UNIVERSITÀ DEGLI STUDI DI NAPOLI

"FEDERICO II"

DIPARTIMENTO DI FARMACIA

CORSO DI DOTTORATO IN SCIENZE DEL FARMACO

XXXIV CICLO

Development of a new food supplement with anti-inflammatory activity for the relieve of the symptoms of benign prostate hypertrophy

Relatore

Chiar. ma Prof.ssa Maria Daglia

Candidato

Dott. Raffaello Masselli

Coordinatore

Ch.ma Prof.ssa Maria Valeria D'Auria

Summary

1. Introduction

The prostate (or prostate gland) is a small, rounded organ that is part of the male genital system. Located in the pelvis, just below the bladder and in front of the rectum, this exocrine gland surrounds the upper part of the urethra. The main function of prostate is the contribution to the sperm production, as it secretes a part of the seminal fluid released during ejaculation. The prostate as an adjunct organ to the reproductive system, is susceptive to internal and external environmental factors at hormonal, biochemical, micronutrient and genetic level.

Benign Prostatic Hypertrophy (BPH) is one of the most common conditions affecting men at a late age. This condition predominantly affects older men. About 75% of men over 50 year of age suffer from BPH related symptoms. Moreover, 20-30% of men above 80 years of age require surgical intervention for the management of BPH. [1], [2] From an histological point of view, the American Urological Association (AUA) defines BPH as "*a disease process characterized by stromal and epithelial cell Hypertrophy begin in the periurethral zone of the prostate*". [3]

The presence of BPH in older men is strongly linked to the development of lower urinary tract symptoms (LUTS), which are defined by several symptoms including urgency, nocturia, frequency, dysuria, difficulty in emptying the bladder, difficulty in initiating micturition, and weak or interrupted stream during micturition. Although some LUTS are defined as "LUTS independent of BPH", BPH and its downstream effects lead to chronic LUTS in many men. BPH with LUTS has also been linked to erectile dysfunction (ED). [4]

A wide variety of treatments are available for BPH, including pharmacological therapies, phytotherapies (alpha blockers, 5-alpha reductase inhibitors (5-ARIs), phospodiesterase-5 (PDE5) inhibitors), minimally invasive therapies and surgery.

The alpha blockers relax the muscles of the prostate and the bladder neck to relieve symptoms, 5-ARIs have been shown to shrink the prostate and prevent additional growth and PDE5 inhibitors improve prostate symptoms through the increase of the flow rate. [5]

Nowaday, trying to relieve the symptoms of BPH, dietary supplements based on plant extracts obtained from *Pygeum africanum* Hook.f. and *Serenoa repens* (W. Bartram) Small, with peripheral antiandrogenic action, or containing *Cucurbita pepo L.* seed oil, that some studies indicate to be active in the prevention of BPH, are produced. Literature data show that the *Epilobium angustifolium L*., traditionally used in the European Countries for the treatment of BPH, whose active ingredient is oenothein B, has antioxidant and anti-inflammatory activity.

Given the chemical and biological properties of *E. angustifolium* highlighted by the scientific community, this PhD project aims to characterize *E. Angustifolium* extract and test them with *in vitro*, *in vivo,* and clinical studies.

2. Prostate

The prostate is a solid, uneven, and median organ, located in the small pelvis between the base of the bladder at the top, the urogenital diaphragm at the bottom, behind the pubic symphysis, and in front of the rectal ampulla. It has glandular and fibromuscular components and surrounds the first portion of the urethra (prostatic urethra) into which it pours its secretion (prostatic juice) through numerous excretory ducts during ejaculation. In the posterosuperior part, it is also crossed, obliquely, by the ejaculatory ducts.

2.1 Shape, position, and relationships

The prostate has a chestnut shape with a base at the top and apex at the bottom, with an oblique axis downwards and forwards; forms with the axis of the urethra that crosses it at an angle of about 20 °. Of consistency and reddish-gray color, it grows during puberty to reach around 20-25 years of weight 8-10 g, and dimensions of 3 cm in height, 4 cm in width at the base and 2.5 cm in thickness. In old age, the organ can undergo hypertrophy, with an increase in volume and difficulty in urination due to compression of the prostatic urethra. It has an upper base (bladder face), an anterior face, a posterior face, two inferior-lateral surfaces and an apex.

The bladder face has a triangular shape with rounded corners, with one posterior and two anterior sides converging forward. It is extensively in relation to the neck of the bladder and near the anterior corner shows the point of penetration of the urethra, while posteriorly, near the posterior face, a transversal shower (hilum of the prostate) is dug into which the ejaculatory ducts penetrate. The surface between the entrance of the urethra and the hilum appears a little raised and corresponds to the so-called middle lobe. The almost vertical front face is uniformly convex. while the posterior face, oblique downwards and forwards, is crossed in the middle by a median furrow which divides it into a right and a left lateral lobe. The inferolateral surfaces are rounded; the apex, trunk, surrounds the urethra at its exit point from the prostate. The prostate is covered by a fibrous sheath (prostatic fascia) containing a venous plexus (prostatic) which represents a thickening of the visceral pelvic fascia and which contracts with neighboring organs. The anterior surface of the prostatic fascia is separated from the pubic symphysis by a space of about 2 cm in which the pudendal venous plexus and branches of the internal pudendal arteries are located, immersed in the adipose tissue at the top, the fascia is fixed to the pubic bones with puboprostatic ligaments; towards

the apex of the prostate the prostatic fascia joins with the superficial fascia of the striated sphincter muscle of the urethra, with the deep transverse muscle and with the tendon center of the perineum. The inferolateral surfaces are in a relationship with the pubic fossae of the levator muscle with the interposition of the vesicoprostatic venous plexus.

The posterior surface of the prostate sheath, avascular, is called the rectovesical fascia and results from the fusion of the peritoneal sheets of the deepest portion of the rectovesical cavity, which in the fetus comes to interpose between the prostate anteriorly and the rectal ampulla posteriorly. The rectovesical fascia continues upward on the posterior surface of the seminal vesicles, the vas deferens, and the ejaculatory ducts until it reaches the floor of the Douglas cavity; at the bottom it is fixed to the tendon center of the perineum; laterally it joins the posterior bladder ligament. The close contact of the posterior surface of the prostate with the anterior face of the rectal ampulla allows the prostate to be palpated by rectal exploration.

The bladder face of the prostate adheres forward to the area of the internal urethral orifice and the bladder trigone, posteriorly it is in relation to the ejaculatory ducts, the deferential ampullae, and the apex of the seminal vesicles.

Finally, the apex of the prostate is in contact with the urogenital diaphragm, which is crossed by the urethra. Below the visceral pelvic fascia and its derivative is the capsule of the prostate. Starting from the posterior surface of the prostate, below a plane passing through the seminal colliculus, the capsule deepens with a median septum that separates the lateral lobes continuing with the urethral crest. In addition, the capsule deepens with various fibromuscular septae that envelop the glandular parenchyma. The smooth muscle component that infiltrates the septa increases significantly ventrally to the urethra. Here the muscle bundles are arranged transversely and continue upwards with the bladder musculature. On the surface of this muscle layer, there are muscle fibers

2.2 Structure

The prostate can be divided into lobes based on the distribution of the glands and the relationships with the organs that pass through it. We recognize an anterior lobe placed in front of the urethra and formed by a few small glands, a middle lobe, located between the urethra and the ejaculatory ducts, of variable size and in some cases devoid of glands; a right and a left lateral lobe, voluminous and posterior to a passing plane for the ejaculatory ducts. The prostate parenchyma is represented by about 30-50 branched tubuloalveolar (otricular) glands. They collect in 15-30 excretory ducts that flow mainly into the prostatic sinuses of the urea between prostate, placed on the sides of the seminal colliculus, Each gland constitutes an irregularly conical lobule, with apex facing the urethra, separated from the contiguous lobules through the fibromuscular septa from the prostatic capsule. Each excretory duct has considerable ramifications, with secreting characteristics and of a tubular or otricular appearance near the outlet in the urethra, with characteristics of convoluted secondary ducts on which tubular or otricular adenomers open towards the base of the lobule. The adenomers have a very large lumen and contain secretions in which amylaceous bodies or prostatic concretions can be found, represented by spherical bodies with a lamellar structure, often calcified. These are frequent formations in elderly subjects, ranging in diameter from 0.2 to 2 mm. produced by the precipitation of macromolecular components of the prostatic secretion, and therefore of calcium salts.

Near the urethra the excretory ducts are lined with stratified cylindrical epithelium, while more distally the epithelium is simple cylindrical and continues directly in the secreting epithelium of the adenomeres. The latter is cylindrical, with numerous apical secretory granules and abundant cytoplasmic basophilia based on the index of a developed granular endoplasmic reticulum. Interspersed with these elements there are some non-secreting basal cells.

The prostatic secretion, which constitutes about 15-30% of seminal fluid, has a milky appearance, is slightly acidic (pH 6.4) and contains numerous enzymes (acid phosphatase beta-glucuronidase, amylase, fibrinolysin, protease), prostaglandins, spermine and spermidine, immunoglobulins, zinc and citric acid.

The glandular epithelium of the prostate is the target of androgens. In correspondence of the epithelium and the prostate stroma, testosterone is converted into the most active form, dihydrotestosterone, by the action of an enzyme (5-a-reductase).

The smooth muscle component present in the stroma surrounding the adenomeres and excretory ducts determines, by contracting, causing episodic emptying of the organ at the time of ejaculation.

3. Benign Prostatic Hypertrophy (BPH)

3.1 Etiology

Nowaday, the mechanism underlying the onset of BPH is not clearly defined, however, three possible hypotheses for its development have been proposed. The first hypothesis is based on the role of androgenes, estrogens and growth factors. Prostate cells can convert approximately 90% of testosterone (TE) to dihydrotestosterone (DHT) via 5-alpha reductase. DHT binds to androgen receptors with greater affinity than TE, which stimulates protein synthesis and the growth of prostate cells. [6], [7] DHT builds up in the prostate when TE levels are low. [8] The binding of DHT to the receptor stimulates the synthesis of growth factors to an abnormal proliferation of prostate cells. [9] Estrogens act synergistically with androgens in the development of BPH through multiple mechanisms, including apoptosis, aromatase expression, and paracrine regulation via prostaglandin E2. [10]

The second hypothesis is based on the presence of a small percentage of androgen-independent prostate cells that can self-renew in case of androgen deficiency. [11]

The third hypothesis concerns the interaction between stroma and epithelium, which can convert TE into DHT inducing the production of various growth factors responsible for cell proliferation, apoptosis and secretion activity of both the stromal (autocrine transmission) and epithelial (paracrine secretion) portions. Recent studies have also highlighted the role of inflammation, which can be a key factor in the progression and aggravation of the BPH symptoms.

3.2 Diagnosis

The diagnosis of BPH occurs following a urological examination. The first examination necessary to identify its presence is digital rectal exploration, that is, the examination of the prostate performed through rectum. This control is recommended every year from the age of 50, especially in subjects having familiarity with prostate cancer. This exam is usually associated with instrumental tests such as [12]: transrectal ecography to detect the size of the prostate. In addition, in case of urinary difficulties, flowmetry is conducted which measures the speed and strength of the urinary jet. These controls are accompanied by 1) a venous sampling to find out the prostate specific antigen (PSA) value, which, although not a specific marker for cancer, may make it necessary to require more in-depth tests to exclude a possible concomitant tumor) and 2) a urinalysis. To complete the diagnosis, tests such as uroflowmetry are appropriate, to objectively quantify the characteristics of the urinary jet and ultrasound of the abdomen, to exclude complications such as calculations, diverticula, dilations of the kidneys or ureters and to identify the presence of residual urine in the bladder after urinating.

The use of different questionnaires for the assessment of symptoms of the lower urinary tract (Low Urinary Tract Syntoms - LUTS) is suggested. The voiding diaries are particularly useful for the evaluation of subjects with nocturia and / or symptoms of the filling phase.

Among the most significant questionnaires, the International Prostate Symptom Score (IPSS) is a questionnaire consisting of 8 questions, seven of which investigate the symptoms related to urination and the last one concerns the quality of life (QoL) perceived by the subject. The final score obtained places the subject within one of the four main groups: "asymptomatic" (0 points), "Mildly symptomatic" (1-7 points), "moderately symptomatic" (8-19 points) and "severely symptomatic" (20-35 points). Limitations of using this questionnaire include the lack of evaluation ion of incontinence, post-voiding symptoms and disorders caused by each symptom separately. [13]

4. Therapeutic approaches to BPH

4.1 Pharmacological therapy

4.1.1 Alfa-Blockers

Alpha 1-adrenoceptors are present on the stromal smooth muscle of the prostate and on the bladder neck. The blocking of alpha 1-adrenergic receptors causes relaxation of the stromal smooth muscle which acts on the dynamic component of BPH and thus improves flow. Unlike 5-alpha reductase inhibitors, alpha-1 lytics have a much faster effect and therefore subjects experience urinary benefits very early. [14]

The most frequent adverse effects of alpha-1-lytic drugs are asthenia, dizziness and orthostatic hypotension. The vasodilation effects are more pronounced for doxazosin and terazosin, while they are less common with alfuzosin and tamsulosin. Subjects with cardiovascular comorbidities or under treatment with vasoactive drugs may be more exposed to the vasodilatory effects of α1 lytic drugs. On the contrary, the frequency of orthostatic hypotension with the selective α1A receptor antagonist silodosin is comparable to placebo. [15]

4.1.2 5-Alpha Reductase Inhibitors

The effects of androgens on the prostate are mediated by dihydrotestosterone (DHT), which is converted from testosterone by the enzyme 5α reductase. Two 5α reductase inhibitors (5-ARI) are available for clinical use: dutasteride and finasteride. Finasteride inhibits only type 2 5α reductase, while dutasteride inhibits both type 1 5α reductase and type 2 5α reductase with similar potency. The 5α reductase inhibitors act by inducing apoptosis of prostate epithelial cells, resulting in a reduction in prostate size by approximately 18-28% and a reduction in circulating levels of approximately 50% after 6-12 months of treatment. The mean reduction in prostate volumes and PSA values may be even more pronounced for longer treatments. Chronic treatments reduce dihydrotestosterone concentration by approximately 70% with finasteride and 95% with dutasteride. However, prostate dihydrotestosterone concentration is similarly reduced (85-90%) with both drugs. [16]

The most important side effects of 5-ARI are associated with sexual function and include decreased libido, erectile dysfunction and, less frequently, ejaculation disorders such as retrograde ejaculation, anejaculation or reduced volume of the ejaculate. However, the incidence of sexual disorders and other side effects is low. [17]

4.1.3 Antimuscarinics

The detrusor muscle is innervated by parasympathetic nervous fibers whose main neurotransmitter is acetylcholine which stimulates the muscarinic receptors (M cholinergic receptors) present on smooth muscle cells. Muscarinic receptors are also present on other types of cells such as urothelial cells in the bladder, epithelial cells of the salivary glands and in the central and peripheral nervous system. There are 5 subtypes of muscarinic receptors (M1-M5) of which M2 and M3 are predominant in the detrusor muscle. M2 receptors are

more numerous but M3 receptors are functionally most important in healthy bladders. Antimuscarinic effects can also be induced or modulated through other cell types such as urothelial cells in the bladder or through the central nervous system. The antimuscarinic drugs used in the treatment of overactive bladder and in the LUTS of the bladder filling phase are the following: darifenacin hydrobromide (darifenacin), fesoterodine fumarate (fesoterodine), oxybutynin hydrochloride, propiverdine hydrochloride (propiverdin), solifenacin succinate. Transdermal preparations of oxybutynin have also been developed and evaluated in clinical studies. Those who fail antimuscarinic treatment may be considered for the use of mirabegron (a beta-3 adrenergic receptor agonist), which causes detrusor relaxation. [18] Thus, the combination of an alpha blocker and an alpha reductase inhibitor is often used to improve urination symptoms. This is supported by studies confirming the efficacy of combination therapy versus monotherapy.

Side effects associated with antimuscarinic drugs include dry mouth (up to 16%), constipation (up to 4%), difficulty urinating (up to 2%), nasopharyngitis (up to 3%) and dizziness (up to 5%). [18]

4.1.4 5-phosphodiesterase inhibitors

5 phosphodiesterase (PDE5I) inhibitors cause an intracellular increase in cyclic guanosine monophosphate (cGMP), reducing the tone of the smooth muscle of the detrusor, prostate and urethra. Nitric oxide and phosphodiesterases can also alter reflexes in the spinal cord and neurotransmission in the urethra, prostate or bladder. [21] In addition, chronic therapy with PDE5I appears to increase blood perfusion and oxygenation in the lower urinary tract. [22] Lastly, PDE5I can reduce chronic inflammation in the prostate and bladder. [23] The exact mechanism of the PDE5I on lower urinary tract symptoms remains to be clarified. Regarding the available drugs, although clinical studies have been conducted with many 5

phosphodiesterase inhibitors in LUTS subjects, only tadalafil (at the dose of 5 mg once daily) was authorized for the treatment of men with LUTS. Several randomized clinical trials have shown that PDE5I reduce IPSS, the symptoms of the phase filling and bladder emptying phase and improve the QoL of subjects. In many trials, however, the maximum urinary flow rate (Qmax) did not differ significantly from placebo. A recent meta-analysis has shown how PDE5I, in particular tadalafil 5 mg, improves the scores of the IPSS and the International Index of Erectile Function (IIEF) but not the Qmax. [24] The use of tadalafil 5 mg improves IPSS by 22-37% and the improvement can be evident within a week from the beginning of the treatment. [25] An improvement in the IPSS score of three or more points was observed in 59.8% of subjects treated with tadalafil within one week and in 79.3% of subjects within four weeks of treatment. [26] The maximum study duration was 52 weeks. A subgroup analysis of the resulting data from four randomized clinical trials demonstrated a significant reduction in LUTS, independently from the severity, age and previous use of α-lytics or PDE5I, the levels of testosterone and prostate volume. [27] A recent *post-hoc* analysis of data from four randomized clinical trials have shown that tadalafil is also effective in men with cardiovascular risk factors or comorbidities, except in subjects taking more than one antihypertensive drug. The use of diuretics may contribute to the subject's perception of lack of efficacy. [28] In men sexually active aged> 45 with LUTS / BPH and erectile dysfunction, tadalafil has been shown to be effective in improving both conditions.

An integrated analysis of data from four randomized placebo-controlled clinical trials showed that the overall improvement in the IPSS score was largely associated with direct treatment effects (92.5%, $p < 0.001$) rather than indirect (7.5%, $p = 0.32$) through the improvement of the Index of Erectile Function IIEF-EF. [29] Another analysis demonstrated a small but significant increase in Qmax without affecting post-void residual (PVR). [30] The combination of 5 phosphodiesterase inhibitors and α-blockers was also evaluated. A

meta- analysis of five randomized clinical trials (two studies with tadalafil 20 mg, two with sildenafil 25 mg and one with vardenafil 20 mg), have shown that combined therapy significantly improves IPSS (-1.8), the IIEF (+3.6) and Qmax (+1.5 ml / sec) compared to therapy with α-blockers alone. [24] The effects of tadalafil 5 mg in combination with finasteride 5 mg were evaluated in a randomized clinical trial, versus placebo for a period of 26 weeks. The combination of tadalafil and finasteride showed an early improvement in urinary symptoms ($p < 0.022$ after 4, 12 and 26 weeks), with a significant improvement of filling and emptying symptoms and QoL. Furthermore, the combination therapy was well tolerated and improved erectile function. [31] To date, only 5 mg tadalafil has been authorized for use in the treatment of LUTS, while data on the combination of PDE5Is with other drugs used in LUTS are emerging.

The side effects of PDE5Is in subjects with LUTS include redness of the face, gastroesophageal reflux, headache, dyspepsia, back pain and nasal congestion. [24] The dropout rate due to side effects for tadalafil was 2.0% [24] and did not differ by age, LUTS severity, testosterone levels or prostate volume in the overall analysis of the data. [32]

5. Surgical Treatment

Despite the advent of new technologies, the intervention of TURP (Transurethral Resection of the Prostate) has remained for more than 9 decades the milestone for the treatment of LUTS / BPH. The identification of a more effective and safer alternative treatments is frequently hindered by methodological limitations of the studies examined, including the presence of inadequate follow-up. Internationally, the short, medium and long-term follow-up times for subjects undergoing surgical treatments are 12, 36 and 36 months, respectively.

For this reason, doctors should inform subjects that randomized clinical trials on the long-term efficacy of surgical interventions are lacking.

5.1 Transurethral resection of the prostate and transurethral incision of the prostate

The purpose of transurethral resection of the prostate is to remove tissue from the transition area of the prostate. The purpose of the transurethral incision of the prostate is to incise the neck bladder without removing the tissue. This second approach is used in selected cases, especially when the prostate volume is lower than 30 mL without the third lobe.

In a recent analysis of 20 contemporary clinical trials with a follow-up of up to 5 years, TURP was shown to increase mean Qmax (+ 162%), decrease IPSS (-70%), QoL score (-69 %), and the PVR (-77%). [33] TURP surgery offers lasting results as shown by studies with a follow-up of 8-22 years. There are no similar data on the duration of any other surgical treatment for BPH. A study with a mean follow-up of thirteen years reported a significant and prolonged decrease in most urinary symptoms and an improvement in urodynamic parameters. The failures were associated with detrusor dysfunction rather than the redevelopment of BPH. A meta-analysis of the data of ten randomized clinical trials (RCTs) found equivalent improvements in LUTS between the two procedures, while insignificant reductions in mean Qmax, in the case of transurethral incision of the prostate (TUIP). In this meta-analysis, one of the inclusion criteria for 5 of the 8 studies examined was the size of the prostate which should be <30 ml and for 3 of the 8 <60 ml. The need for a second surgery, usually a re-TURP, is represented by a constant annual rate of about 1-2%. A review analysing 29 clinical studies found a re-intervention rate of 2.6% after a mean follow-up of 16 months. In a large-scale study of 20,671 men, the rates overall re-surgery (re-TURP, urethrotomy, and bladder neck incision) were 5.8%, 12.3%, and 14.7%, respectively, at one, five, and eight years of follow-up, and the respective incidence of re-TURP was 2.9%, 5.8%

and 7.4%. A meta-analysis of six studies showed that reintervention was more common after TUIP (18.4%) than after TURP (7.2%).

6. Phytotherapeutic approaches to BPH

6.1 *Cucurbita pepo* L.

