Effect of Willow herb (*Epilobium angustifolium* L.) extract on gene expression of selected P450 cytochromes in rat liver – preliminary study

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Summary

One of alternative strategies in prevention and therapy of Benign Prostatic Hyperplasia (BPH) is the usage of a number of traditional herbal plants. In recent years, there is an increasing interest in some members of the *Epilobium* genus in the treatment of BPH. So far there is no data about molecular mechanisms of action of *Epilobium angustifolium* L.

extracts in liver. The aim of this study was to investigate the influence of the standardized water extract from the *Epilobium angustifolium* L. on expression level of selected P450 cytochromes encoding genes (CYP3A1 and CYP7A1) in rats liver tissues, which protein products are involved in steroids metabolism. The most abundant fraction of active compounds obtained from the extract was a phenolic fraction (24.36% m/m phenolic compounds and small amounts of flavonoglycosides – 0.91% and sterols – 0.09%). The plant extract showed the strongest and significant inhibitory CYP3A1/2 mRNA expression effect in rat liver. Our results suggest that a standardized *E. angustifolium* extract can regulate transcription level of investigated *CYP* genes in a different manner since a strong inhibition of CYP3A1 mRNA expression and slight induction of CYP7A1 may be a result of different extract action on the investigated CYP's transcription machinery in rat liver cells.

Key words: *Benign Prostatic Hyperplasia (BPH), medicinal herbal plants, phytotherapy, Willow herb, Epilobium angustifolium, cytochrome P450, CYP3A1, CYP7A1, expression, interaction, synthetic drugs*

INTRODUCTION

Benign Prostatic Hyperplasia (BPH) is a common illness affecting 50% of men in age of 60 and 90% of men older than 90 years [1]. The results of many studies suggest that this illness developes with aging. As the prostate enlarges, the layer of tissue surrounding it stops it from expanding, leading to the gland to press against the urethra, narrowing it inner light and in consequence leading to occurrence of lower urinary tract symptoms (LUTS) i.e. poor urine stream, hesitancy, terminal dribbling, incomplete voiding, overflow incontinence, dysuria, nocturia and some sexual disorders negatively affecting men [1]. The aetiology of BPH is not fully understood. However, the similarity between BPH and the embryonic morphogenesis of the prostate has led to the hypothesis that BPH may result from a "reawakening" of embryonic induction processes in adulthood [2]. An important role in the prostate enlargement plays the steroid hormones equilibrium disturbance (androgens and estrogens) and an elevated level of the 5α-dihydrotestosterone (DHT) converted from testosterone by enzyme steroid 5α-reductase (3-oxo-5-alpha-steroid 4-dehydrogenase) occurring in two isoforms: type 1 and type 2 [2].

Classical therapeutic approach in BPH is the inhibition of 5α-reductase and aromatase activity and as a result is the inhibition of human prostate cells proliferation.

The use of synthetic drugs show a satisfactory effectiveness but their long-term use leads to high risk of adverse effects. An alternative strategy in the therapy of BPH, especially at the beginning of its progression as well as in prevention comprises the usage of a number of traditional herbal plants e.g.: *Serrenoa repens*, *Pygeum africanum*, *Urtica dioica, Cucurbita pepo* and *Zea mays*. In recent years there is an increasing interest in some plants of *Epilobium* sp. *Epilobium* genus (*Onagraceae* family) which contains about 185 species of flowering plants, native to temperate

