Influence of *Epilobium angustifolium* and *Serenoa repens* extracts on cytochrome 2D2 and 3A1 expression level in rats

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S u m m a r y

Benign prostatic hyperplasia (BPH) is a common disease affecting aging males. In recent years, as alternative strategy for the prevention and therapy of BPH, there is an growing interest in usage of plant derived remedies i.e. from *Serenoa repens* (sabal palm) and some

plants from the *Epilobium* genus. The aim of this study was to investigate the influence of standardized *Epilobium angustifolium* L. and *Serenoa repens* extracts on expression level of CYP3A1 and CYP2D2 mRNAs in rats. Testosterone and standardized *Epilobium angustifolium* or commercial *Serenoa repens* extracts were given for 21 days to castrated male Wistar rats. The levels of CYP2D2 and CYP3A1 mRNAs expression were analyzed by real-time quantitative PCR using specific target primers.
We have observed a slightly increased level of CYP2D2 mRNA in animals treated with testosterone and both plant extracts (by 4.6% in rats receiving *Epilobium angustifolium* extract and by 25.29% in animals treated with extract from *Serenoa repens*) and the CYP3A1 mRNA level by 11.02% in rats treated with *Serenoa repens* extract. In rats receiving *E. angustifolium* extract a 20.22% decrease of CYP3A1 mRNA expression level was observed.
Results from our work showed that standardized plant extracts from *Epilobium angustifolium* and *Serenoa repens* have differentially influenced on CYP3A1 and CYP2D2 mRNA expression level in a rat liver.

Key words: *herbal plants, phytotherapy, Serenoa repens, sabal palm, Willow herb, Epilobium angustifolium, cytochrome P450, CYP3A1, CYP2D2, Benign Prostatic Hyperplasia (BPH)*

INTRODUCTION

Benign prostatic hyperplasia (BPH) is a common illness affecting 50% of men aged 60 and 90% men older than 90 [1]. One of typical therapeutic approaches in BPH treatment is the inhibition of human prostate cells proliferation throught the inhibition of the 5α-reductase activity by using i.e. a competitive and selective inhibitor of 5α-reductase type 2 enzyme – finasteride [2].

In recent years the drugs of plant origin have been introduced to be the basis for a strategy directed to the prevention of lifestyle diseases, including BPH, supporting a classical pharmacotherapy. The fact that in the social consciousness herbal remedies can provide an alternative and safe therapeutic strategy due to a long tradition of herbal products is significant. Herbal raw materials such as: *Serenoa repens, Pygeum africanum, Cucurbita pepo, Zea mays, Urtica dioica, Secale cerelae* and others are commonly used in the prevention and symptomatic treatment of BPH. Recently, the interest has been growing in plants of the genus *Epilobium* (especially *Epilobium parviflorum, E. angustifolium E. hirsutum*) due to their common occurrence in Central Europe. The mechanism of action of these herbal extracts is not fully understood, but have been generally described to inhibit the 5α-reductase and aromatase enzymes, possessing antiandrogenic and antiestrogenic activities [3-6] anti-inflammatory properties [7] and capable to inhibit of cell proliferation [5, 8-9]. Also recent evidences suggest that the use of *Serenoa repens* fruit extract (saw palmetto, sabal palm) leads to improve the urinary function accompanying BPH and a wide spectrum of its activity [10]. The comparison of *Serenoa repens* extract with tamsulosin showed also that the first one demonstrates a beneficial therapeutic advantages with few adverse effects [11].

The advancement in knowledge in pharmacognosy and pharmacology provides scientific evidence that natural therapies and plant derived dietary supplements are not always a safe alternative to traditional pharmacotherapy [12]. The awareness on the possibility of occurrence of food-drug and herbal-drug interactions is very important in maintaining the safe and effective phytotherapy. The risk of these appearances intensifies with increasing consumption of natural products for medicinal purposes, a simultaneous and a long-term intake of food and prescribed medicaments [12]. These adverse effects have been reported to occur when a particular food or herbal compounds alter the activity of a drug-metabolizing enzyme belonging especially to cytochromes (CYP) superfamily which are crucial in the metabolism of drugs, foreign chemicals, nutriceuticals and other xenobiotics [12-14]. The clinical interactions between herbal remedies, food ingredients and co-administered drugs on cytochromes level have been observed in many herbal medicines, i.e. St. John's wort [15], garlic [16], ginseng [17], and ginkgo [18,19]. The CYPs most active for drug metabolism are those belonging to the CYP2C, CYP2D and CYP3A subfamilies [20,21]. CYP3A4 (in rats CYP3A1 isoform) is the most abundantly expressed in the liver and is involved in the metabolism of about 50% used drugs [21]. An orthologous of human's CYP2D6, is a rat's CYP2D1 enzyme and also the CYP2D2 enzyme that metabolizes one of the marker substrates of CYP2D6 – dextrometorphan [22].