C. pepo is a plant belonging to the Cucurbitaceae family. It is an annual plant grown mainly in Central and South America, also called pumpkin or Duba. Pumpkin seed oil is mostly used in Traditional Medicine for its antioxidant and anti-inflammatory activities in the treatment of BPH and related urinary symptoms

6.1.1 Chemical Composition and Preclinical Studies

The chemical composition of Pumpkin seed consists of alpha-tocopherol, proteins, carbohydrates, fatty acids (palmitic, stearic, oleic and linoleic), non-essential amino acids (cucurbitin) and Δ5-Δ7-Δ8-phytosterols (including sitosterol, stigmasterol, etc.) [34]. The Δ7- sterols have a chemical structure similar to DHT and can competitively inhibit the binding of DHT to its receptors. However, the active substance has not yet been identified and the data related to the mechanism actions are limited and inconclusive. Some studies performed on rats show that pumpkin seed extracts can decrease prostate enlargement and testosterone/prazosin-induced protein synthesis [40], inhibiting testosterone-induced hypertrophy [35], and improves bladder function (i.e. tonic action, sphincter relaxation) and urethra. [36], [37] The mechanism of action of pumpkin seed oil should be 5alpha-reductase inhibition.

6.1.2 Clinical Studies

A multicenter clinical study carried out on thousands of subjects with BPH treated with pumpkin seed extract capsules (500 mg) demonstrated that there was an improvement in IPSS and an improvement in QoL. [38] DHT levels decreased significantly and there was an improvement in BPH, urinary flow symptoms (i.e., bladder emptying time, residual volume, daily micturition frequency, and nocturia) have also been found in subjects using a combination of pumpkin seeds and *S. repens*. This combination (320 mg / day of each medicinal plant) was also effective in Korean subjects in improving IPSS, QoL, serum PSA levels and maximal urinary flow, while there was no difference in prostate volume. [39] Furthermore, the recent multiple association involves the use of *C. pepo* seed oil (160 mg), *E. parviflorum* extract (500 mg of dry grass), lycopene (2.1 mg), *P. africanum* (15 g of dry stem) and *S. repens* (660 mg of dry leaf). During three months of treatment, this herbal preparation induces a reduction in IPSS and in the frequency of day and night urination in subjects with a medical diagnosis of BPH. [40] However, further studies to confirm the effectiveness of *C. pepo* in the treatment of BPH are needed. Regarding the safety of this plant, there is no indication of serious side effects, although it could be responsible for mild gastrointestinal problems (e.g. indigestion, diarrhoea) [36], loss of electrolytes (probably due to its diuretic properties) [41], and alteration of the International Normalized Ratio (INR, an indicator of prothrombin time). The latter effect makes *C. pepo* contraindicated in case of concomitant anticoagulant therapy.

6.2 Epilobium Spp.

Epilobium is a genus of perennial herbaceous plants that belongs to the Onagraceae family. This family is composed of more than 200 species distributed in Europe, Asia, Africa, Australia, America, Tasmania and New Zealand. The most common species include *E. parviflorum, Epilobium hirsutum, Epilobium rosmarinifolium, and Epilobium*

angustifolium. [42] The commonly used part of the plant for the production of extracts is the aerial part, especially the flowered ends. Traditional Medicine considers *Epilobium* spp to be useful for fever, rheumatic complications, headaches and as general pain relief, although these uses are not supported by scientific evidence.

6.2.1 Chemical composition and preclinical studies

Extracts from the aerial parts of *Epilobium angustifolium, Epilobium parviflorum and Epilobium hirsutum* are traditionally used in Europe for disorders associated with the prostate. Among these, *E. angustifolium* is the most commonly used, which has interesting properties against BPH and a characteristic phytocomplex. The phenolic compounds of *E. angustifolium* are the main responsible for the scavenger capacity, i.e. neutralizing the free radicals responsible for tissue aging and increasingly produced during organic damage. A number of studies have confirmed the scavenging superiority of *E. angustifolium* over vitamin C, protecting prostate tissues from oxidative stress induced by the infiltration of phagocytes for inflammatory or immune reasons. A second activity of *E. angustifolium* complementary to the antioxidant one, is the anti-inflammatory effects. The main polyphenols present in *E. angustifolium* are enotein B, a di-meric ellagitannin, quercitin-3-O-glucuronide and myricetin-3-O-rhamnoside. Among these, enotein B represents the most potent inhibitor of LNCaP cell proliferation, PSA secretion and arginase activity. These extracts significantly reduce the values of the PSA marker and inhibit the activity of arginase, the enzyme that converts L-arginine to L-ornithine and urea. The inhibition of the arginase allows to maintain normal cellular levels of L-arginine, a sub-layer of nitric oxide synthase, and to regulate the relaxation of the smooth muscles of both male and female genes, improving sexual responsiveness. In addition, the ellagitannins present in the *E. angustifolium* extracts are metabolized by the intestinal microbiota to urolithins, derivatives

of ellagic acid. Among these, urolithin C is also an effective inhibitor of LNCaP cells, PSA and arginase. [43]

6.2.2 Clinical studies

Although *Epilobium spp*. extracts are used in numerous food supplement and cosmetic, To date, only one clinical study has been performed to evaluate the efficacy and safety of ProstateEZE Max, an orally dosed herbal preparation containing Cucurbita pepo, Epilobium parviflorum, lycopene, Pygeum africanum and Serenoa repens in the management of symptoms of medically diagnosed benign prostate hypertrophy (BPH). [40]

6.3 *Lycopersicum esculentum* Mill.

L. esculentum (also known as *Solanum lycopersicum* L.) belongs to the Solanaceae family. *L. esculentum* is an annual herbaceous plant native to the area between Mexico and Peru. It is commonly used as a food (tomato-based foods: tomato paste, tomato sauce, tomato soups, etc.). Generally, tomato fruit and tomato-based products provide the human body with about 85% of lycopene, while the remaining 15% is acquired from the consumption of watermelon, apricot, peach, papaya and red berries. [44]

6.3.1 Chemical composition and preclinical studies

L. esculentum contains glycoalkaloids (alpha-tomatin), salicylates, flavones (apigenin and luteolin), polyphenolic compounds (quercetin, myricetin, kaempferol, naringenin and chlorogenic acid) and other carotenoids (phytoene and phytofluene). [45] All the green parts are toxic, as they contain solanine, a steroid glycocaloid, which is not eliminated even by cooking processes. Ripe fruit is rich in nutrients, including amino acids, minerals (e.g. potassium) and vitamins (A, B, C, D, E, K and folate). One substance that has aroused great

interest among researchers is a long-chain polyunsaturated acyclic carotenoid, which is called lycopene. It contains 11 conjugated double bonds, arranged linearly in a trans configuration [46], while most of the lycopene found in prostate tissue has a cis conformation. [47]

The cis form is preferably captured by benign and malignant prostatic tissues and it is produced during the cooking process. [47] Since lycopene has been correlated with a low frequency of adenocarcinoma [48], since 2003 it has been added to most multivitamin products as a cancer prevention agent and has become the most widely used carotenoid, among men aged 45-65. [79] In addition to lycopene, tomato contains lycopene cyclase (the enzyme that catalyzes the conversion of lycopene in β-carotene). The mechanisms of action, which have been proposed for the control of adenocarcinoma, as well as adenoma, include the inhibition of growth factor-induced cell proliferation, interference in cell cycle progression from phase G0 / G1 to phase S, modulation of the COX pathway, down-regulation of type 1 5-alpha-reductase, pro-apoptotic activity (potentially due to alpha-tomatin) [49], inhibition of the synthesis of the androgen receptor (which leads to a reduction in PSA) and the antioxidant activity. [50] This latter property consists in the ability of lycopene (compared to other carotenoids) to capture oxygen radicals otherwise responsible for DNA damage. [46] Other substances involved in the antioxidant effect are the polyphenols aglycones of the tomato (quercetin, naringenin and kaempferol), also responsible for the anticarcinogenic effects and inhibition of cell proliferation, in a dose-dependent manner. [47]

The *in vivo* results have conflicting evidence related to the effect of tomato (or pure lycopene) on the reduction of plasma testosterone level. This could be explained by the duration of treatment, the differences in animal species or lycopene content. [51] However, as experimental studies show that the tomato extract is more active in inhibiting

carcinogenesis than lycopene alone [52], this carotenoid does not appear to be the only constituent of tomato that has effects on the prostate.

In vivo preclinical evaluation demonstrated good safety, with LD50 values up to 5000 mg / kg and no acute dermal toxicity up to 2000 mg / kg body weight.

6.3.2 Clinical studies

A meta-analysis evaluated 26 studies with 17,517 cases of prostate cancer (PCa) reported by 563,299 participants. Although the inverse association between lycopene consumption and PCa risk was not found in all studies, there was a trend that with higher lycopene intake, there was a reduced incidence of PCa. - dose-response analysis showed that higher lycopene consumption was linearly associated with a reduced risk of PCa with a threshold between 9 and 21 mg / day. [53]

However, further studies are needed to determine the mechanism of action by which lycopene reduces the risk of PCa onset and whether there are other factors in tomato products that could potentially reduce the risk and progression of PCa.

6.4 *Pinus Pinaster* Aiton

P. pinaster is an evergreen tree of the Pinaceae family that finds its ideal habitat near the coasts of the Mediterranean Sea. The antioxidant activity of the flavonoids extracted from *Pinus Pinaster* could be the basis of the anti-inflammatory and antiplatelet activity both thanks to the structure of the flavonoids and to their ability to penetrate the lipid membrane of the cell. [54]

6.4.1 Chemical composition and preclinical studies

The essential oil contains α and β-pinenes with antiseptic properties, esters of borneol, D-limonene, aldehydes, and oleoresin. It also contains diterpenes, coumarins and flavonoids. The needles are rich in vitamin C and flavonoids. The main and active component is the β-sitosterol, with a chemical structure similar to that of cholesterol.

Oligomeric proanthocyanidins extracted from the bark of *P. pinaster* have long been studied in *in vitro* experiments and on animal models for their antioxidant properties. Proanthocyanidins are high molecular weight polymers that are formed by the oxidative condensation of the monomeric unit flavan-3-ol (+) catechin and (-) epicatechin) with flavan-3,4-diols, first in dimers and subsequently in oligomers. They are called "proanthocyanidins" because if they are lysed with acid treatment, they give rise to anthocyanidins such as e.g. cyanidine. Oligomeric proanthocyanidins (OPCs- Oligomeric proanthocyanidins) are water-soluble short-chain polymers. Among the beneficial effects of proanthocianidins, one of the most important is the anti-inflammatory activity linked to the ability to prevent the activation of NF-kB and AP-1, nuclear transcription factors over-expressed in conditions of oxidative stress and in the presence of cancer cells. [55] The OPCs extracted from *P. pinaster* are able to decrease the symptoms of chronic inflammation. *In vitro* studies show anti-inflammatory effects probably due to the inhibition of peroxide production by macrophages. Furthermore, animal studies show that OPCs significantly inhibit the formation of proinflammatory cytokines, interleukin 1-beta (IL-1β) and tumor necrosis factor-alpha (TNFα) in models stimulated with LPS. [56]

6.4.2 Clinical studies

A randomized, double-blind, placebo-controlled study was conducted to evaluate the efficacy and safety of β-sitosterol (130 mg / day, for 6 months). In treated subjects there was a significant improvement in QoL, PVR, IPSS and in Qmax. [57] A systematic review also found significant improvement in urinary symptoms due to β –sitosterol. [58] Other authors reported contradictory results regarding the efficacy of β-sitosterol, also in association with other plants and substances, where there was a slight improvement although not statistically significant, therefore further clinical studies are needed. The side the effects induced by β -sitosterol are generally mild; however, impotence and gastrointestinal problems are presented to a greater extent compared with subjects in the placebo group. [58], [59]

6.5 *Pygeum africanum Hook. f.*

P. africanum (also called *Prunus africana* or African plum) is an evergreen tree native of the Central and South American mountains, the Madagascar and Comoros islands and the Gulf of Guinea. From whose bark a lipophilic is obtained an extract with pharmacological properties. It was used for the first time in Europe in 1969, attributing to it an anti-inflammatory activity mediated by the inhibition of chemotactic activity of leukotrienes and the inhibition of fibroblast proliferation and that were translated into histological and physiological modifications of the prostate gland. [60]

6.5.1 Chemical composition and preclinical studies

P. africanum composition consists of phytosterols (β-sitosterol, β-sitosteryl glucoside, β-sitostenone), fatty acids, triterpenoid pentacyclic acids (ursolic, oleanolic and their homologs), alcohols (ndocosanol and its derivatives), esters of ferulic acid, linear acetogenins, nonacosane, hentriacontane, glucopyranosyl ester of benzoic acid and β-D-

glucopyranoside of diphenyl methyl alcohol, lignan isolariciresinol- 9-hydroxy-7,8-dimethyl ether and proanthocyanidins. [61], [62] Two other important constituents isolated from the bark of *P. africanum* are N-Nbutylbenzenesulfonamide (NBBS) with anti-androgenic activity, and atraric acid, a phenolic compound, whose mechanism of action is to inactivate the androgen receptor by inhibiting its nuclear translocation.

In vitro studies in rat prostatic stromal cells (fibroblasts) have shown that *P. africanum* inhibits both its basal growth and that stimulated by EGF, IGF-I and bFGF (direct activators of protein-kinase C) as well as by TPA and PDBu, this inhibition being more powerful in the case of cells stimulated with EGF, an effect that the authors did not attribute to cytotoxicity. These data, extrapolated to the clinical setting, could suggest an inhibition of prostate growth in men. [63] Other experimental observations contributed to reinforce this concept. Thus, another study in human leukocytes showed that *P. africanum* produces a significant inhibition in the production of 5-lipoxygenase metabolites (5-HETE, 20-COOH LTB4, LTB4 and 20-OH LTB4). *In vitro* antiproliferative activity and apoptosis could also be observed in stromal cells (fibroblasts). After the administration of *P. africanum*, SMA + cells, more abundant in BPH, showed greater marking with apoptosis immunohistochemical techniques. The down regulation of the expression of TGFB1, determined through Real-Time PCR, and an inhibition of the FGF2 signal were also observed. The addition of mitogens decreased the sensitivity to *P. africanum* in cell cultures. [64]

In an interesting study performed in rabbits with induced partial obstruction of bladder emptying, 25 experimental animals were grouped into blocks of 5 groups to which different doses of *P. africanum* (groups 1-3), placebo (group 4) and control (group 5) for 3 weeks, in order to study the response of this drug on detrusor contractile dysfunction (hypertrophic response) after 2 weeks of induced partial obstruction, although no changes in detrusor mass were observed in the groups treated with *P. africanum*, this had a significant protective effect

on the contractile response to various forms of stimulation such as electrical and induced by the administration of betanechol and KCl. [65] This experiment was reproduced by the same research team with 65 rabbits with similar methodology, in order to study the induced changes on metabolic activity, specifically the enzymatic activity of citrate synthetase (marker of mitochondrial function) and calcium-ATPase (marker of sarcoplasmic reticulum activity). Again, a protective effect of *P. africanum* was observed on the contractile response and on metabolic activity in the partially obstructed bladder in this model. [66] Other investigations attributed antiandrogenic activity to *P. africanum*; In a 7-week rat study with DHT, simultaneous addition of *P. africanum* reduced the obstructive effects of DHT. [67]

6.5.2 Clinical studies

Currently, the subject's profile is one of the main factors influencing the type of medicine chosen. *P. africanum* has been used in younger subjects with less severe symptoms and smaller prostates. [59] Most of the studies on *P. africanum* used a dose of 100 mg daily and for this reason a similar daily dose is recommended in clinical practice. In the meta-analysis of the Cochrane Database of Systematic Reviews [68], the adverse effects of *P. africanum* were minimal and comparable with the placebo. The dropout rate was 13% for *P. africanum* and 11% for placebo. In addition, the safety profile was excellent, without observing adverse effects such as ejaculatory dysfunction, erectile dysfunction, decreased libido or hypotension. The improvement registered in the IPSS score resulted to be similar to other alternatives supplements. Compared to other therapeutic alternatives and according to some clinical trials, the treatment with *P. africanum* has less efficacy [59], although in other studies and in routine clinical practice the improvement in symptomatology is similar to that of other treatments. These data lead to go on with the studies and use this extract for the treatment of subjects with mild / moderate symptoms. In these subjects, *P. africanum* could

show advantages such as its safety profile, and absence of the typical adverse effects of other therapeutic alternatives, such as hypotension, ejaculatory dysfunction or alterations in libido. [69]

6.6 *Secale cereal* L. pollen

S. cereale is an annual or biennial herbaceous plant belonging to the Graminaceae family and is widespread in temperate zones. It is also known by the names of rye, common rye and rye pollen. The parts of the plant with activity consist of the pollen's extract. The extract is obtained from a microbial digestion of the pollen of various plants, in particular of *S. cereale* from Sweden and Switzerland. The extraction process involves the sequential use of water and organic solvents.

6.6.1 Chemical composition and preclinical studies

The common presence in the pollen extract of proteins, carbohydrates, vitamins, minerals and β-sterols (in the acetone-soluble fraction) has been reported [70], however the active principle is not yet known. Furthermore, the composition of commercial products (tablets and capsules) is highly variable, making interpretation of the results difficult. Several mechanisms of action have been proposed for pollen extracts, including: relaxation of the urethral sphincter smooth muscle tone [71], increased bladder muscle contraction [72], increased apoptosis in epithelial cells of the prostate gland, 5alpha-reductase inhibition, increased serum and prostate zinc levels, blockade of alpha-adrenergic receptors and inhibition of the biosynthesis of prostaglandins and leukotrienes . An *in vivo* study has shown that *S. cereale* pollen extract therapy can retard the growth of prostate cancer by increasing apoptosis of tumor cells in animal models but cannot yet be recommended as an effective treatment for prostate disorders. [73]

6.6.2 Clinical studies

A recent randomized clinical trial indicated that long-term administration of 750 mg of rye pollen extract (Cernilton®) can improve BPH symptoms and prevent clinical progression better and faster than just 375 mg of the same product. [74] A total of 240 subjects who had IPSS values greater than 7 were treated orally with 750 mg of Cernilton® for the first year and 375 mg for the next three. After the first year of Cernilton® treatment, prostate volume, the incidence of urinary retention and the need for surgery were reduced. On the other hand, many other parameters (i.e., IPSS, prostate volume, urine residue, post-micturition, Qmax) only improved after 4 years of treatment. [74] However, no adverse effects were observed. A previous randomized multicenter clinical trial conducted versus placebo in 906 subjects with BPH showed that, after 6 months of treatment, Cernilton® has the same efficacy as alpha1-antagonists and 5-alpha-reductase inhibitors in the improvement of IPSS, QoL, Qmax and residual urine. This effect was greater in subjects with an elevated IPSS baseline. In reverse, Cernilton® was less effective than 5alpha-reductase inhibitors in the decrease in the size of the prostate. Pollen extract is also effective in men with inflammatory prostatitis and chronic pelvic pain syndrome in improving overall symptoms, pain and QoL, without significant side effects. [75] Common side effects deriving from the pollen extract use are allergy, which includes respiratory reactions, skin hypersensitivity, and gastrointestinal symptoms.

6.7 *Serenoa repens* (W. Bartram) Small

S. repens or Sabal serrulata is a thorny dwarf palm belonging to the Arecaceae family. It is native to the United States, where it is known as saw palmetto, and grows in sandy soils. It is a bushy palm that can grow up to 4 meters in height. The extract of *Serenoa repens*, used

for medicinal purposes, is obtained from ripe and dried fruits, whose main constituents are free fatty acids and their corresponding ethyl esters, phytosterols and lipids.

6.7.1 Chemical composition and preclinical studies

The liposterolic extract of *S. repens* consists of a complex mixture of long-chain fatty alcohols, fatty acids and their glycerides (oleic, caprylic, myristic, lauric, stearic, palmitic, linoleic acid), carbohydrates (galactose, arabinose and uronic acid), phytosterols (beta-sitosterol, betasitosterol 3 O-glucoside, beta-sitosterol-3-O-diglucoside, campestrol, cycloartenol), flavonoids (isoquercetin, kaempferol-3-O-glucoside), resins, tannins, pigments, essential oils and organic acids (caffeic, chlorogenic and anthranilic acid). [76] The saw palmetto extract (SPE) contains mainly saturated and unsaturated fatty acids (90%), among which the main constituents are oleic and lauric acids; both represent more than 50% of the SPE content. In addition, there are also myristic, palmitic and linoleic acids. [77] Phytosterols and free fatty acids are thought to be the pharmacologically active constituents. The *in vitro* results suggest two probable mechanisms of action: the non-competitive inhibition of 5alpha-reductase, which leads to a decrease in the prostatic DHT content, and the inhibition of the binding of DHT to androgen receptors, present in the cytosol of prostate cells. [78]

6.7.2 Clinical studies

As far as clinical studies are concerned, *S. repens* appears to be useful in treatment of mild and moderate BPH if subjects are treated with standard products (90% free fatty acids), at a dose of 160 mg twice a day, for at least three months. [79] On the other hand, it is contraindicated in advanced BPH with severe urinary retention and should not be used without consulting a physician. [76] The clinical efficacy of *S. repens* appeared to be superior

to placebo and comparable to 5alpha-reductase inhibitors and alpha1-antagonists. Various studies are discussed below. A meta-analysis of 18 controlled clinical trials conducted for periods ranging from 4 to 48 weeks, have shown the therapeutic efficacy of *S. repens* extracts to be significantly superior to placebo and similar to finasteride. [80] Favorable effects on IPSS, LUTS, urinary flow, nocturia and dysuria have also been reported. [81] However, other studies have not come to the same conclusion, indicating negative results on the improvement of urinary symptoms associated with BPH. [82] Furthermore, *S. repens* does not appear to reduce prostate size and urinary flow measures [83], nor does it change the levels of prostate specific antigen (PSA), testosterone, DHT, follicle-stimulating hormone (FSH), estradiol, and luteinizing hormone (LH). [84] Compared to finasteride (5 mg/g), *S. repens* (320 mg / day) produces similar improvements in urinary flow, IPSS, and QoL. Finasteride appeared to have a slightly greater effect on peak urinary flow, whereas SPE affected libido and impotence significantly less. [85]

Another systematic review and meta-analysis was performed in 2020 to compare *S. repens* with tamsulosin in the treatment of benign prostatic Hypertrophy (BPH) after a treatment course of at least 6 months. This meta-analysis included four studies involving 1,080 subjects (543 in the *S. repens* group and 537 in the tamsulosin group). The results were as follows: compared to tamsulosin, *S. repens* had the same positive effect in the treatment of BPH in terms of IPSS, QoL, Qmax, PVR, and PSA, with the exception of PV, which did not decrease. As regards the side effects, *S. repens* was well tolerated compared to tamsulosin especially in ejaculation disorders and decreased libido. [86]

6.8 *Urtica Dioica* L.

U. dioica belongs to the Urticaceae family, native to Europe, Asia, North Africa and North America, and is the best known and most widespread of the genus Urtica. Nettle root extract

is widely used in Europe for the treatment of enlarged prostate. More than 20 clinical studies have shown that alone or combined with other plants, it improves the clinical symptoms of benign prostatic hypertrophy and prostatitis. The German Commission E and the European Scientific Cooperative on Phytotherapy (ESCOP) recommend its specific use to relieve urinary symptoms associated with BPH.