and subarctic regions of both hemispheres [3]. Most species are known by the common name Willow herb. Tea or ethanolic extracts from fresh aerial parts of this plant are commonly used in folk medicine for the treatment of prostatic disorders, including BPH, and inflammatory states of urethra as well as mucous membranes of stomach and intestines [4, 5]. The pharmacological and clinical standardization of commercially available *Epilobii* herba (Willow herb) is still difficult. The mechanism of action of these natural herbal drugs is not clear but have been generally ascribed to the inhibition of 5α-reductase and aromatase enzymes, antiandrogenic and antiestrogenic activities, anti-inflammatory properties and inhibition of cell proliferation [6-8]. Biologically active compounds of *Epilobium* sp. extracts are not well known because Willow herb products usually consist of mixtures from various species with different phenoloid content, often only partially identified. However, there has been identified the presence of sterols, triterpens, fatty acids, polyphenols (including macrocyclic tannins – oenothein A and B), flavonoids (quercetin, myricetin, kaempferol) [9-11]. Recent years gave us detailed information on the polyphenol composition of some *Epilobium* species. Bazylko et al. have determined using HPTLC-densitometry, the amounts of oenothein B (OeB) and quercetin glucuronide (QG) in aqueous extract of *Epilobii angustifolii herba* which were 152.46±4.92 and 22.07±1.38 mg g(-1), respectively [12]. The LC–MS/MS examination of five *Epilobium* species: *E. parviflorum* (Schreb.), *E.angustifolium* (L.), *Epilobium montanum* (L.), *Epilobium tetragonum* (L.), and *Epilobium roseum* (L.) and two commercially available *E. parviflorum* samples performed by Hevesi et al. resulted in detection of 20 diverse and distinctive components, including 16 determined till now, in various combinations and ratios. The most remarkable differences have been observed between *E. angustifolium* and other species. Exclusively *E. angustifolium* contained flavonol-glucuronide components (quercetinglucuronide and kaempferol-glucuronide), not present in any other *Epilobium* examined species [13].

The most abundant fraction of active compounds obtained from the extract used in this study was a phenolic fraction (24.36% m/m phenolic compounds expressed as gallic acid, there were also small amounts of flavonoglycosides – 0.91% flavonoglycoside m/m compounds expressed as quercetin and sterols – 0.09% m/m sterol m/m compounds expressed as ß-sitosterol detected).

Polyphenols are abundant micronutrients in our diet and evidence for their role in the prevention of degenerative diseases is emerging. Their bioavailability differs greatly from one polyphenol to another [14]. Polyphenols are chemical substances found in plants characterized by the presence of more than one phenol unit or building block per molecule. In general, these compounds are divided into condensed tannins (CTs) [also known as proanthocyanidins (PAs)] and hydrolyzable tannins (HTs) [gallic acid esters of glucose and other sugars - ellagitannins (ETs) and gallotannins (GTs)] [15, 16]. In *in vitro* and in *in vivo* experiments macrocyclic ellagitannins (oenothein A and oenothein B), derived from various species of the *Epilobium* genus have exhibited the inhibition property of two enzymes 5α-reductase and aromatase that are crucial for the development of BPH, and have

a potential antitumor activity on prostatic epithelial (PZ-HPV-7), LNCaP, astrocytoma (1321N1) as well as mammary epithelial (HMEC) cell lines [17-21]. Experiments performed by Schepetkin et al. specify the immunomodulatory effect mechanism of *Epilobium angustifolium* extracts (oenothein B is considered to be the primary component responsible for phagocyte activation) [22]. Methanolic and aqueous *Epilobium angustifolium* L. (Willow herb) extracts showed in human prostate Sk-N-SH and PC3 cells inhibitory activity against some metallopeptidases (NEP, ACE and APN) being a potential marker for cells proliferation [23].

Moreover, it has been proved in *in vitro* model that aqueous acetone extract from *Epilobium parviflorum* strongly inhibits COX-1 and COX-2 activity which are the isoforms of enzyme involved in pro-inflammatory prostaglandins biosyntesis. Dried extract of *E. angustifolium* obtained by evaporating a commercially available tincture has revealed also a potential analgesic property [24].

So far there is no data about molecular mechanisms of action of *Epilobium angustifolium* L. extracts in the liver.