The fact that the molecular mechanism of action of these herbal extracts on cytochromes level is not fully understood encouraged us to investigate the influence of standardized dried water extract of *Epilobium angustifolium* L. and lipidosterolic extract of *Serenoa repens* (Permixon) on expression level of CYP3A1 and CYP2D2 mRNAs in rat's liver tissues.

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 Research Institute of Medicinal Plants.

Animals treatment

Adult male Wistar rats weighing between 170 and 250 g, four weeks old, were housed in plastic cages at the Department of Pharmacology, Poznań University of Medical Sciences. Animals 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

divided by randomization into 6 groups marked with symbols: K1, K2, T, TE, TF (each group consisted of 10 animals). All substances were administrated for 21 consecutive days.

The group K2 (control for finasteride treated animals) was treated with an arachidonic oil with dimethyl sulfoxide (DMSO) [40 mg/kg, each 7th day, s.c.] and PEG 300 (2:8) [50 mg/kg, each 3th day, s.c.]. Another control group – K1 – was treated with an arachidonic oil with H2O and PEG 400 [50mg/kg/day; p.o.]. Group T received testosterone (*Testosteronum prolongatum* 100 mg/ml, Jelfa; 40mg/kg/day, 3 times, each 7th day; s.c). dissolved in arachidonic oil [8]. Mixed group – TE represents 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.]). Mixed group – TF – received both testosterone in abovementioned dose and finasteride (Proscar tabl. 5 mg, Merck Sharp&Dohme, 0,2% water solution + PEG 300 (2:8) [50 mg/kg/3 days; p.o.]. To animals from the last group – TS – testosterone in abovementioned dose and reference to *E. angustifolium* extract plant derived drug from *Serenoa repens* – Permixon was administrated [Permixon, Pierre Fabre Medicament, 100mg/kg/day; p.o]. Sixteeen hours after the last administration, rats were decapitated and liver tissue 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 from the rat livers 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 use. The 1 μg of total RNA from all samples was reverse-transcribed into cDNA using SuperScript™ III First-Strand Synthesis System (Life Technologies, USA) and oligo$(dT)_{20}$ primer (Life Technologies, USA) 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 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 CYP3A1, CYP2D2 and GAPDH expression measurement are described in table 1. Primer specificity was verified by 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. RT-PCR was carried out in a reaction volume of 10 μl reaction mixture for quantification of SRD5AR1, 2 and GAPDH mRNA contained 1 μl RT product and 1 μl SYBR Green master mix. Each PCR set was monitored by measuring the increase in fluorescence by the 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 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 RT-PCR analysis . Primers for GAPDH, CYP3A1, CYP2D2 were designed using the Oligo 6.0 software (National Biosciences)

analyzed gene	primer sequence (5' → 3')	product size (bp)	RT-PCR conditions			
			Cycle	denaturation	annealing	extension
CYP2D2F CYP2D2R	GGGAGGTTACTGGTTCTGG ATGAGGCTGTCTGTGATGTC	184	38	95ºC, 6 s	57ºC, 4 s	72ºC, 8 s
CYP3A1F CYP3A1R	CATCTTTGGAGCTGGATTTG CCATTCAGTGAGGTGTCC	200	35	95ºC, 8 s	55ºC, 8 s	72ºC, 8 s
GAPDH F GAPDH R	GATGGTGAAGGTCGGTGTG ATGAAGGGGTCGTTGATGG	108	28	95ºC, 4 s	56ºC, 8 s	72ºC, 8 s

Statistical analysis

The results were expressed as means ±SEM. Statistical significance of the difference between the control and experimental group was assessed by SPSS 14.0 software using one-way ANOVA test (IBM Corporation, USA) and Fischer LSD post-hoc test. The values of $p<0.05$ were considered as a statistically significant difference.

