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ORIGINAL PAPER

Serenoa repens extracts: In vitro study of the 5α-reductase activity in a co-culture model for Benign Prostatic Hyperplasia

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Summary ***Objectives. Benign Prostatic Hyperplasia (BPH) is a form of benign tumor that occurs in humans mainly with ageing. It affects more than 50% of over 50 years old males and it is characterized by an increased synthesis of dihydrotestosterone (DHT), due to the 5α-reductase activity. The BPH therapeutic approach mainly uses 5α-reductase inhibitors, such as the active compounds present in the extracts deriving from species Serenoa repens. Many lipido-sterolic extracts are available on the market, which are obtained with different solvents, among them ethanol is recognized as non-toxic and has less handling risks than hexane. The purpose of the present experimental study was to investigate in-vitro the potency of an ethanol extract of S. repens comparing it with an n-hexane one.***

Materials and methods. Two different lipido-sterolic extracts of S. repens have been tested: ethanol extract and n-hexane extract, two batches for each one. The inhibitory action of the extract was evaluated estimating in-vitro the activity of enzyme 5α-reductase type I (5α-RI), which was mainly active under the experimental condition of pH 7.5. DHT amount, synthesized from testosterone (1 μM), was evaluated in a co-culture model of epithelial cells and fibroblasts resulting from prostatic biopsy of a patient with BPH.

Results. The analysis of the resulting dose-response curves showed that the entire S. repens extracts inhibited the 5α-RI showing no difference between the two kinds of extract or between the batches. The resulting IC50 values were the following: 8.809 (95% CI = 5.133-15.56) and 9.464 (95% CI = 5.094-18.27) for ethanol extracts; 11.08 (95% CI = 6.389-19.98) and 12.72 (95% CI = 7.758-21.53) for n-hexane extracts.

Conclusions. The potency of ethanol extracts of S. repens was comparable with the one of n-hexane extracts.

KEY WORDS: Benign Prostatic Hyperplasia; Dihydrotestosterone; Ethanol extracts; In-vitro study; 5α-reductase; Serenoa repens.

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INTRODUCTION

Benign Prostatic Hyperplasia (BPH) is a form of benign tumor that occurs in humans mainly with ageing. In fact, it affects more than 50% of over 50 years old males, with an incidence rate directly proportional to age (1). It is characterized by an increased synthesis of *dihydrotestosterone* (DHT) starting from testosterone, due to the action of enzyme 5α-reductase, which leads to an increase of the prostate size and causes various disorders, especially in the lower urinary tract (1, 2). The approach for the treatment and therapy of BPH is mainly based on the use of inhibitors of 5α-reductase enzyme, in particular natural inhibitors, such as the active compounds present in the alcoholic extracts deriving from species Serenoa repens, a typical palm common in the Subtropical sandy soils and in the southern coasts of the United States. Although the sure mechanism of action of *S. repens* is not yet fully understood, numerous mechanisms have been proposed (3) and the presence of specific fatty acids (saturated and unsaturated fatty acids) and phytosterols in its alcoholic extracts confers upon *S. repens* an anti-inflammatory and above all antiproliferative action on prostatic tissue (4). Both a systematic Cochrane review of the literature (5) and a meta-analysis (6) assessed the safety profile and the clinical efficacy of *S. repens* in the treatment of symptoms in patients with BPH. These studies have shown an improvement of urinary tract disorders, with a mild to moderate effect and, therefore, a lack of therapeutic equivalence between *S. repens* extracts of different brands and between extracts of a single brand, but belonging to different manufacturing batches (5, 6). Many lipido-sterolic extracts are commercially available, which were obtained with different solvents, among them ethanol is recognized as non-toxic and it has less handling risks than hexane (7, 8). The purpose of the present experimental study was to investigate in-vitro the potency of an ethanol extract of *S. repens* comparing it with an n-hexane one. In particular, the activity of enzyme 5α-reductase was estimated in-vitro concerning the inhibitory action of the extracts, using co-cultures of epithelial cells and fibroblasts resulting from prostatic biopsy of a patient with BPH.