It is known that the mammalian cytochrome P450 (CYP) superfamily containing 17 *CYP* gene families, comprises about 60 genes encoding enzymes that are crucial in metabolism of drugs, foreign chemicals, arachidonic acid and eicosanoids, cholesterol, bile-acid, vitamin D(3) synthesis and metabolism, retinoic acid hydroxylation [25, 26]. Modulation of the cytochrome P450 (CYP450) activity in liver is of great importance because of their meaning in the human metabolism. The most active CYPs for drug metabolism are those in the CYP2C, CYP2D and CYP3A subfamilies [27, 28]. CYP3A4 is the most abundantly expressed in the liver and is involved in the metabolism of about 50% of clinically used drugs [24]. An analogue of human CYP3A4 in rats is a CYP3A1 protein, whereas CYP7A1 (cholesterol 7-alpha-hydroxylase; EC=1.14.13.17) is a protein involved in the biochemical reactions of steroids [29].

The aim of this study was to investigate the influence of a dried standardized water extract of *Epilobium angustifolium* L. of performed from aerial parts of plant, on expression level of selected rat cytochrome P450 encoding genes, which protein products are involved in steroids metabolism, mainly CYP3A1 and CYP7A1.

Result obtained from this experiment can give us an interesting information on molecular mechanisms of action of this herbal plant extract on selected CYP's involved in steroids metabolism in liver.

MATERIALS 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 ß-sitosterol and 0.01% m/m tannin compounds expressed as pyrogallol) was obtained in The Branch of Medicinal Plants of the Institute of Natural Fibres and Medicinal Plants.

Animals treatment

Adult male Wistar rats weighing 170–250 g, four-six week old, were housed in the plastic cages at the Department of Pharmacology, Poznań University of Medical Sciences. 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 few days prior to experiment. All the rats were divided by randomization into 5 groups marked with symbols: K, E, T, TE, TF (7 animals in each group). All substances were administrated for 21 consecutive days.

The control group (designated as K) was treated with an arachidonic oil with dimethyl sulfoxide (DMSO)[0.1 mg/kg/day, s.c.] and PEG 300 (2:8) [5 mg/kg/3 days, s.c.]. Group E received 40 mg/kg/day (p.o.) of *Epilobium angustifolium* standardized dried water extract, group T was treated with testosterone (*Testosteronum prolongatum* 100 mg/ml, Jelfa) dissolved in arachidonic oil in a dose of 0.1 mg/kg/day (s.c.). The mixed group TE represents a combined treatment of rats with testosterone [0.1 mg/kg/day, s.c.) and extract of *Epilobium angustifolium* (40 mg/kg/day, p.o.). The mixted group TF received both testosterone [0.1 mg/kg/day, s.c.] and finasteride (Proscar tabl. 5 mg, Merck Sharp & Dohme, 0.2% water solution + PEG 300 (2:8) (5 mg/kg/3 days) in a dose of 0.1 mg/kg/day, s.c.].

Sixteen hours after last administration, rats were decapitated and liver samples were immediately frozen in liquid nitrogen and stored at –80ºC until use.

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 of Laboratory Animals in Poznań (No. 54/2007).

RNA isolation and reverse transcription reaction

Total RNA isolation from the rat livers was carried out using TriPure Isolation Reagent (Roche) according to 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). The RNA samples were stored at –80°C until have been used. The 1 μg of total RNA from all samples was reverse-transcribed into cDNA using SuperScript™ III First-Strand Synthesis System (Invitrogen) and oligo$(dT)_{20}$ primer according to manufacturer's protocol. Obtained cDNA samples were stored at -20°C or used directly for the real-time PCR (RT-PCR) reaction.