RESULTS

The expression level of each selected gene was normalized *vs* expression level of house-kept gene coding GAPDH mRNA. The analysis of CYP2D2 and CYP3A1 mRNA expression profile in all treated groups of animals *vs* control groups (K1, K2) has revealed a small increase of CYP2D2 only in animals treated with testosterone and *Serenoa repens* (TE) in comparison of their level in all testosterone treated groups (T, TE, TS, TF) with K1 and K2 control animals (fig. 1). The administration with testosterone alone, with *Epilobium angustifolium* extract ($p<0.05$) or with finasteride ($p<0.05$) caused a decline of CYP2D2 mRNA expression *vs* control animals, by 23, 9, and 42%, respectively. In testosterone administrated animals, together with both plant extracts, a small increase of level of CYP2D2 mRNA (4,6; 25,29% in TE and TS groups in comparison with testosterone treated rats) was observed. ($p<0.05$) Only simultaneous treating with testosterone and finasteride caused decrease of its level by 33.3% ($p<0.01$).

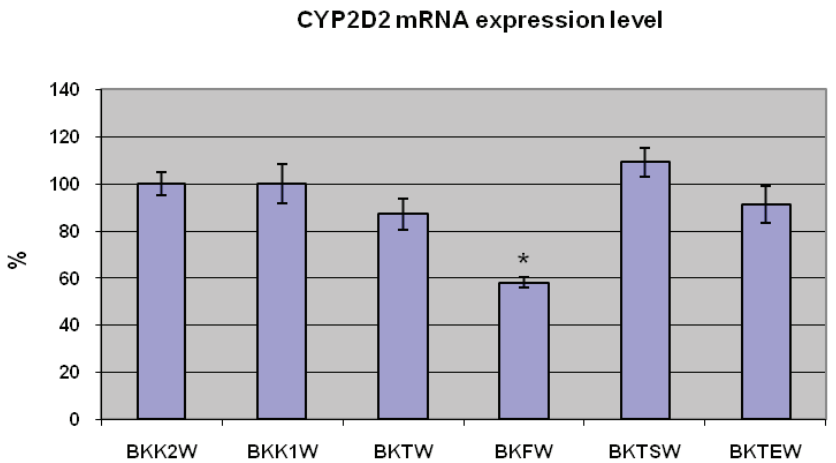

Figure 1.
Effect of extract from *Epilobium angustifolium* on CYP2D2 mRNA expression level in liver of rats.
Abreviations:
BKK1W, BKK2W – control groups
BKTW – group of animals treated with testosterone
BKTF – group of animals treated with testosterone and finasteride
BKTSW – group of animals treated with testosterone and *E. angustifolium* extract
BKTEW – group of animals treated with testosterone with *Serenoa repens* extract
* $p<0.05$ as compared to the control group

Differential expression profile was observed in example of CYP3A1 mRNA. In all treated groups there was an induction of its level in comparison with K1 and K2 controls, by 172, 117, 202 and 608% in T, TE, TS, TF ($p<0.01$) groups, respectively (fig. 2). Finasteride caused the largest elevation of CYP3A1 mRNA expression level (6.08-fold more vs. "K2" control group, and more than 4-fold than simultaneous administration of testosterone and *Serenoa repens* extract) ($p<0.01$). Simultaneous administration of testosterone and *Epilobium angustifolium* extract caused a 20.22% decrease of CYP3A1 mRNA expression level in comparison with testosterone alone treated animals ($p<0,01$), while the *Serenoa repens* extract and finasteride induced its expression level by 11.02 and 260.29%, respectively ($p<0.01$). These results show that the synthetic drug caused the strongest induction of the CYP3A1 mRNA transcription vs. control groups (K1 and K2) and testosterone administrated rats.

