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Original article

Epilobium angustifolium L. extract with high content in oenothein B on benign prostatic hyperplasia: A monocentric, randomized, double-blind, placebo-controlled clinical trial

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ABSTRACT

Benign prostatic hyperplasia (BPH) is a common condition in adult men. Especially in Europe, increasing attention has been focused on *E. angustifolium* extracts (EAEs), which are widely used for their positive effects on the symptoms of BPH, although human clinical trials are limited. The aim of this monocentric, randomized, double-blind, placebo-controlled clinical trial is to evaluate if a daily intake of hard, gastric-resistant capsules containing a chemically characterized EAE (500 mg) for 6 months may allow a significant improvement in symptoms in subjects with BPH. This study was conducted in 128 adult men, randomly assigned to receive either EAE food supplement (N = 70) or placebo (N = 58), who underwent four visits (baseline = t0, after 15 days = t1, after 2 months = t2 and after 6 months = t3) in an outpatient setting to evaluate post-void residual (PVR) and prostate volume (PV) by means of prostate ultrasound, prostate-specific antigen (PSA) and neutrofile/lymphocyte ratio (N/L), nocturia before the clinical visits and International Prostate Specific Score (IPSS) registered by the physicians. EAE food supplement induced a significant decrease in the PVR and consequently nocturia improving the quality of life as suggested by the decrease of IPSS. No subjects reported adverse effects related to oral intake of EAE food supplement. Moreover, EAE food supplement did not show hepatic or renal toxicity. In conclusion, EAE food supplements can be used in subjects with BPH, to improve their quality of life and general renal function.

Abbreviations: ACE, Angiotensin-converting enzyme; AEs, Adverse events; ALT, Alanine transaminase; AP-N, Aminopeptidase N; AST, aspartate transaminase; AUR, Acute urinary retention; BOO, Bladder outflow obstruction; BPH, Benign prostatic hyperplasia; BR, bilirubin; CHE, Cholinesterase; CRE, Creatinine; CRF, Case report form; CUR, Chronic urinary retention; DDA, Data dependent acquisition; DHT, Dihydrotestosterone; EAE, *E. angustifolium* extracts; EGF, Epidermal growth factor; EMA, European medicinal agency; ESI, Electrospray source; FTMS, Fourier transform mass spectrometry; GFR, Glomerular Filtration Rate; GLMM, Generalized linear mixed models; HRMS, High resolution mass spectrometry; IGF, Insulin-like growth factor; IPSS, International prostate specific score; IT, Ion trap; L, Lymphocyte; MS, Mass spectrometry; MS/MS, Tandem mass spectrometry; N, Neutrofile; NEP, Neutral endopeptidase; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; PDA, Photo diode array; PSA, Prostate-specific antigen; PV, Prostate volume; PVR, post-void residual; RP, Reverse phase; TE, Testosterone; UHPLC, Ultra performance liquid chromatography.

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1. Introduction

The American Urological Association Guidelines define benign prostatic hyperplasia (BPH) as a histologic diagnosis referring to the proliferation of smooth muscle and epithelial cells within the prostatic transition zone, often occurring in the second prostate growth phase [1], which starts at about 25 years of age and continues during much of a man's life [2].

Gontero et al. estimated that BHP is present in 70% of adult males, and yet the epidemiology of this condition is not well defined [3]. Through a systematic research of major scientific databases, a recent meta-analysis of 30 studies published by *Nature* in 2017, estimates a global BPH prevalence of 26.6%, ranging from 14.0% in individuals up to 40%, and 36.8% in individuals aged 80 and older [4].

As the prostate enlarges, the gland presses against and pinches the urethra. The bladder wall becomes thicker and may lose the ability to empty completely, leaving some urine in the bladder. The narrowing of the urethra can cause acute (AUR) and chronic urinary retention (CUR), the most important complications associated with BPH [2]. The first one is a serious complication, which requires hospitalization, while the second may cause other complications including recurrent urinary tract infection, formation of bladder calculi, hematuria, and damage to the bladder wall and kidneys. The most common symptoms of BHP include incomplete bladder emptying, nocturia (i.e., the need to urinate two or more times per night), dribbling at the end of urinary stream, incontinence or leakage of urine, the need to strain when urinating, a weak urinary stream, a sudden urge to urinate, a slowed or delayed urinary stream, painful urination, and blood in the urine. Moreover, there is an important association between benign prostatic hyperplasia/bladder outflow obstruction (BPH/BOO) and male erectile dysfunction. Literature data obtained from clinical trials suggest that the incidence of these complications is low, but, unfortunately, this is enough for them to occur regularly in real life [5].

To date, the mechanism underlying the onset of BPH is not clearly defined. However, as reported by Allkanjari et al., three possible hypotheses have been proposed [6]. The first is based on the role of androgens, estrogens and growth factors. Prostate cells can convert about 90% of testosterone (TE) to dihydrotestosterone (DHT) through 5-alpha reductase. The latter binds to androgen receptors with higher affinity than TE, and seems to act directly by stimulating protein synthesis and prostate cell growth [7,8]. DHT accumulates in the prostate even when TE levels are low [9]. The binding of DHT to the receptor further stimulates the synthesis of growth factors (e.g., epidermal growth factor - EGF and insulin-like growth factor- IGF), leading to abnormal prostate cell proliferation [10]. Estrogens act in synergy with androgens in the development of BPH, through multiple mechanisms including apoptosis, aromatase expression and paracrine regulation via prostaglandin E2 [11]. The second hypothesis is based on the presence of a small percentage of androgen-independent prostate cells that can self-renew in androgen-deficient conditions [12]. The third theory regards the interaction between stroma and epithelium, which can convert TE into DHT allowing the production of various growth factors responsible for cell proliferation, apoptosis, and secretion activity of both stromal (autocrine transmission) and epithelial portions (paracrine secretion) [13,14]. Growing evidence has more recently highlighted the role of inflammation, which may represent an important factor in influencing prostatic growth and progression of symptoms [15–18] although the beneficial activity of anti-inflammatory agents has not yet been sufficiently investigated.

The main pharmacological treatments for BPH are α-blockers and 5-α-reductase inhibitors. The former act as 1-α-adrenergic receptor antagonists, which relax bladder neck muscles and prostate muscle fibers making urination easier. The latter target 5-α-reductase, increasing DHT affinity for androgen receptors. The clinical use of these drugs in the treatment of BPH may put patients at high risk of adverse events including erectile dysfunction, orthostatic hypotension, dizziness and tachycardia [19].

Due to the adverse effects of drug therapies currently used in BPH, which particularly affect the subject's quality of life, increasing attention has been focused on the study of biological activities of vegetable extracts for the treatment of urinary tract dysfunctions, which could be used to alleviate the symptoms occurring in BPH, especially in the early stages (i.e., *Serenoa repens* (W. Bartram) Small, *Pygeum africanum* Hook. f., *Urtica dioica* L., *Cucurbita pepo* L., and *Epilobium* spp).

Epilobium angustifolium L. is an erect stem herbaceous plant belonging to the *Onagraceae* family. The roots and aerial parts of *E. angustifolium* are used in traditional Chinese medicine for the treatment of traumatic injuries, localized inflammation and disorders related to the menstrual cycle. In Europe, preparations based on the aerial parts of *E. angustifolium* are used in the treatment of prostatic disorders. Recent research suggests that *E. angustifolium* yields positive effects on the inflammation of urethra and prostate, as well as micturition problems [20]. In the monograph on *E. angustifolium* published by the European Medicinal Agency (EMA), many European Union countries were found to have used it for over 30 years, thus meeting the requirements for "traditional use" with the following indications: "*Relief of lower urinary tract symptoms related to benign prostatic hyperplasia, after serious conditions have been excluded by a medical doctor*" [21].

