.05$ ). The percentage prostate weights changes are summarized in table nr 2.

Table 2.

Percentage changes in rat prostate weights between different groups (n=10)

Group	Median weight of prostates [mg]	Percentage change in weight compared to the control group
*K1	633.8 ± 101.2	100%
*K2	747.8 ± 52.9	100%
E	602.2 ± 40.3	80.5%
*T	1538.6 ± 112.8	205.8%
*TE	1476.3 ± 88	195.9%
*TF	1416.7 ± 96.8	192%
*TFE	1155.6 ± 77.5	175.1%
Comparison of the groups compared to controls [%]		
T/K2		in. 105.8%
E/K2		dec. 19.5%
TE/T		dec. 4.1%
TF/T		dec. 8%
TFE/T		dec. 24.9%

“in” – increase of prostate weight; “dec” – decrease of prostate weight. K1, K2 – control groups; E – animals administered with *E. angustifolium* extract; T – testosterone administered animals; TE – testosterone + *E. angustifolium* administered rats; TF – testosterone with finasteride administered animals; TFE – testosterone with *E. angustifolium* and finasteride administered animals; \*  $p < 0.05$ .

## Influence of the *Epilobium angustifolium* extract on the SRC kinase mRNA expression in rat prostate ventral lobe

In rats receiving *E. angustifolium* extract an over 2.5-fold induction (by ca. 251%) of SRC kinase mRNA transcription was observed, in comparison to control animals. The induction with testosterone caused the strongest elevation of mRNA level – over 94.3-fold vs. the control group. The induction of this mRNA expression caused by tested plant extract was nearly 94-times (ca. 94092%) weaker than its increase caused by testosterone injection. The concomitant administration of rats with testosterone and finasteride caused the strongest inhibition of SRC mRNA transcription – a ca. 96% reduction of its level was observed, in comparison to testosterone (control) administered animals ( $p < 0.05$ ). In rats receiving

testosterone and the plant extract a ca. 90% decrease of mRNA level was observed vs. testosterone-induced animals ( $p < 0.05$ ), while in testosterone-induced animals receiving concomitantly *E. angustifolium* extract and finasteride the observed reduction reached 87.3% vs. testosterone (control) administered rats ( $p < 0.05$ ). The difference in mRNA transcription inhibition between testosterone-induced rats receiving *E. angustifolium* extract alone or with finasteride was 2.2%.

## DISCUSSION

Despite the growing number of studies explaining the mechanism of action of *Epilobium* sp. extract in prostate cells, conducted mainly in *in vitro* models [1, 3, 5, 27-29], the question whether the aqueous extract from the herb of *Epilobium angustifolium* acts in enlarged prostate cells via one of members of androgen non-genomic signaling pathway remained without answer. Several studies have shown the influence of various extracts or fractions of *Epilobium* sp. on 5- $\alpha$ -steroid reductase and aromatase activity [13, 14], caspase 3 [6], metalloproteinases [30] and other [31].

Up to day, no studies were aimed to determine the potential role of the SRC kinase in the molecular mechanism of action of *E. angustifolium* extract or its bioactive metabolites were carried out. Rat prostate weights changes with studied mRNA transcription profile changes in each examined group of animals strongly indicates a hormonal (androgen)-dependence of prostate gland. In all analyzed groups induced by testosterone an elevation of SRC kinase mRNA transcription was observed, in comparison to control animals (not receiving the testosterone) ( $p < 0.05$ ). In our opinion, the observed increase may probably be due to an excess of exogenous testosterone in prostate gland cells strongly involved in a nongenomic signaling pathway via i.e. inducing studied SRC kinase mRNA transcription changes. The answer to the question whether the elevated levels of SRC mRNA in all testosterone induced enlarged prostate ventral lobes is associated with changes of SRC kinase activity (phosphorylation) requires further experiments.

Observations of rat prostate weights changes along with changes of studied mRNA transcription profile in each examined group of animals strongly indicates a hormonal (androgen)-dependence of prostate gland. In all analyzed groups induced by testosterone an elevation of SRC kinase mRNA transcription was observed, in comparison to control animals (not receiving the testosterone) ( $p < 0.05$ ). In our opinion, the observed increase may probably be due to an excess of exogenous testosterone in prostate gland cells strongly involved in a nongenomic signaling pathway via i.e. inducing studied SRC kinase mRNA transcription changes. The answer to the question whether elevated levels of SRC mRNA in all testosterone induced enlarged prostate ventral lobes is associated with changes of SRC kinase activity (phosphorylation) requires further experimental work. So far, no studies were aimed to determine the potential role of the SRC kinase in the

molecular mechanism of action of *E. angustifolium* extract or its bio-active metabolites were carried out.

