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Prostatic therapeutic efficacy of LENILUTS®, a novel and multi-active principles formulation

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Abstract: Lower Urinary Tract Symptoms (LUTs) in men are usually associated to benign prostatic hyperplasia (BPH), a non-malignant prostate enlargement. Unfortunately, BPH etiology is still unclear. Recent works highlighted a relevant inflammation role in BPH onset and development. Consequently, to complement the 5-α reductase (and α-adrenergic receptor agonists-based therapy, an anti-inflammatory therapy should be devised. To reduce multi-drug treatment potential adverse effects, plant extracts-based therapies are becoming increasingly common. Serenoa repens, the main phytotherapic treatment for BPH, is not sufficient to front the multi-faceted BPH etiology. In response to that, a novel, multiple phytotherapic agents-based formulation, LENILUTS®, was developed. In the present work, we compared, with an in vitro approach, the prostatic safety and efficacy of LENILUTS® with a commercial formulation, based only on Serenoa repens, and a 5αR inhibitor, Dutasteride. Furthermore, preliminary in vitro experiments to investigate LENILUTS® active principles bioaccessibility and bioavailability were performed. Our results showed a better prostatic safety and therapeutic efficacy of LENILUTS®, compared to the commercial formulation and Dutasteride, with an increased anti-inflammatory, and pro-apoptotic activity, and a stronger inhibitory effect on the key enzyme 5αR and Prostatic-Specific Antigen (PSA) release. Limited bioaccessibility and bioavailability of LENILUTS® active principles were highlighted. Considering the obtained results, LENILUTS® formulation is more promising for BPH and LUTs therapy compared to Serenoa repens only-based formulations, but further efforts should be devised to improve active principles bioaccessibility and bioavailability.

Keywords: urology; BPH; LUTs; LNCaP; prostate; dietary supplements; delivery system

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1. Introduction

Lower Urinary Tract Symptoms (LUTS) is a group of urinary symptoms triggered by an obstruction, abnormality, infection or irritation of the lower urinary tract (i.e. urethra, bladder, bladder neck, urinary sphincter and/or prostate (in men)), negatively affect aging men lifestyle [1]. LUTS can be categorized as being related to urine storage (urinary frequency, urinary urgency, etc.) or voiding (obstruction) (hesitancy, weak or intermittent stream, etc.) and present themselves as various voiding dysfunctions [2]. While both men and women could be affected, LUTS is most often diagnosed in men affected by a benign enlargement of the prostate, known as Benign Prostatic Hyperplasia (BPH). BPH is an enlargement of the prostate gland, typically in the central zone, which is the zone of the prostate surrounding the urethra. This enlargement, in turns, puts pressure on urethra, increasing outlet resistance, leading to LUTS as a consequence [3]. At present, it is

generally agreed that BPH, is a consequence of a androids-mediated cell proliferation [4], in particular of its smooth muscle component, which contraction is responsible for many BPH-related symptoms, such as LUTS [5,6]. However, as pointed out by the Medical Therapy of Prostatic Symptoms study [7], a prominent role for inflammation in BPH insurgence and development was proposed [8,9]. Taken together, these evidences seem suggest a complex etiology for BPH, which treatment should require a multidrug-based approach. Accordingly, BPH-related LUTS is presently treated with a combination of 5αR inhibitors, which block conversion of testosterone to dihydrotestosterone, α-adrenergic receptor agonists that favor smooth muscle relaxation, and plant extracts or phytotherapic agents [10,11] that can adjuvate and amplify the mentioned activities. Indeed, formulations based on plant extracts are by far the most popular approach used in the medical management of BPH-induced LUTS [12,13]. Among them, Serenoa repens, extracted from saw palmetto tree berries, is the most popular one [14–16]. However, considering its multi-faceted origin [17,18], a formulation based on multiple active principles would be likely preferable in BPH-mediated LUTS treatment. To test this hypothesis, a novel formulation, LENILUTS®, in which anti-inflammatory (beta-sitosterol, BS [19,20], Curcuma longa CL [21,22] and oligomeric proanthocyanidins, OPC [23,24]) and anti-oxidant and antibacterial (BS [25,26], CL [27,28] and OPC [29,30]) active principles are blended, was compared to a commercially available, Serenoa repens oil mono-component based formulation. The effect of LENILUTS® and the commercial formulation on different prostatic parameters such as inflammation, 5αR inhibitory activity, prostate-specific antigen (PSA) release and smooth muscle activity were investigated with an in vitro approach, based on a prostate in vitro model. Finally, preliminary in vitro tests, based on an integrated system composed of an in vitro human digestive and an intestinal epithelium model, were performed to determine LENILUTS® bioaccessibility and bioavailability.

2. Materials and Methods

2.1 Materials

High glucose Dulbecco's Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI) 1640 Medium, Hanks' Balanced Salt Saline (HBSS), non-essential amino acids (NEAA), L-glutamine, Penicillin-Streptomycin mix, lipopolysaccharide (LPS), diclofenac, dihydrotestosterone (DHT), staurosporine (STS) and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich (St Louis, MO, USA). LNCaP androgen-sensitive human prostate adenocarcinoma cell line (ATCC® CRL-1740™), Caco-2 human colorectal adenocarcinoma cells (ATCC® HTB-37™) and THP-1 (ATCC® TIB-202™) were purchased from ATCC (Manassas, VA, USA). CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) and Apo-ONE® Homogeneous Caspase-3/7 Assay were purchased from Promega (Madison, WI, USA). Oxygen Radical Antioxidant Capacity (ORAC) Assay kit was purchased from Cell Biolabs (San Diego, CA, USA). Transwell® insert were purchased from Millipore (Burlington, MA, USA). Fetal bovine serum (FBS) was purchased from Euroclone (Milan, IT). Interleukin 1β (IL-1β), Tumor Necrosis Factor α (TNF-α), PSA and DHT ELISA kit were purchased from R&D Systems, PeproTech (London, UK), Abcam (Cambridge, UK) and Cloud Clone (Katy, TX, USA) respectively.