Preclinical evidence suggests that *E. angustifolium* extracts and their active components, mainly the ellagitannin oenothein B, exert beneficial effects on prostate health through a complex mechanism of action involving the regulation of androgen levels, inhibition of prostate-specific antigen (PSA) synthesis, and anti-proliferative and pro-apoptotic activities [22–24]. In 2004, Kiss et al. demonstrated the activity of *E. angustifolium* in the inhibition of metalloproteinases (i.e., neprilisyn, angiotensin-converting enzyme, and aminopeptidase N). The deregulation of the levels of these metalloproteinases has been correlated with the development of BPH [25]. In a preclinical study published in 2019, in which a rat model of BPH was induced with testosterone propionate, several of the previously proposed mechanisms of action of *E. angustifolium* were observed, such as down-regulation of androgen levels, suppression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) expression and reduction of inflammatory response and oxidative stress [20]. In 2013, the results of a randomized, double-blind, placebo-controlled, phase II trial were published, showing the efficacy and safety of a traditional herbal medicinal product based on *E. angustifolium*, *S. lycopersicum*, *P. africanum* and *S. repens*, on BPH symptoms (by means of *International Prostate Specific Score,* IPSS) and reduction of night-time urinary frequency [26].

To date, there is no clinical evidence in the literature on the efficacy of *E. angustifolium* extract alone against BPH. Thus, considering that prostatic inflammation plays an important role in BPH and recent evidence suggests that prolonged treatment in CD1 mice with a chemically characterized *E. angustifolium* extracts (EAE), standardized to contain $\geq$ 15% oenothein B, reveals the formation of urolithins A and B which are known to exert anti-inflammatory activity [28], the aim of this monocentric, randomized, double-blind, placebo-controlled clinical trial is to evaluate if a daily intake of a food supplement based on EAE for a period of 6 months may allow a significant improvement in symptoms and urinary flow in subjects with BPH. In addition, using the latest generation of LC-MS instrumentation, the metabolic profile of EAE was further analyzed to deepen the knowledge on the chemical composition of the phytocomplex.

2. Material and methods

2.1. Food supplement based on E. angustifolium extract and placebo

The food supplement and placebo used in this study consisted of hard, gastric-resistant capsules containing EAE (500 mg) and magnesium stearate (5 mg) as a sliding agent, and microcrystalline cellulose (350 mg) and magnesium stearate (5 mg), respectively. EAE,

standardized to contain ≥ 15% oenothein B, is a commercial food supplement ingredient (ENOTprost®) produced by EPO S.r.l. (Milan, Italy). According to the manufacturer, EAE complies with European specifications for contaminants and microbiological limits. EAE food supplement and placebo capsules, produced by Sorgente del Benessere S.r.l. (Fiuggi, Italy), were packaged in white containers of 60 capsules each. Both treatments (EAE food supplement and placebo) were indistinguishable in appearance, color, and flavor. The control of the net weight of EAE food supplement and placebo capsules was managed by means of Metrostat statistical software, in agreement with Italian law (Legge 25 ottobre 1978 n. 690) and standard UNI ISO 2859.

2.2. *E. angustifolium* extract analysis by UHPLC-LTQ Orbitrap

Chromatographic analysis of the EAE extract was performed by means of UHPLC-LTQ Orbitrap. The EAE sample was collected in a 2 ml eppendorf microtube and solubilized in 1 ml of methanol/water (80:20 v/v). 500 µl were filtered through 0.22 µm Minisart RC 4 membrane filter and analyzed. UHPLC-HRMS analyses were performed on a Shimadzu Nexera UHPLC system, consisting of a CBM-20A controller, two LC-30 CE dual-plunger parallel-flow pumps, a DGU-20 AR5 degasser, an SPD-M20A photo diode array detector, a CTO-20A column oven, and a SIL-30AC autosampler. The system was coupled online to an LTQ-Orbitrap XL (Thermo Scientific, Bremen, Germany) equipped with an electrospray source (ESI).

For RP-UHPLC analyses, a Kinetex® EVO C18 150 mm × 2.1 mm, 2.6 µm (100 Å) (L × I.D, particle size, Phenomenex®, Bologna, Italy) column was employed at a flow rate of 0.4 ml/min. The mobile phases consisted of A) 0.1% HCOOH in H2O and B) ACN plus 0.1% HCOOH v/v. Analysis was performed in gradient as follows: 0–25.0 min, 2–30% B; 25.01–30.0 min, 30.01–98% B; 98% B hold for 5 min; returning to initial conditions in 0.1 min. The column oven was set to 45 °C, and 2 µl were injected. PDA detection parameters were: sampling rate 12 Hz, time constant 0.160 s and chromatograms extracted at 280 and 330 nm. LC data elaboration was performed using LCMS solution® software (Version 3.50.346, Shimadzu).

MS detection was performed in negative mode as follows: spray voltage was set to − 3.5 kV, sheath gas arbitrary units 40, auxiliary gas arbitrary units 12, and capillary temperature 250 °C. MS/MS spectra were collected in data-dependent mode (DDA), over the *m/z* range of 150–2000, at 30,000 resolution. All MS/MS spectra were collected using a collision energy of 35% and an isolation window of 2 *m/z*, minimum signal threshold 100, and monoisotopic precursor enabled. Ion trap and Orbitrap maximum ion injection times were set to 50 and 100 ms, respectively. Automatic gain control was set to 2×10^5 for full FTMS scan and 3×10^4 ions for IT. Dynamic exclusion was enabled with a repeat count of 1 and a repeat duration of 30 s. Preview mode was enabled for the FTMS master scan. The instrument was tuned using a Thermo negative ion calibration solution. Thermo RAW data files were converted in mzXML and were aligned using the open-source software MZmine2.

Metabolite annotation was based on accurate mass measurement, MS/MS fragmentation pattern and comparison with *in silico* spectra by the following software: SIRIUS ver.4.01 (https://bio.informatik.uni-jena.de/sirius/) and the Mass bank of North America (MoNA).

2.3. Clinical trial design

A monocentric, randomized, double-blind, placebo-controlled clinical trial was performed by Samnium Medical Cooperative (Benevento, Italy) to evaluate the effects of the EAE food supplement on an adult male population (mean age ± SD, treated group: 67 ± 10, placebo group: 64 ± 10), suffering from BPH diagnosed through prostate ultrasound, blood tests and IPSS score.

The study was double-blind, both for the investigating physician and for the enrolled subjects. The participants received oral and written information regarding the study before they gave their written consent. Protocol, letter of intent of volunteers, and synoptic documents regarding the study were submitted to the Scientific Ethics Committee of ASL Benevento, Italy. The study was approved by the Committee (protocol number 10534 of 24/01/2020) and carried out in accordance with the Helsinki declaration of 1964 (as revised in 2000). This study is listed on the ISRCTN registry (www.isrctn.com) with ID ISRCTN18705154 (doi.org/10.1186/ISRCTN18705154).

Table 1
Analyses planned at t0, t1, t2, and t3.

