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EXPERIMENTAL PAPER

Influence of *Epilobium angustifolium* extract on 5α-reductase type 2 and MAPK3 kinase gene expression in rats prostates

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Summary

The aim of this study was to investigate the influence of standardized *Epilobium angustifolium* L. extract [100 mg/kg/day, p.o.] on the expression level of 5α-reductase type 2 (*Srd5ar2*) mRNA and *Mapk3* mRNA a representative of non-genomic xenobiotics signaling pathway. It was shown that plant extract from the *E. angustifolium* showed a slight tendency to reduce prostate weight in hormonally induced animals ($p>0.05$) and in testosterone induced animals receiving both, extract and finasteride ($p<0.05$). Finasteride in rats induced by testosterone caused a smaller decrease in the level of mRNA 5α-steroid reductase 2 (*SRd5ar2*), than in rats treated with the hormone and studied plant extracts. In general, an increase in the amount of *MAPK3* mRNAs in testosterone-induced groups of rats receiving tested plant extract with or without finasteride was observed, while the expression of type 2 5α-steroid reductase decreased ($p<0.05$). Further experimental studies should be performed in order to understand the molecular basis of interactions, the efficacy and safety of tested plant extracts.

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

INTRODUCTION

Benign prostatic hyperplasia (BPH) and lower urinary tract symptoms (LUTS) associated with these disease are very serious health and socio-economic problems of modern society. Due to numerous side effects, in consequence of BPH/LUTS long-term pharmacotherapy [1, 2], there is a growing interest in searching for alternative forms of therapy, in particular with use of preparations of plant plant origin [3-6].

One of herbal materials traditionally used in form of infusions in case of prevention and complementary treatment in early stages of benign prostatic hyperplasia (BPH) are *Epilobium* sp. representatives [6-9]. The most popular European species of this genus are species as follows: *E. angustifolium* L., *E. parviflorum* Schreb. and *E. hirsutum* L. [9-12]. Individual members of the *Epilobium* genus and extracts obtained from these plants are very well phytochemicaly characterized [6, 11-15]. The vast majority of studies highlightes an antiproliferative, antiinflammatory and antioxidant potential of *Epilobium* sp. representatives [16-23]. The influence of *Epilobium* sp. various extracts, fractions and their bioactive metabolites on androgen/estrogen-related enzymes and apoptosis pathways proteins, i.e. 5α-steroid reductase and aromatase [24-25], caspase 3 [11], estrogen receptors [26] and several metaloproteinases activities [14,27] has been described previously. These and other androgen-dependent proteins, usually directly related to the activity of the androgen receptor (AR), belong to so-called genomics signaling pathway [28-29]. However, the molecular mechanism of action of any *Epilobium* sp. preparations, their bio-active metabolites

on the activity of the genes or proteins involved in a second one, a non-genomic signaling pathway of steroid hormones in prostate gland cells is still not known, in particular in a model organism. This cellular signaling pathway is characterized by speed indicating a lack of transcription and translation from androgen-responsive genes [29-32]. In this signaling cascade a number of protein kinases are involved, including *MAPK* (*ERK1/2*) kinases (in rats*: MAPK3*/*MAPK1*, respectively) [28-29, 33-34].

In this study, an attempt was made to assess the impact of a crude aqueous extract from *Epilobium angustifolium* leaves on 5α-reductase type 2 mRNA (SRd5ar2 – a prostate enzyme isoform crucial for the BPH development, generating dihydrotestosterone from the testosterone) [35-37] and MAPK3 kinase mRNA (a representative of one of the non-genomic cellular signal transduction proteins) [33-34] in testosterone-induced rats prostate ventral lobes. Subsequently, observed expression changes were compared with changes in rats prostates weight. Understanding the molecular basis of interactions caused by tested plant extracts in a rat model of hormone-induced prostatic hyperplasia may, in the 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 aqueous extract from of *Epilobium angustifolium* herb (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 β-sitosterol and 0.01% m/m tannin compounds expressed as pyrogallol) was obtained from the Institute of Natural Fibers and Medicinal Plants, Poznań, Poland).