2.2 Formulation Composition

The comparative efficacy evaluation on prostate and smooth muscle *in vitro* models was performed between LENILUTS® and commercially available formulation (CF), whose compositions are detailed in Supplementary Materials 1 (**SM1**), and Dutasteride. Both of formulations were resuspended in dimethyl sulfoxide (DMSO).

2.3 Methods

2.3.1 Cell cultures

LNCaP cell culture

The LNCaP cells (human prostate cancer line) were kept at 37 °C in a humidified atmosphere with 5% CO2 in complete cell culture medium (RPMI-1640 medium supplemented with 10% FBS and 1% Penicillin-Streptomycin mix) from passage 25 to 40. For propagation, cells were subcultivated by trypsinization every 7 d when 80–90% confluent, seeded at a density of 1 X 10^4 cell/cm^2 and medium changed every other day. LNCaP cells were seeded at a density of 1 X 10^5 cells/cm^2 in 96- and 6-well plates for vitality and anti-inflammatory experiments and allowed to adhere for 2 days prior to experiments. A lower cell density (5 X 10^4 cells/cm^2) was used for prostate-specific antigen (PSA) experiments, performed in 24-well plates.

THP-1 cell culture

Human THP-1 monocytes were cultured at a density of 5 x 10^5 cells/mL and maintained in cell culture complete medium (RPMI-1640 medium with glutamate supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin) in 5% CO_2 humidified atmosphere. Cells were subcultured twice a week. A concentration of 500 nM of phorbol myristate acetate (PMA; Sig-ma-Aldrich, MO, USA) was applied for 24 h to induce macrophage differentiation. At the end of the exposure, the differentiation inducing-medium was replaced with complete cell culture medium and cells cultured for an additional 24 h. Conditioned medium was prepared by seeding 6 X 10^6 cells in 75 cm^2 flask, followed by macrophage differentiation and treatment with 1 ng/mL LPS for 6 h. At the end of the LPS treatment, medium was recovered and stored at -80 °C until use.

Caco-2 cell culture

The Caco-2 cells (human colon adenocarcinoma cells) were seeded in adhesion flask in cell complete medium (high glucose DMEM, 10% heat inactivated FBS, 1% Non-Essential Amino Acids, 4 mM L-glutamine and 1% penicillin/streptomycin mix) at a density of 2 X 10^3 cell/cm^2 in and kept at 37 °C and 5% CO2 in a humidified incubator ((passage 32 to 42). Cells were subcultivated by trypsinization every 7 d when 80–90% confluent and seeded at a density of 2000 cells/cm^2. The medium was refreshed every other day.

2.3.2 Evaluation of LENILUTS® antioxidant activity

The antioxidant activity of LENILUTS® was evaluated using the ORAC test, following the indications provided on a commercial kit (Cell Biolabs). Briefly, the ORAC test for LENILUTS® was conducted in accordance with the protocol contemplated for food samples, considering both the hydrosoluble and liposoluble components of the formula. In brief, a LENILUTS® tablet was crushed to a powder and the formula was weighed and resuspended in water. After centrifugation to precipitate the fraction not dissolved in the water, the supernatant composed of the water-soluble fraction (hydrophilic fraction) was removed. The pellet was once again resuspended in water, and, after centrifugation, the supernatant collected was added to the hydrophilic fraction. The pellet remaining after the aforementioned steps was resuspended in absolute acetone and shaken at room temperature for 30 minutes. After centrifugation, the supernatant (lipophilic fraction) was removed. The hydrophilic and lipophilic fractions were adequately diluted in the reaction buffer, and 25 μL of solution was used in each reaction to determine antioxidant activity. After a 30-minute pre-incubation phase at 37 °C, a free radical initiator is added, and the reaction is incubated at 37 °C for 60 minutes, during which fluorescence is monitored at one-minute intervals, using excitation and emission wavelengths of 480 and 520 nm respectively. For both hydrophilic and lipophilic fraction, the final result was calculated and expressed as μmol Trolox equivalents per gram of sample. Finally, the ORAC value results is composed of from the sum of the ORAC values obtained from the hydrophilic and lipophilic fractions.

2.3.3 Evaluation of the impact of tested formulations, Dutasteride and Diclofenac on LNCaP cells

To evaluate LENILUTS®, CF, Dutasteride and anti-inflammatory drug Diclofenac impact on human prostatic cellular model, and to determine their higher, non-toxic concentration, a dose-response curve experiment on LNCaP cells was performed. Briefly, LNCaP cells were treated with increasing concentrations of LENILUTS® (from 0 to 2000 μg/mL), CF (from 0 to 4000 μg/ml) and Dutasteride (from 0 to 372.5 μg/mL) for 6 and 24 h. Diclofenac treatment (from 0 to 1591 μg/mL) was limited to 6 h, corresponding to the duration of anti-inflammatory activity experiments. At the end of incubation time, LNCaP cells vitality was determined by MTS assay, according to the manufacturer's instruction. Obtained dose-response curves were fitted with OriginLab software and half-maximal effective concentration (EC50) calculated.