It is well known that finasteride is a weak, reversible inhibitor of 5- $\alpha$ -steroid reductase type 1 (*Srd5aR1*) and a potent, time-dependent inhibitor of type 2 protein [32-35]. Although there have been recent advances in developing mechanistic models for the 5- $\alpha$ -steroid reductase inhibition by finasteride and subsequent effects on the prostate [36-38] the impact of this drug on the studied kinase activity remained unknown. In our study we have observed a strong inhibition (by ca. 96%) of the SRC mRNA transcription in animals receiving finasteride with testosterone vs. rats receiving the testosterone only (control) ( $p < 0.05$ ). The transcriptional regulation of SRC kinase gene by finasteride is still unknown, so our results are the first published. We have observed also a strong inhibition of studied mRNA expression level in testosterone-induced rats receiving *E. angustifolium* extract ( $p < 0.05$ ), however, this effect was weaker than the inhibition of transcription caused by finasteride in animals administered with hormones ( $p < 0.05$ ). We did not observe, however, any positive feedback between studied plant extract and finasteride in the inhibitory activity ( $p < 0.05$ ).

Furthermore, extract from *E. angustifolium* at tested dose showed only a slight tendency to reduce prostate weight in testosterone-induced rats ( $p > 0.05$ ), as well as in rats administered concomitantly with testosterone and finasteride ( $p < 0.05$ ) (fig. 1). In our opinion, it is possible that the strengthening of *E. angustifolium* extract inhibitory effect by finasteride may be due to a potential synergistic interaction between present in this herbal substance bio-active metabolites belonging to the fraction of flavonoids and/or tannins. The explanation of such relationships requires further experimental work.

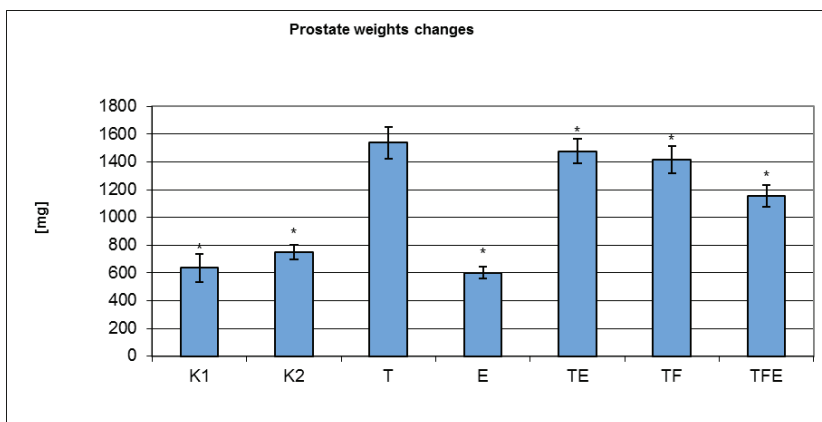


Figure 1.

Rat prostate weights changes [mg] in different groups (n=10)

K1, K2 – control groups; E – animals administered with *E. angustifolium* extract; T – testosterone administered animals; TE – testosterone + *E. angustifolium* administered rats; TF – testosterone with finasteride administered animals; TFE – testosterone with *E. angustifolium* and finasteride administered animals; \* $p < 0.05$ .



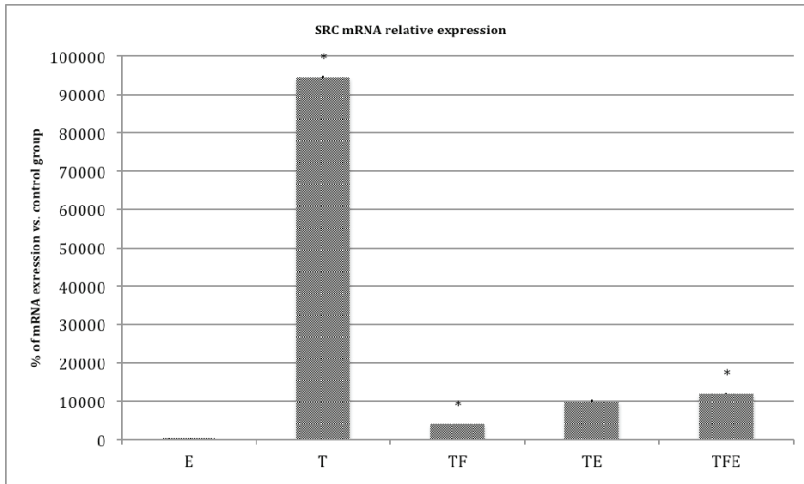


Figure 2.