Animals treatment

Adult male four-week-old Wistar rats (180–250 g) were housed in plastic cages at the Department of Pharmacology, Poznan University of Medical Sciences. Animals were kept in a climate-controlled room with twelve-hour light/dark cycle and allowed the access to commercial rat chow and tap water *ad libitum*. They were acclimatized for at least a few days prior to experiment. All rats were 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 H2O and PEG400 [50mg/kg/day; p.o.]. One group was administered with a dried aqueous extract of *Epilobium angustifolium* [100 mg/kg/day, p.o.]. Following four groups of rats were testosterone-induced in order to increase prostate mass. Induced control rats received a testosterone (*Testosteronum prolongatum*, 100 mg/ml, Jelfa; 40mg/kg/day, 3 times, each 7th day; s.c). dissolved in arachidonic oil [38]. A "mixed" group of rats represented a combined treatment 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 tbl., Merck Sharp & Dohme, 0,2% water solution + PEG300 (2:8) [50 mg/kg/3 days; p.o.]. In animals from the last group testosterone (in above mentioned dose), finasteride 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 –80ºC 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 measured by a measuring the absorbance at 260 and 280 nm in a spectrophotometer (BioPhotometer Eppendorf). RNA samples were stored at –80°C until used. The 1 μg of total RNA from all samples was reverse-transcribed into cDNA using Transcriptor cDNA First Strand Synthesis Kit (Roche, Germany) and oligo(dT)20 primer (Roche, Germany) according to manufacturer's protocol. Obtained cDNA samples were stored at –20°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 *5Ra5ar2, MAPK3,* and a reference gene - GAPDH expression measurement are described in a table 1 (tab. 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 μl 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.

Genes/ Primers	Primer sequence (5' → 3')	Product size (bp)	qRT reaction conditions			
			Cycle	Denaturation	Annealing	Elongation
GAPDHF	GATGGTGAAGGTCGGTGTG	108	30	95ºC, 4 s.	56ºC, 8 s.	72ºC, 8 s.
GAPDHR	ATGAAGGGGTCGTTGATGG					
Srd5ar2F	AGAACTGGGAATGGAGGAA	210	45	95ºC, 4 s.	58ºC, 6 s.	72ºC, 8 s.
Srd5ar2R	AGAATTATCTAAACCGTCGAG					
MAPK3F	ACGACCACACTGGCTTTCTT	120	45	95ºC, 8 s.	58ºC, 5 s.	72ºC, 8 s.
MAPK3R	GATTTGGTGTAGCCCTTGGA					

All primers were designed using the Oligo 6.0 software (National Biosciences).
F – forward primer
R – reverse primer

Statistical analysis

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

RESULTS

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

It was shown that the testosterone induction caused the largest increase of rat prostates weight (induction over 100%) ($p>0.05$), while the administration with the *E. angustifolium* extract caused a statistically insignificant 19.5% decrease of prostate weights ($p<0.05$), as compared to control group, respectively. In testosterone-induced rats the presence of the studied extract did not affect prostate weights (a reduction of only ca. 4% was observed) ($p<0.05$), in comparison to control testosterone-induced rats. In animals administered with the testosterone and finasteride also there was no significant reduction in prostates weight (a decrease by 8% *vs.* testosterone administered rats) ($p<0.05$). The administration of finasteride-tested plant extract in animals injected with testosterone-induced the rise in prostate weights by ca. 25%, in comparison to the only testosterone-induced rats ($p<0.05$). The percentages of prostate weights changes are summarized in table 2.

Table 2.

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

Group	Median weight of prostates [mg] ± [SEM]	Percentage change in weight compared to 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 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; Data were presented as mean ±SEM in each group. *$p<0.05$ as compared with control group (one-way ANOVA test)