2.3.4 Prostate-specific anti-inflammatory activity

The prostate-specific anti-inflammatory activity of tested formulations and drugs was evaluated in a LNCaP cell-based prostatic epithelium in vitro model, with a two-step protocol: i) 2 h pre-treatment of the prostatic epithelium in vitro model with the highest, non-toxic concentrations of formulations and drugs and ii) a 4 h exposure to inflammatory stimulus in presence of formulations and drugs. In vitro prostatic model inflammation was achieved with the method described by Carmen and colleagues [31]. At the end of the treatment, LNCaP cells were washed with DPBS, scraped in ice-cold PBS, centrifugated and lysed by sonication in lysis buffer. Following centrifugation at 10000 g for 15 min, the level of interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) cytokines in obtained supernatants were quantified by commercial ELISA (Enzyme-Linked Immunosorbent Assay) kits, following the manufacturer's instructions.

2.3.5 LENILUTS® pro-apoptotic activity

The pro-apoptotic activity of LENILUTS® pro-apoptotic activity was evaluated with a commercial fluorimetric assay, based on caspases 3/7 activation. The activated caspases selectively cleave a specific substrate, making it fluorescent (Ex: 499 nm, Em: 521 nm), linking the intensity of the produced fluorescence to the cell apoptotic process activation. To investigate the correlation between the anti-inflammatory and pro-apoptotic activity, the same experimental setup described above for the anti-inflammatory activity was applied. The experiments were conducted following the manufacturer's instructions. STS, a cell death inducer, was used as positive control for apoptosis at 1μM.

2.3.6 5α-R activity

The formulations impact on 5αR activity was assessed following the protocol described by Assinder [32]. Briefly equal amounts of the same LNCaP cell lysate (i.e. same total protein content) were incubated for 16 h at 37 °C under agitation with LENILUTS® (750 μg/mL) and CF (100 μg/mL), in presence of 5αR cofactor NADPH (100 μM) and the substrate testosterone (100 μM). Dutasteride was used as a positive control (93.1 μg/mL), while no testosterone-incubated cell lysate was considered as negative control. At the end of the incubation period, the reactions were blocked with ice and the DHT content of the different lysate measured by ELISA assay, according to the manufacturer's indications.

2.3.7 Measurement of PSA secretion by LNCaP prostatic cells

The effect of LENILUTS®, CF and Dutasteride on PSA secretion was evaluated in LNCaP prostatic cells, following the protocol described by Kampa and colleagues [33], in presence and absence of DHT (10 nM), an androgen known to increase PSA release. Secreted PSA levels were measured with a commercial ELISA kit, following the

manufacturer's instructions. Results were expressed as percentage of secreted PSA in cells treated with different formulations compared to control.

2.3.8 *In vitro* digestion process

A single dose of each formulation listed in SM1 was digested with an in vitro digestion system composed of three compartments (i.e. oral, gastric and intestinal compartment) and simulating the physiological human digestion. Briefly, the formulations were incubated in saliva-simulating fluid at 37 ± 1 °C for 5 min, rotating head-over-heels at 55 rpm to simulate the peristaltic movements. Then, gastric juice-simulating fluid (pH 1.3 ± 0.1) was added to the mixture and the pH adjusted to 2.5 ± 0.5 with NaOH (1 M) or HCl (37%). As for the oral compartment, the digesta was maintained under head-over-heel rotation at 37 °C for 2 h. Subsequently, duodenal juice (pH 8.1 ± 0.1), bile (pH 8.2 ± 0.1) and sodium bicarbonate were added and the pH adjusted to 6.5 ± 0.5. Head-over-hell rotation was kept for another 2 h. For simulated digestive fluids composition refer to [34]. Once completed the digestion process, beta-sitosterol and oligomeric proanthocyanidins (OPAs) bioaccessibility were determined by high pressure liquid chromatography (HPLC), while curcumin was quantified with a spectrophotometric approach.

2.3.9 Curcumin determination

Curcumin was determined spectrophotometrically. Briefly, when resuspended in the organic solvent dimethyl sulfoxide (DMSO), curcumin shows an absorption peak at 420-430 nm (SM2A), while, when excited at 420 nm, curcumin produces an emission peak comprised between 520 and 550 nm (SM2B). Considering its peculiar spectral properties, the concentration of curcumin was determined by exciting at 420 nm and measuring the fluorescence intensity at 545 nm. The resulting values were interpolated with a linear calibration curve, obtained with different concentrations of a curcumin standard. Such approach ensures a lower limit of detection (LOD) compared to the standard HPLC approach (50 ng/mL compared to 0.05 mg/mL) and allows for the determination of curcumin in the intestinal epithelium.

2.3.10 *In vitro* model of human intestinal epithelium

Absorption and bioavailability of beta-sitosterol, curcuminoids and OPAs were determined using an in vitro model of human intestinal epithelium based on Caco-2 cells. Briefly, Caco-2 cells were seeded at a density of 1.5 X 10^5 cells/cm^2 on 1 μm pore size Transwell® polytetrafluoroethylene inserts and left to mature and differentiate for 21 days. In this peculiar environment, characterized by the compartmentalization typical of the Transwell® system, Caco-2 cells acquire the morpho-functional features of the mature enterocyte (presence of microvilli, tight junctions and P-glycoprotein). Absorption experiment were performed between 21- and 28-days post seeding.

2.3.11 Digested formulations' impact on the viability of the intestinal epithelium

The impact of digested formulations on the viability of the intestinal epithelium was evaluated by adding serially-diluted digested formulations in digestive fluids in the apical compartment and incubating for 3 hours. In the basolateral compartment, HBSS was added and digestive fluids (without formulations) were added to the apical side as a negative control. At the end of the exposure period, monolayers were washed with prewarmed HBSS and viability of intestinal epithelia was evaluated with MTS assay, according to manufacturer's instructions. The absorbance at 490 nm was determined with a micro-plate reader (Synergy4, Biotek) and cell viability (%) was expressed as the ratio of the absorbance in the treated groups to that in the control (untreated) group. Active principles' bioavailability experiments were performed using non-toxic concentrations determined by dose-response curves.