6.8.1 Chemical composition and preclinical studies

U. dioica leaves contain sterols (beta-sitosterol, hydroxy-sitosterol), flavonoids (rutin, kaempferol, quercetin), minerals (calcium, potassium), tannins, acids (salicylic, malic) and amines (histamine). [36] it has been identified that, in the root, there are lectins (mixture of isolectins from 0.2 to 0.6%), polysaccharides (glucans, glucogalacturonans, arabinogalactan acid), hydroxycoumarins (scopoletin), ceramides, lignans (secoisolariciresinol-9-O-glucoside, (-) 3,4-divanylyltetrahydrofuran, neo-olivile), sterols and their glycosides (beta-sitosterol, stigmasterol, campesterol), phenols (p-hydroxy benzaldehyde), monoterpendiols and them glycosides, fatty triterpenes, phenylpropane (homovanyl alcohol and its 41-O-glycoside). [87]

Some fractions of nettle root extract inhibit the growth of human prostate cells, but the mechanism of this action which may involve lectins remains unknown. Some research indicates that nettle root can interfere with or block hormone-chemical processes involved in the development of BPH. In some clinical studies, nettle root extract has been shown to stop the conversion of testosterone to dihydrotestosterone (by inhibiting the enzyme necessary for its conversion) and to bind directly to the Sex Hormone Binding Globulin (SHBG), preventing them from the binding to other hormones.

Other studies suggest the possibility of an interaction between substances present in the nettle root extracts and serum proteins that bind androgens (such as SHBG), the interaction

that would lead to a change in the concentration of free androgens. Nettle root extract can prevent SHBG already bound to a hormone from attaching to receptor sites in the prostate. [88]

In a number of papers, German researchers have identified a component of nettle root, (-) - 3,4-divanillitetrahydrofuran, which has a strong binding affinity for SHBG. [89] Nettle root extract can reduce the production of estrogen (estradiol and estrone) by inhibiting the activity of aromatase. At least five components of the root have to low inhibitory activities of aromatase, thereby reducing the conversion of androgens to estrogen. [90] It has also been suggested that steroids or other hydrophobic components of nettle root extracts inhibit the activity of the Na + , K (+) ATPase membrane of the prostate, causing prostate growth arrest. [91] The antiproliferative effect on prostate cancer cells from a methanolic extract was observed in an *in vivo* model and on an in vitro system. [92] An aqueous extract inhibits adenosine deaminase (ADA) activity of prostate tissue. [93]

6.8.2 Clinical Studies

Studies involving a total of 15,000 men with BPH have shown that nettle root extract caused significant improvements in prostate size, urinary frequency, nocturnal urination and post-urination residue. [94] Thus, 41 subjects with BPH were enrolled in a double-blind placebo-controlled study. Subjects had a maximum urinary flow of 15 mL / second and a mean score of 18.2 on the IPSS international prostate symptom score). An IPSS score of 0 to 7 is considered to be mildly symptomatic, 8 to 19 as moderately symptomatic, and 20 to 35 as highly symptomatic.

At the end of the study, maximal urinary flow was increased by 66.1% (10.9 to 18.1 mL / second) in the nettle root extract group versus 36.6% (12.3 to 16, 8 ml / second) the placebo

group. Mean IPSS scores were doubled in the treated group (18.2 to 8.7) compared to placebo (17.7 to 12.9). [95]

A multicentre, randomized, double-blind, placebo-controlled study focused on 146 subjects with BPH followed for one year. They randomly took 459 mg per day of nettle root extract or placebo. The results showed that the extract could be considered as a safe therapeutic option for BPH, especially for reducing irritating symptoms and for its antiproliferative effect. [96]

In a double-blind placebo-controlled study in Iran, 558 men received a placebo or nettle root extract for six months. At the end of this six-month period, subjects on placebo received nettle extract. All continued treatment for a total of 18 months. The results highlighted that nettle extract was more effective than placebo in all measures of the severity of benign prostatic hypertrophy. [97]

7. Clinical trials in the field of food supplements

At global and national level, the last decades have been marked by a significant growth of the area of market characterized by the use of food supplements [98] whose regulatory framework is different at the international level. This situation was accompanied by an important growth in clinical trials on foods to support the safety, health claim and biomedical validity of these food products / ingredients inevitably placing, especially for the Food Business Operators (FBO), significant procedural issues.

Such experiments must be based on a correctly formulated scientific rationale, which consider the type of food to be tested, and be conducted inspired by the general principles of the Good Clinical Practice (GCP) and Good Laboratory Practice (GLP).

Given the lack of homogeneity of the regulatory framework regarding the conduct of clinical studies of food supplements, standard operating procedures (SOP), for the correct implementation of the clinical studies are strongly requested. Thus, a in the following section of this thesis, a summary of the activities to be carried out to perform a clinical study in accordance with the guidelines of the Italian Ministry of Health is reported. [99]

8.1 Step of a clinical trial of food supplement

8.1.1. Study, organization, and planning

The first step necessary for the drafting of the experimental protocol consists in carrying out a bibliographic search through the object of the clinical study, choosing works of high scientific value based on the impact factor, number of citations and quartile of the Journal. Furthermore, it is necessary to evaluate the presence of other clinical studies carried out on the food supplement or food supplement ingredient in the registries that are a primary register of the WHO International Clinical Trials Registry Platform (ICTRP) that includes the minimum acceptable 24-item trial registration dataset or in ClinicalTrials.gov, which is a data provider to the WHO ICTRP.

Before the study begins it is necessary to consider the feasibility of the study based on personnel, equipment, space, subject enrollment (number and type). This procedure is used to specify the total number of subjects enrolled in the study, the method and the enrollment period. Economic agreements and insurance policies with external promoter, and intellectual property of the data must be specified. Procedures for randomization, stratification and maintenance of the blind state should be established, if applicable. To make the documents referring to the study always available, a "Study File" has to be organized to archive them.

Sharing of information within the team involved in the study is fundamental for the correct execution of the clinical trial. If not all procedures can be carried out internally, any external laboratories must be evaluated and the price of the collaboration established.

The presentation to the Ethics Committee and the organization of the necessary materials are agreed jointly with the promoter of the study.

Before the study and enrollment begin, it is ensured that all personnel involved in the study are familiar with the protocol, the Investigator's Brochure, the Case report Form (CRF), and any other clinical or nursing procedures specific to the study.

Following acceptance by the Ethics Committee, meetings of the research group will be organized on a monthly or weekly basis.

At the end of the study all study activities (subject follow-up, filling in the CRF) all the required activities must to be completed as agreed.

An official communication will be sent to certifying the end of the studies and the results of the clinical study will be published within 12 months of the end of the study (Reg. 536/2014). [100]

8.1.2 Staff involved in the study: responsibilities definition

The Principal Investigator (PI) is responsible of the good management study. He must be professionally qualified, have training and research experience, and familiarity with the topics of the study. He's also up to ensure that all the Ethics Committee requirements are observed. He can delegate his daily functions at one or more colleagues to specify in the "Delegation Log". In a multicentric study is defined as the "Study Coordinator".

A staff member involved in the clinical trial will act as an intermediary among the PI, the laboratories where the analyses are carried out, and the promoter of the study.

8.1.3 Study Files

The PI or the person appointed by him is responsible for the Study File preparation and update containing all the documents of the study. The Study File must be identified by the protocol code, promoter's name and the principal investigator's name, and must contain the following original documents:

- Investigator's Brochure or Characteristic Product Summary.
- Study protocol, signed and dated by PI, including any amendments.
- Protocol Synopsis, that is the protocol summary.
- Case Report Form (CRF) that is the tool used to collect data from each participating subject.
- Experimental product Technical sheet containing:
  - Product quality data;
  - Any other clinical studies conducted previously.
- Recruitment arrangements in which it is included the study planification, inclusion and exclusion criteria and the results of the power analysis;
- Material to hand to the subjects:
  - Informative sheet and consent file
  - nutritional supplement
- Informative sheet and agreement module to be read and signed to the enlisted subject.
- Documents concerning the economic aspects of the study and the agreement signed between the parties.
- Documentation for insurance policy.
- Ethics Committee and expert authority's consent.
- "Delegation log" compiled with CV's, signed and dated, of all the staff involved in the study.

- Reference normal/range values for the established exams of the protocol and the certification of laboratories carrying out the exams.
- Concerning documentation to the experimental product management.
- Person Identification code list (Identification code list).
- Recruitment person register (Enrolment Log).
- Evaluated person register (Screening Log).
- Randomization list (where applicable)
- Decoding procedure for blind studies (where applicable, to be used in case of emergency)
- Completed CRF.
- Concerning documentation to monitoring visits.
- Study progress report and final report.

8.1.4 Local level's specific guidelines

The PI is responsible of the compliance of the requirements established by the Sponsor and Ethics Committee, before, during and at the end of study. This responsibility also extends to the post-study period.

8.1.5 Protocol writing and review

The preparation and review process of an internal protocol or the evaluation of a protocol prepared by external promoter is a crucial aspect that must be carefully considered. It is important that the used definitions are clear and unambiguous in all sections of the protocol, even in the case of common pathologies. In the Regulation of Good Clinical Practices, the protocol must consist in the following information:

- Background e rationale;

- Aim (main and secondary aim);

- Study plan (monocentric or multicentric, observational or interventional, if randomized, blind study, etc.);

- Number of people to enlist (total and number of people for each center);

- Inclusion and exclusion criteria;

- Treatment plan;

- Possible concomitant therapies and/or not allowed pharmacological treatments;

- Procedures under the protocol (diagnostic and follow-up tests);

- Randomization and blinding procedures, where applicable;

- During of the study (recruitment and total duration).

- Determination closing study (if different from the last person enrolment);

- Sample size estimation/power calculation (sample/power);

- Statistical analysis;

- Management of experimental products (labeling, accounting, traceability of the batch, etc.)

- Ownership of the data and publication policy of the results;

- Procedures for study monitoring;

- The list of participant centers.

The protocol must be identified by:

- Title;

- Alphanumeric code;

- Version number and data number.

The PI has the responsibility to check the protocols and update them in case of modifications (adding version and data). Eventual critical issues must be discussed with promoter before the approval.

8.1.6 Documentation amendments

During the study, modifications of the clinical trial (i.e. inclusion criteria too restrictive, unexpected adverse events, unclear information sheet, findings in the compilation of CRF) could be requested.

The PI may indicate the requirement of an amendment, even if the final decision to amend a document is entrusted to the promoter.

The amendments may be presented in a separate document (identified by version and data as well as by code/number of amendment) to be added to the existing protocol (adding a legible and clear note indicating the existence of the amendment). But if the amendment regards more aspects, it is recommended to rewrite the entire document, updating the version and data, and filed a copy of the previous version.

If the amendments of the protocol may be transmitted to the subjects involved in the study, it is necessary to produce a new version or add an addendum to the information sheet and informed consent document for the subjects.

An amendment can be significant or not. The D.M. of 21/12/2007 define [101]:

Substantial, an amendment which impacts on the security, or on the: physical/mental integrity, ethical aspect of the trial, scientific value of the study, direction or management of the study, quality or safety of each experimental product used in the study.

Therefore a significant amendment must be submitted to the evaluation of the coordinator center and different participating centers Ethics Committee, and it must be implement only after receiving the written agreement.

The not significant amendment, instead, must be notified only to the C.E. except for the case of significant urgent amendment (necessary for the subjects protection), that must be notified to C.E and instantly implemented in all the centers.

The PI must sign the amendments only after carefully checking, and make sure the staff involved are promptly informed about any modification.

8.1.7 Drafting and reviewing of data sheet (CRF)

This procedure should be used before the beginning of the study, in order to prepare/review the data sheet and identify any findings.

Aim of CRF is to collect relevant data in a specific format, in compliance with the Regulatory requirements and Protocol. CRF allows an efficient and complete development, analysis and reporting of data and have a match on the feasibility of the protocol.

In the drafting of CRF it is recommended to follow the flow-chart of the protocol, in which the order of the study's procedures has to be respected to facilitate the compilation.

The CRF must contain only the data required by protocol and must be analyzed.

The CRF must also provide always a section, usually in the early pages, for the evaluation of inclusion/exclusion criteria, sections for the registration of concomitant therapies and possible adverse events and, at the end, a section concerning the possible premature interruption of the study. At the end of CRF, there is a space for the consent (signature and date) of the PI.

It may be useful to include the CRF instructions for the compilation and correction of the data and the list of abbreviation. The CRF can be organized in paper or electronic form (e-CRF) and must be finalized before the beginning of the study.

8.1.8 Investigator's brochure (IB)

The Investigator's Brochure (IB) must contain all the information about the food supplement subject of the study, including data related to pharmacokinetic and pharmacodynamic in the animals, and the results of previous clinical studies.

Before the beginning of the trial with a food supplement, all the investigators (principal investigator and co-investigators) must know the contents of IB.

A copy of IB must be always available in the Study File for the consultation.

The IB contains very sensitive data. Only people involved in the study can have access to IB.

8.1.9 Subject's number evaluation

In the Good Clinical Practices, the enrollment subjects' number must be clearly calculated. The biostatistics calculate the sample size according to a specific method based on the plan of the project; the motivations of selection must be specified in the section of protocol related on the statistic methodology.

8.1.10 Obligation to Ethics Committee (CE)

As defined of the art. 6 of D.Lgs. 211 of 24/06/2003 [102] , the Ethics Committee (CE) must promulgate its opinion before the beginning of the clinical trial.

Before the beginning of the study the PI is responsible for the presentation of request to the Ethics Committee centers which participated in the trial; for multicentric studies the Coordinator Centre must be identified; the consent of the study by satellite center's Ethics Committee is subordinated to Ethics Committee of Coordinator Centre. The way and time for the submission of the consent request are reported on the site of C.E. involved in the clinical study.

After the analysis of the study, the Ethic Committee issue an opinion that could be: positive, positive under condition, suspensive or contrary.

In case of positive opinion under condition or suspensive opinion, it is necessary to attach to the documents of the study (protocol, informative sheet, etc.) the modifications required by C.E. and present again to it the changed documents.

During the study, the investigator must communicate to the C.E. any amendment regarded to protocol and/or other documents about the study (Informative Sheet, informed consent, Investigator's Brochure, etc.) and adverse severe or unexpected events. Furthermore the PI must communicate the eventual and important deviations from the protocol during the study. The C.E. will make an opinion on the substantial amendments and will take note of non-substantial amendments. The investigator must communicate to C.E. the enrollment date of first subject and send a relation about the progress state of the study each 12 months at least.

At the end of the study the investigator must inform the C.E. about the end of the study or, if applicable, its failure.

8.1.11 The enrollment of the subjects

The recruitment of the subject is based on different moments, which:

- Identification of the subjects based on inclusion and exclusion criteria;
- Requirement and achievement of the informed consent;
- Screening of the subjects;
- Randomization, where expected;
- Other verifications, if necessary.

Each potential candidate must be exposed to the procedure of the recruitment. Before to start the enrollment, the investigator can draw up a list "Potential Enrollments" which contain the list of all potential enrollment subjects, where containing if the subject take part to the study or not (explaining the exclusion). This list allows the comparison of a potential population

to which indeed enrollment; and it is useful in case of emerge bias doubts during evaluation of the data.

Based on the protocol the enrollment can be sequentially or in different steps. At the enrollment time, an identification code will be assigned to each subject, drawing up the “Identification code list of the subjects” (Identification list). The code assigned to a subject that leave the study can’t be reuse for another subject also immediately after the sign of the consensus.

The “Identification code list of the subjects” establish that are included, next to the code of the subject, the following data: name, surname, date of birth, date of sign of the informative sheet. The subject is considered enlisted when he signs the informative sheet. After the enlistment of the subjects, the trial and all the procedures reported in the protocol begin.

8.1.12 Signature of the informed consent

The request and the obtaining of the written informed consent of the subject represent a very delicate moment of the study. The subject's consent must be obtained before any procedure of the study is implemented.

The signature of the informed consent must be preceded by an interview with the subject, during which the investigator (or person delegated by him through the "Delegation Log") informs with completeness the subject regarding all the aspects related to the study. If a relative/friend of the subject is present, it is desirable that he takes part in the conversation.

The language used during the interview must be simple, non-technical, and easy to understand for the subject, and it is equally essential to respond clearly to any questions posed.

8.1.13 Randomization and stratification

The term randomization mean the casual position of the subjects in two or more groups of treatments.

In a controlled clinical trial, the randomization is used for an equilibrium of the confused factors, known or not, between treatment's groups. In presence of one or more confused factors, it is possible to organize a stratification randomization, for which the balance between groups with different treatments must be guaranteed.

Usually in a multicentric study, the randomization is stratified for each center.

The randomization procedure must be specified in the protocol and the PI must approve it from an ethical and clinical point of view.

The randomization list is generated using a validated system, based on a pseudo-random number generator, in order to the assignment to the treatment group is reproducible and unpredictable.

Generally, the block randomization technique is used. The access to the codes must be controlled and documented by a register.

The list of randomizations must not be note at the investigators and must be conserved in a different place to the Study File.

8.1.14 Trial report

At the end of the study, after the statistical analysis, the Trial Report is prepared. The Trial Report must be written also in case of the study is not completed.

It must be draft (within 12 months of the end of the study) according to the European guidelines (CPMP / ICH / 137/95) [103] and it must contain:

- Sponsor name;

- Identification data of the study and experimental products;
- List of participating centers and investigators;
- Design of the study;
- Aims;
- Enlistment subjects;
- Inclusion and exclusion criteria;
- Treatment;
- Efficacy and security evaluation;
- Statistical analysis;
- Conclusion.

The repost must be send at all the interested Ethics Committee.

9. Aim of the research

Plant extracts are commonly used as ingredients in food supplements aimed at wellness management and add-on therapies for the prevention and treatment of a number of pathologies, including both communicable and noncommunicable diseases. [98]

Prostate problems such as BPH [10], [104] are one of the ailments often treated with plant-based products, especially in the early stages of development before pharmacological therapy, as these products exert beneficial effects, improving the quality of life through the partial reduction of symptoms [105], [106] with low adverse effects in comparison with the current pharmacological treatments for BPH. [107]-[108]

Due to the global diffusion of this condition among men, the search for alternative and safe treatments for prostate disorders is becoming an urgent need. [109] The most studied and used vegetable extracts are represented by *Serenoa repens (W. Bartram) Small, Prunus Africana (Hook. f.) Kalkman* and *Urtica dioica L.*. [110]–[114] In addition to these plants, growing evidence suggests the possible role of *Epilobium angustifolium L*. [115], which is used for wounds and skin diseases in Traditional Medicine [116] and, especially in European Countries, is used in the treatment of the symptoms of BPH. [117]–[120] Although some *in vitro* and *in vivo* studies showed its biological properties such as antioxidant, anti-inflammatory, antitumor, antimicrobial, and immunomodulatory agent [119], the knowledge on its chemical composition, bioaccessibility and bioavailability and efficacy in the remission of the symptoms of BPH is limited. Thus the aim of this project was to study the metabolic profile of a commercial *E. angustifolium* extract (EAE), used in food supplements suggested for the improvement of BPH symptoms. Moreover, the *in vitro* chemical degradation in oragastric and duodenal fluids and *in vivo* bioavailability of *E. angustifolium* metabolites following acute and prolonged treatment with EAE were investigated. In

addition, its antioxidant activity was studied using an *in vitro* model system. Finally, given the promising results obtained from these preclinical studies a monocentric, randomised, double-blind, placebo-controlled clinical trial was performed 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 the recruited subjects suffering for an mild/moderate BPH.

10. Epilobium angustifolium L. extract: chemical characterization, *in vitro* bioaccessibility, *in vivo* bioavailability and *in vitro* preclinical studies

10.1 Introduction

Benign prostatic Hypertrophy (BPH) is a common condition as men get older. An enlarged prostate gland can cause uncomfortable urinary symptoms, such as blocking the flow of urine out of the bladder. It can also cause bladder, urinary tract or kidney problems.

BPH is a condition mainly characterized by a proliferation of both stromal and epithelial cells of the prostate with an alteration of the periurethral area. [10], [104]

A wide variety of treatments are available for BPH, including pharmacological therapies, minimally invasive therapies and surgery, but these are not free from side effects.

Mild/moderate BPH is often treated with plant-based products before pharmacological and surgical therapy [108] and are considered an alternative approach to avoid the possible adverse effects of these therapies. [121],[122] Nowaday, trying to prevent these diseases, dietary supplements based on plant extracts obtained *Serenoa repens (W. Bartram) Small, Prunus Africana (Hook. f.) Kalkman and Urtica dioica L.* [110]–[114]

In addition to these plants, there has been a growing interest over the past few decades in *Epilobium angustifolium L*. for its possible activity against BPH. The *Epilobium* genus belongs to the Onagracee family and includes over 200 species spread across temperate and cold regions [115], Some *in vitro* and *in vivo* studies have focused their attention on possible positive effects of *E. angustifolium* in the treatment of BPH. [117]–[120] The main classes of polyphenols occurring in *E. angustifolium* are phenolic acids, flavonoids and ellagitannins [123], and the beneficial effects of *E. angustifolium* have been ascribed to these. [124], [125]

The main ellagitannin in *E. angustifolium* is oenothein B, which has shown *in vitro* and *in*

vivo biological activities such as antioxidant, anti-inflammatory, inhibitory enzyme activity, antitumor, antimicrobial, and immunomodulatory activities. [119]

While *E. angustifolium* is used against BPH, especially in Europe, little is known about its bioaccessibility and bioavailability. So, there is an urgent need to perform these studies to eventually support subsequent clinical trials, which could evaluate the safety and efficacy of *E. angustifolium* in prostate ailments. Thus, the aim of this investigation was to study the metabolic profile of a commercial *E. angustifolium* extract (EAE), used in food supplements suggested for the improvement of BPH symptoms. Moreover, the *in vitro* chemical degradation in oral-gastro- duodenal fluids and *in vivo* bioavailability of *E. angustifolium* metabolites following acute and prolonged treatment with EAE were investigated. In addition, its antioxidant activity was studied using an *in vitro* model system.