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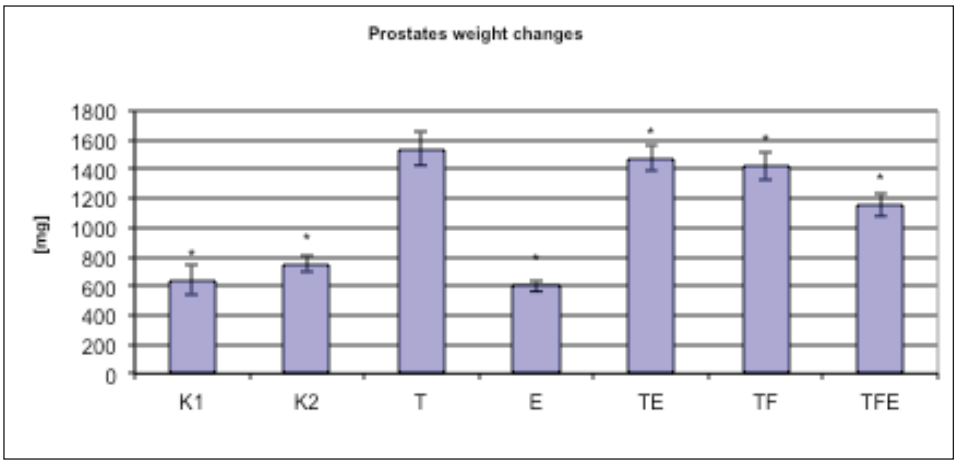

Figure 1.

Rat prostates weight 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. Data presented as mean ±SEM in each group. $^{*}p<0.05$ as compared to control group (one-way ANOVA test)

Influence of *Epilobium angustifolium* extract on 5α-steroid reductase type 2 mRNA expression in rat prostate ventral lobe

In rats receiving *Epilobium angustifolium* extract a statistically insignificant strong induction (over 41-fold) of the 5α-steroid reductase type 2 mRNA (*SRd5ar2*) transcription was observed, in comparison to the control group ($p>0,05$). The highest induction of SRd5ar2 mRNA expression was observed in testosterone-induced rats – over 1244-fold vs. the control group ($p<0.05$). Also in animals administered both with testosterone and studied extract, a decrease of SRd5ar2 mRNA level (by ca. 88%) was observed, in comparison to testosterone-only induced rats (control) ($p<0.05$). The inhibitory effect of *E. angustifolium* extract was more potent than inhibition of *SRd5ar2* mRNA transcription observed in testosterone-induced rats receiving finasteride (reduction ca. 61%) ($p<0.05$). Surprisingly, the administration with *E. angustifolium* extract and finasteride in testoterone induced rats caused a similar reduction (by ca. 81%) of the mRNA transcription, when compared to rats injected with testosterone ($p<0.05$). The differences of the mRNA level in each analyzed group were presented in a figure 2.

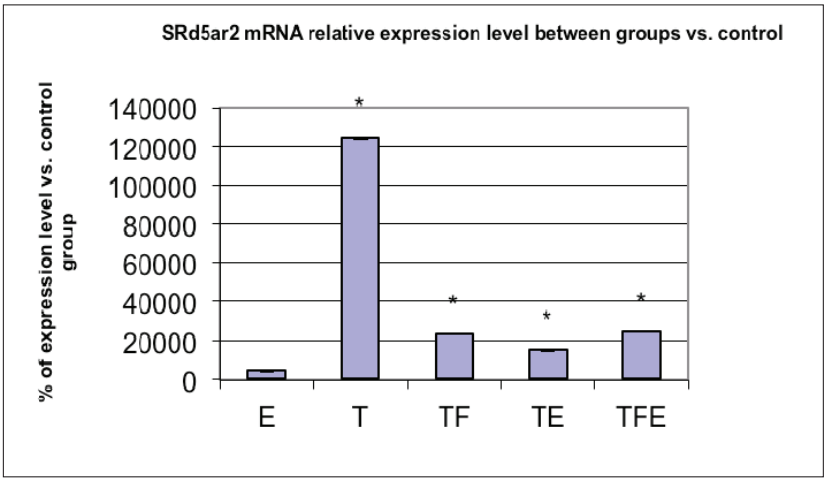

Figure 2.

Changes in mRNA expression level of 5α-steroid reductase type 2 (5ar2) in the ventral lobe of rat prostates influenced with *Epilobium angustifolium* extract.