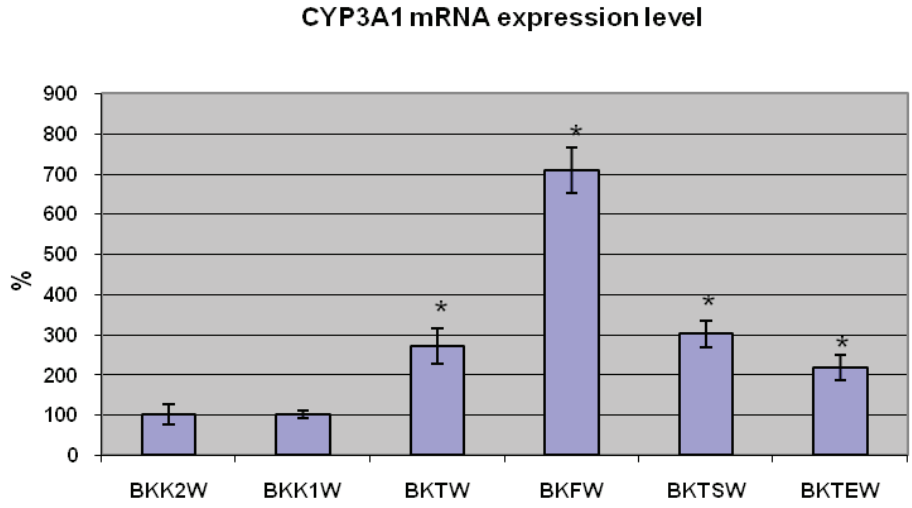

Figure 2.
Effect of extract from *Epilobium angustifolium* on CYP3A1 mRNA expression level in liver of rats.
Abbreviations:
BKK1W, BKK2W – control groups
BKTW – group of animals treated with testosterone
BKTF – group of animals treated with testosterone and finasteride
BKTSW – group of animals treated with testosterone and *Serenoa repens* extract
BKTEW – group of animals treated with testosterone and *E. angustifolium* extract
* $p<0.05$ as compared to the control group

DISCUSSION

The use of complementary alternative medicine (CAM) is increasing rapidly in West Europe countries [21]. The usage of plant derived drugs or dietary supplements develops mainly due to hormonal manipulation. Synthetic drugs such a α-adrenergic blockers and 5α-reductase inhibitors may often cause undesirable side effects [23,24]. Modulation of the cytochrome P450 (CYP450) activity in liver is of great importance due to their role in the human metabolism in the presence of xenobiotics, prescribed drugs, popular phytotherapeutics and dietary supplements [19]. 4-Azasteroids, represented i.e. by finasteride used in this experiment, are one of the extensively studied and clinically analyzed azasteroidal 5α-reductase inhibitors [2].

A few recent studies revealed an inhibitory property of finasteride on aromatase (CYP19) activity in human brain, placenta [25] and cardiac microsomes [26] as well as potential risk of interactions connected with an concurrent intake of *Serenoa repens* extracts with synthetic drugs [27, 28]. For example, results from Markowitz et al. revealed that the *Serenoa repens* extract (320-mg capsule once daily) administered orally for 14 days followed by dextromethorphan and alprazolam administered orally did not alter activity of CYP3A4 and 2D6 in healthy male and female volunteers. This may suggest that this plant extract can be recommended in doses that do not alter the disposition of coadministered drugs primarily dependent on the CYP2D6 or CYP3A4 activity [27]. Phenotypic analysis determined for CYP3A4, CYP1A2, CYP2E1, and CYP2D6 have also revealed that longer (28 days with a 30-day washout period) supplementation with this plant extract appears to be at a minimal risk for CYP-mediated herb-drug interactions in humans [28]. Results from an *in vitro* study obtained by Yale et al. showed that *Serenoa repens* extract may be a potent inhibitor of the metabolic activity of tested CYP3A4, 2D6, and 2C9 [29] which is partially inconsistent with the results obtained by our staff, where simultaneous administration of *Serenoa repens* extract with testosterone caused an elevation of CYP3A1 expression level by 11,02% in comparison to testosterone alone administered rats. According to our knowledge there is no published data on potential interactions between administrated standardized extract of *Serenoa repens* or *Epilobium angustifolium* with testosterone (one of the androgens crucial for the BPH progression) and CYP2D2 and 3A1 transcripts expression regulation in a rat liver. In our opinion these different effects may result from the different research model and analysis methodologies used by our group and by Yale et al.