10.2 Materials and methods

10.2.1 Chemicals and reagents

One batch of commercial EAE, standardized to contain ≥ 15 % oenothein B, was provided by EPO S.R.L. (Milan, Italy). All the compounds used for *in vitro* oral, gastric and duodenal digestion processes are reported below: potassium chloride (KCl), dihydrogen potassium phosphate (KH2PO4), sodium carbonate (NaHCO3), magnesium chloride (MgCl2), ammonium carbonate (NH4)CO3, calcium chloride (CaCl2), sodium chloride (NaCl), hydrochloric acid (HCl), sodium hydroxide (NaOH). All were provided by Carlo Erba (Milan, Italy). Pancreatin from a porcine pancreas (extract of pig bile), α-amylase from Bacillus licheniformis, pepsin from porcine gastric mucosa and porcine bile extract, formic acid solution (1 M), water, methanol, acetonitrile LC–MS grade, sodium monobasic dehydrated phosphate (NaH2PO4 2H2O), tryptone, cysteine, sodium sulphite (Na2S),

resazurin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), lipopolysaccharides from Escherichia coli O55:B5, and dimethyl sulfoxide (DMSO) were sourced from Sigma-Aldrich, Merck KGaA (Milan, Italy). All the media and reagents for cell culture were purchased from Gibco (Milan, Italy).

10.2.2 E. angustifolium extract analysis by UHPLC-LTQ Orbitrap

EAE stock solution was prepared, accurately weighing 200 mg of extract and making up the volume with a solution 50:50 v/v acidified water (0.1 % v/v formic acid) and methanol. From the stock solution, 1 mL was picked up and filtered before the analysis (0.45 μm and 0.20 Minisart RC 4 membrane filters). Chromatographic analysis of the EAE extract was performed by means of UHPLC-LTQ Orbitrap. 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 analysed by Liquid Chromatography-Mass Spectrometry.

UHPLC-HRMS analyses were performed on a Shimadzu Nexera UHPLC system, consisting of a CBM-20A controller, two LC-30AD dual-plunger parallel-flow pumps, a DGU-20 AR5 degasser, an SPD-M20A photo diode array detector, a CTO-20A column oven, 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. Column oven was set to 45 °C, 2 μL were injected. PDA detection parameters were:

sampling rate 12 Hz, time constant 0.160 s and chromatograms were extracted at 280 and 330 nm. LC data elaboration was performed by the LCMS solution® software (Version 3.50.346, Shimadzu).

MS detection was performed in negative mode as follows: spray voltage was set at -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 × 105 for full FTMS scan and 3 × 104 ions for IT. Dynamic exclusion was enabled with a repeat count of 1 and a repeat duration of 30 s. Preview mode for FTMS master scan was enabled. The instrument was tuned using a Thermo negative ion calibration solution. Thermo RAW data files were converted in mzXML and were aligned by the open source MZmine2.

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

10.2.3 In vitro bioaccessibility of E. angustifolium extract by simulated orogastric and duodenal digestion processes

To verify the effect of orogastric and duodenal digestion processes on the chemical composition of EAE we followed a protocol by Milekus et al. with some modifications. [126] In brief, 5 g of EAE were dissolved in 3.5 mL of previously prepared simulated salivary fluid (SSF). The same procedure was followed for the blank sample using 5 mL of water instead of EAE. Then, 0.5 mL (1500 U/mL) of fresh α-amylase solution were added

to both samples. In the end, water was added to reach a final volume of 10 mL and the samples were incubated for 2 min at 37 ◦C. The bolus obtained in the previous phase was mixed with 7.5 mL of simulated gastric fluid (SGF) and 1.6 mL (25,000 U/mL) of fresh pepsin, the pH was then adjusted to 2.00 ± 0.02 using 1 M HCl. The samples were brought up to 20 mL volumes and the mixture was incubated at 37 ◦C for 2 h in a shaking water bath. In two further samples, EAE and blank were treated to simulate only the duodenal step of digestion. For these, 5 g of EAE or 5 mL of water were added to 20 mL of water to simulate the gastric chyme. Subsequently, 5 mL of freshly pancreatin (800 U/mL) were added with 2.5 mL of fresh bile mixture (160 mM) to reach a final volume of 32.5 mL. The samples were finally made up to a 40 mL final

volume, pH was adjusted to 7.00 ± 0.02 using 1 M NaOH and incubated 37 ◦C for 2 h. At the end of the digestion process, orogastric digested (OGD) and duodenal digested (DD) samples were freeze dried and maintained at 4 ◦C prior to analysis in the experimental conditions reported above.

10.2.4 In vivo bioavailability of E. angustifolium extract metabolites after acute and prolonged treatment

10.2.4.1 Animals

The *in vivo* bioavailability of *E. angustifolium* constituents was investigated in the plasma of mice after acute and prolonged administration of EAE. For this purpose, CD1 (Harlan-Nossan) male mice (10–14 weeks of age, 25–30 g of weight) were used for all experiments. The animals were housed in cages (5–7 animals per cage) under standard conditions of light (with a 12-h light/dark cycle), temperature (22 ± 1 ◦C) and relative humidity (60 ± 10 %), for at least a week before starting the experiments. Food and water were available ad libitum. Groups of 5–7 animals were used for each experiment. All animal care and experimental

procedures were carried out in compliance with international and national law and policies, according to the guidelines on animal experimentation as indicated by the D.L. 116/92. All procedures were carried out to minimize the number of animals used (n = 7 per acute treatment group and n = 5 prolonged treatment group) and their suffering. [127], [128]

The experimental animals were acutely orally administered (gavage) with a 0.6 mg/mouse dose of EAE, corresponding to a dosage approximately double that expected for humans, considering that the daily intake in humans ranges from 500 to 750 mg. Blood samples were collected, applying the intracardiac procedure in enflurane-anaesthetized mice using syringes pre-filled with 3.8 % sodium citrate (1: 9 blood ratio) at 5, 10 and 20 min, so as to mimic acute administration. Whole blood was then left to settle for 24 h, and the supernatant obtained after centrifugation at 4000 rpm for 4 min (plasma) was then collected and used as the blood sample on which subsequent analytical investigations were carried out.

The same blood-collection procedure was followed for the prolonged treatment, after daily administration of 0.6 mg/mouse of EAE by oral gavage for a total of 2 weeks. The blood collection was performed at 24 h, 1 week and 2 weeks after initiation of the treatment. In this case, whole blood was left to settle for 24 h, centrifuged, and then plasma was separated from cellular material (pellet). Plasma and pellet samples were then submitted to analytical investigation.

10.2.4.2 UHPLC-MS/MS analysis of mouse plasma after acute treatment

Blood samples collected from each group of mice (5, 10, and 20 min) were pooled and processed before analysis. In brief, 50 µL of plasma were treated with 150 µL of ice-cold acetonitrile acidified with 98 % HCN and 2% HCOOH. After centrifugation for 15 min at 16,000 rpm (Eppendorf®, Hamburg, Germany) and evaporation of solvent with nitrogen, the supernatants were solubilized in methanol and analyzed.

UHPLC-MS/MS analyses for quantitative evaluation of *E. angustifolium* metabolites in plasma samples were performed on a Shimadzu Nexera UHPLC system, consisting of two LC 30 CE pumps, a SIL 30AC autosampler, a CTO 20AC column oven, and a CBM 20A controller. The system was coupled online to a triple quadrupole LCMS 8050 (Shimadzu, Kyoto, Japan) equipped with an electrospray source (ESI).

The separation was performed on an ACQUITY UPLC® BEH C18 column (L × ID) 50 mm × 2.1 mm, 1.7 μm (Waters, Milford, USA) employing as mobile phases: A) 0.1 % CH3COOH in H2O and B) CAN plus 0.1 % CH3COOH, with the following gradient: 0–1.0 min, 5–70% B; 1.01–2.0 min, 70–78 % B; 2.01–2.50 min 78-100 %B; 100 % B hold for 1 min; returning to initial conditions in 0.1 min. The flow rate was set to 0.5 mL/min. Column oven was set to 45 ◦C, and 5 μL of extract were

injected.

The ESI source was operated in the negative mode. MS/MS analyses were conducted in selected reaction monitoring (SRM) mode, using the following transitions: kaempferol; 285.00 > 117.00 (quantifier ion), Q1 pre bias 10.0 V, collision energy: 44.0 V, Q3 pre bias 21.0 V; quercetin, 301.00 > 151.15 (quantifier ion), Q1 pre bias 11.0 V, collision energy:22.0 V, Q3 pre bias 30.0 V. Dwell time, 25 msec. Interface temperature, desolvation line temperature, and heat block temperature were set, respectively at 300 ◦C, 250 ◦C and 400 ◦C. Nebulizing gas, drying (N2) and heating gas (air) were set, respectively, at 3, 10 and 10 L/min. Quercetin and Kaempferol were selected as external standards. Stock solutions (1 mg/mL) were prepared in methanol and calibration curves were obtained for a concentration range of 5–100 ng/mL, with five concentration levels and triplicate analysis performed for each level. (R2 = 0.9991).

Repeatability was established by triplicate injections of sample and solutions at low, medium, and high concentration levels of the calibration curve, with the same

chromatographic conditions and analyst both on the same day and within two consecutive days, showing good retention time and ng/mL extract repeatability with maximum CV% values ≤ 0.07 and 8.02, respectively. Limits of detection (LODs) and quantification (LOQs) were calculated from the ratio between the standard deviation (SD) and the analytical curve slope multiplied by 3 and 10, respectively, obtaining values for kaempferol LOD: 0.66 ng/mL, LOQ: 2.22 ng/mL; and for quercetin LOD: 1.06 ng/mL, LOQ: 3.55 ng/ mL. All samples were analyzed in triplicate.

10.2.4.3 UHPLC-MS/MS analysis of mouse plasma after prolonged treatment

Plasma and pellet samples underwent the same extraction procedure and UHPLC-MS/MS analysis reported in section. Urolithins were detected with the following transitions: urolithin A; 227.00 > 198.00 (quantifier ion), Q1 pre bias 10.0 V, collision energy: 44.0 V, Q3 pre bias 21.0 V; urolithin B, 211.00 > 167.15 (quantifier ion), Q1 pre bias 11.0 V, collision energy: 22.0 V, Q3 pre bias 30.0 V. Dwell time, 25 msec. Urolithin A and urolithin B were selected as external standards. Stock solutions (1 mg/mL) were prepared in methanol and calibration curves were obtained for a concentration range of 0.5–50 ng/mL, with five concentration levels and triplicate analysis performed for each level. (R2 = 0.999). Repeatability was established by triplicate injections of sample and solutions at low, medium, and high concentration levels of the calibration curve, with the same chromatographic conditions and analysts both on the same day and within two consecutive days, showing good repeatability with maximum CV% values ≤ 2.92. Limits of detection (LODs) and quantification (LOQs) were calculated by the ratio between the standard deviation (SD) and analytical curve slope multiplied by 3 and 10, respectively, obtaining values for urolithin A LOD: 0.01 ng/mL, LOQ: 0.02 ng/mL; and for urolithin B LOD: 0.89 ng/mL, LOQ: 0.29 ng/mL. All samples were analyzed in triplicate.

10.2.5 In vitro antioxidant activity of E. angustifolium extract

10.2.5.1 LNCaP-FGC clone

LNCaP-FGC clone from the European Collection of Cell Cultures (ECACC 89,110,211) was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin and 100 µg/mL of streptomycin (complete medium). Cells were maintained at 37 ◦C in a 5% CO2 incubator and the medium was replaced every 48 h. The trypsinization process for LNCaP cells was always performed at 70–80 % confluence.

10.2.5.2 Cell viability test

A stock solution of EAE, submitted to *in vitro* duodenal digestion, was prepared at 5 mg/mL using the complete medium. The viability MTT test for LNCaP was set up, seeding 1.5 × 105 cells per well in a 24 plate at per well at passage 26 (p26). After 48 h, cells were treated at the following concentrations: 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL e 15.62 µg/mL. After 24 h of treatment the solution was removed and the cells were gently washed with PBS prior to the addition of the MTT at a final concentration of 1 mg/mL. After 4 h of incubation the medium was removed, the cells were lysed with DMSO, and the cell viability was verified, reading the absorbance at 570 nm using an Envision microplate reader (Perkin Elmer, Waltham, Massachusetts, United States).

10.2.5.3 *In vitro* evaluation for antioxidant properties of EAE

Pre-treatment was performed using an EAE solution at a concentration of 32.5 µg/mL, starting from the stock solution. LNCaP cells were seeded at 5 × 105 at p26, and after 48 h the complete medium was removed and the cells washed with PBS. The solution was replaced with the pre-treatment solution for 2 h at 37 ◦C in 5% CO2, after which the medium was removed and the cells washed again. Cells were collected after trypsinization and

centrifuged. The supernatants were removed and the cell pellets were resuspended in 300 μL of PBS and centrifuged to mechanically break the cell membranes. The solutions were frozen at - 80 ◦C for subsequent analysis.

10.2.5.4 Protein Quantification

To quantify intracellular Superoxide Dismutase-1 (SOD-1) (AB119520) and Catalase (CAT) (AB171572) we used a Human ELISA kit purchased from Abcam (Milan, Italy) following the manufacturer's instructions. Samples were diluted 1:120 for SOD-1 analysis, while CAT samples were not diluted. Total protein quantification was performed before the ELISA assay to normalize data on total soluble protein content. Fluorometric quantification was assessed with Qubit 4.0 (Thermo Fisher Scientific, Waltham, MA, USA), using the Qubit™ Protein Assay Kit (Invitrogen, Waltham, MA, USA).

10.2.6 Statistical analysis

Data are reported as mean ± standard deviation (SD). Statistical comparisons between the two groups (Control vs Treated) were conducted using T tests to determine significance, which was set to $p < 0.05$. For each well, a technical triplicate measurement was obtained and averaged. Statistical analyses were performed using Prism Graphpad 8 (San Diego, CA, USA).

10.3 Results

10.3.1 Metabolic profile of EAE

The first step was the chemical characterization of the commercial hydroalcoholic extract, obtained from the aerial parts of *E. angustifolium*, standardized to contain ≥ 15 % oenothein

B. The extract was analysed by means of UHPLC- LTQ Orbitrap to obtain the metabolic profile **Fig. 1A**. 42 compounds were annotated by UHPLC-HRMS as shown in **Fig. 1B e Table 1**. The identification of all compounds reported was carried out by accurate mass and fragmentation pattern comparison against reference MS/MS spectra reported *in silico* and in previous literature. The 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 putatively identified compounds were 8 organic and phenolic acids, 1 sugar, 1 tannin, 3 ellagitannins and 29 flavonoids as reported in Table 2. The employment of high-resolution mass spectrometry resulted in a higher number of identified compounds in the *E. angustifolium* extract with respect 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 [129], we identified 20 compounds consisting in 2 sugars, seven organic and phenolic acids, one ellagitannin, and ten flavonols. In another paper on the antioxidant activities and active chemical constituents from *E. angustifolium*, 28 compounds were identified as phenolic compounds and flavonoids by LC-MS/MS. [130] 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) a large number of variously substituted metabolites glycosylated (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"-ferulylhexoside), two kaempferol-p-coumaroylhexoside).

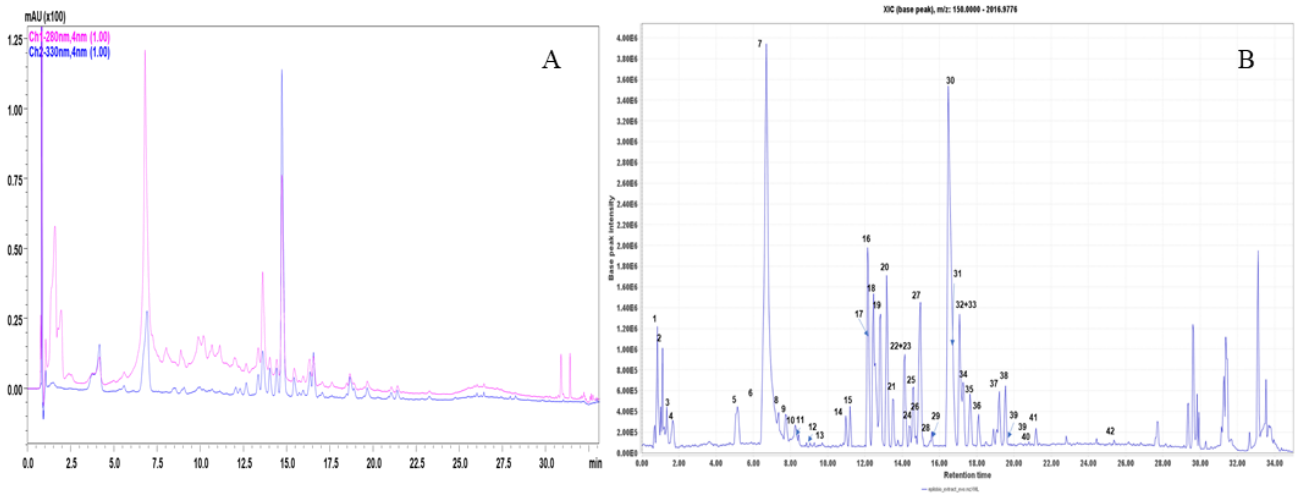

Figure 1 *RP-UHPLC chromatograms of EAE with UV-detection registered at λ 280 nm and 330 nm.* ***A)*** *and base peak chromatogram (BPC)* ***B)*** *with corresponding peak annotation.*

Peak number	RT (min)	Proposed structure	Molecular Formula	*m/z* $[M-H]^-$	MS/MS	Error (ppm)
1	0.84	Caffeic acid 4-O-hexoside	C12H22O11	341.10896	179.0245; 113.0587; 143.0246	0.30
2	1.03	Shikimic acid	C7H10O5	173.04556	137.0381; 111.1149	0.40
3	1.12	Galloylhexose	C13H16O10	331.06674	191.0562	1.00
4	1.67	Gallic acid	C7H6O5	169.01429	125.0134	0.50
5	5.15	Chlorogenic acid	C16H18O9	353.08746	191.0537; 135.0401	0.80
6	6.46	Gemin D	C27H22O18	633.07263	301.0704	-1.01
7	6.70	Oenothein B	C68H48O44	1567.14417	765.1010; 935.1260; 633.4009	1.60
8	6.79	p-coumaroylquinic acid isomer	C16H18O8	337.09280	191.0562	-0.20
9	7.73	Chlorogenic acid isomer	C16H18O9	353.08752	191.0537; 135.0401	0.90
10	8.25	Oenothein A	C102H72O66	1175.6093 [M-2H]2-	765.1010; 935.1260; 633.4009	1.60
11	8.29	3-feruloylquinic acid	C17H20O9	367.10286	193.0408; 173.0130	-1.04
12	9.04	p-coumaroylquinic acid	C16H18O8	337.09235	191.0562	-0.10
13	9.28	Tannic acid	C27H24O18	635.08838	465.1876	-0.90
14	10.96	Caffeoyl quinic acid -3-O-hexoside	C21H20O13	479.08223	316.0802	-1.09

15	11.20	Myricetin-3-O-hexoside	C21H20O13	479.08237	316.0802	-1.12
16	12.13	Galloylquercetin	C28H24O16	615.09845	463.0809; 301.0906	-1.02
17	12.41	Quercetin-3-O-pentoside	C19H14O12	433.04062	301.1205	-1.04
18	12.46	Galloylquercetin	C28H24O16	615.09851	463.0809; 301.0906	-1.02
19	12.83	Quercetin-3-O-hexoside	C21H20O12	463.08728	301.1288	-1.07
20	13.16	Quercetin-3-O-hexoside	C21H20O12	463.08731	301.1279	-0.98
21	13.49	Kaempferol galloyl hexoside	C28H24O15	599.10336	447.3458; 313.1143; 285.1048	-1.04
22	14.14	Quercetin-3-O-pentoside	C20H18O11	433.07681	301.0614	-1.90
23	14.19	Kaempferol-3-O-hexoside	C21H20O11	447.09253	284.0121; 255.1	-1.70
24	14.37	Quercetin-3-O-pentoside	C20H18O11	quinic .07690	301.0614	-1.80
25	14.61	Kaempferol galloyl hexoside	C28H24O15	599.10358	447.3458; 313.1143; 285.1048	-1.04
26	14.74	Myricetin-methylether-hexoside	C21H18O14	493.06177	317.0300	-1.10
27	14.98	Kaempferol-3-O-hexoside	C21H20O11	447.09254	284.0121; 255.1483	-1.70
28	15.49	Kaempferol 3-O-arabinoside	C20H18O10	417.08203	284.0187	-1.70
29	15.59	Myricetin-3-O-caffeoyl-hexoside	C30H26O16	641.11346	479.1309; 317.1401	-2.00
30	16.47	Quercetin-glucuronide	C21H18O13	477.06689	301.1463	-1.10
31	16.84	Rutin	C27H30O16	609.12427	463.0871; 301.1127	0.10
32	17.08	Kaempferol-7-O-rhamnoside	C21H20O10	431.09763	285.1060	-1.07
33	17.17	Quercetin 3'-hexoside-7-acetate	C23H22O13	505.09805	301.1793; 329.2003	-1.30
34	17.26	Quercetin-3-O-caffeoylhexoside	C30H26O15	625.11932	463.0875; 479.2256	1.10
35	17.64	Kaempferol-glucuronide	C21H18O12	461.07166	285.0398	-1.80
36	18.10	Quercetin	C15H10O7	301.03493	179.0153	-1.50
37	18.48	Quercetin-p-coumaroylhexoside	C30H26O14	609.12424	463.0275; 447.3030	-1.20
38	19.22	Quercetin-p-coumaroylhexoside	C30H26O14	609.12421	463.0275; 447.3030	0.10

39	19.45	Quercetin 3-(6"-ferulylhexoside)	C31H28O15	639.13470	463.0213; 301.1146	-1.30
40	20.81	Kaempferol-p-coumaroylhexoside	C30H26O13	593.12933	447.2656; 285.1403; 255.1559	-1.02
41	21.18	Kaempferol-p-coumaroylhexoside	C30H26O13	593.12952	447.2656; 285.1403; 255.1559	-0.90
42	25.37	Dimethylquercetin	C17H14O7	329.06636	314.1575	0.80

Table 1 *Identified compounds in EAE according to the retention time (RT), compound, molecular formula, m/z and MS/MS.*

10.3.2 Influence of in vitro simulated oro-gastric and duodenal digestion processes on EAE chemical composition

To evaluate the *in vitro* bioaccessibility of EAE, the extract was submitted to *in vitro* simulated orogastric and duodenal digestion processes and then the digested extracts were analysed to verify the stability of its main components. **Table 2** shows the mean peak area reduction percentage after orogastric digestion, revealing a degradation process when compared with the peak area registered before digestion. In particular, a great percentage reduction was determined for kaempferol-hexoside (84.17 %), kaempferol-p-coumaroyl glucoside (52.31 %) and dehydro catechin type B (59.78 %), while organic acids appear to be less sensitive to the orogastric environment, especially malic acid and gallic acid with a percentage peak reduction below 2%. For the other polyphenols, the peak reduction ranged from 16.48 % to 34.63 %. As far as oenothein B is concerned, oro-gastric digestion led to a reduction of about 17 %. A similar degree of degradation was registered following duodenal digestion. Kaempferol-hexoside (81.87 %) was found to be one of the compounds most sensitive to

duodenal digestion. As far as oenothein B is concerned, duodenal digestion led to a reduction of over 20 % **Table 2**.