Clinical visits	Analyses
t0 (baseline)	PV, PVR, PSA, N/L, CRE, BR direct/indirect/total, Protrombine, AST, ALT, CHE, GFR
t1 (15 days)	CRE, BR direct/indirect/total, Protrombine, AST, ALT, CHE, GRF
t2 (2 months)	PSA, N/R, CRE, BR direct/indirect/total, Protrombine, AST, ALT, CHE, GFR
t3 (6 months)	PV, PVR, PSA, N/R, CRE, BR direct/indirect/total, Protrombine, AST, ALT, CHE, GFR

Prostate volume (PV), post-void residual volume (PVR), prostate-specific antigen (PSA), neutrofile/lymphocyte ratio (N/L), creatinine (CRE), bilirubin (BR direct/indirect/total), prothrombin, aspartate transaminase (AST), alanine transaminase (ALT), cholinesterase (CHE).

The clinical trial duration was 7 months. Participants underwent four visits (baseline = t0, after 15 days = t1, after 2 months = t2, and after 6 months = t3) in an outpatient setting. For the baseline visit (t0) information on the sociodemographic, clinical and symptomatologic characteristics of the subjects were collected and reported in the case report form (CRF). In particular, bladder post-void residual volume (PVR) and prostate volume (PV) were obtained by prostate ultrasound; prostate-specific antigen (PSA), and neutrofile/lymphocyte ratio (N/L) were derived from blood tests analyzed by Unisannio Lab (San Giorgio del Sannio, BN, Italy); number of urinations during the night before the clinical visits, and IPSS score were registered by the physicians.

At the end of the baseline visit, the randomization sequence was generated using STATA 16 software (Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC) and the randomization list was kept hidden. Subjects were assigned to each treatment groups (EAE food supplement or placebo groups) by simple randomization (about 1:1 allocation ratio). Neither stratification nor blocking were used. The allocation sequence was kept hidden from the physician recruiting and evaluating participants using progressively numbered, opaque, sealed and stapled envelopes. The corresponding envelopes were opened only after the enlisted participants completed all baseline assessments. The EAE food supplement group was provided one hard gastro-resistant capsule per day for 6 months; it contained 500 mg of EAE, corresponding to 2 g of aerial parts of *E. angustifolium,* according to the indications of the Assessment Report on *E. angustifolium* L. and/or *Epilobium parviflorum* Schreb., herba [20]. The placebo group received one hard gastro-resistant capsule per day containing 350 mg of microcrystalline cellulose for six months. Hard gastric-resistant capsules containing EAE or microcrystalline cellulose were made unrecognizable by identical color, shape and taste. The white plastic container used for both EAE food supplement treatment and placebo was not recognizable. 60 capsules per white plastic container were given to both EAE food supplement and placebo groups during the baseline visit (t0). The rest of the treatment (i.e., 4 containers) were given to the subjects during the t2 visit (after 2 months of treatment).

Clinical visits were carried out at t1 (after 15 days of treatment) to monitor the possibility of kidney and liver toxicity, t2 (after 2 months of treatment), and t3 (after 6 months of treatment). After each clinical visit, all data were compiled in the CRF by the physicians.

In detail, the specific analyses carried out are shown in Table 1.

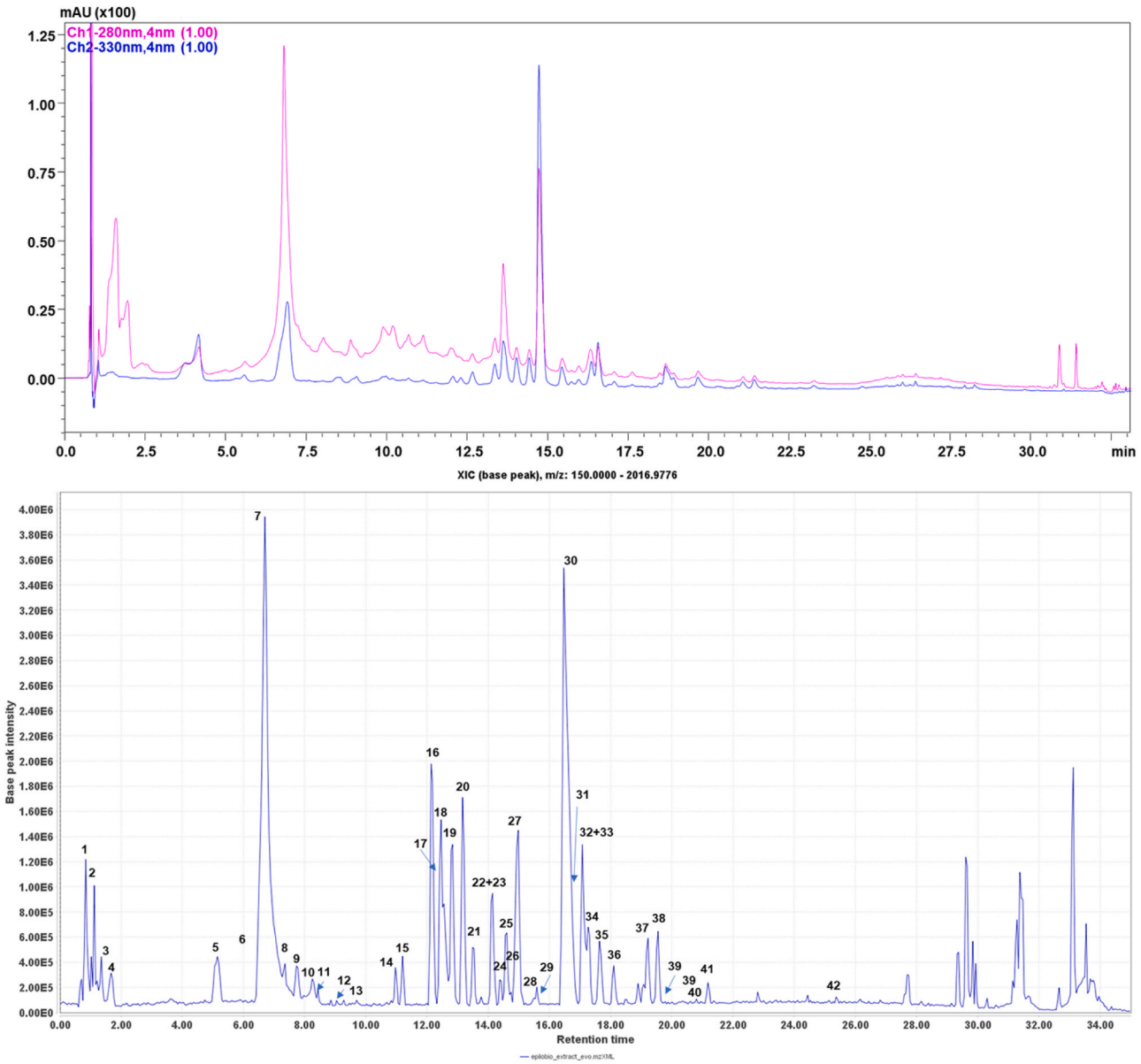

Fig. 1. RP-UHPLC chromatograms of EAE with UV-detection registered at λ 280 nm and 330 nm. A) and base peak chromatogram (BPC) with corresponding peak annotation (B).