2.3.12 Evaluation of beta-sitosterol, curcumin and OPAs bioavailability

Based on dose-response curve information and their posology, digested formulations were added to the apical side of the in vitro intestinal epithelium, while HBSS buffer supplemented with 1% BSA was placed in the basolateral compartment. Due to the lipophilicity of formulation active components, 1% BSA was added to the basolateral compartment for improving their bioavailability. According to the literature [35], the addition of BSA improves the correlation between absorption occurring in Caco-2 cell monolayer and humans. *In vitro* intestinal epithelia were exposed to the digested formulations containing 12.1 mg/mL of LENILUTS® for 1 and 3 h solutions, and beta-sitosterol, curcuminoids and OPCs were measured in both apical (lumen) and baso-lateral (serosal) compartments by HPLC and spectrophotometric approach respectively. ,. Bioavailability was then calculated and expressed as percentage of absorption (%) compared to the active principles amount initially loaded in the apical (i.e. luminal) compartment, and concentration (ng/mL), derived from three independent experiments.

2.3.13 Evaluation of post-intestinal absorption Caco-2 monolayer barrier integrity and viability

After exposure to digested formulations, the Caco-2 monolayer viability and barrier integrity were evaluated. Briefly, once the incubation with the digested formulations was over, the Caco-2 monolayer were washed and equilibrated in pre-warmed HBSS for 30 min. Then, Caco-2 monolayer barrier integrity was evaluated with an ERS2 Voltohmmeter (Millipore), equipped with a chopstick electrode, by measuring the trans-epithelial electrical resistance (TEER). TEER values are the average of three measurements taken at different points in the well in order to have information as representative of the monolayer as possible. Lucifer Yellow, a fluorescent polar tracer unable to pass through intact tight junctions, was used to assess the paracellular permeability of Caco-2 monolayers. Paracellular permeability was measured by adding 100 μg/mL LY in HBSS in the apical compartment and 1.5 mL of HBSS in the basolateral compartment. Then, after 1 hour incubation, the basolateral fractions were collected and their fluorescence measured with a spectrofluorometer (Synergy 4, Biotek).

The following formula was used to calculate the apparent permeability coefficient (Papp, cm/s):

$$\boldsymbol{Papp}=(\Delta C\ V)/(\Delta t\ A\ C0)$$

where ΔC/Δt is the flow of the molecule being transported across the monolayer during the incubation time (mM/s), V is the volume of the basolateral compartment (cm^3), A is the area of the membrane (cm^2), C_0 is the initial concentration of the molecule in the apical compartment. Finally, cell viability was evaluated by using MTS as-say as described above.

2.3.14 Statistical analysis

Experiments were performed in triplicate and results presented as average ± standard deviation. Results were statistically analysed by t-test or one-way ANOVA test in case of 3 or more experimental groups, using OriginLab software (OriginLab Corporation, MA, US) and a p value of ≤0.05 was considered significant.

3. Results

3.1 Antioxidant activity

The antioxidant action of the LENILUTS® was determined using an ORAC test. Table 1 shows the hydrophilic, lipophilic and total ORAC values, expressed as μmol TE/g of

formula ± standard deviation (SD). The results show that the formula has an antioxidant action, mainly due to its liposoluble components.

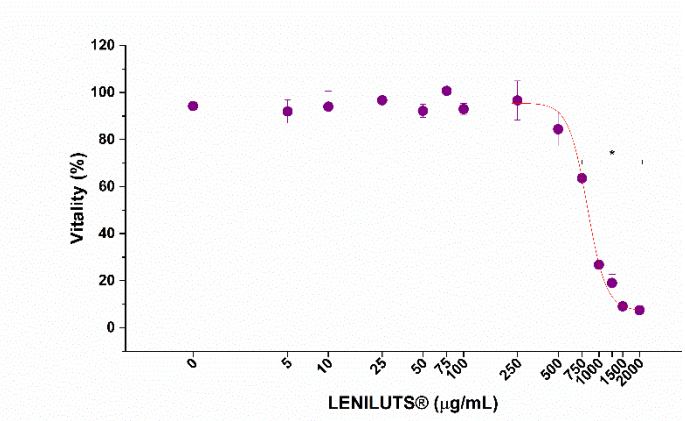
Table 1. Hydrophilic, lipophilic and total ORAC values of the product LENILUTS®, expressed as micromole Trolox equivalents per gram of formula (µmol TE/g) ± standard deviation (SD).