Peak	Compound	Orogastric digestion (%) ± SD*	Duodenal digestion (%) ± SD*
1	Hexose	6.08 ± 0.18	24.14 ± 0.14
2	Disaccharide	23.03 ± 0.98	24.14 ± 0.39
3	Gluconic acid	9.86 ± 0.15	13.97 ± 0.12
4	Quinic acid	10.01 ± 0.18	18.59 ± 0.13
5	Malic acid	1.73 ± 0.09	9.63 ± 0.14
6	Gallic acid	1.92 ± 0.09	32.11 ± 0.29
7	Oenothein B	16.80 ± 0.29	20.66 ± 0.47
8	Chlorogenic acid	20.11 ± 1.13	45.34 ± 0.69
9	Chlorogenic acid	13.02 ± 0.45	35.58 ± 0.26
10	Dehydro catechin type B	59.78 ± 2.91	98.07 ± 2.12
11	Chlorogenic acid	18.63 ± 0.35	35.58 ± 0.42
12	Myricetin-hexoside	20.50 ± 0.42	34.35 ± 0.59
13	Quercetin-galloyl-hexoside	16.48 ± 0.19	12.05 ± 0.15
14	Quercetin-hexoside	24.48 ± 0.27	24.75 ± 0.24
15	Quercetin-3-O-pentoside	34.63 ± 0.79	13.25 ± 0.17
16	Kaempferol-hexoside	84.17 ± 4.29	81.87 ± 5.21
17	Kaempferol-3-O-rhamnoside	26.27 ± 0.89	23.50 ± 0.29
18	Kaempferol-p-coumaroylglucoside	52.31 ± 1.97	19.26 ± 0.16
19	Myricetin-3-O-glucuronide	31.43 ± 0.56	27.11 ± 0.36
20	Quercetin-glucuronide	22.54 ± 0.71	11.83 ± 0.24

Table 2 *Mean relative peak area reduction percentage (% ± SD) of identified EAE compounds after orogastric digestion process and after duodenal digestion process.*

10.3.3 *In vivo* bioavailability of EAE metabolites after acute and prolonged treatment

Aiming to detect *E. angustifolium* metabolites occurring in mouse blood samples after acute treatment, 5 mice were treated with EAE (5, 10, and 20 min).

The experimental animals were acutely orally administered (gavage) with a 0.6 mg/mouse dose of EAE, corresponding to a dosage approximately double that expected for humans, considering that the daily intake in humans ranges from 500 to 750 mg.

The pooled and processed plasma samples were analyzed by means of a validated UHPLC-MS/MS method, which revealed the presence of kaempferol and quercetine as reported in **Fig. 2A**. The maximum concentration (Cmax) was reached after 10 min for both polyphenols, at 569.34 ± 0.25 ng/mL for kaempferol and 1076.02 ± 0.54 ng/mL for quercetin. Moreover, urolithins were not detected after acute treatment. **Fig. 2B and C** report an example of a SRM chromatogram of the transition ions 285.00 > 117.00 and 301.00 > 151.15 corresponding to kaempferol and quercetin, respectively.

As the analysis of the metabolic profile confirmed that EAE contains oenothein B, which belongs to the chemical class of ellagitannins, hydrolysable tannins generally converted into urolithins by the intestinal microbiota and after acute treatment urolithins were not found in mouse blood samples, 7 mice were treated with EAE at the dose of 0.6 mg/mouse for 2 weeks of treatment. Following prolonged treatment of mice with EAE, both plasma and pellets (so as to evaluate the metabolite content of red blood cells) obtained from the centrifugation of blood samples collected after 24 h, 1 and 2 weeks, were analyzed immediately after culling by means of a validated UHPLC-MS/MS method to determine EAE metabolites, especially urolithins. Urolithins A and B were identified and quantified in all plasma samples after 24 h, 1 and 2 weeks, while they were determined in pellet samples only after 1 and 2 weeks of EAE treatment **Table 3**. **Fig. 3A** shows the total concentrations of urolithin A and urolithin B. The results show that after 24 h of EAE treatment, the concentration of urolithin B is higher than that of urolithin A ($Df = 8$; $p < 0.05$), while after 1 and 2 weeks their concentrations are similar. Moreover, Plasma, pellet, and whole blood sample concentrations of both metabolites increased with the ongoing duration of treatment, confirming that urolithins are produced at concentrations higher than the limit of detection (LOD) only after prolonged treatment.

An example of a SRM chromatogram of urolithins A and B, obtained from a plasma sample, is reported in **Fig. 3B** where the two molecules were monitored by the respective SRM transitions, 227- 198 for urolithin A, and 211-167 for urolithin B.

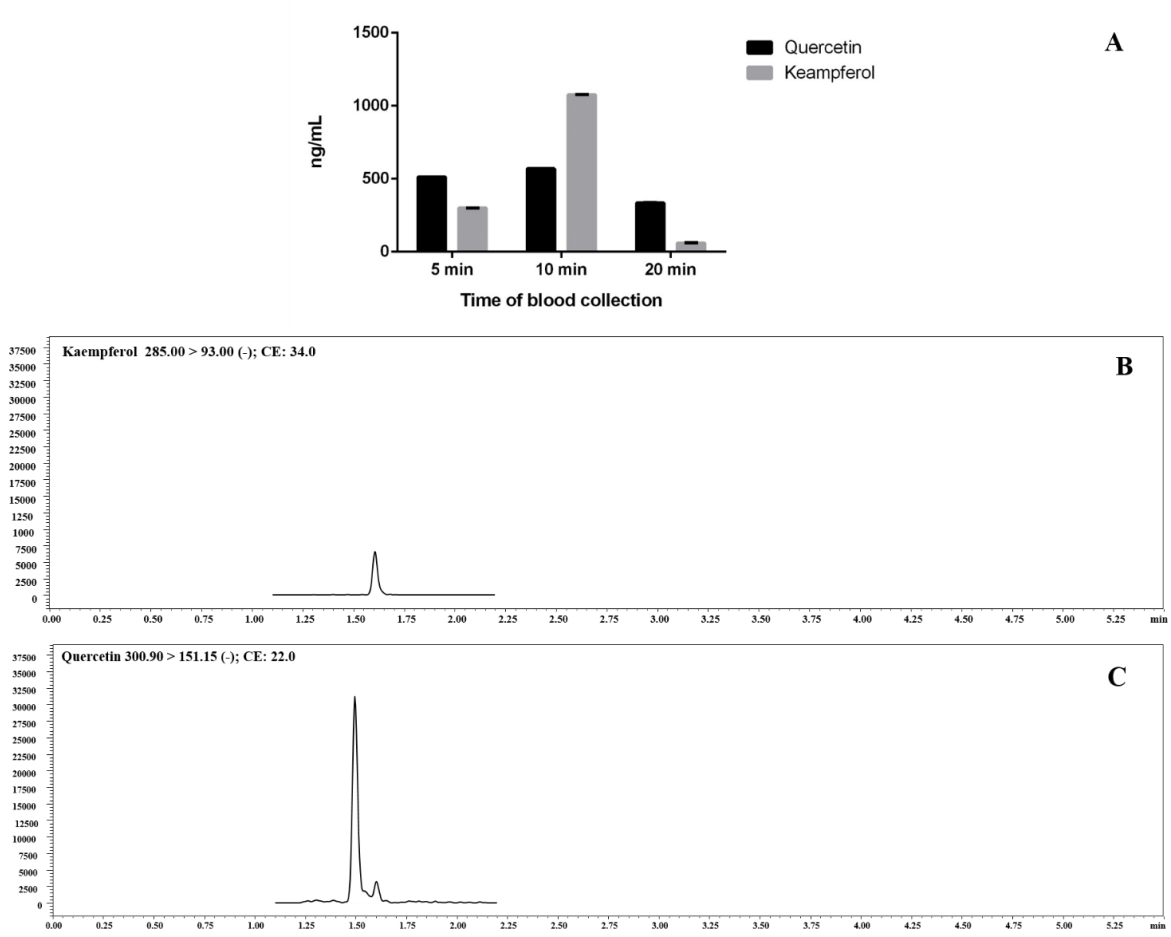

Figure 2 *In vivo bioavailability of EAE components after acute treatment of CD1 mice.* ***A)*** *Mean concentration (ng/mL) ± SD of quercetin and kaempferol in CD1 mouse plasma samples after 5, 10 and 20 min from the administration of EAE.* ***B)*** *SRM chromatogram of ion transition 285.00 > 93.00 corresponding to kaempferol and* ***C)*** *SRM chromatogram of ion transition 300.90 > 151.15 corresponding to quercetin, detected in CD1 mouse plasma samples after 10 min from the administration of EAE by oral gavage.*

Sample	Urolithin A			Urolithin B		
	Time point			Time point		
	24 h	1 week	2 weeks	24 h	1 week	2 weeks
			Plasma			
Plasma 1	1.50 ± 0.692	6.98 ± 0.15	6.00 ± 0.228	5.94 ± 0.379	9.30 ± 0.127	8.75 ± 0.093
Plasma 2	3.81 ± 0.713	2.94 ± 0.873	0.17 ± 0.207	7.18 ± 0.064	8.91 ± 0.342	2.34 ± 0.304
Plasma 3	3.61 ± 0.531	3.74 ± 0430	6.63 ± 0.261	7.50 ± 0.148	6.27 ± 0.285	7.35 ± 0.111
Plasma 4	0.73 ± 0.522	0.64 ± 0.198	5.05 ± 0.524	6.23 ± 0.086	3.89 ± 0.140	7.80 ± 0.085
Plasma 5	3.09 ± 0.653	8.92 ± 0.292	6.97 ± 0.719	11.16 ± 0.185	11.29 ± 0.094	8.14 ± 0.192
Mean ± SD	2.54 ± 1.361	6.46 ± 3,297	4.96 ± 2.777	7.60 ± 2.091	7.93 ± 2.881	6.87 ± 2.586
			Pellet			
Pellet 1	-	4.57 ± 0.225	6.91 ± 0.222	-	4.21 ± 0.034	9.44 ± 0.063
Pellet 2	-	4.64 ± 0.227	8.58 ± 0.184	-	7.08 ± 0.174	8.99 ± 0.191
Pellet 3	-	9.38 ± 0.516	7.82 ± 0.302	-	10.26 ± 0.083	8.46 ± 0.331
Pellet 4	-	8.85 ± 0.098	9.12 ± 0.402	-	8.83 ± 0.051	9.26 ± 0.068
Pellet 5	-	6.95 ± 0.142	11.58 ± 0.521	-	10.00 ± 0.118	12.06 ± 0.299
Mean ± SD	-	6.87 ± 2.263	8.80 ± 1.761	-	8.07 ± 2.498	9.642 ± 1.401

Table 3 *Concentration of urolithin A and urolithin B in plasma and pellet samples at time point 24 h, 1 week and 2 weeks***.**

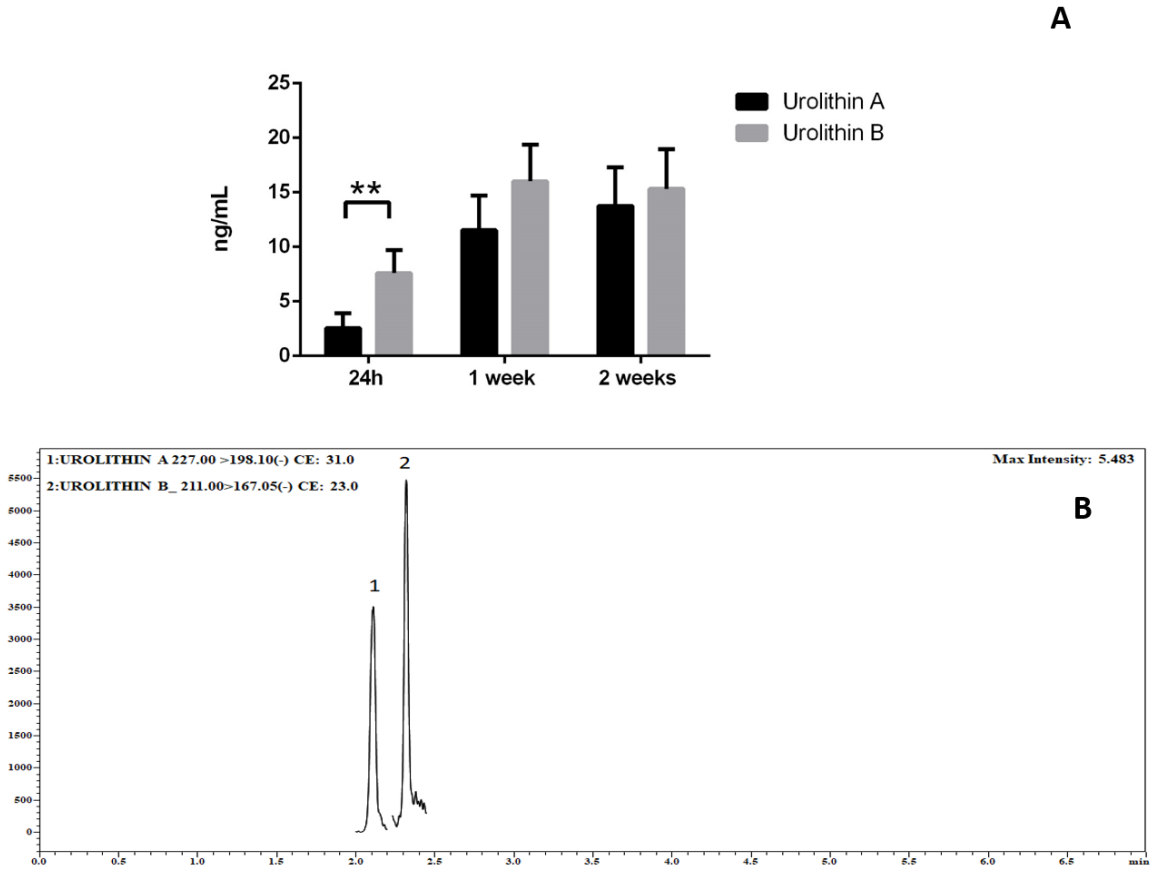

Figure 3 *In vivo bioavailability of urolithin A and B after prolonged treatment of CD1 mice.* ***A)*** *Concentration of urolithin A and urolithin B in plasma samples at 24 h, 1 week and 2 weeks. (Df = 8 p < 0.05).* ***B)*** *An example of SRM chromatogram of urolithins, with peak 1 corresponding to ion transition 227.00 > 198.00 (urolithin A) and peak 2 corresponding to ion transition 211.00 > 167.05 (urolithin B) detected in CD1 mouse plasma samples after prolonged administration of EAE by oral gavage.*

10.3.4 In vitro antioxidant activity

To evaluate the in vitro antioxidant activity of duodenal digested EAE treatment on LNCaP cells, first, the maximum non-cytotoxic concentration of EAE was established. LNCaP cell viability after 24 h of treatment with duodenal digested EAE is reported in **Fig. 4A**.

Thus, the maximum non-cytotoxic concentration (which determines a cell viability of 78.45 ± 8.13%), selected for use in the subsequent experiments, was found to be 31.25 µg/mL.

The mean superoxide dismutase 1 (SOD-1) concentration (ng/µL) was calculated using total protein content for normalization (ng SOD-1/mg soluble proteins). The quantification of soluble protein is reported in section 2.7 (data not shown). **Fig. 4B** reports the mean SOD-1

concentrations found in LNCaP cells subjected to treatment with duodenal digested EAE (expressed as mean ± SD), compared to the control group mean (Df = 16; P = 00,010). The results show a statistical difference between treated and control groups. In more detail, the control group showed a mean SOD-1 concentration of 111.81 ± 32.39 ng/mg, while the treated group exhibited a mean SOD-1 concentration of 157.24 ± 10.43 ng/mg. The same procedure was also followed for the determination of catalase (CAT) antioxidant protein; however, no statistical differences were found between the control and treated groups (**Fig. 4C**).

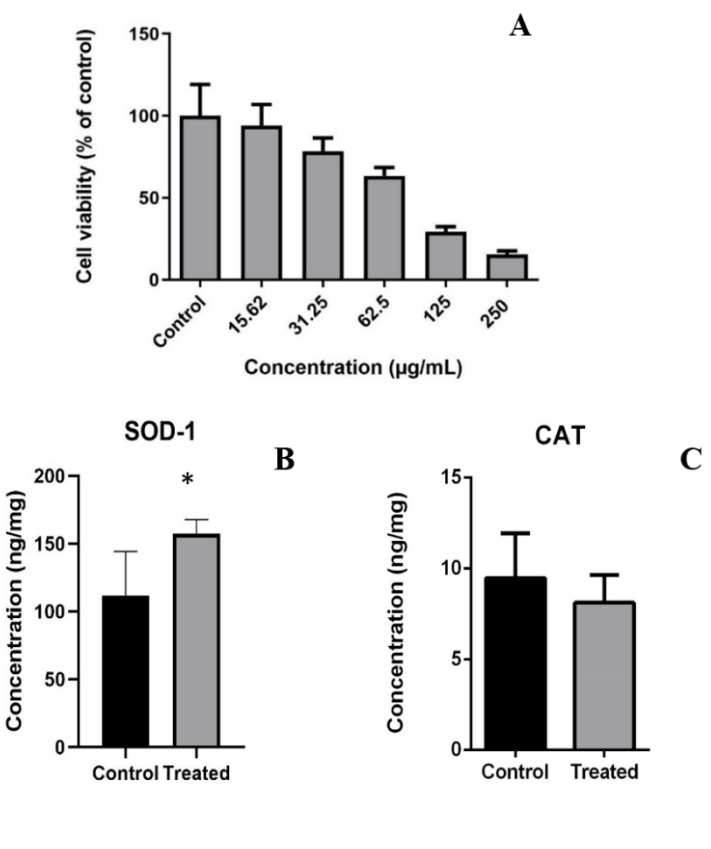

Figure 4 *In vitro antioxidant activity of EAE.* ***A)*** *LNCaP cell viability expressed as percentage compared to the control ± SD, achieved with different concentrations of duodenal digested EAE after a cell treatment of 24 h.* ***B)*** *Mean concentration of SOD-1, expressed in ng/mg of soluble protein ± SD) detected in control and treated LNCaP cells. Results are normalized with the total protein content. (Df = 16; p < 0.05).* ***C)*** *Mean concentration of CAT, expressed in ng/mg of soluble protein ± SD detected in control and treated LNCaP cells. Results are normalized with the total protein content. (Df = 16; p > 0.05).*

10.4 Discussion

In this study the dry extract produced by the aerial parts of *E. angustifolium*, obtained through a hydroalcoholic extraction and standardized to contain ≥ 15 % oenothein B, was studied for its chemical profiling, in vitro bioaccessibility, in vivo bioavailability and in vitro antioxidant activity.

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, oenothein A and oenothein B).

The EAE phytocomplex suffered from degradation in a similar way after the orogastric digestion and duodenal digestion. It is important to highlight that flavonols are known to be bioactive compounds, able to play an important role in the prevention of oxidative stress and different inflammatory-based diseases [131], and the protective activity of *E. angustifolium* extracts against prostate ailments is ascribed to oenothein B. Therefore, the results of digestion provide very important information, useful for proceeding with the selection of suitable coating agents in order to preserve the bioactive components, especially flavonols and oenothein B, key molecules for prostate health. EAE metabolites were studied after *in vivo* acute and prolonged treatment of mice. According to literature data, polyphenol metabolism may vary. Supporting this, quercetin and kaempferol, which have a very fast metabolism, were immediately found in plasma and pellet samples after acute treatment, while urolithins A and B were only detected after prolonged treatment. Urolithins are generally derived from hydrolysable tannins by the intestinal microbiota and are not common in nature, although they can be found in plants rich in ellagitannins. These molecules are produced from ellagic acid after the loss of two lactones. Lactase and decarboxylase are responsible for the conversion from ellagic acid to urolithins. In particular,

the formation of urolithin M-5 is promoted after decarboxylation of ellagic acid, and the consequent removal of a hydroxyl group determines the formation of tetrahydroxy (D; M-6), trihydroxy (C; M-7) dihydroxy (iso-A; A) and monohydroxy urolithins (iso-B; B). [132] The anti-inflammatory activity of urolithins on BPH and prostate health is documented in literature with *in vitro* and *in vivo* studies. [132]–[135] Therefore, the activity of EAE may also be related to the anti-inflammatory action of urolithins. The literature data on the studies of urolithins in mice are poor and this is the first paper that underlines the presence of urolithins in blood samples after the oral administration of E. angustiflium extract. Another investigation focused its attention on the presence of ellagic acid in the plasma of mice after oral administration of 0.8 mg pomegranate extract. [136] In this case, clearance of ellagic acid occurred in 2 h, while intraperitoneal administration extended the presence of this molecule in the blood by 6 h. The tissue distribution of ellagic acid suggests its presence in mouse prostate only after intraperitoneal administration. Urolithins were not detected in tissues after both administrations of pomegranate extract. However, the oral and intraperitoneal administration of 0.3 mg urolithins A underline the distribution of this molecule and their metabolites in different tissues, including prostate. This evidence suggests the possible distribution of urolithins in the prostate after their formation and sustains the slow fermentation of ellagitannins or ellagic acid into urolithins.

In fact, 6 h after oral administration it is not possible to confirm the presence of urolithins in tissues.

SOD-1 represents one of the fundamental endogenous antioxidants defences, acting on superoxide anions. Our results obtained in LNCaP cells, a widely used model system for BPH, suggest that EAE treatment is able to improve the first line of cellular defence. In fact, EAE cell treatment induced a statistically significant difference in SOD-1 concentration between treated and control groups. In line with our findings on EAE, literature data show

that *Epilobium* spp. extracts have good antioxidant properties, exerting high inhibitory activity against reactive oxygen species (ROS) inducing an increase in SOD activity in both *in vitro* and *in vivo* studies [132]. Karakurt et al. [136] treated Wistar rats with an intraperitoneal administration of *Epilobium hirsutum L.* at 37.5 mg/Kg and 10 mg/Kg of ellagic acid dose for 9 days. The results show an increase in SOD-1 activity in liver of the treated group, compared with control. Deng et al. [119] instead recorded an increase in the SOD and glutathione peroxidase (GPx) activity in Sprague Dawly rat prostate after oral administration of butanol extract of *E angustifolium L.* in a dose-dependent manner (100 mg/Kg; 200 mg/Kg; 300 mg/Kg).