MATERIALS AND METHODS

Samples

Two extracts, belonging to commercial brands *SABA® (Lampugnani Farmaceutici)* and *PERMIXON® (Pierre Fabre Pharma)*, supplied with marketing authorization in

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several *EU Member States (including Italy)* and belonging to the class of drugs employed to counter the increase in prostatic volume in males with BPH, have been tested. The two branded drugs contained the lipid-sterolic extract of *S. repens* (320 mg/soft capsule) as active ingredient and particularly, ethanol extract (*SABA®*) and hexane extract (PERMIXON®). Two different batches of each brand were tested: SABA® (G08363 and G08364); PERMIXON® (F 11917 and G07340), mentioned below as "*samples*".
The content of one soft capsule was weighed (10 mg) and dissolved in N-hexane; then the solvent was evaporated to leave the solid extract that was dissolved in ethanol (70%) to give a concentration of 10 mg/ml.
This stock solution was further diluted in appropriate media to provide a working solution of 1 mg/ml (9).

Co-culture cell model

Two types of primary cells were used: epithelial cells and fibroblasts, previously isolated from prostatic tissue obtained during a biopsy from a patient with established BPH. Informed consent of the patient was obtained. Primary cultures of fibroblast and epithelial cells were obtained as previously described (2). Both the epithelial and fibroblast cells were cultured separately in flasks with culture medium, complete with fetal bovine serum (10%), DMEM and RPMI 1640 respectively, and kept in an incubator in a humidified atmosphere (37°C and 5% CO_2). Corresponding to the third generation step, both the epithelial cells and fibroblasts, were transferred into multiwell plates of 12 wells provided with *Transwell®* (*3470 Clear-Corning*), or supports consisting of a microporous polystyrene membrane. This enabled to keep the two cell populations separate but, at the same time, to allow their interactions.
In particular: epithelial cells were sown in the number of 50,000 on the bottom of each well; on the contrary, fibroblasts were sown in the number of 5,000 on the microporous polystyrene membrane. Co-cultures were maintained in DMEM:RPMI-1640 (1:1 v/v) media + 10% fetal calf serum at 37°C in 5% CO_2 (10).
Furthermore, in order to verify that the fibroblasts were correctly adhering to the membrane, the quantitative technique TEER (*Transepithelial Transendothelial Electrical Resistance*) was applied; this technique enabled to examine the integrity of the cell junctions (tight junctions) by measuring the resistance that the monolayer of fibroblasts opposed to the passage of electric current (11).
Measurement of the trans-epithelial resistance value, expressed in Ohms and normalized for the polystyrene membrane area (Ω/cm²), was performed using a voltmeter (*Millicell® ERS-2, Merck Millipore*) equipped with two electrodes of different length (data not reported).

5α-reductase activity assay

The co-culture cells were pre-treated for a period of four days with a non-toxic concentration (10 µg/ml) of each sample (*SABA®: G08363 and G08364; PERMIXON®: F 11917 and G07340*). Afterwards the cells were harvested by trypsinization, centrifuged and the pellet was suspended in RPMI-1640, supplemented with 10% fetal calf serum, and then counted. The pH value of the medium solution was 7.85, an optimum value for the activity of 5 α-reductase type I (12). Cell suspensions were added into tubes containing testosterone (1 µM) as substrate, a NADPH-regenerating system (0.5 mM glucose-6-phosphate, 0.06 U glucose-6-phosphate dehydrogenase, 50 µM NADPH) (H+ donor) (13) and different concentrations of each sample: 1 mg/ml, 100 µg/ml, 10 µ /ml, 5 µg/ml, 1 µg/ml, 0.1 µg /ml, 0.01 µg/ml; these concentrations were chosen basing on the cytotoxicity results, previously obtained in laboratory applying the MTT test (14)) and in agreement with data present in the literature (9, 10).
The tubes were then incubated at 37° C for 30 min in a stirring water bath. The reaction was stopped by dipping the tubes into liquid nitrogen (9, 10, 12).
The 5α-reductase type I activity was assessed by measuring the conversion of testosterone to *dihydrotestosterone* (DHT) applying a qualitative/quantitative Enzyme-Linked Immunosorbent Assay (*Abnova KA1886*), as previously described (15).
Enzyme activity is expressed as a percentage of the control. The conversion of 1 µM of testosterone in the absence of inhibitors is defined as 100% activity (2.95-3.63 nmol of DHT/10^6 cells/min for type I isoenzyme).
Dose-effect response curves were analyzed using a sigmoid maximum-effect model with a variable slope (Graphpad, Prism7).
Inhibitory potency was assessed by estimating the IC50 value that represented the concentration (µg / ml) capable of determining the 50% of the maximal effect (enzymatic inhibition). The highest IC50 value was then divided by the IC50 values obtained for each sample for the *relative potency* (RP) (the relative potency value = 1 was considered the lowest value).