2.4. Study population

Participants (128 male subjects: 70 in the treated group and 58 in the placebo group) were recruited by the Samnium Medical Cooperative (Benevento, Italy). The subjects were recruited following these inclusion criteria: no clinically significant deviation in laboratory tests; history of BPH for at least one year, IPSS score ≤ 25, prostate volume ranging from 25 cc to 200 cc, no medication intake for BPH prior to baseline assessment and during the study, PVR $\leq 300 \pm 2$ ml and serum total PSA lower than 4 ng/ml. Subjects with the following criteria were excluded from the study: acute or chronic disease that could interfere with the study or endanger the subject; use of any of the following concomitant drugs: immunosuppressants, anticoagulants, α-blockers, 5α-reductase inhibitors, antipsychotics, chemotherapy drugs, drugs for dementia, male hormone replacement therapy and drugs for overactive bladder, atonic and/or neurogenic bladder; bladder neck contracture; acute prostatitis; bladder calculosis; urinary tract infection more than once in the last 12 months; prostate or bladder cancer; history of pelvic trauma or surgery; clinically significant kidney or hepatic insufficiency; microscopic hematuria that was not evaluated by a urologist and not attributed to BPH; any condition that might interfere with the subject's ability to give informed consent, to comply with study instructions, to provide an objective evaluation of his or her symptoms, or that might confuse the interpretation of study results; those considered unsuitable for participation by the physician.

2.5. Evaluated variables

As a sociodemographic characteristic, the age of the participants was registered in the CRF. The primary endpoint was to investigate the efficacy of a 6 month-daily dose of EAE food supplement to reduce PVR and PV in subjects with BPH, assessed at baseline (t0) and after 6 months (t3). A BPH diagnosis was made by the physician based on prostatic ultrasound, PSA assay and IPSS score.

As secondary outcomes, assessment of symptomatology reduction using a validated symptomatology scale, such as IPSS score, number of urinations during the night before each clinical visit, N/L ratio, and PSA assay were evaluated (t0, t2, t3).

Table 2
Identified compounds in EAE according to the retention time (RT), compound, molecular formula, *m/z* and MS/MS.

Peak number	RT (min)	Proposed structure	Molecular Formula	*m/z* [M-H]$^-$	MS/MS	Error (ppm)
1	0.84	Caffeic acid 4-O-hexoside	$C_{12}H_{22}O_{11}$	341.10896	179.0245; 113.0587; 143.0246	0.30
2	1.03	Shikimic acid	$C_7H_{10}O_5$	173.04556	137.0381; 111.1149	0.40
3	1.12	Galloylhexose	$C_{13}H_{16}O_{10}$	331.06674	191.0562	1.00
4	1.67	Gallic acid	$C_7H_6O_5$	169.01429	125.0134	0.50
5	5.15	Chlorogenic acid	$C_{16}H_{18}O_9$	353.08746	191.0537; 135.0401	0.80
6	6.46	Gemin D	$C_{27}H_{22}O_{18}$	633.07263	301.0704	-1.01
7	6.70	Oenothein B	$C_{68}H_{48}O_{44}$	1567.14417	765.1010; 935.1260; 633.4009	1.60
8	6.79	p-coumaroylquinic acid isomer	$C_{16}H_{18}O_8$	337.09280	191.0562	-0.20
9	7.73	Chlorogenic acid isomer	$C_{16}H_{18}O_9$	353.08752	191.0537; 135.0401	0.90
10	8.25	Oenothein A	$C_{102}H_{72}O_{66}$	1175.6093 [M-2H]2-	765.1010; 935.1260; 633.4009	1.60
11	8.29	3-feruloylquinic acid	$C_{17}H_{20}O_9$	367.10286	193.0408; 173.0130	-1.04
12	9.04	p-coumaroylquinic acid	$C_{16}H_{18}O_8$	337.09235	191.0562	-0.10
13	9.28	Tannic acid	$C_{27}H_{24}O_{18}$	635.08838	465.1876	-0.90
14	10.96	Caffeoyl quinic acid -3-O-hexoside	$C_{21}H_{20}O_{13}$	479.08223	316.0802	-1.09
15	11.20	Myricetin-3-O-hexoside	$C_{21}H_{20}O_{13}$	479.08237	316.0802	-1.12
16	12.13	Galloylquercetin	$C_{28}H_{24}O_{16}$	615.09845	463.0809; 301.0906	-1.02
17	12.41	Quercetin-3-O-pentoside	$C_{19}H_{14}O_{12}$	433.04062	301.1205	-1.04
18	12.46	Galloylquercetin	$C_{28}H_{24}O_{16}$	615.09851	463.0809; 301.0906	-1.02
19	12.83	Quercetin-3-O-hexoside	$C_{21}H_{20}O_{12}$	463.08728	301.1288	-1.07
20	13.16	Quercetin-3-O-hexoside	$C_{21}H_{20}O_{12}$	463.08731	301.1279	-0.98
21	13.49	Kaempferol galloyl hexoside	$C_{28}H_{24}O_{15}$	599.10336	447.3458; 313.1143; 285.1048	-1.04
22	14.14	Quercetin-3-O-pentoside	$C_{20}H_{18}O_{11}$	433.07681	301.0614	-1.90
23	14.19	Kaempferol-3-O-hexoside	$C_{21}H_{20}O_{11}$	447.09253	284.0121; 255.1	-1.70
24	14.37	Quercetin-3-O-pentoside	$C_{20}H_{18}O_{11}$	quinic.07690	301.0614	-1.80
25	14.61	Kaempferol galloyl hexoside	$C_{28}H_{24}O_{15}$	599.10358	447.3458; 313.1143; 285.1048	-1.04
26	14.74	Myricetin-methylether-hexoside	$C_{21}H_{18}O_{14}$	493.06177	317.0300	-1.10
27	14.98	Kaempferol-3-O-hexoside	$C_{21}H_{20}O_{11}$	447.09254	284.0121; 255.1483	-1.70
28	15.49	Kaempferol 3-O-arabinoside	$C_{20}H_{18}O_{10}$	417.08203	284.0187	-1.70
29	15.59	Myricetin-3-O-caffeoyl-hexoside	$C_{30}H_{26}O_{16}$	641.11346	479.1309; 317.1401	-2.00
30	16.47	Quercetin-glucuronide	$C_{21}H_{18}O_{13}$	477.06689	301.1463	-1.10
31	16.84	Rutin	$C_{27}H_{30}O_{16}$	609.12427	463.0871; 301.1127	0.10
32	17.08	Kaempferol-7-O-rhamnoside	$C_{21}H_{20}O_{10}$	431.09763	285.1060	-1.07
33	17.17	Quercetin 3'-hexoside-7-acetate	$C_{23}H_{22}O_{13}$	505.09805	301.1793; 329.2003	-1.30
34	17.26	Quercetin-3-O-caffeoylhexoside	$C_{30}H_{26}O_{15}$	625.11932	463.0875; 479.2256	1.10
35	17.64	Kaempferol-glucuronide	$C_{21}H_{18}O_{12}$	461.07166	285.0398	-1.80
36	18.10	Quercetin	$C_{15}H_{10}O_7$	301.03493	179.0153	-1.50
37	18.48	Quercetin-p-coumaroylhexoside	$C_{30}H_{26}O_{14}$	609.12424	463.0275; 447.3030	-1.20
38	19.22	Quercetin-p-coumaroylhexoside	$C_{30}H_{26}O_{14}$	609.12421	463.0275; 447.3030	0.10
39	19.45	Quercetin 3-(6''-ferulylhexoside)	$C_{31}H_{28}O_{15}$	639.13470	463.0213; 301.1146	-1.30
40	20.81	Kaempferol-p-coumaroylhexoside	$C_{30}H_{26}O_{13}$	593.12933	447.2656; 285.1403; 255.1559	-1.02
41	21.18	Kaempferol-p-coumaroylhexoside	$C_{30}H_{26}O_{13}$	593.12952	447.2656; 285.1403; 255.1559	-0.90
42	25.37	Dimethylquercetin	$C_{17}H_{14}O_7$	329.06636	314.1575	0.80

2.6. Statistical analysis

The sample size calculation was made using three 1-β power values equal to 0.95 and a significance level $\alpha = 0.05$. The sample size was determined to be 130 participants, allowing for a 15% drop out rate.