Product	Hydrophilic ORAC (µmol TE/g ± SD)	Lipophilic ORAC (µmol TE/g ± SD)	Total ORAC (µmol TE/g ± SD)
LENILUTS®	32.5 ± 2.2	506.5 ± 61.2	539.0 ± 63.4

3.2 Impact of LENILUTS®, CF, Dutasteride and diclofenac on the in vitro prostatic model

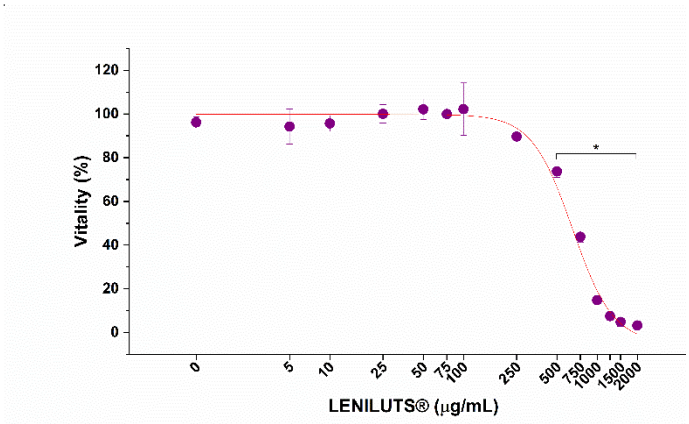
Before comparing the efficacy of LENILUTS® with its commercial competitor CF and Dutasteride we investigated their safety on in vitro prostatic model through dose-response toxicological analysis, considering 6 and 24 h as relevant exposure times. In vitro prostatic model vitality is significantly reduced, following 6 and 24 h treatment, at 750 µg/mL and 500 µg/mL of LENILUTS® respectively (Figure 1A and 1B), while CF completely abrogates the prostatic model vitality at 250 µg/mL, independently of exposure time (Figure 1C and 1D). Concerning Dutasteride, no adverse effects on the LNCaP-based in vitro prostatic model were observed following 24 h at all tested concentrations. (Figure 1E). However, since there are known cases in the literature of the depositing of crystals .with possible toxic effects for the cells at concentrations higher than 5.29 µg/Ml, this maximum concentration was used for the subsequent assays on the in vitro prostate model. Finally, since diclofenac is used as a positive control in inflammation experiment, its impact on in vitro prostatic model was also evaluated (SM3). EC50 value are reported in SM4. Based on cytotoxicity results, 6 h exposure-efficacy tests were performed with 500 µg/Ml of LENILUTS®, 100 µg/Ml of CF, 5,3 µg/Ml of Dutasteride and 32 µg/Ml of Diclofenac, while for 24 h exposure-efficacy a concentration of 250 µg/Ml, 100 µg/Ml and 5,3 µg/Ml was applied for LENILUTS®, CF and Dutasteride respectively. To further investigate its efficacy, lower concentrations of LENILUTS® were considered (250 µg/Ml and 100 µg/Ml at 6 and 24-hour respectively).

6 h



(A)

24 h



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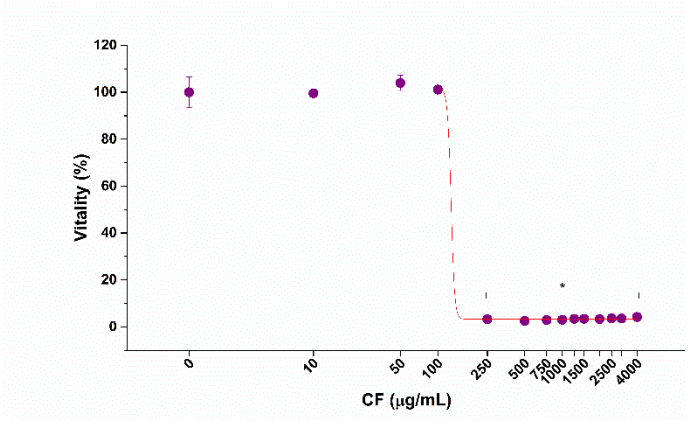

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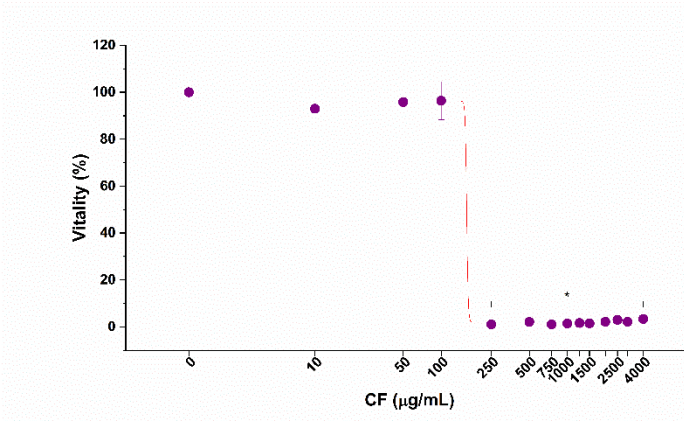

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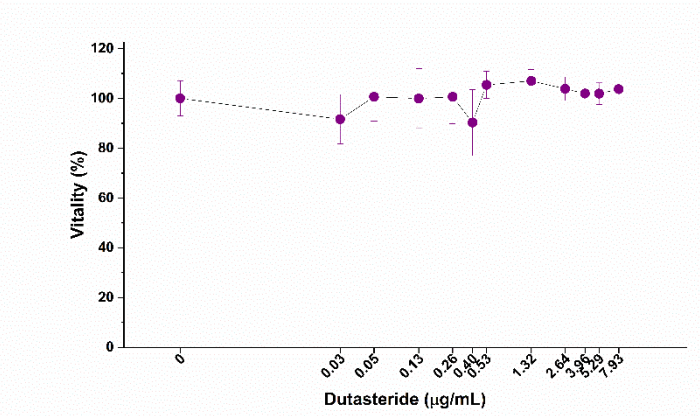


(E)

Figure 1. Impact of LENILUTS® (A and B), CF (C and D) and DutasteriI(E) on *in vitro* prostatic model vitality, following 6 and 24 h exposure. * p<0.05

3.3 Effect of LENILUTS®, CF and Dutasteride treatment on pro-inflammatory cytokine release from the in vitro prostatic model