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

11. 1 Introduction

The American Urological Association Guideline has defined benign prostatic Hypertrophy (BPH) as a histologic diagnosis that refers to the proliferation of smooth muscle and epithelial cells within the prostatic transition zone, often occurring in the second prostate growth phase , which starts at about 25 years of age and continues during much of man's life. [3]

Although, Gontero et al. estimated that BHP is present in 70 % of adult males, to date the epidemiology of this condition is not well defined. [137] Through systematic research on the major scientific databases, a recent meta-analysis of 30 studies, published in 2017, estimates the global BPH prevalence of 26.6 %, ranging from 14.0 % in individuals up to 40.0 and 36.8 % in individuals aged 80 and older. [138]

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), being the most important complications associated with BPH. [139] The first one is a serious complication, which requires hospitalization, while the second one may cause other complications including recurrent urinary tract infection, formation of bladder calculi, haematuria, and damage to 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, blood in the urine. Moreover, there is an important association between benign prostatic Hypertrophy/bladder outflow obstruction (BPH/BOO) and male erectile dysfunction. Although literature data obtained from clinical trials suggest that the incidence of these complications resulted to be low, unfortunately they occur regularly in real life. [140]

To date, the mechanism underlying the onset of BPH is not defined. However, as reported by Allkanjari et al., three possible hypotheses have been proposed. [141] The first one is based on the role of androgens, oestrogens and growth factors. Prostate cells can convert about 90 % of testosterone (TE) to dihydrotestosterone (DHT) by 5-alpha reductase. The latter binds to androgen receptors with higher affinity than TE and it seems to act directly by stimulating protein synthesis and prostate cell growth. [142], [143] DHT accumulates in the prostate even when TE levels are low. [144] 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 cells proliferation. [145] In addition, oestrogens act in synergy with androgens in the development of BPH, with multiple mechanisms including apoptosis, aromatase expression and paracrine regulation via prostaglandin E2. [146] 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. [147] The third theory regards the interactions 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 sides, stromal (autocrine transmission) and epithelial portions (paracrine secretion). [148], [149] More recently, growing evidence has highlighted the role of inflammation, which may represent an important factor in influencing prostatic growth and progression of symptoms. [150]–[153]

The main pharmacological BPH treatments are α-blockers and 5-α-reductase inhibitors. The first ones are 1-α-adrenergic receptor antagonists, which relax bladder neck muscles and prostate muscle fibres, making urination easier. The second ones target 5-α-reductase increasing DHT affinity for androgen receptors. Although the adverse effects of these drugs do not occur frequently, they can lead to erectile dysfunction, and induce hypotension, dizziness and tachycardia. [119]

Due to the adverse effects of drug therapy currently used in BPH, which particularly affects 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 the early stage of BPH (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 family *Onagraceae*. 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 suggested that *E. angustifolium* showed positive effects on the inflammation of urethra and prostate, as well as micturition problems. [154] In the monograph on *E. augustifolium* published by the European Medicinal Agency (EMA), it resulted to be used in many European Union countries for more than 30 years, meeting the requirements for "traditional use" for the following indications: "*Relief of lower urinary tract symptoms related to benign prostatic Hypertrophy, after serious conditions have been excluded by a medical doctor*". [155]

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) [156]–[158] synthesis, and anti-proliferative and pro-apoptotic activity . In 2004, Kiss et al. showed the activity of *E. angustifolium* in the inhibition of metalloproteinases (i.e., neprilisyn, angiotensin-converting enzyme, and aminopeptidase N). A deregulation of the levels of these metalloproteinases has been correlated with the development of BPH [40], [159]. A preclinical study published in 2019, confirmed in a rat model, in which BPH was induced with testosterone propionate, several of the previously proposed mechanisms of action of *E. angustifolium*, 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. [154] In 2013, the results of a randomised, 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 night-time urinary frequency reduction. [160]

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 the prolonged treatment in CD1 mice with a chemically characterized *E. angustifolium* extracts (EAE), standardized to contain ≥ 15 % oenothein B, reveals the formation of urolithins A and B, which are known to exert anti-inflammatory activity [129], the aim of this monocentric, randomised, 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.

11.2 Material and methods

11.2.1 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 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's specifications, EAE complies with European specifications for contaminants and microbiologic limits. 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 were determined by means of Metrostat statistical software, in agreement with Italian law (Legge 25 ottobre 1978 n. 690) and standard UNI ISO 2859.

11.2.2 Clinical trial design

A monocentric, randomised, double-blind, placebo-controlled clinical trial was performed by Samnium Medical Cooperative (Benevento, Italy) to evaluate the effects of 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 concerning 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).

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 outsubject setting. At baseline visit (t0) information on the sociodemographic, clinical and symptomatologic characteristics of the subjects was collected and reported in the case report form (CRF). In particular, post-void residual (PVR) and prostate volume (PV) were obtained by prostate ultrasound; prostate-specific antigen (PSA), neutrofile/lymphocyte ratio (N/L) derived from blood tests analysed by Unisannio Lab (San Giorgio del Sannio, BN, Italy); urinations number during the night before the clinical visit, 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 randomisation (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. To the EAE food supplement group, one hard gastro-resistant capsule per day was given for 6 months; it contains 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]. To the placebo group one hard gastro-resistant capsule per

day containing 350 mg of microcrystalline cellulose was given for six months. Hard gastric-resistant capsules containing EAE or microcrystalline cellulose were made unrecognizable by identical colour, shape and taste. The white plastic container used both for EAE food supplement treatment and placebo was not recognisable. To EAE food supplement and placebo groups 60 capsules per white plastic container were given 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 a possible occurrence 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 4**.

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

Table 4 *Analyses planned at t0, t1, t2, and t3. 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).*

11.2.3 Study population

Participants (128 subjects: 70 in treated group and 58 in placebo group) were recruited by the Samnium Medical Cooperative (Benevento, Italy). The male 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 ≤300 ± 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 dangerous for 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 instr0ns, to provide an objective evaluation of his or her symptoms, or that might confuse the interpretation of study results; those considered unsuitable for the participation by the physician.

11.2.4 Evaluated variables

As sociodemographic characteristic, the age of the participants was registered in the CRF. The primary endpoint was to investigate the efficacy of a 6 months-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).

11.2.5 Statistical analysis

The sample size calculation was made using three 1-β power values equal to 0.95 and a significance level α = 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 supplement 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 subject entered the model as random intercept to account for unexplained variation at individual level (σ^2_{ind}) after we controlled for the explanatory variables. We run 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 urinations during night 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 package lme4 (Bates et al. 2015) and MuMIn (Barton 2016) in R ver. 3.2.4 (R core Team 2016), and otherwise stated, data reported are means ± standard errors.

11.2.6 Tolerance and safety assessment

In the clinical trial, hepatic and renal toxicity tests have been studied. 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.

11.3 Results

11.3.1 Clinical trial

The study flow chart is reported in **figure 5** according to the CONSORT PRO reporting guideline. The participants in the treated 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 5**.

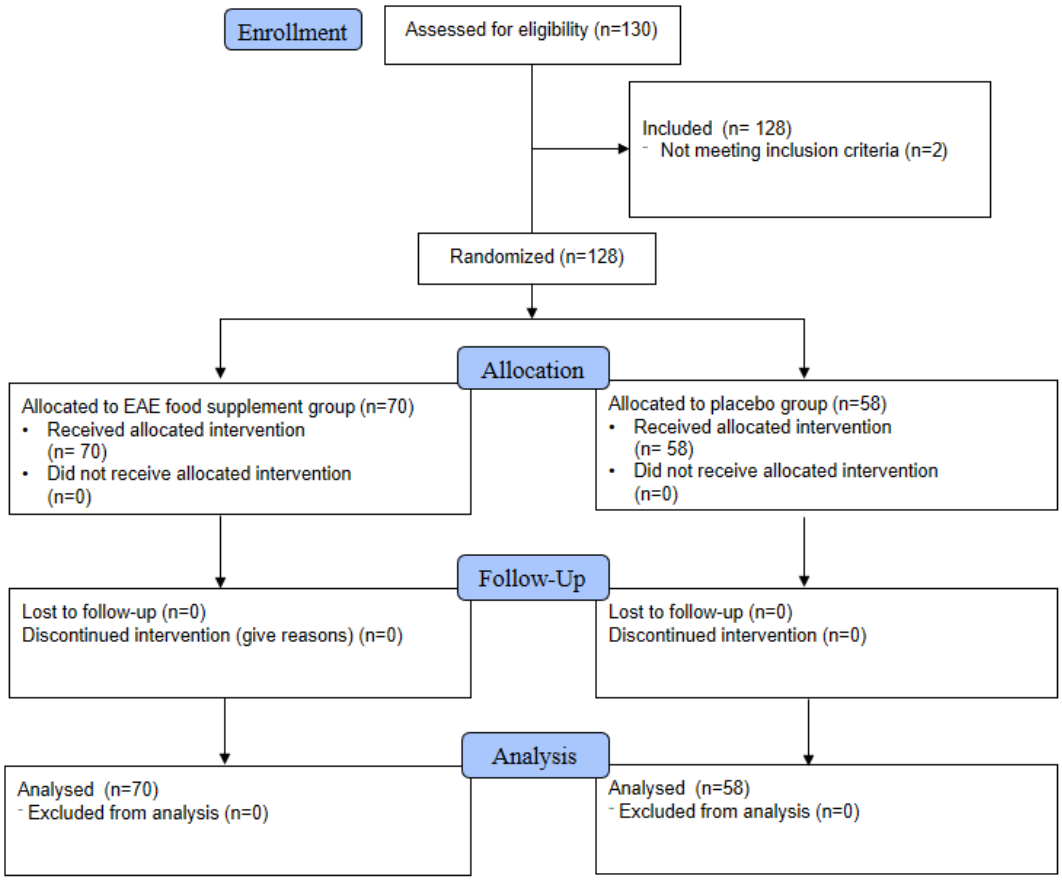

Figure 5 *CONSORT flow diagram.*

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/1,73mq)	62.3 ± 1.4	69.8 ± 2.2
CRE (mg/dl)	1.19 ± 0.02	1.10 ± 0.03

Table 5 *Characteristics of the study population: demographic and clinical data at baseline.*

The study revealed that three response variables (IPSS, PVR, and the number of urinations during night) changed in the EAE food supplement group with respect to the placebo group between the beginning (t0) and the end (t3) of the clinical trial **Table 6**. Indeed, the GLMM analysis showed that the treatment × measure interaction was statistically significant for IPSS, PVR, and the number of urinations during night, but not for the PV **Table 7**. In more detail, PV values did not significantly change either between t0 and t3 or between EAE food supplement and placebo groups **Table 7, Figure 6**, but depending on the age of the subjects. In fact, as expected, in both groups, older individual had higher PV ($\beta=0.16 \pm 0.03$, $t_{125}=4.611$, $P<0.001$, **Figure 6**).

As far as IPSS score is concerned, between t0 and t3, in the EAE food supplement group, it significantly decreased ($\beta=-1.9 \pm 0.2$, $t_{126}=7.89$ $P<0.001$ **Figure 6**), while in the placebo group it slightly increased ($\beta=+0.6 \pm 0.2$, $t_{126}=2.36$ $P=0.02$, **Figure 6**). As well as for PV,

the IPSS score significantly grew with increasing subjects' age, irrespective of the treatment (β=1.43 ± 0.49, t_{125}=2.888, P=0.005, **Figure 6**).

The Poisson GLMM for the PVR value showed that in EAE food supplement group the frequency of subject with a low residual urine volume in the bladder significantly increased while decreasing subjects with residual urine volume higher than 100 µl (β=-0.17 ± 0.03, Z=5.792, P<0.001, **Figure 7**). The opposite pattern was instead observed in the placebo group (β=0.12 ± 0.03, Z=3.419, P<0.001, **Figure 7**).

A similar pattern of response was observed for the number of urinations during night (**Figure 8**): in the EAE food supplement group, the frequency of subjects without urination increased, while decreased that of the subjects urinating more than once per night (β=-0.41 ± 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 groups, the frequency of urination increased significantly with the age of the subjects (β=0.19 ± 0.07, Z=2.561, P=0.010). In detail, the number of subjects did not urinate per night increased by 21.7% in the treated group, whereas it decreased by 10.2% in the placebo group. Similarly, the number of subject urinating less than once per night increased by 10.1% in the treated group, and only by 3.7% 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 it remained unchanged in the placebo group.

Means, standard errors and ranges of each biochemical variable for EAE food supplement and placebo groups are reported in **table 8**. The GLMM analyses did not find any significant effect of the experimental treatment on all biochemical variables (statistics not shown), with the exceptions of N/L. In detail, the N/L was significantly higher in the placebo group in comparison with the EAE food supplement group ($F_{1,125}$=5.893, P=0.017, **Figure 8**), but did not change between t0 and t3 ($F_{1,126}$=1.191, P=0.27)

At the end of the treatments, all participants were included in the analysis in 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, including renal and hepatic toxicity, and the principal investigator judged that the application of *E. angustifolium* capsules was considered well tolerated.

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)

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

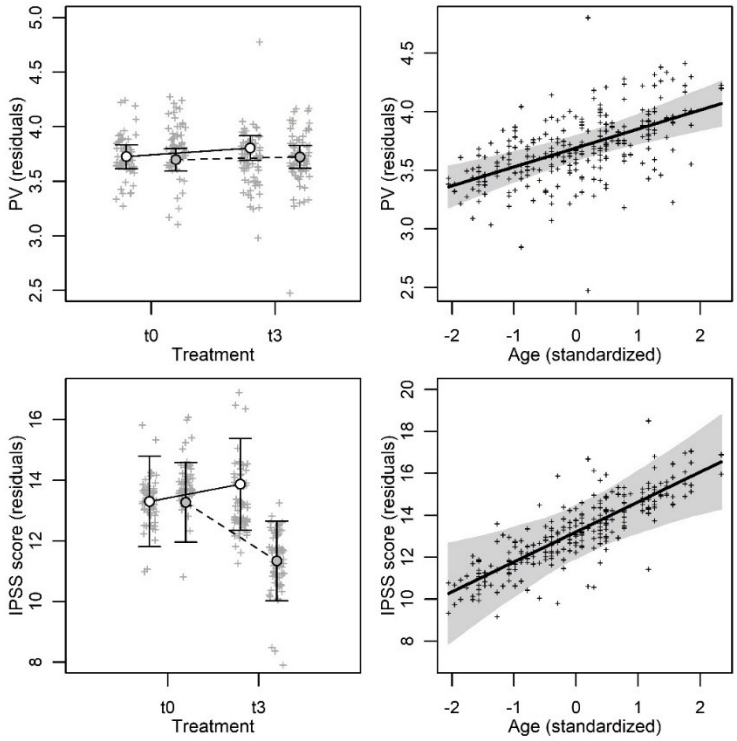

Figure 6 Effects of the treatment (white circles: placebo group, gray circles: EAE food supplement group) and age of subjects on the PV and IPSS score predicted by GLMMs. Bars and grey areas are for 95% confidence intervals of the estimate

Effect	F*/χ^2**	Df	P
PV*			
Treatment	0.612	1,125	0.43
Measure	1.921	1,126	0.17
Age	21.26	1,125	<0.001
Treat. × Meas.***	0.482	1,126	0.49
IPSS*			
Treatment	1.666	1,125	0.20
Measure	17.39	1,126	<0.001
Age	8.341	1,125	0.004
Treat. × Meas.	58.67	1,126	<0.001
VR**			
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**			
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

Table 7 *Fixed effects of the GLMMs used for the four response variables.*

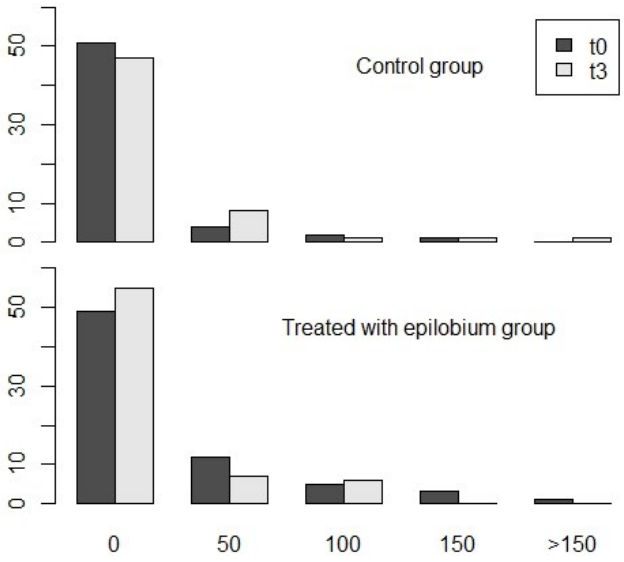

Figure 7 *Frequency distributions of the PVR in the two experimental groups at t0 and t3 as predicted by the GLMM.*

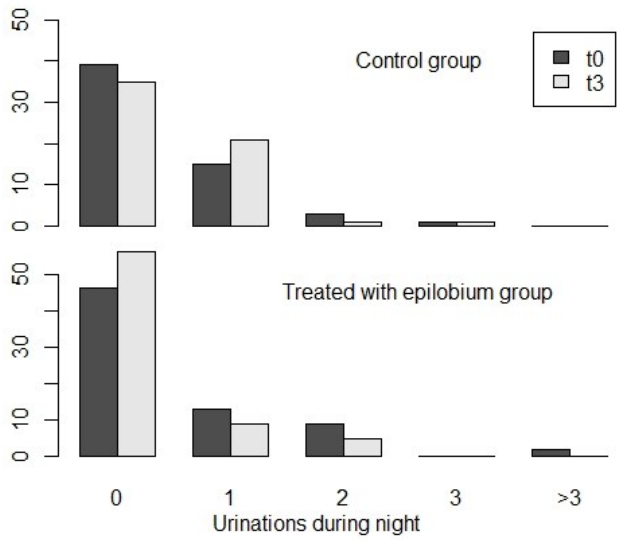

Figure 8 *Frequency distributions of urinations during night in the two experimental groups measured at t0 and t3 as predicted by GLMM.*

	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)

Table 8 *Descriptive statistics (mean ± SE, range) for the four response variables measured at t0 and t3 in the two experimental groups.*

11.4 Discussion

BPH is a condition mainly characterized by a proliferation of both stromal and epithelial cells of the prostate with an alteration of periurethral area responsible for symptoms that can strongly affect the quality of life. [161] The symptoms of BPH are often very mild at first, but they become more serious if PBH is not treated.

Although literature data on the effect of *E. angustifolium* against PBH are limited, this plant is used, especially in Europe, in the early stage of BPH. Therefore, 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 allow a significant improvement in symptoms, and the improvement of urinary flow. In fact, in the treated group the frequency of subjects without urinations during 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 regarding PVR value. In fact, in EAE food supplement group the frequency 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 µl. Finally, the quality of life of the treated subjects was improved as in the EAE food supplement group, IPSS score significantly decreased while in the placebo group it slightly increased.

This work has limitations and strengths. The main limitation is represented by the fact that it was not possible to follow up the subject after the 6 months of treatment being impossible to learn about longer term effects after EAE food supplement treatment.

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 PBH suggesting a significant reduction of nocturia. This result is very remarkable for the impact on wellness and health, as nocturia is a serious therapeutic problem with serious impact on the quality of sleep leading to sleep disorders, decreased quality of life and depression. [3] In addition, nocturia can be a cause of falls and hip fractures, which, in turn, increase the disability and mortality rate. [3] 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 out 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. Symptom score less than or equal to 7 indicates mild symptoms, symptom score range 8-19 indicates moderate symptoms, and symptom score range 20-35 indicates severe symptoms, as reported by Barry et al. [3] 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 EAE food supplement and highlighting the protective effect of this supplementation. Finally, as far as tolerance and safety assessment are concerned, *E. angustifolium* food supplement is well tolerated and it not showed hepatic and renal toxicity. This study confirms the safety of *E. angustifolium*, as no subjects reported AEs related to *E. angustifolium* treatment.

12. Conclusions

In conclusion, the metabolic profile of EAE reveals the presence of a rich phytocomplex with different polyphenol species including oenothein B. Many compounds have been identified that had never previously been found within *E. angustifolium* extracts. All polyphenols occurring in the extract suffer from degradation after *in vitro* simulated orogastric and duodenal digestion processes, suggesting the need to administer the extract in gastro-protected dosage forms for the preservation of the phytocomplex to improve the bioavailability of EAE components.

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 acute treatment of EAE in CD1 mice confirms the presence of flavonol aglycones and the absence of urolithins. In contrast, the prolonged treatment reveals the formation of urolithins A and B suggesting that the gut fermentation process requires time to promote the conversion of ellagic acid or ellagitannins into urolithins. The antioxidant potential of EAE was confirmed, underlying the increased expression of SOD-1.

The results of the monocentric, randomized, double-blind, placebo-controlled clinical trial 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.

References

[1] C. G. Roehrborn *et al.*, "The Influence of Baseline Parameters on Changes in International Prostate Symptom Score with Dutasteride , Tamsulosin , and Combination Therapy among Men with Symptomatic Benign Prostatic Hypertrophy and an Enlarged Prostate : 2-Year Data from the CombAT ," vol. 55, pp. 461–471, 2009, doi: 10.1016/j.eururo.2008.10.037.

[2] J. K. Parsons and C. Kashefi, "Physical Activity , Benign Prostatic Hypertrophy , and Lower Urinary Tract Symptoms," vol. 53, pp. 1228–1235, 2008, doi: 10.1016/j.eururo.2008.02.019.

[3] M. J. Barry *et al.*, "The American Urological Association symptom index for benign prostatic Hypertrophy," *J. Urol.*, vol. 148, no. 5 I, pp. 1549–1557, 1992, doi: 10.1016/S0022-5347(17)36966-5.

[4] C. De Nunzio, C. G. Roehrborn, K. Andersson, K. T. Mcvary, and C. Gratzke, "Erectile Dysfunction and Lower Urinary Tract Symptoms," *Eur. Urol. Focus*, vol. 3, no. 4–5, pp. 352–363, 2017, doi: 10.1016/j.euf.2017.11.004.

[5] M. Rossanese *et al.*, "Medical treatment for benign prostatic Hypertrophy : Where do we stand ?," 2019, doi: 10.1177/0391560319859785.

[6] L. Saporta, I. A. Aridogan, N. Erlich, and D. Yachia, "Objective and subjective comparison of transurethral resection, transurethral incision and balloon dilatation of the prostate. A prospective study," *Eur. Urol.*, vol. 29, no. 4, pp. 439–445, 1996, doi: 10.1159/000473793.

[7] Amin-ul-Haq, S. Khan, M. A. Jan, and T. A. Khan, "Transurethral resection versus transurethral incision of the prostate: A subjective and objective analysis," *J. Postgrad. Med. Inst.*, vol. 21, no. 1, pp. 40–45, 2007.

[8] C. B. IJsselmuiden and R. R. Faden, "The New England Journal of Medicine

Downloaded from nejm.org on January 31, 2011. For personal use only. No other uses without permission. Copyright © 1992 Massachusetts Medical Society. All rights reserved.," vol. 326, 1992.