Results of our work shows also that steroids, similarly to testosterone and finasteride, differentially influence CYP3A1 and CYP2D2 expression level in a rat liver tissue. From our point of view, their expression profile changes may be a consequence of competitive influence of mixture of bio-active compounds in *E. angustifolium* and *Serenoa repens* extracts on transcriptional machinery regulating the expression of CYP3A1 and CYP2D2 genes, an in-

teraction between those compounds and/or testosterone with transcription factors or direct interaction with examined enzymes. Result from our study are in connection with findings obtained by other researchers in which androgen metabolism mediated by a CYP3A subfamily proteins i.e. in the liver and prostate microsomes were observed. For example, it was shown that this enzymes hydroxylate testosterone and dehydroepiandrosterone to less active metabolites, which might by of a great importance for the pharmacotherapy of prostatic disorders, especially prostate cancer [30-31]. Other experiments have revealed an association between the testosterone hydroxylations at 6β-, 2β-, 15α- and 15β-positions and CYP3A isoform(s) occurrence [32-34]. Hence, the testosterone oxidation measurement became one of the methods for the cytochromes activity evaluation and its 6 β-hydroxylase activity analysis in hepatocytes microsomes is often used as a marker of CYP3A activity [35-37]. The study of scientific literature did not reveal the hepatic CYP3A1 and CYP2D2 gene expression analysis attempts in testosterone-induced prostate growth in animal model. This paper provides interesting indications explaining the responsiveness of CYP3A1 and CYP2D2 transcription activity in the presence of this androgen.

CONCLUSION

Our results indicate that extracts from *Epilobium angustifolium* and *Serenoa repens* administered in a dose of 100 mg/kg b.w. (p.o.) seems to be risk-free interaction inducing activity of CYP2D2 and CYP3A1 isoenzymes. Further *in vivo* experiments need to be undertaken, especially a long-term studies, in order to understand the molecular mechanisms of action of this or other herbal extracts in the prostate tissue. A real-time quantitative PCR method seems to be sensitive, effective and very helpful tool for the evaluation of efficacy and safety of prophylaxis and the treatment of benign prostatic hyperplasia disorders on molecular level using i.e. standarized extracts of *Epilobium* sp. and other phytotherapeutics, especially of similar phytochemical profile as those used in our experiment. Results from this experiment may be very helpful for physicians prescribing drugs and phytotherapeutics for patients with BPH, especially in determining a potential risk of interactions between abovementioned extracts with conventional drugs metabolized by these enzymes.

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WPŁYW EKSTRAKTÓW Z *EPILOBIUM ANGUSTIFOLIUM* I *SERENOA REPENS* NA POZIOM EKSPRESJI CYTOCHROMÓW 2D2 I 3A1 W MODELU SZCZURZYM

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

Łagodny rozrost gruczołu krokowego jest powszechnym schorzeniem występującym u starzejących się mężczyzn. W ostatnich latach wzrosło zainteresowanie roślinami leczniczymi należącymi do gatunku boczni piłkowanej (*Serenoa repens)* i rodzaju wierzbownica (*Epilobium* sp.), które mogą stać się alternatywną strategią profilaktyki i leczenia BPH. Celem pracy było ustalenie wpływu standaryzowanych wyciągów z *Epilobium angustifolium* i *Serenoa repens* na ekspresję cytochromów CYP3A1 i 2D2 u szczurów. Kastrowanym samcom szczurów rasy Wistar podawano przez okres 21 dni testosteron i badane, standaryzowane wyciągi roślinne. Zmiany poziomu ekspresji mRNA CYP3A1 i 2D2 mierzono techniką PCR w czasie rzeczywistym z użyciem starterów specyficznych dla cytochromów. Stwierdzono nieznaczne podwyższenie poziomu ekspresji mRNA CYP2D2 u zwierząt otrzymujących testosteron i oba wyciągi roślinne (podwyższenie o 4,6% u szczurów otrzymujących

wyciąg z *Epilobium angustifolium* i o 25,29% u zwierząt otrzymujących wyciąg z *Serenoa repens*) i wzrost poziomu ekspresji mRNA CYP3A1 o 11,02% u zwierząt otrzymujących wyciąg z *Serenoa repens*. U szczurów otrzymujących wyciąg z *Epilobium angustifolium* stwierdzono obniżenie poziomu ekspresji mRNA CYP3A1 o 20,22%. Wyniki naszej pracy wykazały, że standaryzowane wyciągi z *Epilobium angustifolium* i *Serenoa repens* w zróżnicowany sposób wpływają na poziom transkrypcji CYP3A1 i CYP2D2 mRNA w wątrobie szczura.

Słowa kluczowe: *roślinne surowce lecznicze, fitoterapia, palma sabalowa, Serenoa repens, wierzbownica wąskolistna, Epilobium angustifolium, cytochrom P450, CYP3A1, CYP2D2, łagodny przerost prostaty (BPH).*