In recent years, a direct correlation between inflammation and BPH development was highlighted [8, 9, 10]. LENILUTS®, CF and Dutasteride anti-inflammatory activity was assessed by measuring pro-inflammatory cytokine (IL-1β and TNF-α) release. Diclofenac, an anti-inflammatory drug, was used as positive control. As shown in Figure 2A and SM5, a significant reduction in IL-1β release, compared to inflamed and untreated in vitro prostatic model (11.0 ± 0.0 fold-change), was observed for LENILUTS® at both tested concentrations (about 5 and 1 fold-change at 250 and 500 µg/mL respectively), CF (7.7 ± 0.1 fold change) and diclofenac (9.7 ± 0.1 fold change) following 6 h treatment. LENILUTS®-induced IL-1β release reduction is significantly higher compared to CF, Dutasteride and diclofenac, with the latter being the less effective. LENILUTS® anti-inflammatory activity was further confirmed by TNF-α release (Figure 2B and SM5). Indeed, conversely to CF and diclofenac, LENILUTS®, compared to the inflamed, untreated control, reduced TNF-α release by 30 and 90 % at 250 and 500 µg/mL respectively. The higher LENILUTS® anti-inflammatory activity, compared to CF, is probably due to the synergic effect of the

phytotherapic agents contained in the formulation, such as Pinus spp. [19,20,23,24] and Curcuma longa [36,37].

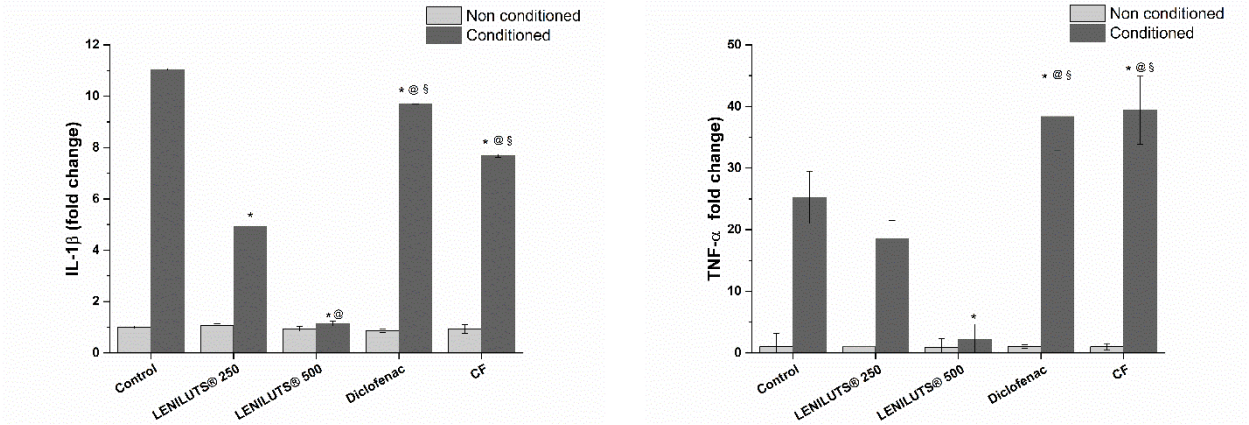


Figure 2. IL-1β (A) and TNF-α (B) release variation in inflamed LNCaP-based in vitro prostatic model before and after treatment with LENILUTS®, CF and Diclofenac (positive control). compared to control (Ctrl; untreated LNCaP cells). *p<0.05 vs Control, @ p<0.05 vs LENILUTS 250, § p<0.05 vs LENILUTS 500**.**

3.4 Pro-apoptotic activity of LENILUTS®, CF and Dutasteride

As detailed in the previous paragraph, BPH is the most common cause of LUTS development. This prostate enlargement is mainly due to the uncontrolled proliferation of prostatic cells. As a consequence, a formulation able to contain such proliferation, via cell death mechanisms like apoptosis and/or necrosis, may slow down the BPH onset and development. Considering the underlying inflammatory processes, it is also fundamental for such formulation to affect cell proliferation in an inflamed environment. To this aim we investigated the ability of LENILUTS®, CF and Finasteride to induce apoptosis and necrosis in both non-inflamed and inflamed in vitro prostatic model. Furthermore, to investigate the possible correlation between anti-inflammatory activity and cell death induction, the same inflammation protocol was maintained. The activation of key enzymes in the apoptotic signaling cascade (caspase 3 and 7) was used to assess pro-apoptotic activity induction by considered formulations and drugs.

LNCaP prostatic cells treated with 250 and 500 μg/mL of LENILUTS® showed, respectively, a 1.7 ± 0.2 and 3.9 ± 0.0 fold-change in caspase 3/7 activation compared to control while a lower, yet significant, increase was observed also for CF (1.9 ± 0.1 fold-change compared to control respectively) (Figure 3A and SM6). As shown in Figure 3B and SM6, the same trend was maintained in inflamed condition, even if with a milder caspase 3/7 activation (9.4 ± 1.2, 3.6 ± 0.5 and 7.9 ± 0.6 fold-change compared to inflamed, untreated in vitro prostatic model for LENILUTS®, CF and Finasteride respectively). As expected, the positive control of pro-apoptotic activity, staurosporine (STS), effectively induces apoptosis in both inflamed and non-inflamed condition.