The effect of the treatment with EAE food supplements on the response variables for the primary and secondary outcomes of the study (i.e., PV, PVR, urinations during night, and IPCC score) was assessed by generalized linear mixed models (GLMM) including treatment (EAE food supplement vs placebo), measure (t0 vs t3), and age of the subject (standardized) as explanatory variables. We also added the treatment × measure interaction to account for differential effects of the treatment between t0 and t3. The subjects entered the model at a random intercept to account for unexplained variation at the individual level (σ^2_{ind}) after we controlled for the explanatory variables. We ran an independent model for each response variable. The PV (after log transformation) and IPSS were normally distributed and consequently the distribution error of the two corresponding GLMMs was set as Gaussian. By contrast, both PVR and nightly urinations followed a Poissonian distribution, and consequently the distribution error of the two corresponding GLMMs was set as Poisson.

Biochemical variables were analyzed using a GLMM with the same predictors as for the four main response variables. All variables were normally distributed or achieved normality after log transformation (i.e., PSA, N/L, AST, ALT, and BR total). Analyses were performed using the lme4 [34] and MuMIn [35] packages in R ver. 3.2.4 (R core Team 2016), and unless otherwise stated, data are reported as means ± standard error.

2.7. Tolerance and safety assessment

Hepatic and renal toxicity tests were undertaken throughout the clinical trial. In particular, blood tests to evaluate creatinine (CRE), bilirubin (BR direct/indirect/total), prothrombin, aspartate transaminase (AST), alanine transaminase (ALT), Glomerular Filtration Rate (GFR), and cholinesterase (CHE) were performed at t0, t1, t2, t3.

For the evaluation of tolerance and safety of the intervention (EAE food supplement), adverse events were monitored throughout the intervention period through spontaneous reporting of adverse events (AEs) by the participants to the relative physicians. At the end of the intervention period all subject data were evaluated by the principal investigator to determine the presence or absence of AEs.

3. Results

3.1. E. angustifolium extract chromatographic analysis

In this study a dry hydroalcoholic extract produced from the aerial parts of *E. angustifolium*, standardized to contain ≥15% oenothein B, was used. The extract was analyzed by means of UHPLC- LTQ Orbitrap to obtain its metabolic profile (Fig. 1). 42 compounds were annotated by UHPLC-HRMS as shown in Table 2. The identification of all compounds

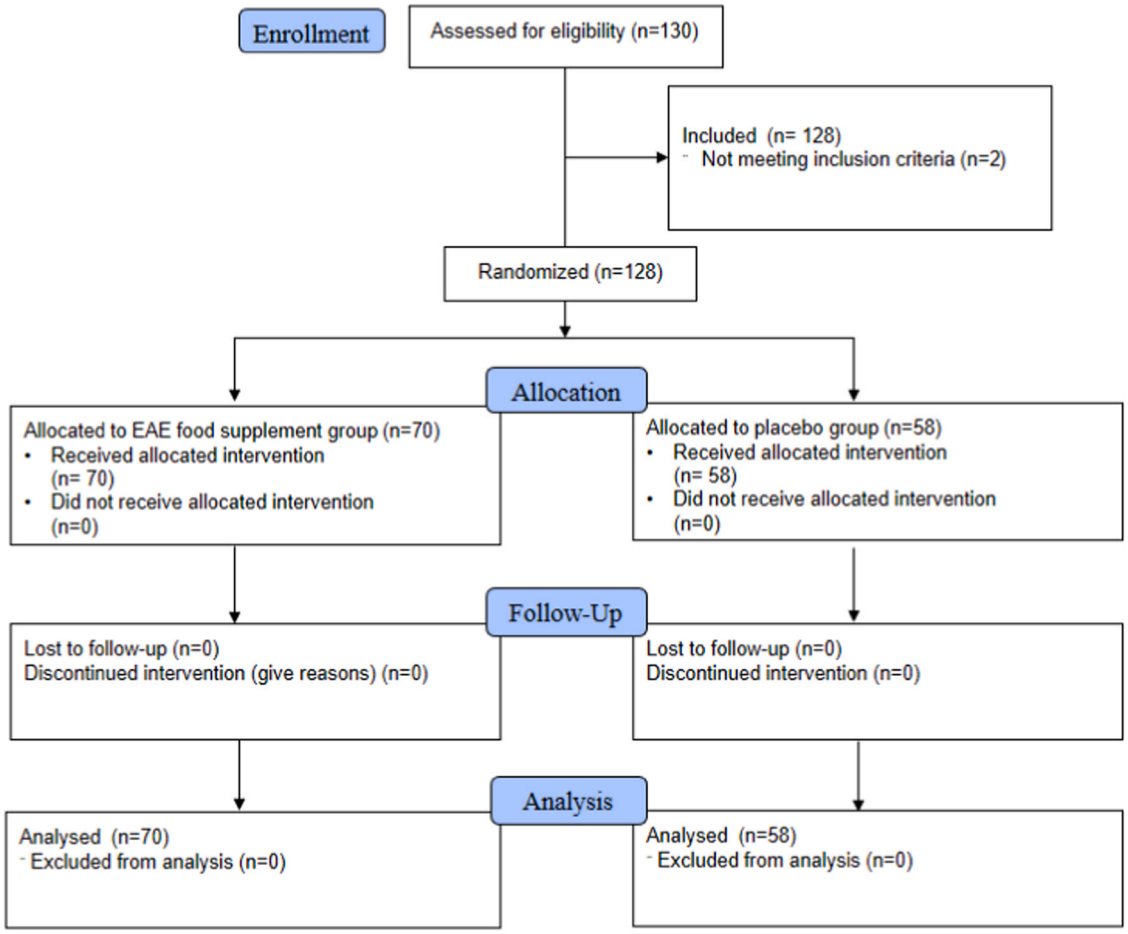

Fig. 2. CONSORT flow diagram.

Table 3
Characteristics of the study population: demographic and clinical data at baseline.

Characteristic/ Clinical data	Treated (n = 70)	Untreated (n = 58)
Age	67 ± 10	64 ± 10
Ethnic origin: All Europeans	70	58
PV (cc)	45.2 ± 2.4	44.1 ± 3.1
PVR (ml)	40.4 ± 7.3	28.0 ± 5.4
Urinations during night	1.2 ± 0.1	1.1 ± 0.1
IPSS	13.4 ± 0.7	13.0 ± 0.8
PSA (ng/ml)	1.7 ± 0.2	2.6 ± 0.8
N/L (%)	2.2 ± 0.1	1.9 ± 0.1
AST (U/L)	22.1 ± 0.7	22.1 ± 0.9
ALT (U/L)	21.7 ± 1.0	21.6 ± 1.3
BR (direct/indirect) (mg/dl)	0.24 ± 0.03	0.35 ± 0.05
BR total (mg/dl)	0.88 ± 0.05	0.84 ± 0.05
Protrombine (Inr)	1.01 ± 0.01	1.01 ± 0.01
CHE (U/L)	7983 ± 175	8150 ± 163
GFR (ml/min/1,73 mq)	62.3 ± 1.4	69.8 ± 2.2
CRE (mg/dl)	1.19 ± 0.02	1.10 ± 0.03

reported was carried out by accurate mass and fragmentation pattern comparison against reference MS/MS spectra reported *in silico* and in previous literature. UHPLC-MS/MS analysis of the phytocomplex has led to the identification of various metabolites, both aglycone and glycosylated, and the presence of ellagitannins (such as oenothein A and B). Among the putatively identified compounds were 8 organic and phenolic acids, 1 sugar, 1 tannin, 3 ellagitannins and 29 flavonoids as reported in Table 2.