Percentage in SRC kinase mRNA expression level in the ventral lobe of rat prostates under the influence of *Epilobium angustifolium* extract

E – animals administered with *E. angustifolium* extract; T – testosterone administered animals; TE – testosterone + *E. angustifolium* administered rats; TF – testosterone with finasteride administered animals; TFE – testosterone with *E. angustifolium* and finasteride administered animals; \* $p < 0.05$ . All groups (n = 10) were normalized vs. control.

## CONCLUSIONS

Changes of rat prostate weights observed in our experimental model as well as studied level of mRNA in each examined group of animals strongly indicates a hormonal (androgen)-dependence of prostate gland.

Observed in our experimental model statistically insignificant slight tendency to the reduction of prostate weights in hormonally induced animals treated with *E. angustifolium* extract ( $p > 0.05$ ) and in testosterone-induced animals receiving both, extract and finasteride ( $p < 0.05$ ); a weak inhibition of 5- $\alpha$ -steroid reductase 2 (*Srd5ar2*) mRNA synthesis in testosterone-induced rats; inversely proportional relationship between *Srd5ar2* and *MAPK3* mRNA expression profile of in both testosterone-induced groups of animals receiving *E. angustifolium* extract with or without finasteride due to the presence of the exogenous testosterone used in this study and potential interactions between bio-active metabolites of these extracts and testosterone or finasteride can be studied. The evaluation of efficacy and safety of studied plant extract in the prevention and symptomatic treatment of BPH requires further research.

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#### ZMIANY LICZBY TRANSKRYPTÓW KINAZY SRC W BRZUSZNYM PŁACIE PROSTATY SZCZURÓW INDUKOWANYCH HORMONALNIE POD WPŁYWEM WYCIĄGU Z ZIELA *EPILOBIUM ANGUSTIFOLIUM*

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## Streszczenie

Celem pracy było zbadanie wpływu standaryzowanego ekstraktu z zioła *Epilobium angustifolium* L. [100 mg/kg/dzień, p.o.] na poziom transkrypcji mRNA kinazy SRC - przedstawiciela androgenozależnego, niegenomowego szlaku sygnalizacji komórkowej w brzusznych płacie prostaty indukowanych hormonalnie, kastrowanych szczurów. We wszystkich grupach szczurów indukowanych testosteronem zaobserwowaliśmy wzrost liczby transkryptów kinazy SRC, w porównaniu do zwierząt kontrolnych ( $p < 0.05$ ). U szczurów indukowanych hormonalnie finasteryd wykazał najsilniejsze właściwości hamujące transkrypcję mRNA ( $p < 0.05$ ). U zwierząt otrzymujących testosteron wraz z wyciągiem z *E. angustifolium* stwierdziliśmy obniżenie liczby badanych transkryptów o blisko 90%, w porównaniu ze zwierzętami z grupy kontrolnej, indukowanymi testosteronem ( $p < 0.05$ ). U szczurów otrzymujących jednocześnie testosteron i ekstrakt roślinny łącznie z finasterydem redukcja poziomu mRNA kinazy SRC sięgała 87.3% ( $p < 0.05$ ). Nie zaobserwowaliśmy dodatniego sprzężenia w inhibicji transkrypcji badanego mRNA u szczurów otrzymujących badany ekstrakt wraz z finasterydem ( $p < 0.05$ ). Istnieje potrzeba przeprowadzenia dalszych badań zmierzających do wyjaśnienia molekularnego podłoża interakcji, mechanizmu działania oraz skuteczności badanego wyciągu roślinnego.

**Słowa kluczowe:** indukowany hormonalnie rozrost prostaty, szczury, BPH, *Epilobium angustifolium*, kinaza SRC, niegenomowy szlak sygnalizacji wewnątrzkomórkowej zależnej od receptora androgenowego