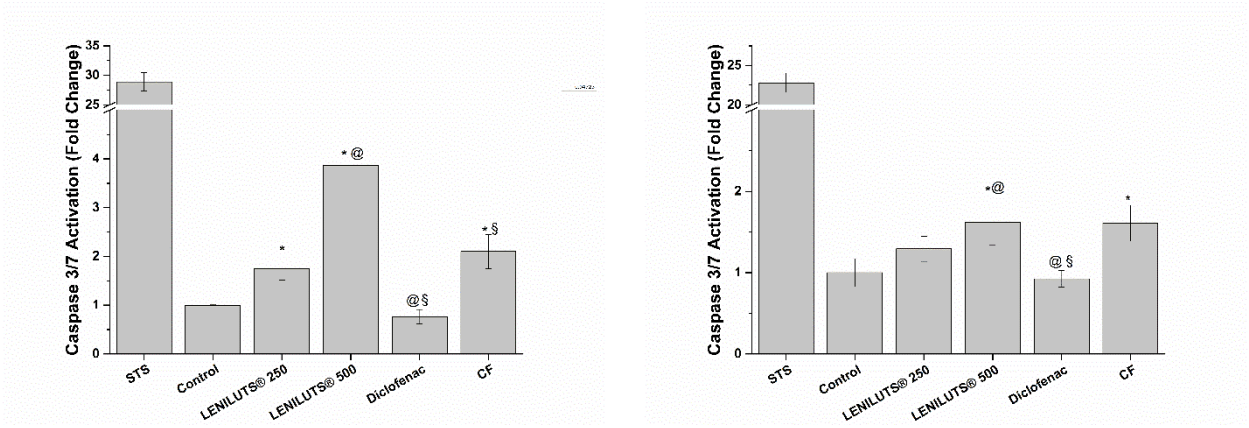


Figure 2. Caspase 3/7 activation compared to non-inflamed, untreated LNCaP cells (A, Ctrl) and inflamed, untreated LNCaP cells (B, Ctrl) following 6 h treatment with LENILUTS®, CF, Finasteride and staurosporine (STS, positive control). *p<0.05 vs Control, @ p<0.05 vs LENILUTS 250, § p<0.05 vs LENILUTS 500.

The activation of the apoptotic process in LNCaP prostatic cells by LENILUTS®, in inflamed or non-inflamed condition, may be explained by the curcumin presence among the active principles of the formulation. Indeed, curcumin and its active principles (i.e. curcuminoids), are well-known to induce apoptosis in tumoral cells [37]. Similarly, the S. repens extract contained in CF formulation is endowed with pro-apoptotic properties [38].

3.5 Impact of LENILUTS®, CF and Dutasteride treatment on in vitro prostatic model 5αR activity

The 5αR is fundamental in the insurgence and development of some prostatic pathologies (i.e. BPH). Indeed, it stimulates PSA production through dihydrotestosterone, a hormone characterized by a higher androgenic activity compared to testosterone itself [39]. As such, 5αR activity inhibition may be indicative of a positive effect at prostatic level. -As shown in Figure 4 and SM7, the addition of testosterone to 5αR, in presence of NADPH, stimulated the enzyme activity, leading to an increase in DHT synthesis compared to control. LENILUTS® showed to be more effective in reducing DHT production than CF (51.0 and 24.2 % reduction for LENILUTS® 250 µg/mL and CF respectively).

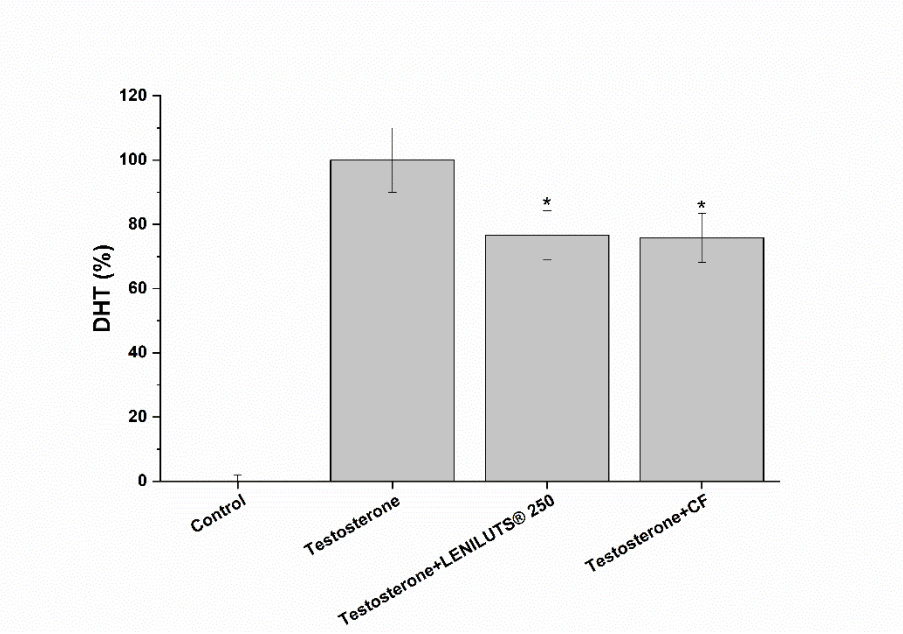


Figure 3. Percentage of DHT produceD by 5αR from testosterone reduction, calculated compared to control, in presence or absence of LENILUTS® and CF. *p<0.05 vs Testosterone

3.6 Effect of LENILUTS®, CF and Dutasteride on the release of PSA.

PSA is a protein released from the prostatic epithelium and its increase in the bloodstream is associated with the development of prostatic pathologies, like the BPH. As such, PSA is a useful marker to assess the potentially positive effect of a formulation at the prostatic level. Following stimulation with androgenic hormone DHT, a significative increase in PSA release (361.2 ± 11.9 % increase compared to untreated control) was observed in the prostatic in vitro model (Figure 5, SM8). Both LENILUTS® (500 μg/mL) and Dutasteride (5.9 μg/mL) significantly reduced PSA release, with LENILUTS® 500 μg/mL being the most effective (145.7 ± 12.5 % and 189.3 ± 19.7 % PSA release respectively, compared to 465.4 ± 31.8 % PSA release of the DHT-stimulated control) (Figure 5, SM8). Conversely, no decrease in PSA release from DHT-stimulated LNCaP cells was observed for CF. Effect of LENILUTS®, CF and Dutasteride on non DHT-stimulated LNCaP cells PSA release is reported in SM9 and SM10.