Statistical analysis

The nonlinear-regression curves were analyzed applying two different *Linear Mixed Models*, one to analyze the commercial brands (*SABA® vs PERMIXON®*) and one for the batches. The analyses were performed with software R.

Results

All the samples of *S. repens* extracts (F 11917; G07340; G08363; G08364) were found to be capable of inhibiting 5α-reductase (5α-RI) in a prostatic co-cultured epithelial and fibroblast cells, as shown by dose-effect curves in Figure 1 (enzyme activity (%) as a function of the logarithm of concentrations).
Regarding the potency of each sample, evaluated by IC50 value (Table 1) and highlighted by the relative potency (Table 2), *SABA®* (IC50 = 8.809 and 9.464) showed a higher efficacy on 5α-RI than *PERMIXON®* (IC50 = 11.08 and 12.72), but there were non-significant differences between the two brands and batches, as mentioned below.
Regarding the comparison between the two brands (*SABA® vs PERMIXON®*), the analysis of deviance (Type III tests) for 5α-RI data set showed that the model was non-significant (Response: enzyme; Chisq Df Pr (> Chisq); (Intercept) 28.3600 1 1.007e-07 ***; Brand 2.9228 1 0.08734; Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1).

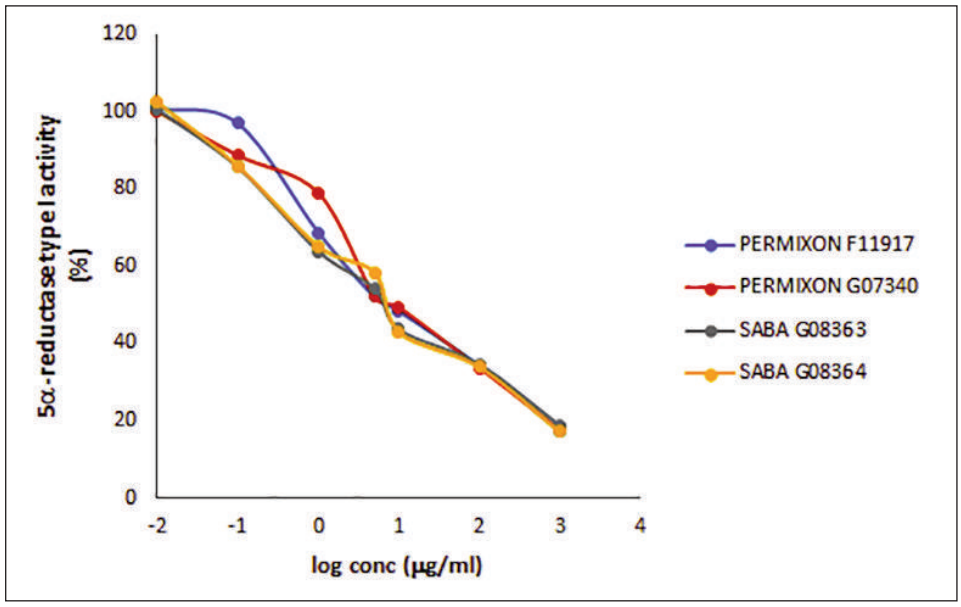

Figure 1.
Inhibition of 5α-reductase type I. Enzyme activity, depending on the logarithm of concentrations, is expressed in percentage as compared with the positive control (100% of enzyme activity converting testosterone 1µM, in the absence of inhibitors).