Real-time PCR assay

The gene expression level was analyzed by real-time quantitative PCR reaction using a LightCycler TM Instrument (Roche, Germany) and a LightCycler Fast Start DNA Master SYBR Green I kit (Roche Applied Science) according to the instructions of the manufacturer. All primers sequences were designed using the Oligo 6.0 software (National Biosciences), based on the sequence entries in the Genbank and synthesized from TIB Molbiol (Poland). Primers sequences and specific PCR reaction conditions used for CYP3A1, CYP7A1 and GAPDH expression measurement are described in table 1 and 2, respectively. Primer specificity was verified by assessment of a single PCR product on agarose gel and single temperature dissociation peak (melting curve analysis). Real-time PCR was evaluated within the linear range of product amplification. The GAPDH was used as a housekeeping gene (endogenous internal standard) for normalization (tab. 3). The relative quantification for any gene given was expressed as a signal relative to average signal value for the internal standard. The RT-PCR was carried out in a reaction volume of 10 μl reaction mixture for quantification of CYP1A1/2, CYP7A1 and GAPDH mRNA containing 1 μl RT product and 1μl SYBR Green master mix. Each PCR set was monitored by measuring the increase in fluorescence by binding of SYBR Green I dye to the generated double-stranded cDNA. 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. The data were evaluated using LightCycler Run 5.32 software (Roche Applied Science). Each PCR run has included a no-template control to detect potential contamination of reagents.

Table 1.

Sequences of oligonucleotide primers used for the RT-PCR analysis. GAPDH – glycerylaldehydes phosphate 3-dehydrogenase encoding gene (housekeeping gene)

isoforms	GenBank accession number	forward primer sequence (5'→3')	reverse primer sequence (5'→3')	product size (bp)
CYP3A1	NM_173144	TGGTAATAGACTTGAGAGAG	GGGCAGATATACATAAGGA	196
CYP7A1	X_17595	CTGTGTTCACTTTCTGAAGCC	CCCAGGCATTGCTCTTTGAT	120
GAPDH	NM_017008	GATGGTGAAGGTCGGTGTG	ATGAAGGGGTCGTTGATGG	108

Table 2.

RT-PCR reaction conditions for rat CYP3A1 and CYP7A1 mRNA quantification

gene	**CYP3A1**		
program	denaturation		
segment No.	temperature target (°C)	hold time (sec)	cycles number
1	95	600	1
program	amplification		
1	95	15	
2	56	5	35
3	72	6	
program	melting curve		
1	95	0	
2	70	15	1
3	99	0	
program	cooling		
1	40	30	1
CYP7A1			
program	denaturation		
1	95	600	1
program	amplification		
1	95	10	
2	60	4	35
3	72	10	
program	melting curve		
1	95	0	
2	70	15	1
3	99	0	
program	cooling		
1	40	30	1

Table 3.

RT-PCR reaction conditions for rat housekeeping gene (GAPDH) mRNA quantification

gene	**GAPDH**		
program	denaturation		
segment No.	temperature target (°C)	hold time (sec)	cycles number
1	95	600	1
program	amplification		
1	95	8	
2	56	6	35
3	72	6	
program	melting curve		
1	95	0	
2	70	15	1
3	99	0	
program	cooling		
1	40	30	1

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 software using one-way ANOVA test (SPSS Inc.) and Fischer LSD post-hoc test. The values of $p<0.05$ were considered as a statistical significant difference.

RESULTS

In this study we tried to investigate cellular molecular machinery reaction involved in steroids metabolism after the administration of standardized dried water *Epilobium angustifolium* L. extract alone or together with testosterone or synthetic drug – finasteryde in rat liver tissues. The expression level of each selected gene was normalized *vs.* expression level of housekeeping gene mRNA–GAPDH.

The results showed that CYP3A1 and CYP7A1 expression level changes in all groups of animals were different (fig. 1, 2). In animals treated with E, T, TE and TF for 21 days it was indicated a decrease of CYP3A1 mRNA expression level in comparison to control group (ANOVA $(4.30)=3.56$; $p<0.02$). The plant extract showed the strongest inhibitory of CYP3A1 mRNA expression (63.7% reduction of expression level *vs*. control group), whereas the other groups of rats did not statistically differ when compared with control animals. Moreover, the combined extract and testosterone administration caused an insignificant decrease of CYP3A1 mRNA expression level (15.27% reduction *vs*. control group). On the other hand, the treatment with all investigated substances did not show general statistical difference (ANOVA $(4.30)=0.81$; $p>0.527$). It was found that only TF produced inhibitory trend of CYP7A1 mRNA, whereas in all remaining groups a slight induction of CYP7A1 expression was observed. An increased expression of CYP7A1 mRNA liver in healthy rat was also observed by Del Bas et al. which is explained by increased bile acids elimination and the presence of procyanidins in an extract from graped seeds studied by this group [30]. These results suggest that *E. angustifolium* extract may modulate the CYP3A1 mRNA transcription in a different manner than testosterone or finasteride.