Table 4
Descriptive statistics (mean ± SE, range) for the four response variables measured at t0 and t3 in the two experimental groups.

Response variable	Placebo group (n = 58)		EAE food supplement group (n = 70)	
	t0	t3	t0	t3
PV	44.1 ± 3.1 (17–143)	50.4 ± 4.6 (15–200)	45.2 ± 2.4 (13–100)	47.2 ± 2.8 (10–118)
IPSS	13.0 ± 0.8 (2–25)	13.6 ± 0.7 (2–25)	13.4 ± 0.7 (3–25)	11.5 ± 0.6 (1–25)
PVR	28 ± 5.4 (0–200)	31.4 ± 5.2 (0–210)	40.4 ± 7.3 (0–296)	39.5 ± 8.2 (0–360)
Urinations during the night before clinical visits	1.1 ± 0.1 (0–4)	1.3 ± 0.1 (0–4)	1.2 ± 0.1 (0–5)	0.8 ± 0.1 (0–3)

3.2. Clinical trial

The study flow chart is reported in Fig. 2, according to the CONSORT PRO reporting guideline [27]. The participants in the EAE food supplement and placebo groups had similar sociodemographic characteristics and clinical data, with no significant differences. The baseline characteristics of the subjects for each group are summarized in Table 3.

The study revealed that three response variables (IPSS, PVR, and the number of urinations during night) changed between the EAE food supplement group and the placebo group between the beginning (t0) and the end (t3) of the clinical trial (Table 4). Indeed, GLMM analysis showed that the treatment × measure interaction was statistically significant for IPSS, PVR, and the number of urinations during night, but

Table 5
Fixed effects of the GLMMs used for the four response variables.

Effect	F[a]/χ^2[b]	Df	P
PV[a]			
Treatment	0.612	1125	0.43
Measure	1.921	1126	0.17
Age	21.26	1125	< 0.001
Treat. × Meas.[c]	0.482	1126	0.49
IPSS[a]			
Treatment	1.666	1125	0.20
Measure	17.39	1126	< 0.001
Age	8.341	1125	0.004
Treat. × Meas.	58.67	1126	< 0.001
VR[b]			
Treatment	0.211	1	0.64
Measure	4.399	1	0.036
Age	1.820	1	0.18
Treat. × Mis.	40.84	1	< 0.001
Number of Urinations[b]			
Treatment	2.253	1	0.13
Measure	0.855	1	0.35
Age	6.559	1	0.010
Treat. × Meas.	6.174	1	0.013

***Poissonian distribution.
[a] Models with Gaussian error distribution.
[b] models with Poissonian error distribution.
[c] Treatment x Measures.

not for PV (Table 5). In more detail, PV values did not significantly change either between t0 and t3 or between EAE food supplement and placebo groups (Table 5, Fig. 3A), but were dependent on the age of the subjects. In fact, as expected, older individual in both groups had higher PV ($\beta = 0.16 \pm 0.03$, $t_{125} = 4.611$, $P < 0.001$, Fig. 3B).

As far as the IPSS score is concerned, it decreased significantly between t0 and t3 in the EAE food supplement group ($\beta = -1.9 \pm 0.2$, $t_{126} = 7.89$, $P < 0.001$ Fig. 3C), while it slightly increased in the placebo group ($\beta = +0.6 \pm 0.2$, $t_{126} = 2.36$, $P = 0.02$, Fig. 3C). Similarly to PV, the IPSS score significantly increased with increasing subjects' age, irrespective of the treatment ($\beta = 1.43 \pm 0.49$, $t_{125} = 2.888$, $P = 0.005$, Fig. 3D).

The Poisson GLMM for the PVR value showed that in the EAE food supplement group the number of subjects with a low residual urine volume in the bladder significantly increased, decreasing the proportion of subjects with residual urine volumes higher than 100 μl ($\beta = -0.17 \pm 0.03$, $Z = 5.792$, $P < 0.001$, Fig. 4). The opposite pattern was instead observed in the placebo group ($\beta = 0.12 \pm 0.03$, $Z = 3.419$, $P < 0.001$, Fig. 4).

A similar pattern of response was observed for the number of urinations during the night (Fig. 5): in the EAE food supplement group, the frequency of subjects without urination increased, while decreasing the number of subjects urinating more than once per night ($\beta = -0.41 \pm 0.17$, $Z = 2.408$, $P = 0.016$). No significant change between t0 and t3 occurred in the placebo group. As expected, regardless of the group, the frequency of urination increased significantly with the age of the subject ($\beta = 0.19 \pm 0.07$, $Z = 2.561$, $P = 0.010$). In detail, the number of subjects not urinating overnight increased by 21.7% in the treated group, whereas it decreased by 10.2% in the placebo group. More interestingly, the number of subjects urinating three or more times per night was completely wiped out in the treated group but remained unchanged in the placebo group.

Means, standard errors and ranges for each biochemical variable of EAE food supplement and placebo groups are reported in Table 6. The GLMM analyses did not find any significant effects of the experimental treatment on any of the biochemical variables (statistics not shown), with the exception of N/L. In detail, the N/L was significantly higher in the placebo group in comparison with the EAE food supplement group ($F_{1125} = 5.893$, $P = 0.017$), but did not change between t0 and t3 ($F_{1126} = 1.191$, $P = 0.27$).

At the end of the treatment, all participants were included in the analysis of the groups to which they were originally assigned (intention-to-treat analysis). During 6 months of treatment, no subjects reported adverse effects (AEs) related to oral intake of *E. angustifolium* food supplement, and the principal investigator judged that the treatment with *E. angustifolium* capsules was considered well tolerated. Moreover, as shown by the results reported in Table 6, *E. angustifolium* food supplement did not exert hepatic or renal toxicity.

4. Discussion

In this study the dry extract from the aerial parts of *E. angustifolium*, obtained through hydroalcoholic extraction and standardized to contain ≥ 15% oenothein B, was used as ingredient for the food supplement in hard gastro-resistant capsule dosage form. The extract consists of three main polyphenolic families: flavonoids (i.e., myricetin, quercetin, kaempferol, hyperoside, isoquercetin, quercitrin and myquelianin), phenolic acids (shikimic, gallic chlorogenic, caffeic, and ferulic acids), and tannins (tannic acid, gemin D, oenothein A, oenothein B). The employment of high-resolution mass spectrometry resulted in a higher number of identified compounds in the *E. angustifolium* extract compared to previous studies carried out with low resolution MS devices, where a lower number of compounds was determined. In particular, in a recent study aimed to investigate the in vitro bioaccessibility and in vivo bioavailability of EAE [28], we identified 20 compounds consisting of 2 sugars, seven organic and phenolic acids, one ellagitannin, and ten flavonols. In another paper on the antioxidant activities and active chemical constituents present in *E. angustifolium*, 28 compounds were identified as phenolic compounds and flavonoids by LC-MS/MS [29]. In the present investigation, new compounds occurring in EAE were found for the first time i.e. ellagitannins (oenothein A and gemin D), a tannin (tannic acid), shikimic acid, two flavonol galloyl (galloylquercetin), a dimethoxyflavone (dimethyl quercetin) and a large number of variously substituted glycosylated metabolites (i.e caffeic acid-4-O-hexoside, galloylhexoside, caffeoylquinic acid 3-O-hexoside, myricetin-methyetherhexoside, kaempferol-3-O-arabinoside, myricetin-3-O-caffeoylhexoside, rutin, kaempferol-7-O-rhamnoside, quercetin-3-O-caffeoylhexoside, kaempferol glucuronide, quercetin 3-(6''-feruylhexoside), two kaempferol-p-coumaroylhexoside).