[9] E. M, C. EB, B. PF, F. RO, G. JM, and C. R, "Benign prostatic Hypertrophy as a progressive disease: a guide to the risk factors and options for medical management," *Int. J. Clin. Pract.*, vol. 62, no. 7, pp. 1076–1086, Jul. 2008, doi: 10.1111/J.1742-1241.2008.01785.X.

[10] C. G. Roehrborn, "Pathology of benign prostatic Hypertrophy," *Int. J. Impot. Res.*, vol. 20, no. SUPPL. 3, Dec. 2008, doi: 10.1038/IJIR.2008.55.

[11] R. RP *et al.*, "Urodynamic effects of the combination of tamsulosin and daily tadalafil in men with lower urinary tract symptoms secondary to benign prostatic Hypertrophy: a randomized, placebo-controlled clinical trial," *Int. Urol. Nephrol.*, vol. 45, no. 1, pp. 39–43, Feb. 2013, doi: 10.1007/S11255-012-0317-7.

[12] L. Educazionale, "Linee Guida EAU per il trattamento dei sintomi non neurogeni del basse vie urinarie maschili," 2019.

[13] S. Gravas *et al.*, "EAU Guidelines on Management of Male Lower Urinary Tract Symptoms Benign Prostatic Obstruction (BPO)," 2018.

[14] M. C. Michel and W. Vrydag, "α1-, α2- and β-adrenoceptors in the urinary bladder, urethra and prostate," *Br. J. Pharmacol.*, vol. 147, no. S2, pp. S88–S119, Feb. 2006, doi: 10.1038/SJ.BJP.0706619.

[15] M. M. Barendrecht, R. P. Koopmans, J. J. M. C. H. D. E. L. A. Rosette, and M. C. Michel, "Treatment of lower urinary tract symptoms suggestive of benign prostatic Hypertrophy : the cardiovascular system," 2005, doi: 10.1111/j.1464-410X.2005.05487.x.

[16] G. Andriole *et al.*, "Dihydrotestosterone and the prostate: The scientific rationale for

5α-reductase inhibitors in the treatment of benign prostatic Hypertrophy," *J. Urol.*, vol. 172, no. 4 I, pp. 1399–1403, 2004, doi: 10.1097/01.ju.0000139539.94828.29.

[17] M. J. Naslund and M. Miner, "A review of the clinical efficacy and safety of 5α-reductase inhibitors for the enlarged prostate," *Clin. Ther.*, vol. 29, no. 1, pp. 17–25, 2007, doi: 10.1016/j.clinthera.2007.01.018.

[18] G. ER, S. PK, T. H, and P.-G. J, "Efficacy and safety of oxybutynin topical gel 3% in subjects with urgency and/or mixed urinary incontinence: a randomized, double-blind, placebo-controlled study," *Neurourol. Urodyn.*, vol. 34, no. 1, pp. 37–43, Jan. 2015, doi: 10.1002/NAU.22504.

[19] H. K, B. M, J. G, and J. U, "Safety and efficacy of tolterodine extended release in men with overactive bladder symptoms and presumed non-obstructive benign prostatic Hypertrophy," *World J. Urol.*, vol. 25, no. 6, pp. 627–633, Dec. 2007, doi: 10.1007/S00345-007-0212-5.

[20] R. CG *et al.*, "The effects of combination therapy with dutasteride and tamsulosin on clinical outcomes in men with symptomatic benign prostatic Hypertrophy: 4-year results from the CombAT study," *Eur. Urol.*, vol. 57, no. 1, pp. 123–131, Jan. 2010, doi: 10.1016/J.EURURO.2009.09.035.

[21] G. F, Ü. S, M. M, B. L, K. J, and V. L, "The mechanism of action of phosphodiesterase type 5 inhibitors in the treatment of lower urinary tract symptoms related to benign prostatic Hypertrophy," *Eur. Urol.*, vol. 63, no. 3, pp. 506–516, Mar. 2013, doi: 10.1016/J.EURURO.2012.09.006.

[22] R. Mancina, M. Gacci, L. Vignozzi, and M. Carini, "Phosphodiesterase Type 5 Expression in Human and Rat Lower Urinary Tract Tissues and the Effect of Tadalafil on Prostate Gland Oxygenation in Spontaneously Hypertensive Rats," pp. 2746–2760, 2011, doi: 10.1111/j.1743-6109.2011.02416.x.

[23] V. L *et al.*, “PDE5 inhibitors blunt inflammation in human BPH: a potential mechanism of action for PDE5 inhibitors in LUTS,” *Prostate*, vol. 73, no. 13, pp. 1391–1402, Sep. 2013, doi: 10.1002/PROS.22686.

[24] G. M *et al.*, “The use of a single daily dose of tadalafil to treat signs and symptoms of benign prostatic Hypertrophy and erectile dysfunction,” *Res. reports Urol.*, vol. 5, no. 1, pp. 99–111, Apr. 2013, doi: 10.2147/RRU.S31580.

[25] M. Oelke, R. Shinghal, A. Sontag, S. K. Baygani, and F. Craig, “SC,” *J. Urol.*, 2014, doi: 10.1016/j.juro.2014.11.094.

[26] O. M, G. F, M. V, X. L, C. D, and V. L, “Monotherapy with tadalafil or tamsulosin similarly improved lower urinary tract symptoms suggestive of benign prostatic Hypertrophy in an international, randomised, parallel, placebo-controlled clinical trial,” *Eur. Urol.*, vol. 61, no. 5, pp. 917–925, May 2012, doi: 10.1016/J.EURURO.2012.01.013.

[27] D. CF *et al.*, “Tadalafil administered once daily for lower urinary tract symptoms secondary to benign prostatic Hypertrophy: a 1-year, open-label extension study,” *BJU Int.*, vol. 107, no. 7, pp. 1110–1116, Apr. 2011, doi: 10.1111/J.1464-410X.2010.09687.X.

[28] V. C *et al.*, “Impact of cardiovascular risk factors and related comorbid conditions and medical therapy reported at baseline on the treatment response to tadalafil 5 mg once-daily in men with lower urinary tract symptoms associated with benign prostatic Hypertrophy: an integrated analysis of four randomised, double-blind, placebo-controlled, clinical trials,” *Int. J. Clin. Pract.*, vol. 69, no. 12, pp. 1496–1507, Dec. 2015, doi: 10.1111/IJCP.12722.

[29] B. GB *et al.*, “Direct effects of tadalafil on lower urinary tract symptoms versus indirect effects mediated through erectile dysfunction symptom improvement:

integrated data analyses from 4 placebo controlled clinical studies," *J. Urol.*, vol. 191, no. 2, pp. 405–411, Feb. 2014, doi: 10.1016/J.JURO.2013.09.057.

[30] R. CG, C. C, O. M, C. D, E. A, and V. L, "Effects of tadalafil once daily on maximum urinary flow rate in men with lower urinary tract symptoms suggestive of benign prostatic Hypertrophy," *J. Urol.*, vol. 191, no. 4, pp. 1045–1050, 2014, doi: 10.1016/J.JURO.2013.10.074.

[31] C. G. Roehrborn *et al.*, "Efficacy and Safety of the Coadministration of Tadalafil Once Daily with Finasteride for 6 Months : A Randomized , Double-Blind , Placebo Controlled Study in Men with Lower Urinary Tract Symptoms and Prostatic Enlargement Secondary to Benign Prostatic Hyp," no. November, pp. 1–7, 2013, doi: 10.1016/j.juro.2013.09.059.

[32] P. H *et al.*, "Efficacy and safety of tadalafil 5 mg once daily for lower urinary tract symptoms suggestive of benign prostatic Hypertrophy: subgroup analyses of pooled data from 4 multinational, randomized, placebo-controlled clinical studies," *Urology*, vol. 82, no. 3, pp. 667–673, Sep. 2013, doi: 10.1016/J.UROLOGY.2013.05.005.

[33] M. A *et al.*, "Five-year follow-up of feedback microwave thermotherapy versus TURP for clinical BPH: a prospective randomized multicenter study," *Urology*, vol. 69, no. 1, pp. 91–96, Jan. 2007, doi: 10.1016/J.UROLOGY.2006.08.1115.

[34] S. E. A. Badr *et al.*, "Chemical composition and biological activity of ripe pumpkin fruits (Cucurbita pepo L.) cultivated in Egyptian habitats," *Nat. Prod. Res.*, vol. 25, no. 16, pp. 1524–1539, 2011, doi: 10.1080/14786410903312991.

[35] M. Gossell-Williams, A. Davis, and N. O'Connor, "Inhibition of testosterone-induced Hypertrophy of the prostate of Sprague-Dawley rats by pumpkin seed oil," *J. Med. Food*, vol. 9, no. 2, pp. 284–286, Jun. 2006, doi: 10.1089/JMF.2006.9.284.

[36] V. Steenkamp, “Phytomedicines for the prostate,” *Fitoterapia*, vol. 74, no. 6, pp. 545–552, 2003.

[37] E. Pagano, M. Laudato, M. Griffo, and R. Capasso, “Phytotherapy of benign prostatic Hypertrophy. A minireview,” *Phyther. Res.*, vol. 28, no. 7, pp. 949–955, 2014, doi: 10.1002/ptr.5084.

[38] B. Salehi *et al.*, “Cucurbits Plants: A Key Emphasis to Its Pharmacological Potential,” *Molecules*, vol. 24, no. 10, 2019, doi: 10.3390/MOLECULES24101854.

[39] H. Hong, C.-S. Kim, and S. Maeng, “Effects of pumpkin seed oil and saw palmetto oil in Korean men with symptomatic benign prostatic Hypertrophy,” *Nutr. Res. Pract.*, vol. 3, no. 4, p. 323, 2009, doi: 10.4162/NRP.2009.3.4.323.

[40] S. Coulson, A. Rao, S. L. Beck, E. Steels, H. Gramotnev, and L. Vitetta, “A phase II randomised double-blind placebo-controlled clinical trial investigating the efficacy and safety of ProstateEZE Max: A herbal medicine preparation for the management of symptoms of benign prostatic hypertrophy,” *Complement. Ther. Med.*, vol. 21, no. 3, pp. 172–179, Jun. 2013.

[41] L. Dvorkin and K. Y. Song, “Herbs for Benign Prostatic Hypertrophy:,” *http://dx.doi.org/10.1345/aph.1A228*, vol. 36, no. 9, pp. 1443–1452, Jun. 2016, doi: 10.1345/APH.1A228.

[42] Thomson Healthcare (Firm), “PDR for herbal medicines.,” 2007.

[43] S. Granica, J. P. Piwowarski, M. E. Czerwińska, and A. K. Kiss, “Phytochemistry, pharmacology and traditional uses of different Epilobium species (Onagraceae): A review,” *J. Ethnopharmacol.*, vol. 156, pp. 316–346, Oct. 2014.

[44] J. Levy and Y. Sharoni, “The functions of tomato lycopene and its role in human health,” *Foods and Food Ingredients Journal of Japan*, vol. 210, no. 1. pp. 49–56, 2005, [Online]. Available:

http://cms.herbalgram.org/herbalgram/issue62/article2696.html%5Cnhttp://scienceli nks.jp/j-east/article/200505/000020050505A0116283.php.

[45] H. L. Tan *et al.*, "Tomato-based food products for prostate cancer prevention: What have we learned?," *Cancer Metastasis Rev.*, vol. 29, no. 3, pp. 553–568, Sep. 2010, doi: 10.1007/S10555-010-9246-Z.

[46] M. Ebadi, "Book Review: Pharmacodynamic Basis of Herbal Medicine, 2nd Edition," *Ann. Pharmacother.*, vol. 41, no. 9, p. 699, 2006.

[47] J. K. Campbell, K. Canene-Adams, B. L. Lindshield, T. W.-M. Boileau, S. K. Clinton, and J. W. Erdman, "Tomato Phytochemicals and Prostate Cancer Risk," *J. Nutr.*, vol. 134, no. 12, pp. 3486S-3492S, Dec. 2004, doi: 10.1093/JN/134.12.3486S.

[48] "Effect of lycopene on androgen receptor and prostate-specific antigen velocity - 中华医学杂志（英文版）." http://rs.yiigle.com/CN112154201016/566310.htm (accessed Oct. 11, 2021).

[49] S. T. Lee, P. F. Wong, S. C. Cheah, and M. R. Mustafa, "Alpha-tomatine induces apoptosis and inhibits nuclear factor-kappa B activation on human prostatic adenocarcinoma PC-3 cells," *PLoS One*, vol. 6, no. 4, 2011, doi: 10.1371/JOURNAL.PONE.0018915.

[50] H. D and L. QY, "Overview of mechanisms of action of lycopene," *Exp. Biol. Med. (Maywood).*, vol. 227, no. 10, pp. 920–923, 2002, doi: 10.1177/153537020222701013.

[51] A. G. Liu, S. E. Volker, E. H. Jeffery, and J. W. Erdman, "Feeding tomato and broccoli powders enriched with bioactives improves bioactivity markers in rats," *J. Agric. Food Chem.*, vol. 57, no. 16, pp. 7304–7310, 2009, doi: 10.1021/JF901926B.

[52] Z. Polívková, P. Šmerák, H. Demová, and M. Houška, "Antimutagenic effects of lycopene and tomato purée.," *J. Med. Food*, vol. 13, no. 6, pp. 1443–1450, 2010,

doi: 10.1089/JMF.2009.0277.

[53] C. P *et al.*, "Lycopene and Risk of Prostate Cancer: A Systematic Review and Meta-Analysis," *Medicine (Baltimore).*, vol. 94, no. 33, p. e1260, Aug. 2015, doi: 10.1097/MD.0000000000001260.

[54] S. A, S. M, L. M, M. D, B. F, and C. F, "Flavonoids as antioxidant agents: importance of their interaction with biomembranes," *Free Radic. Biol. Med.*, vol. 19, no. 4, pp. 481–486, 1995, doi: 10.1016/0891-5849(94)00240-K.

[55] "FLAVONOIDS IN HEALTH AND DISEASE.," 2019.

[56] Y. GC, D. PD, H. DW, H. CL, and F. TY, "Protective effect of pine (Pinus morrisonicola Hay.) needle on LDL oxidation and its anti-inflammatory action by modulation of iNOS and COX-2 expression in LPS-stimulated RAW 264.7 macrophages," *Food Chem. Toxicol.*, vol. 46, no. 1, pp. 175–185, Jan. 2008, doi: 10.1016/J.FCT.2007.07.012.

[57] K. F. KLIPPEL, D. M. HILTL, and B. SCHIPP, "A multicentric, placebo-controlled, double-blind clinical trial of β-sitosterol (phytosterol) for the treatment of benign prostatic Hypertrophy," *Br. J. Urol.*, vol. 80, no. 3, pp. 427–432, Sep. 1997, doi: 10.1046/J.1464-410X.1997.T01-1-00362.X.

[58] B. MJ and R. CG, "Benign prostatic Hypertrophy," *BMJ*, vol. 323, no. 7320, pp. 1042–1046, Nov. 2001, doi: 10.1136/BMJ.323.7320.1042.

[59] A. Hutchison, R. Farmer, K. Verhamme, R. Berges, and R. V. Navarrete, "The Efficacy of Drugs for the Treatment of LUTS/BPH, A Study in 6 European Countries," *Eur. Urol.*, vol. 51, no. 1, pp. 207–216, Jan. 2007, doi: 10.1016/J.EURURO.2006.06.012.

[60] M. C. Andro and J. P. Riffaud, "Pygeum africanum extract for the treatment of subjects with benign prostatic Hypertrophy: A review of 25 years of published

experience," *Curr. Ther. Res.*, vol. 56, no. 8, pp. 796–817, Aug. 1995, doi: 10.1016/0011-393X(95)85063-5.

[61] "Phytotherapy: A Quick Reference to Herbal Medicine - Francesco Capasso, Timothy S. Gaginella, Giuliano Grandolini, Angelo A. Izzo - Google Libri." https://books.google.it/books?hl=it&lr=&id=ejHpcJXzPTsC&oi=fnd&pg=PA1&ots=sVggcp_95j&sig=N9-uwpKTzokdGXi1Z-C0gWHslzA&redir_esc=y#v=onepage&q&f=false (accessed Oct. 11, 2021).

[62] J. L. Capodice and A. E. Katz, "What subjects take without telling you: Holistic approach for BPH," *World J. Urol.*, vol. 24, no. 4, pp. 378–382, Sep. 2006, doi: 10.1007/S00345-006-0093-Z.

[63] F. Yablonsky,* A R O N I Q U E N I C O M, J. Pierre, and F. Bellah4y, "ANTIPROLIFERATIVE EFFECT OF PYGEUM AFRICANUM EXTRACT ON RAT PROSTATIC FIBROBLASTS," vol. 157, pp. 2381–2387, 1997.

[64] M. Paubert-Braquet *et al.*, "Effect of Pygeum africanum extract on A23187-stimulated production of lipoxygenase metabolites from human polymorphonuclear cells," *J. Lipid Mediat. Cell Signal.*, vol. 9, no. 3, pp. 285–290, 1994.

[65] R. M. Levin *et al.*, "TkE JOURNAL OF u R 0 U X ; Y PROTECTIVE EFFECT OF TADENAN ON BLADDER FUNCTION SECONDARY TO PARTIAL OUTLET OBSTRUCTION," vol. 155, pp. 1466–1470.

[66] R. M. Levin *et al.*, "Effects of tadenan pretreatment on bladder physiology and biochemistry following partial outlet obstruction," *J. Urol.*, vol. 156, no. 6, pp. 2084–2088, 1996, doi: 10.1016/S0022-5347(01)65440-5.

[67] M.-S. Choo, F. Bellamy, and C. E. Constantinou, "Functional evaluation of tadenan on micturition and experimental prostate growth induced with exogenous dihydrotestosterone," *Urology*, vol. 55, no. 2, pp. 292–298, Feb. 2000, doi:

10.1016/S0090-4295(99)00407-0.

[68] W. T, I. A, M. D. R, R. I, and S. G, “Pygeum africanum for benign prostatic Hypertrophy,” *Cochrane database Syst. Rev.*, vol. 1998, no. 1, Jan. 2002, doi: 10.1002/14651858.CD001044.

[69] A. A *et al.*, “Quality of life in subjects with lower urinary tract symptoms associated with BPH: change over time in real-life practice according to treatment--the QUALIPROST study,” *Int. Urol. Nephrol.*, vol. 48, no. 5, pp. 645–656, May 2016, doi: 10.1007/S11255-015-1206-7.

[70] A. C. Buck, “Phytotherapy for the prostate,” *Br. J. Urol.*, vol. 78, no. 3, pp. 325–336, Sep. 1996, doi: 10.1046/J.1464-410X.1996.00104.X.

[71] K. Nakase, K. Takenaga, T. Hamanaka, and M. Kimura, “Inhibitory effect and synergism of cernitin pollen extract on the urethral smooth muscle and diaphragm of the rat,” *Folia Pharmacol. Jpn.*, vol. 91, no. 6, pp. 385–392, 1988, doi: 10.1254/FPJ.91.385.

[72] M. Kimura, I. Kimura, K. Nakase, T. Sonobe, and N. Mori, “Micturition activity of pollen extract: Contractile effects on bladder and inhibitory effects on urethral smooth muscle of mouse and pig,” *Planta Med.*, vol. NO. 2, pp. 148–151, 1986, doi: 10.1055/S-2007-969102.

[73] W. P, B. A, Z. JX, H. G, S. P, and B. A, “Rye bran diet increases epithelial cell apoptosis and decreases epithelial cell volume in TRAMP (transgenic adenocarcinoma of the mouse prostate) tumors,” *Nutr. Cancer*, vol. 53, no. 1, pp. 111–116, 2005, doi: 10.1207/S15327914NC5301_13.

[74] J. Xu, W. Qian, and J. Song, “[A comparative study on different doses of cernilton for preventing the clinical progression of benign prostatic Hypertrophy],” *Zhonghua Nan Ke Xue*, vol. 14, no. 6, pp. 533–537, 2008, [Online]. Available:

http://europepmc.org/abstract/MED/18649754.

[75] W. FM, S. H, L. M, S. J, B. E, and W. W, "A pollen extract (Cernilton) in subjects with inflammatory chronic prostatitis-chronic pelvic pain syndrome: a multicentre, randomised, prospective, double-blind, placebo-controlled phase 3 study," *Eur. Urol.*, vol. 56, no. 3, pp. 544–551, Sep. 2009, doi: 10.1016/J.EURURO.2009.05.046.

[76] "The ABC Clinical Guide to Herbs - American Botanical Council." https://www.herbalgram.org/resources/abc-clinical-guide/ (accessed Oct. 11, 2021).

[77] M. Suzuki *et al.*, "Pharmacological effects of saw palmetto extract in the lower urinary tract," *Acta Pharmacol. Sin.*, vol. 30, no. 3, p. 271, Mar. 2009, doi: 10.1038/APS.2009.1.

[78] C. F and M. A, "Preventing diseases of the prostate in the elderly using hormones and nutriceuticals," *Aging Male*, vol. 7, no. 2, pp. 155–169, Jun. 2004, doi: 10.1080/13685530412331284722.

[79] T. B. Klepser and M. E. Klepser, "Unsafe and potentially safe herbal therapies," *Am. J. Heal. Pharm.*, vol. 56, no. 2, pp. 125–138, Jan. 1999, doi: 10.1093/AJHP/56.2.125.

[80] W. TJ, I. A, S. G, M. R, L. J, and M. C, "Saw palmetto extracts for treatment of benign prostatic Hypertrophy: a systematic review," *JAMA*, vol. 280, no. 18, pp. 1604–1609, Nov. 1998, doi: 10.1001/JAMA.280.18.1604.

[81] Bertaccini A, Giampaoli M, Cividini R, G. Gl, Sanseverino R, and Realfonso T, "SAbal serrulata (Serenoa repens) é eficaz na hipertrofia benigna de próstata-Overview Observational database serenoa repens (DOSSER): overview, analysis and results. A multicentric SIUrO (Italian Society of Oncological Urology) project," *Arch Ital Urol Androl*, vol. 84, no. 3, pp. 117–139, 2012.

[82] T. J. Wilt, A. Ishani, G. Stark, R. MacDonald, J. Lau, and C. Mulrow, “Saw palmetto extracts for treatment of benign prostatic Hypertrophy: A systematic review,” *J. Am. Med. Assoc.*, vol. 280, no. 18, pp. 1604–1609, Nov. 1998, doi: 10.1001/JAMA.280.18.1604.

[83] J. Tacklind, R. MacDonald, I. Rutks, J. U. Stanke, T. J. Wilt, and C. U. Group, “Serenoa repens for benign prostatic Hypertrophy,” *Cochrane Database Syst. Rev.*, vol. 2012, no. 12, pp. 8–10, Jan. 2012, doi: 10.1002/14651858.CD001423.PUB3.