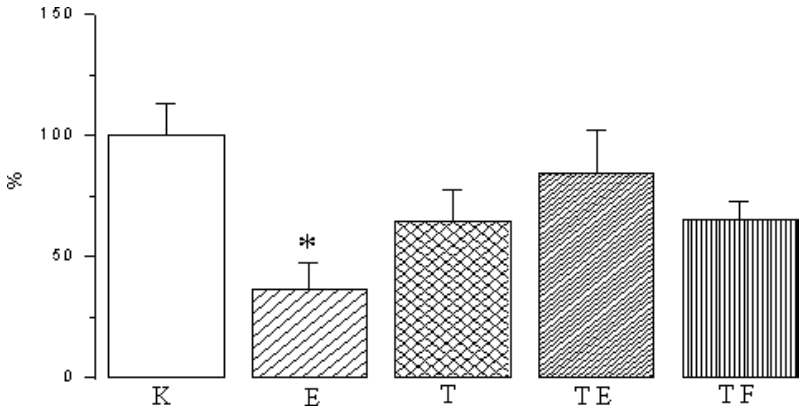

Figure 1. Effect of extract from *Epilobium angustifolium* on CYP3A1 mRNA expression level in liver of rats
Legend:
Control group (K) was defined as 100%. Data were presented as mean ±SEM of 7 rats in each group.
E – group treated with extract from *Epilobium angustifolium* (40 mg/kg/day, p.o.)
T – group treated with testosterone (0.1 mg/kg/day, s.c.)
TE – group treated with testosterone (0.1 mg/kg/day, s.c.) and extract from *Epilobium angustifolium* (40 mg/kg/day, p.o.)
TF – group treated with testosterone (0.1 mg/kg/day, s.c.) and finasteride (50 mg/kg/day, p.o.)
ANOVA (4.30)=3.56; $p<0.02$
* – statistically significant difference vs. K group, $p<0.05$

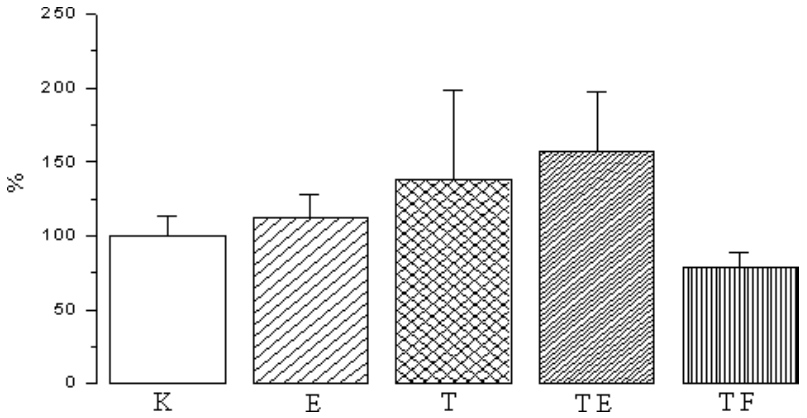

Figure 2. Effect of extract from *Epilobium angustifolium* on CYP7A1 mRNA expression level in liver of rats.
Legend:
Control group (K) was defined as 100%. Data were presented as mean ±SEM of 7 rats in each group.
E – group treated with extract from *Epilobium angustifolium* (40 mg/kg/day, p.o.)
T – group treated with testosterone (0.1 mg/kg/day, s.c.)
TE – group treated with testosterone (0.1 mg/kg/day, s.c.) and extract from *Epilobium angustifolium* (40 mg/kg/day, p.o.)
TF – group treated with testosterone (0.1 mg/kg/day, s.c.) and finasteride (50 mg/kg/day, p.o.)
ANOVA (4.30)=0.81; $p>0.527$,
* - statistically significant difference vs. K group, $p<0.05$