Regarding the comparison between the batches (*F 11917; G07340; G08363; G08364*), the analysis of deviance (Type III tests) showed that the model was non-significant [Response: enzyme; Chisq Df Pr (> Chisq); (Intercept) 28.0176 1 1.202e-07 ***; Batch 2.9326 3 0.4021; Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1].
Regarding the relative potency (Table 2), both *SABA®* and *PERMIXON®* showed comparable potencies between the two batches and *PERMIXON®* showed the lowest values.

Discussion

All the extracts of *S. repens* that we tested, *SABA®* (G08363 and G08364) and *PERMIXON®* (F 11917 and G07340), have shown to inhibit enzyme 5α-reductase in a co-culture model of human prostatic cells (epithelial and fibroblasts), as reported also in the literature (9, 10). Even though the potency of SABA samples of inhibiting the activity of enzyme 5α-reductase type I showed higher values than PERMIXON's values, there were no significant statistical differences between the two brands or the batches. These in-vitro results pointed out that the *S. repens* ethanol extract (*SABA®*) was equivalent to the n-hexane one (*PERMIXON®*).
This conclusion is important because basing on the equivalence of efficacy for both tested extracts, obtained with different extraction methods, it would be better to use organic solvents (ethanol) recognized as environmentally safer and alternative to hexane, which is a solvent obtained from petrochemical sources that can remain in potential traces in edible oils after refining and can be emitted during extraction and recovery and that has been identified as an air pollutant since it can react with other pollutants to produce ozone and photochemical oxidants (7, 8, 16).
Moreover, data that we obtained allow us to speculate on the possible effects of the ethanol extract from *S. repens* on the pathology of BPH: it is known that clinical benefits are generally associated with the free fatty acid content, along with a small contribution from unsaponifiable components (17, 18).
Particularly, the relative inhibitory efficacy of the various free fatty acids seems to depend on the length of the carbon chain and its saturation state (17). For example, lauric acid (short saturated, C12 chain) inhibits both 5 α-reductase type I and II, while oleic acid with a C18 unsaturated chain (C18 Δ9) and linoleic acid (C18 Δ9, 12) have a good activity on type I but not on type II (15). *SABA®* and *PERMIXON®* (19) were analyzed in

Table 1.
Comparative potency of the two extracts on 5α-reductase type I, given by IC50 value and related 95% CI.

	PERMIXON		SABA	
	F11917	G07340	G08363	G08364
IC50 (5α-RI)[1]	11.08	12.72	8.809	9.464
95% CI[2]	6.39 to 19.98	7.76 to 21.53	5.13 to 15.56	5.09 to 18.27

[1]5α-RI: 5α-reductase type I. [2]CI: Confidence Interval.

Table 2.
Relative potency of the various extracts on 5α-reductase type I (considering RP = 1 as the lowest potency).

	5α-RI[1]			
	SABA	SABA	PERMIXON	PERMIXON
Batch	G08363	G08364	F11917	G07340
RP[2]	1.444	1.344	1.148	1

[1]5α-RI: 5α-reductase type I. [2]RP: relative potency.

terms of concentration in free fatty acids, methyl and ethyl esters, long-chain esters and glycerides. These analyses revealed that each of the individual FFAs analyzed was found in similar proportions in the tested products, with lauric and oleic acids present at the highest concentrations in every tested sample and similar concentrations of methyl and ethyl esters were found. Instead, glyceride content was particularly high (420%) in *SABA®* and not in *PERMIXON®*.
Generally, a similar content composition was observed between *SABA®* and *PERMIXON®* (19). So, our results are explained by data reported above: similar content composition is associated with the same potency of ethanol and hexane extracts.

Conclusions

Ethanol extract of *S. repens* has shown in vitro a potency of inhibiting the activity of enzyme 5α-reductase comparable with potency of n-hexane extract of *S. repens*.

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