[84] I. A. Pytel’, N. A. Lopatkin, L. M. Gorilovskiĭ, A. Z. Vinarov, A. V Sivkov, and A. A. Medvedev, “[The results of long-term permixon treatment in subjects with symptoms of lower urinary tracts dysfunction due to benign prostatic Hypertrophy].,” *Urologiia*, no. 2, pp. 3–7, 2004.

[85] J. C. Carraro *et al.*, “Comparison of phytotherapy (Permixon) with finasteride in the treatment of benign prostate Hypertrophy: a randomized international study of 1,098 subjects.,” *Prostate*, vol. 29, no. 4, pp. 231–232, Oct. 1996, doi: 10.1002/(SICI)1097-0045(199610)29:4<231::AID-PROS4>3.0.CO;2-E.

[86] T. Cai, Y. Cui, S. Yu, Q. Li, Z. Zhou, and Z. Gao, “Comparison of Serenoa repens With Tamsulosin in the Treatment of Benign Prostatic Hypertrophy: A Systematic Review and Meta-Analysis.,” *Am. J. Mens. Health*, vol. 14, no. 2, p. 1557988320905407, 2020, doi: 10.1177/1557988320905407.

[87] C. JE, R. BD, W. H, and C. S, “A comprehensive review on the stinging nettle effect and efficacy profiles. Part II: urticae radix,” *Phytomedicine*, vol. 14, no. 7–8, pp. 568–579, Aug. 2007, doi: 10.1016/J.PHYMED.2007.03.014.

[88] H. DJ, K. MS, R. NA, and R. W, “The effect of extracts of the roots of the stinging nettle (Urtica dioica) on the interaction of SHBG with its receptor on human prostatic membranes,” *Planta Med.*, vol. 61, no. 1, pp. 31–32, 1995, doi: 10.1055/S-

2006-957993.

[89] S. M, G. D, and S. G, “Lignans from the roots of Urtica dioica and their metabolites bind to human sex hormone binding globulin (SHBG),” *Planta Med.*, vol. 63, no. 6, pp. 529–532, 1997, doi: 10.1055/S-2006-957756.

[90] G. D and S. G, “Aromatase inhibitors from Urtica dioica roots,” *Planta Med.*, vol. 61, no. 2, pp. 138–140, 1995, doi: 10.1055/S-2006-958033.

[91] H. T, H. M, and O. K, “Effects of stinging nettle root extracts and their steroidal components on the Na+,K(+)-ATPase of the benign prostatic Hypertrophy,” *Planta Med.*, vol. 60, no. 1, pp. 30–33, 1994, doi: 10.1055/S-2006-959402.

[92] K. L, M. HH, L. C, L. H, A. G, and L. JJ, “Antiproliferative effect on human prostate cancer cells by a stinging nettle root (Urtica dioica) extract,” *Planta Med.*, vol. 66, no. 1, pp. 44–47, 2000, doi: 10.1055/S-2000-11117.

[93] D. I, B. H, D. E, S. S, and A. A, “Aqueous extract of Urtica dioica makes significant inhibition on adenosine deaminase activity in prostate tissue from subjects with prostate cancer,” *Cancer Biol. Ther.*, vol. 3, no. 9, pp. 855–857, 2004, doi: 10.4161/CBT.3.9.1038.

[94] “European Scientific Cooperative on Phytotherapy.” https://escop.com/about-escop/ (accessed Oct. 11, 2021).

[95] F. C. Lowe and E. Fagelman, “Phytotherapy in the treatment of benign prostatic Hypertrophy: an update,” *Urology*, vol. 53, no. 4, pp. 671–678, Apr. 1999, doi: 10.1016/S0090-4295(98)00664-5.

[96] S. T and R. H, “[Stinging nettle root extract (Bazoton-uno) in long term treatment of benign prostatic syndrome (BPS). Results of a randomized, double-blind, placebo controlled multicenter study after 12 months],” *Urologe. A*, vol. 43, no. 3, pp. 302–306, Mar. 2004, doi: 10.1007/S00120-004-0532-7.

[97] M. R. Safarinejad, “Urtica dioicafor Treatment of Benign Prostatic Hypertrophy,” *http://dx.doi.org/10.1080/J157v05n04_01*, vol. 5, no. 4, pp. 1–11, Jan. 2009, doi: 10.1080/J157V05N04_01.

[98] A. Santini, G. C. Tenore, and E. Novellino, “Nutraceuticals: A paradigm of proactive medicine,” *Eur. J. Pharm. Sci.*, vol. 96, pp. 53–61, Jan. 2017.

[99] D. D. S. P. E. D. Ministero Della Salute, “Ministero Della Salute,” *Raccomandazioni Clin. Odontostomatol.*, p. 281, 2014.

[100] “EUR-Lex - 32014R0536 - EN - EUR-Lex.” https://eur-lex.europa.eu/legal-content/IT/ALL/?uri=CELEX:32014R0536 (accessed Oct. 17, 2021).

[101] “Gazzetta Ufficiale.” https://www.gazzettaufficiale.it/eli/id/2008/03/03/08A01360/sg (accessed Oct. 17, 2021).

[102] “Gazzetta Ufficiale.” https://www.gazzettaufficiale.it/eli/id/2003/08/09/003G0229/sg (accessed Oct. 17, 2021).

[103] “ICH Topic E 3 Structure and Content of Clinical Study Reports Step 5 NOTE FOR GUIDANCE ON STRUCTURE AND CONTENT OF CLINICAL STUDY REPORTS,” 1996, Accessed: Oct. 17, 2021. [Online]. Available: http://www.emea.eu.int.

[104] G. L. Lloyd, J. M. Marks, and W. A. Ricke, “Benign Prostatic Hypertrophy and Lower Urinary Tract Symptoms: What Is the Role and Significance of Inflammation?,” *Curr. Urol. Rep.*, vol. 20, no. 9, Sep. 2019, doi: 10.1007/S11934-019-0917-1.

[105] I. Paterniti *et al.*, “Effects of different natural extracts in an experimental model of benign prostatic Hypertrophy (BPH),” *Inflamm. Res.*, vol. 67, no. 7, pp. 617–626,

Jul. 2018, doi: 10.1007/S00011-018-1152-9.

[106] T. H. Kim, H. J. Lim, M. S. Kim, and M. S. Lee, “Dietary supplements for benign prostatic Hypertrophy: An overview of systematic reviews,” *Maturitas*, vol. 73, no. 3, pp. 180–185, Nov. 2012, doi: 10.1016/J.MATURITAS.2012.07.007.

[107] R. C. Langan, “Benign Prostatic Hypertrophy,” *Prim. Care - Clin. Off. Pract.*, vol. 46, no. 2, pp. 223–232, Jun. 2019, doi: 10.1016/J.POP.2019.02.003.

[108] J. D. McConnell *et al.*, “The Long-Term Effect of Doxazosin, Finasteride, and Combination Therapy on the Clinical Progression of Benign Prostatic Hypertrophy,” *N. Engl. J. Med.*, vol. 349, no. 25, pp. 2387–2398, Dec. 2003, doi: 10.1056/NEJMOA030656.

[109] K. B. Egan, “The Epidemiology of Benign Prostatic Hypertrophy Associated with Lower Urinary Tract Symptoms: Prevalence and Incident Rates,” *Urol. Clin. North Am.*, vol. 43, no. 3, pp. 289–297, Aug. 2016, doi: 10.1016/J.UCL.2016.04.001.

[110] R. Vela-Navarrete *et al.*, “Efficacy and safety of a hexanic extract of Serenoa repens (Permixon®) for the treatment of lower urinary tract symptoms associated with benign prostatic Hypertrophy (LUTS/BPH): systematic review and meta-analysis of randomised controlled trials and observational studies,” *BJU Int.*, vol. 122, no. 6, pp. 1049–1065, Dec. 2018, doi: 10.1111/BJU.14362.

[111] T. B. Agbabiaka, M. H. Pittler, B. Wider, and E. Ernst, “Serenoa repens (Saw Palmetto): A systematic review of adverse events,” *Drug Saf.*, vol. 32, no. 8, pp. 637–647, 2009, doi: 10.2165/00002018-200932080-00003.

[112] R. Q. Thompson, D. Katz, and B. Sheehan, “Chemical comparison of Prunus africana bark and pygeum products marketed for prostate health,” *J. Pharm. Biomed. Anal.*, vol. 163, pp. 162–169, Jan. 2019, doi: 10.1016/J.JPBA.2018.10.004.

[113] A. K. Jena, K. Vasisht, N. Sharma, R. Kaur, M. S. Dhingra, and M. Karan,

"Amelioration of testosterone induced benign prostatic Hypertrophy by Prunus species," *J. Ethnopharmacol.*, vol. 190, pp. 33–45, Aug. 2016, doi: 10.1016/J.JEP.2016.05.052.

[114] M. R. Safarinejad, " Urtica dioica for Treatment of Benign Prostatic Hypertrophy ," *J. Herb. Pharmacother.*, vol. 5, no. 4, pp. 1–11, Jan. 2005, doi: 10.1080/J157V05N04_01.

[115] P. H. Raven, "Generic and Sectional Delimitation in Onagraceae, Tribe Epilobieae," *Ann. Missouri Bot. Gard.*, vol. 63, no. 2, p. 326, 1976, doi: 10.2307/2395311.

[116] R. Sõukand *et al.*, "Inventing a herbal tradition: The complex roots of the current popularity of Epilobium angustifolium in Eastern Europe," *J. Ethnopharmacol.*, vol. 247, Jan. 2020, doi: 10.1016/J.JEP.2019.112254.

[117] A. Vitalone, J. McColl, D. Thome, L. G. Costa, and B. Tita, "Characterization of the effect of Epilobium extracts on human cell proliferation," *Pharmacology*, vol. 69, no. 2, pp. 79–87, 2003, doi: 10.1159/000072360.

[118] J. P. Piwowarski *et al.*, "Evaluation of the Effect of Epilobium angustifolium Aqueous Extract on LNCaP Cell Proliferation in In Vitro and in Vivo Models," *Planta Med.*, vol. 83, no. 14–15, pp. 1159–1168, Oct. 2017, doi: 10.1055/S-0043-109372.

[119] L. Deng *et al.*, "Evaluation of the therapeutic effect against benign prostatic Hypertrophy and the active constituents from Epilobium angustifolium L.," *J. Ethnopharmacol.*, vol. 232, pp. 1–10, Mar. 2019, doi: 10.1016/J.JEP.2018.11.045.

[120] A. Vitalone, F. Bordi, C. Baldazzi, G. Mazzanti, L. Saso, and B. Tita, "Anti-proliferative effect on a prostatic epithelial cell line (PZ-HPV-7) by Epilobium angustifolium L," *Farmaco*, vol. 56, no. 5–7, pp. 483–489, Jul. 2001, doi: 10.1016/S0014-827X(01)01067-9.

[121] V. Cosentino, A. Militello, and G. Lauria, “Short-term effects of a dietary supplement on lower urinary tract symptoms.,” *Journal of biological regulators and homeostatic agents*, vol. 32, no. 6. Italy, pp. 1557–1563, 2018.

[122] A. Keehn and F. C. Lowe, “Complementary and alternative medications for benign prostatic Hypertrophy.,” *Can. J. Urol.*, vol. 22 Suppl 1, pp. 18–23, Oct. 2015.

[123] S. Jürgenson, V. Matto, and A. Raal, “Vegetational variation of phenolic compounds in Epilobium angustifolium,” *Nat. Prod. Res.*, vol. 26, no. 20, pp. 1951–1953, Oct. 2012, doi: 10.1080/14786419.2011.643310.

[124] A. G. Ramstead, I. A. Schepetkin, M. T. Quinn, and M. A. Jutila, “Oenothein B, a Cyclic Dimeric Ellagitannin Isolated from Epilobium angustifolium, Enhances IFNγ Production by Lymphocytes,” *PLoS One*, vol. 7, no. 11, Nov. 2012, doi: 10.1371/JOURNAL.PONE.0050546.

[125] A. G. Ramstead *et al.*, “Aging influences the response of T cells to stimulation by the ellagitannin, oenothein B,” *Int. Immunopharmacol.*, vol. 26, no. 2, pp. 367–377, Jun. 2015, doi: 10.1016/J.INTIMP.2015.04.008.

[126] M. Minekus *et al.*, “A standardised static in vitro digestion method suitable for food- an international consensus,” *Food Funct.*, vol. 5, no. 6, pp. 1113–1124, 2014, doi: 10.1039/C3FO60702J.

[127] C. Kilkenny, W. Browne, I. C. Cuthill, M. Emerson, and D. G. Altman, “Animal research: Reporting in vivo experiments: The ARRIVE guidelines,” *Br. J. Pharmacol.*, vol. 160, no. 7, pp. 1577–1579, Aug. 2010, doi: 10.1111/J.1476-5381.2010.00872.X.

[128] J. C. McGrath and E. Lilley, “Implementing guidelines on reporting research using animals (ARRIVE etc.): New requirements for publication in BJP,” *Br. J. Pharmacol.*, vol. 172, no. 13, pp. 3189–3193, Jul. 2015, doi: 10.1111/BPH.12955.

[129] M. Dacrema *et al.*, “Metabolic profiling, in vitro bioaccessibility and in vivo bioavailability of a commercial bioactive Epilobium angustifolium L. extract,” *Biomed. Pharmacother.*, vol. 131, p. 110670, 2020, doi: 10.1016/j.biopha.2020.110670.

[130] L. Q. Deng *et al.*, “HPLC-ESI-MS/MS analysis of phenolics and in vitro antioxidant activity of Epilobium angustifolium L.,” *Nat. Prod. Res.*, vol. 32, no. 12, pp. 1432–1435, Jun. 2018, doi: 10.1080/14786419.2017.1344659.

[131] R. García-Villalba, D. Beltrán, J. C. Espín, M. V. Selma, and F. A. Tomás-Barberán, “Time course production of urolithins from ellagic acid by human gut microbiota,” *J. Agric. Food Chem.*, vol. 61, no. 37, pp. 8797–8806, Sep. 2013, doi: 10.1021/JF402498B.

[132] A. González-Sarrías, M. Larrosa, F. A. Toms-Barberán, P. Dolara, and J. C. Espín, “NF-κB-dependent anti-inflammatory activity of urolithins, gut microbiota ellagic acid-derived metabolites, in human colonic fibroblasts,” *Br. J. Nutr.*, vol. 104, no. 4, pp. 503–512, Aug. 2010, doi: 10.1017/S0007114510000826.

[133] J. A. Giménez-Bastida, A. González-Sarrías, M. Larrosa, F. Tomás-Barberán, J. C. Espín, and M. T. García-Conesa, “Ellagitannin metabolites, urolithin A glucuronide and its aglycone urolithin A, ameliorate TNF-α-induced inflammation and associated molecular markers in human aortic endothelial cells,” *Mol. Nutr. Food Res.*, vol. 56, no. 5, pp. 784–796, May 2012, doi: 10.1002/MNFR.201100677.

[134] S. G. Kasimsetty, D. Blalonska, M. K. Reddy, G. Ma, S. I. Khan, and D. Ferreira, “Colon cancer chemopreventive activities of pomegranate ellagitannins and Urolithins,” *J. Agric. Food Chem.*, vol. 58, no. 4, pp. 2180–2187, Feb. 2010, doi: 10.1021/JF903762H.

[135] N. P. Seeram *et al.*, “Pomegranate ellagitannin-derived metabolites inhibit prostate

cancer growth and localize to the mouse prostate gland," *J. Agric. Food Chem.*, vol. 55, no. 19, pp. 7732–7737, Sep. 2007, doi: 10.1021/JF071303G.

[136] S. Karakurt, A. Semiz, G. Celik, A. M. Gencler-Ozkan, A. Sen, and O. Adali, "Contribution of ellagic acid on the antioxidant potential of medicinal plant Epilobium hirsutum," *Nutr. Cancer*, vol. 68, no. 1, pp. 173–183, Jan. 2016, doi: 10.1080/01635581.2016.1115092.

[137] "Prostate Enlargement (Benign Prostatic Hypertrophy) | NIDDK." https://www.niddk.nih.gov/health-information/urologic-diseases/prostate-problems/prostate-enlargement-benign-prostatic-Hypertrophy (accessed Oct. 11, 2021).

[138] P. Gontero, R. S. Kirby, and C. C. Carson, "Problem based urology," *Probl. Based Urol.*, vol. 9781447146346, pp. 1–235, Oct. 2013, doi: 10.1007/978-1-4471-4634-6.

[139] S. W. H. Lee, E. M. C. Chan, and Y. K. Lai, "The global burden of lower urinary tract symptoms suggestive of benign prostatic Hypertrophy: A systematic review and meta-analysis," *Sci. Rep.*, vol. 7, no. 1, Dec. 2017, doi: 10.1038/S41598-017-06628-8.

[140] M. J. Speakman and X. Cheng, "Management of the complications of BPH/BOO," *Indian J. Urol.*, vol. 30, no. 2, pp. 208–213, 2014, doi: 10.4103/0970-1591.127856.

[141] O. Allkanjari and A. Vitalone, "What do we know about phytotherapy of benign prostatic Hypertrophy?," *Life Sci.*, vol. 126, pp. 42–56, Apr. 2015, doi: 10.1016/J.LFS.2015.01.023.

[142] J. B. Anderson, C. G. Roehrborn, J. A. Schalken, and M. Emberton, "The progression of benign prostatic Hypertrophy: Examining the evidence and determining the risk," *Eur. Urol.*, vol. 39, no. 4, pp. 390–399, 2001, doi: 10.1159/000052475.

[143] A. S. Wright, R. C. Douglas, L. N. Thomas, C. B. Lazier, and R. S. Rittmaster, "Androgen-induced regrowth in the castrated rat ventral prostate: Role of 5α-reductase," *Endocrinology*, vol. 140, no. 10, pp. 4509–4515, 1999, doi: 10.1210/ENDO.140.10.7039.

[144] R. S. Kirby, "Medical therapy for benign prostatic Hypertrophy: The road ahead," *Br. J. Urol.*, vol. 79, no. SUPPL. 1, pp. 92–96, 1997, doi: 10.1111/J.1464-410X.1997.TB00808.X.

[145] C. K. M. Ho and F. K. Habib, "Estrogen and androgen signaling in the pathogenesis of BPH," *Nat. Rev. Urol.*, vol. 8, no. 1, pp. 29–41, Jan. 2011, doi: 10.1038/NRUROL.2010.207.

[146] J. T. Isaacs, "Etiology of benign prostatic Hypertrophy," *Eur. Urol.*, vol. 25, no. SUPPL. 1, pp. 6–9, 1994, doi: 10.1159/000475324.

[147] E. S. Grant, T. Brown, A. Roach, B. C. Williams, and F. K. Habib, "In Vitro Expression of Endothelin-1 (ET-1) and the ET A and ET B ET Receptors by the Prostatic Epithelium and Stroma 1 ," *J. Clin. Endocrinol. Metab.*, vol. 82, no. 2, pp. 508–513, Feb. 1997, doi: 10.1210/JCEM.82.2.3724.

[148] D. M. Peehl and R. G. Sellers, "Induction of smooth muscle cell phenotype in cultured human prostatic stromal cells," *Exp. Cell Res.*, vol. 232, no. 2, pp. 208–215, May 1997.

[149] V. C. Mishra *et al.*, "Does intraprostatic inflammation have a role in the pathogenesis and progression of benign prostatic Hypertrophy?," *BJU Int.*, vol. 100, no. 2, pp. 327–331, Aug. 2007, doi: 10.1111/J.1464-410X.2007.06910.X.

[150] B. Chughtai, R. Lee, A. Te, and S. Kaplan, "Role of Inflammation in Benign Prostatic Hypertrophy," *Rev. Urol.*, vol. 13, no. 3, p. 147, 2011, Accessed: Oct. 11, 2021. [Online]. Available: /pmc/articles/PMC3221555/.

[151] G. Gandaglia *et al.*, “The role of prostatic inflammation in the development and progression of benign and malignant diseases,” *Curr. Opin. Urol.*, vol. 27, no. 2, pp. 99–106, 2017, doi: 10.1097/MOU.0000000000000369.

[152] S. Madersbacher, N. Sampson, and Z. Culig, “Pathophysiology of Benign Prostatic Hypertrophy and Benign Prostatic Enlargement: A Mini-Review,” *Gerontology*, vol. 65, no. 5, pp. 458–464, Aug. 2019, doi: 10.1159/000496289.

[153] “Mayo Clinic Essential Guide to Prostate Health | Mayo Clinic Press.” https://mcpress.mayoclinic.org/shop/mens-health-books/mayo-clinic-essential-guide-to-prostate-health/ (accessed Oct. 11, 2021).

[154] E. Medicines Agency, “Assessment report on Epilobium angustifolium L. and/or Epilobium parviflorum Schreb., herba,” Accessed: Oct. 11, 2021. [Online]. Available: www.ema.europa.eu/contact.

[155] B. Ducrey, A. Marston, S. Göhring, R. W. Hartmann, and K. Hostettmann, “Inhibition of 5α-reductase and aromatase by the ellagitannins oenothein A and oenothein B from Epilobium species,” *Planta Med.*, vol. 63, no. 2, pp. 111–114, 1997, doi: 10.1055/S-2006-957624.

[156] L. D *et al.*, “Determination of oenothein B as the active 5-alpha-reductase-inhibiting principle of the folk medicine Epilobium parviflorum,” *J. Nat. Prod.*, vol. 59, no. 5, pp. 490–492, May 1996, doi: 10.1021/NP960231C.

[157] A. Hiermann and F. Bucar, “Studies of Epilobium angustifolium extracts on growth of accessory sexual organs in rats,” *J. Ethnopharmacol.*, vol. 55, no. 3, pp. 179–183, Feb. 1997.

[158] A. Kiss, J. Kowalski, and M. F. Melzig, “Compounds from Epilobium angustifolium inhibit the specific metallopeptidases ACE, NEP and APN,” *Planta Med.*, vol. 70, no. 10, pp. 919–923, Oct. 2004, doi: 10.1055/S-2004-832617.

[159] A. Kiss, J. Kowalski, and M. F. Melzig, “Induction of neutral endopeptidase activity in PC-3 cells by an aqueous extract of Epilobium angustifolium L. and oenothein B,” *Phytomedicine*, vol. 13, no. 4, pp. 284–289, Mar. 2006.

[160] M. Calvert, J. Blazeby, D. G. Altman, D. A. Revicki, D. Moher, and M. D. Brundage, “Reporting of subject-reported outcomes in randomized trials: The CONSORT PRO extension,” *JAMA - J. Am. Med. Assoc.*, vol. 309, no. 8, pp. 814–822, Feb. 2013, doi: 10.1001/JAMA.2013.879.

[161] V. Varilla, R. V. Samala, D. Galindo, and J. Ciocon, “Nocturia in the elderly: A wake-up call,” *Cleve. Clin. J. Med.*, vol. 78, no. 11, pp. 757–764, 2011, doi: 10.3949/CCJM.78A.11025.