Although literature data on the effects of *E. angustifolium* against BPH are limited, this plant is used, especially in Europe. BPH is a condition mainly characterized by a proliferation of both stromal and epithelial cells in the prostate with an alteration of the periurethral area responsible for symptoms that can strongly affect the quality of life [30]. The symptoms of BPH are often very mild at first, but they become more serious if BPH is not treated. In the present study, a monocentric, double-blind, placebo controlled clinical trial was conducted to demonstrate the effects of *E. angustifolium*, with a high content of oenothein B, in subjects with BPH. The results of this clinical study clearly show that a daily intake of *E. angustifolium* for a period of 6 months may yield a significant improvement in symptoms, and an improvement of urinary flow. In fact, in the EAE food supplement group the frequency of subjects without urinations during the night increased, and the subjects urinating more than once per night significantly decreased. On the other hand, the placebo group did not follow the same trend. The same result was achieved for PVR values. In fact, in the EAE food supplement group the number of subjects with a low residual urine volume in the bladder significantly increased, while there was a decreasing frequency of subjects with residual urine volume higher than 100 ml. Finally, the quality of life of the treated subjects was improved, as in the EAE food supplement group the IPSS score significantly decreased while in the placebo group it slightly increased.

The results obtained by this clinical trial are in line with those obtained by Coulson et al. [26] who found significant reductions in IPSS score and night-time urinary frequency in the group treated with the herbal preparation containing *Cucurbita pepo*, *Epilobium parviflorum*,

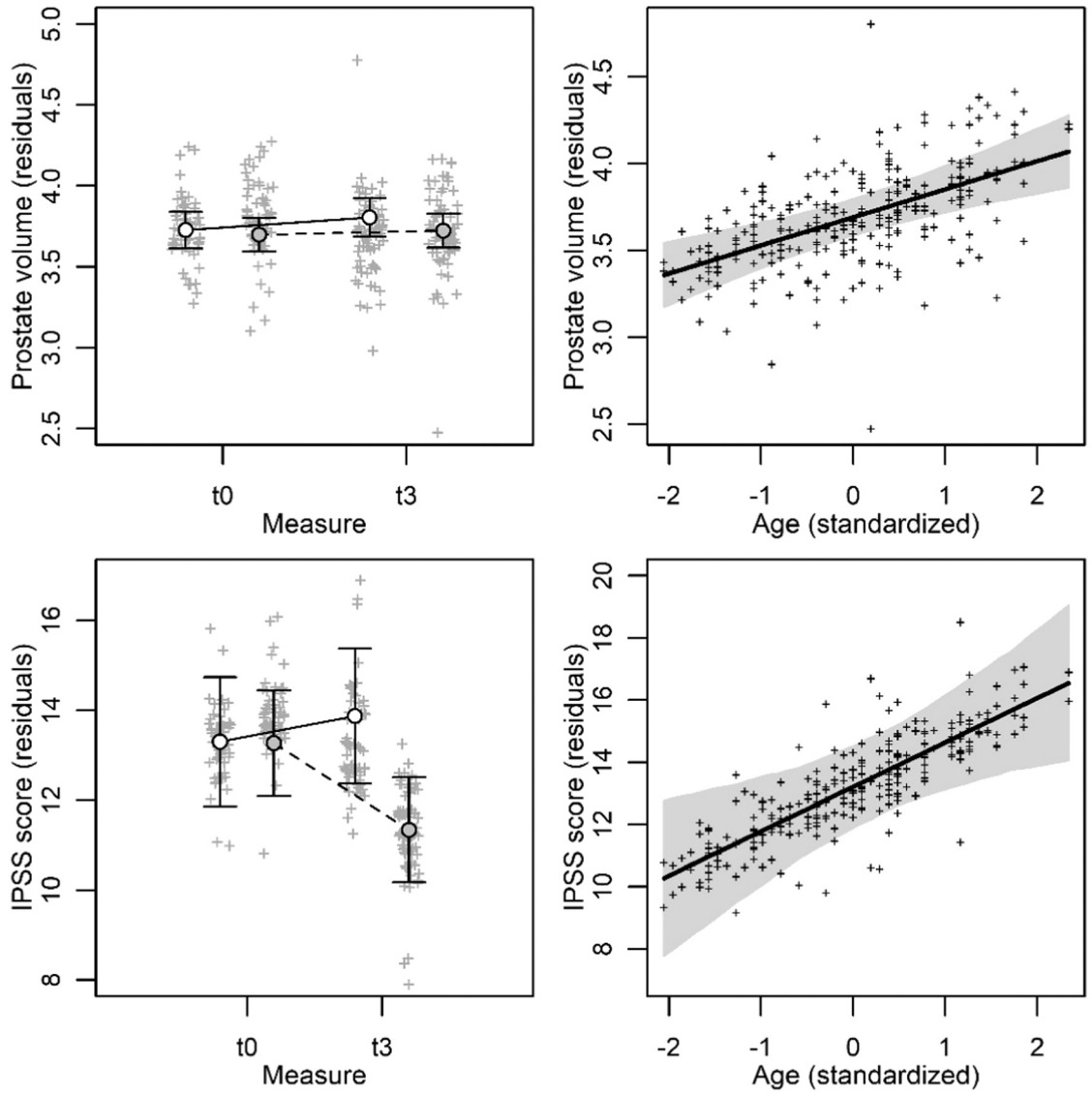

Fig. 3. Variation of prostate volume (PV) and International Prostate Specific Score (IPSS) in response to treatment (EAE food supplement vs placebo) and age of the subject (standardized) as predicted by GLMMs. White circles: placebo group, gray circles: EAE food supplement group; bars and gray areas are for 95% confidence intervals of the estimate coefficients.

lycopene, *Pygeum africanum* and *Serenoa repens* during the 3-months intervention.

This work has limitations and strengths. The main limitation is represented by the fact that the follow up after the 6 months of treatment was not performed, making it impossible to learn about any longer term effects of supplementation.

On the other hand, the major strength of this study is that to the best of our knowledge, it was the first double blind, controlled interventional study on the effects of *E. angustifolium* extract alone on BPH suggesting a significant reduction of nocturia. This result is very remarkable for its impact on wellness and health, as nocturia is a serious therapeutic problem with a serious impact on the quality of sleep, leading to sleep disorders, decreased quality of life and depression [31]. In addition, nocturia can be the cause of falls and hip fractures, which, in turn, increase disability and mortality rates [32]. A second important strength of our study concerns the International Prostate Symptom Score (IPSS), a validated questionnaire to assess BHP symptoms in men with urinary complaints. Each question concerning urinary symptoms allows the subject to choose one of six answers indicating increasing severity of that particular symptom. The answers are assigned points from 0 to 5. The total score can therefore range from 0 to 35 (asymptomatic to very symptomatic). The questions refer to the following urinary symptoms: 1) incomplete emptying, 2) frequency, 3) intermittency, 4) urgency, 5) weak stream, 6) straining, and 7) nocturia. The last question refers to the subject's perceived quality of life. A symptom score less than or equal to 7 indicates mild symptoms, symptom scores ranging 8–19 indicate moderate symptoms, and symptom scores ranging 20–35 indicate severe symptoms, as reported by Barry et al. [33]. In the present clinical trial, the IPSS score significantly decreased by nearly 2 points between t0 and t3 in the treated group and slightly increased (0.6 points) in the placebo group, showing an improvement in the quality of life of the subjects treated with the EAE food supplement and highlighting the protective effect of this supplementation. Finally, as far as tolerance and safety assessment is concerned, *E. angustifolium* food supplement is well tolerated and did not show hepatic or renal toxicity. This study confirms the safety of *E. angustifolium*, as no subjects reported AEs related to *E. angustifolium* treatment.