K – control group; 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; Data were presented as mean ±SEM in each group. $^{*}p<0.05$ as compared with th control group (one-way ANOVA test)

Influence of *Epilobium angustifolium* extract on MAPK3 mRNA expression in rat prostate ventral lobes

In our study, the level of *MAPK3* mRNA in animals administered with testosterone and *E. angustifolium* extract strongly increased - over 18.5-fold and 4.6-fold, respectively ($p<0.05$), in comparison to control animals. The presence of finasteride in testosterone-induced animals caused a decrease of *MAPK3* mRNA level by 37%, in comparison to rats receiving testosterone (control) ($p<0.05$). Surprisingly, the mRNA level increased (by 20%) in testosterone-induced rats administered with studied extract ($p<0.05$). In animals administered with testosterone the strongest induction of *MAPK3* mRNA transcription was observed in rats receiving concomitantly *E. angustifolium* extract and finasteride (induction by ca. 71%), in comparison to rats receiving testosterone ($p<0.05$). The differences of the mRNA level in each analyzed group were presented in a figure 3.

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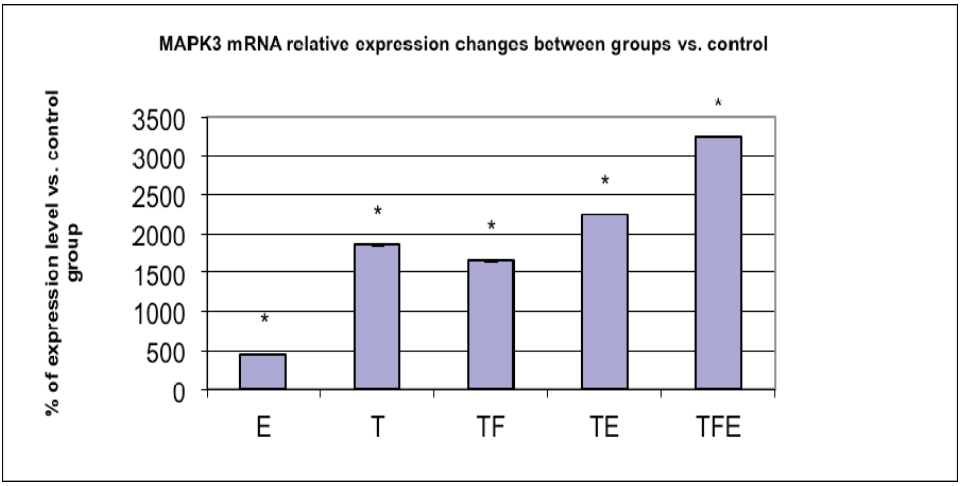

Figure 3.

Fold changes in mRNA expression level of MAPK3 kinase in the ventral lobe of rat prostates influenced by *Epilobium angustifolium* extract.

K – control group; 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. Data were presented as mean ±SEM in each group. $^{*}p<0.05$ as compared to control group (one-way ANOVA test)

DISCUSSION

Our results confirmed the androgen-dependent nature of transcriptional machinery regulating the mRNA synthesis of studied genes, which can be seen in particular in the group of testosterone-induced rats. In general, for all two tested genes (5α-reductase type 2 (*SRd5ar2*) and *MAPK3* kinase), an induction of studied mRNA transcription in the ventral lobe of the prostate vs. the control animals was observed [fig. 2, 3]. Changes in maintaining the ability of prostate cells to proliferate under the influence of the *Epilobium* sp. extracts were conducted mainly in *in vitro* model [9, 13, 17-19, 39-40]. Only several studies have shown the inhibition of the activity of 5α-steroid reductase under the influence of various extracts or fractions of *Epilobium* sp. [24-25].

From our point of view, it is not clear why finasteride in rats administered with testosterone (an experimental induction of prostate growth) caused a smaller decrease in the level of mRNA 5α-steroid reductase 2 (by ca. 61%), than in rats treated with the hormone and studied plant extract (reduction by ca 88%), as compared to the control rats administered only with this androgen ($p<0.05$). It is well known that finasteride is a weakly competitive, reversible inhibitor of 5α-steroid reductase type 1 (*SRd5aR1*) and a potent, time-dependent inhibitor of

type 2 protein (*Srd5aR2*) [41-42]. Although there have been recent advances in developing of mechanistic models of the 5α-steroid reductase inhibition by finasteride and subsequent effects on the prostate [43-45], the impact of this azasteroid on this enzyme transcription regulation is still the subject of the research. On the basis of literature data, we assume that in the case of *E. angustifolium* extract tannins, in particular, oenothein B and/or A may be responsible for these observed activities [15,19,24-25]. It is possible that other bioactive compounds of this plant, like flavonoids and their derivatives, phytosterols and their derivatives as well as free fatty acids (mainly stearic, palmitic, oleic, linoleic, linolenic, and caproic) may cause the inhibition of 5α-steroid reductase 2 mRNA transcription [11, 24-25].