DISCUSSION

Results obtained in this experiment give us an interesting, preliminary information about molecular mechanisms of this herbal plant extract action on selected CYP's involved in steroids metabolism in liver. These results suggest that a standardized *E. angustifolium* extract can regulate transcription level of investigated *CYP* genes in a different manner since a strong inhibition of CYP3A1 mRNA expression and slight induction of CYP7A1 may occur as a result of different extract action on the investigated CYP's transcription machinery in rat liver cells. With regard to fact that half of drugs used in classical and modern pharmacotherapy are metabolized by CYP3A4 protein (CYP3A1 in rats) [31], any modulation of its expression level consequent of combine, simultaneous using of synthetic drugs and herbal plants extracts (as in the case of dried water extract from a herb of *Epilobium angustifolium*) can cause an occurrence of interaction between synthetic and plant derived drugs and dietary supplements.

Further *in vitro* and *in vivo* studies need to be undertaken for understanding the molecular mechanisms of action of the herbal compounds in the liver tissue what could be very helpful for elucidation of efficacy and safety of prophylaxis and treatment of benign prostatic hyperplasia disorder using standardized extracts of *Epilobium* sp.

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WPŁYW EKSTRAKTU Z *EPILOBIUM ANGUSTIFOLIUM* L. NA EKSPRESJĘ WYBRANYCH GENÓW CYTOCHROMÓW P450 W WĄTROBIE SZCZURA. DONIESIENIE WSTĘPNE

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

Alternatywę w terapii i leczeniu łagodnego przerostu gruczołu krokowego (ang. *Benign Prostatic Hyperplasia,* BPH) stanowią produkty lecznicze pochodzenia roślinnego. W ostatnich latach wzrosło zainteresowanie surowcami roślinnymi należącymi do rodzaju *Epilobium* (Onagraceae). Pomimo wzrastającej wiedzy na temat mechanizmów działania wyciągów uzyskiwanych z surowców pochodzących z rodzaju *Epilobium*, w tym *Epilobium angustifolium*, molekularny mechanizm ich działania w tkance wątrobowej nadal pozostaje nieznany. Celem opisanego eksperymentu była zbadanie wpływu standaryzowanego wyciągu wodnego z ziela *Epilobium angustifolium* na poziom ekspresji mRNA wybranych cytochromów z rodziny P450 w tkance wątrobowej. W badanych ekstrakcie najistotniejszą grupę związków biologicznie czynnych stanowiła frakcja polifenoli (24,36%), stwierdzono stosunkowo niewielką ilość flawonoglikozydów (0,91%) i steroli (0,09%). Badany wyciąg

najsilniej hamował poziom ekspresji mRNA CYP3A1. Uzyskane przez nas wyniki wskazują, iż badany wyciąg zmieniał poziom ekspresji mRNA analizowanych cytochromów w odmienny sposób, a obserwowane silne zahamowane ekspresji mRNA CYP3A1 i nieznaczny wzrost CYP7A1 mRNA może być efektem wpływu różnych związków biologicznie czynnych zawartych w ekstrakcie na maszynerię komórkowa regulującą proces transkrypcji obu cytochromów w szczurzej tkance wątrobowej.

Słowa kluczowe: *łagodny przerost prostaty (BPH), roślinne surowce lecznicze, fitoterapia, wierzbownica wąskolistna, Epilobium angustifolium, cytochrom P450, CYP3A1, CYP7A1, ekspresja, interakcje, leki syntetyczne*