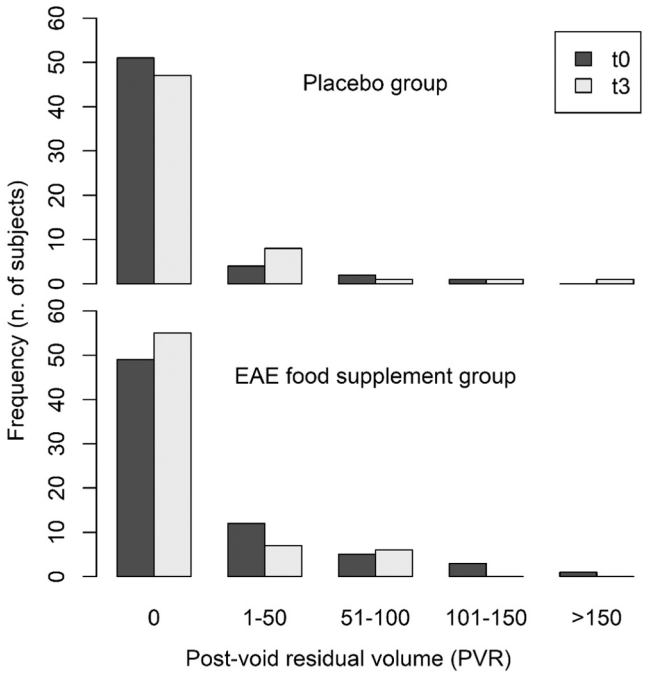

Fig. 4. Frequency distributions (n. of subjects) of the post-void residual volume (PVR) in the two experimental groups at t0 and t3 as predicted by the GLMM.

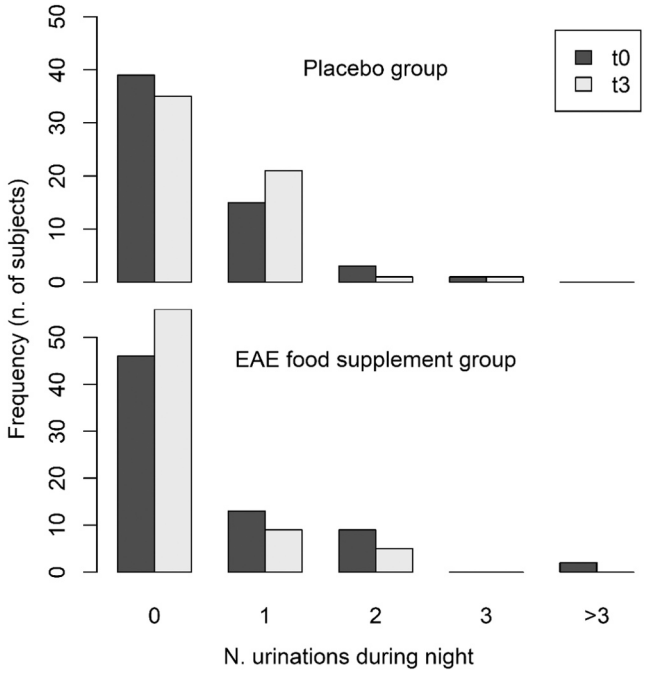

Fig. 5. Frequency distributions (n. of subjects) of urinations during the night in the two experimental groups measured at t0 and t3 as predicted by GLMM.

5. Conclusions

In conclusion, the metabolic profile of EAE reveals the presence of a rich phytocomplex with different polyphenol species, including many compounds not yet identified in *E. angustifolium* extracts. The presence of oenothein A and B, which are converted by gut fermentation into urolithins, whose anti-inflammatory activity is known, suggests that EAE food supplement can exert its beneficial effect against BPH through an anti-inflammatory action. The results showed that *E. angustifolium* food supplements can be used in subjects with BPH, to improve their quality of life by reducing post-void residual volume and consequently nocturia and general renal function without hepatic or renal toxicity.

Table 6
Descriptive statistics (mean ± SE, range) for the four response variables measured at t0 and t3 in the two experimental groups.

	Placebo group (n = 58)		EAE food supplement group (n = 70)	
	t0	t3	t0	t3
PSA	2.6 ± 0.8 (0.3–35.6)	3.2 ± 1.3 (0.2–53.3)	1.7 ± 0.2 (0.1–9.3)	1.9 ± 0.2 (0.1–11.6)
N/L	1.9 ± 0.1 (0.8–3.5)	2.0 ± 0.1 (0.9–3.5)	2.2 ± 0.1 (0.9–5)	2.3 ± 0.1 (1–5.7)
AST	22.1 ± 0.9 (10–49)	21.2 ± 0.7 (10–38)	22.1 ± 0.7 (13–40)	20.8 ± 0.7 (11–44)
ALT	21.6 ± 1.3 (8–50)	23.4 ± 1.4 (2–53)	21.7 ± 1 (9–52)	24.4 ± 1.3 (6–56)
BR(direct/indirect)	0.35 ± 0.05 (0.14–2.45)	0.3 ± 0.04 (0.09–1.33)	0.24 ± 0.03 (0.09–1.92)	0.21 ± 0.01 (0.12–0.33)
BR total	0.84 ± 0.05 (0.14–1.76)	0.80 ± 0.04 (0.37–1.8)	0.88 ± 0.05 (0.3–3.04)	0.84 ± 0.04 (0.33–1.93)
Protrombine	1.01 ± 0.01 (0.80–1.51)	1.02 ± 0.01 (0.80–1.41)	1.01 ± 0.01 (0.85–1.44)	1.04 ± 0.02 (0.85–1.69)
CHE	8150 ± 163 (4625–10921)	9058 ± 179 (5092–12434)	7983 ± 175 (4335–11765)	8928 ± 193 (4646–13030)
GFR	69.8 ± 2.2 (42–124.1)	69 ± 1.7 (43–97)	62.3 ± 1.4 (35–100)	68.1 ± 1.4 (42–102)
CRE	1.10 ± 0.03 (0.49–1.60)	1.11 ± 0.02 (0.8–1.55)	1.19 ± 0.02 (0.79–1.86)	1.11 ± 0.02 (0.78–1.61)

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Author contributions

MDA, MD, and GCT designed the clinical study, analyzed and interpreted the data; MD, CE and VI drafted the manuscript; GB, GC, and ARB conducted the clinical study; CE and RM wrote the documents for the Ethics Committee, monitored the clinical study, and collected and analyzed the data; CS, EMS, and PC set up the chromatographic analysis protocol, were in charge of the analysis of EAE, and wrote the part of the manuscript regarding the chromatographic analysis; RS performed the statistical analysis; GN studied the traditional and safe use of *E. angustifolium* and its safe dosage; VI and GN set up the composition and coordinated the production of EAE food supplement and placebo. All authors revised and gave their final approval for publication.

Conflict of interest statement

GN and VI are employees of EPO srl. None of the academic researchers listed as co-authors served as consultant for EPO srl or received any personal compensation. The research was partially supported by a research contract signed in 2018 between TEFARCO Innova and EPO srl entitled "Studio di un nuovo prodotto anti-infiammatorio per la prevenzione dei disturbi della prostata".

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