As already has been mentioned one of the key representatives of the AR mediated xenobiotics nongenomic signaling pathway are *ERK1/2* kinases [28]. In the *Rattus norvegicus* analogues of human kinases *ERK1/2* are *MAPK3* and *MAPK1*, respectively. The analysis of literature data (Pubmed and Medline databases) has not revealed to us the already published experimental results that may indicate the cause of the impact of studied plant extract on the *MAPK3* gene expression. It is therefore difficult, in our opinion, to clearly explain the general increase in the amount of *MAPK3* transcripts (by a 20%) in testosterone-induced rats receiving *E. angustifolium* extracts, compared with testosterone treated animals) [Fig. 3]. In our point of view that effect can also be explained with the differential influence of various bio-active substances, contained in the extract, on studied mRNA/or on the protein machinery regulating synthesis of these transcripts, which molecular background of interactions is still an issue that needs further research. The results obtained by our team in this subject are the first ever published.

It was shown that *E. angustifolium* extract 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 studies.

CONCLUSIONS

In our experimental model, a statistically insignificant slight tendency to reduce prostate weights in hormonally induced animals by the *E. angustifolium* extract ($p>0.05$) and in testosterone-induced animals receiving both, extract and finasteride ($p<0.05$) is observed. A weak inhibition of 5α-steroid reductase 2 (*Srd5ar2*) mRNA synthesis in rats induced by testosterone treated finasteride. Proportionally inverse relationship between *SRd5ar2* and *MAPK3* mRNA expression profile of in both testosterone-induced groups of animals, receiving *E. angustifolium* extract with

or without finasteride may occur due to the presence of the exogenous testosterone used in this study and potential interactions between bioactive metabolites present in this extract and testosterone or finasteride. The evaluation of efficacy and safety of studied plant extract in the prevention and symptomatic treatment of BPH requires further studies.

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WPŁYW WYCIĄGU Z *EPILOBIUM ANGUSTIFOLIUM* NA EKSPRESJĘ GENÓW 5A-REDUKTAZY TYPU 2 ORAZ KINAZY MAPK3 W SZCZURZYCH PROSTATACH

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S t r e s z c z e n i e

Celem pracy było zbadanie wpływu standaryzowanego ekstraktu z ziela *Epilobium angustifolium* L. [100 mg/kg/dzień, p.o.] na poziom ekspresji mRNA 5α-reduktazy typu 2 (*SRd5ar2*) oraz mRNA kinazy *MAPK3* – przedstawiciela androgenozależnego, nie-genomowego szlaku sygnalizacji komórkowej. W zastosowanym modelu eksperymentalnym wyciąg z z *E. angustifolium* wykazał statystycznie nieistotną, niewielką tendencję do zmniejszania masy prostat u zwierząt indukowanych hormonalnie ($p>0,05$) oraz u szczurów indukowanych testosteronem, otrzymujących zarówno ekstrakt, jak i finasteryd ($p<0,05$). Finasteryd u szczurów otrzymujących testosteron spowodował mniejsze, aniżeli zakładano, obniżenie poziomu mRNA 5α-reduktazy typu 2 (*SRd5ar2*), niż u szczurów, którym podano hormon i badany wyciąg ($p<0.05$). Stwierdziliśmy ponadto, zwiększenie ilości mRNA kinazy *MAPK3* u szczurów indukowanych testosteronem otrzymujących badany ekstrakt, wraz z finasterydem lub bez niego, podczas gdy ekspresja reduktazy w tych grupach uległa zwiększeniu ($p<0,05$). Należy przeprowadzić dalsze badania eksperymentalne w celu zrozumienia molekularnych podstaw oddziaływań, skuteczności i bezpieczeństwa badanych ekstraktów roślinnych.

Słowa kluczowe: *indukowany hormonalnie rozrost prostaty, szczury, BPH, Epilobium angustifolium, ekspresja genów, niegenomowy szlak sygnalizcji wewnątrzkomórkowej zależnej od receptora androgenowego*