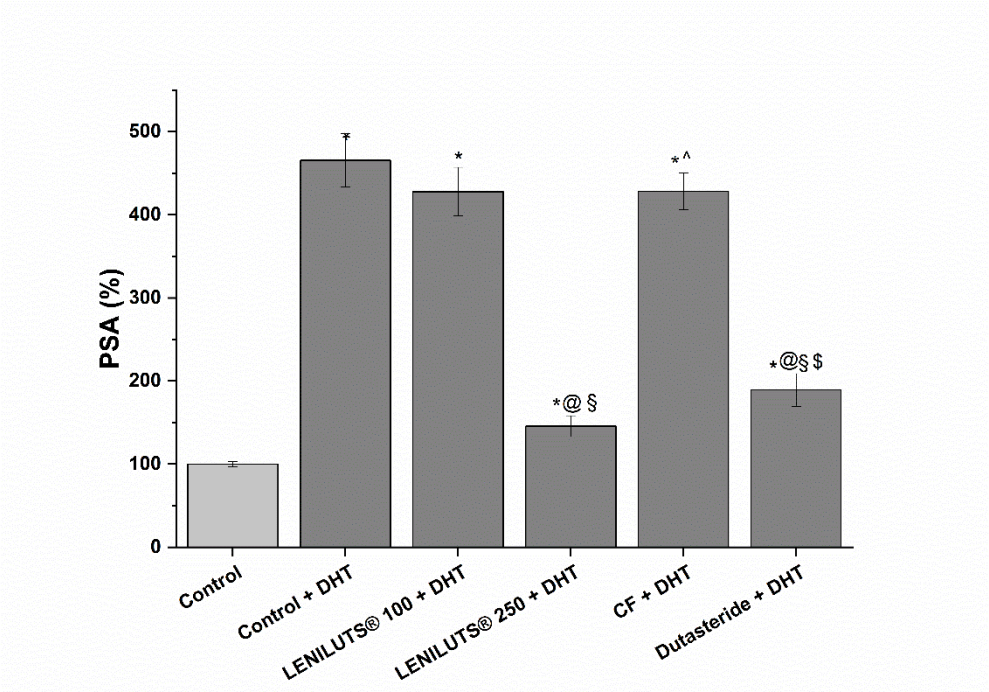


Figure 4. Prostate specific antigen release in LNCaP prostatic cells following stimulation with DHT and treatment with LENILUTS®, CF and Dutasteride. *p<0.05 vs Control, @ p<0.05 vs Control + DHT, § p<0.05 LENILUTS 100 + DHT, ^ p<0.05 vs LENILUTS 250 +DHT, $ p<0.05 vs CF + DHT. 5

3.7 LENILUTS® active principles bioaccessibility

The therapeutic application of active principles of plant origin, such as beta-sitosterol or curcumin, is hindered by their poor solubility in aqueous medium, like the digestive fluids. Even if their solubility during the digestive process is slightly improved by the emulsifying activity of bile salts, they are far from efficient. To investigate its active principle bioaccessibility, we exposed a single dose of LENILUTS® to in vitro digestion procedure mimicking human adulthood with the aim of evaluating the total amount of active principles and the apparent bioaccessible fraction (i.e. soluble part and released from its matrix), which includes the portion available for absorption. As shown in Table 2, the recovery of the active principles, calculated as the ratio between measured and expected active principles content, is 67.0 %, 4.6 % and 3.5 % for curcumin, beta-sitosterol and OPCs respectively, indicating that the active principles stability is affected by the digestive process. As a consequence of degradation and the lipophilic nature of the active principles, the bioaccessible fraction (i.e. supernatants) of the active principles is 5.1 % for curcumin (3.4 mg/dose), 17.1% for beta-sitosterol (0.7 mg/dose) and 26.6 % (0.2 mg/dose) for OPCs.

Table 2. Bioaccessibility of the active principles contained in LENILUTS® formulation following in vitro digestion. Data are expressed as mean ± standard deviation.

Active principle	Recovery (%)	Supernatant (%)
CURCUMIN	67.0 ± 5.4	5.1 ± 0.1
BETA-SITOSTEROL	4.6 ± 0.1	17.1 ± 1.0
OPCs	3.5 ± 0.3	26.6 ±

3.8 Impact of digested LENILUTS® on intestinal epithelium viability

Other than efficacy, therapeutic formulation must respond to another requirement, safety. Indeed, taking into consideration their dose and posology, formulations should not negatively impact the organism. In particular, damages to the intestinal epithelium must be avoided since this may lead to a decrease in absorption efficiency. As such, before

measuring its active principles' bioavailability, the impact of digested LENILUTS® on intestinal epithelium viability and integrity was assessed. To this aim, intestinal monolayers were exposed to increasing concentrations of the formulation bioaccessibile fraction (i.e., supernatant), and dose-response curves were obtained (Figure 6). As emerged from the dose-responses curve, LENILUTS® shows adverse effect on the intestinal epithelium starting from a concentration of 18.2 mg/mL. The formulation highest non-toxic concentration, 12.1 mg/mL, was considered for active principles bioavailability evaluation.

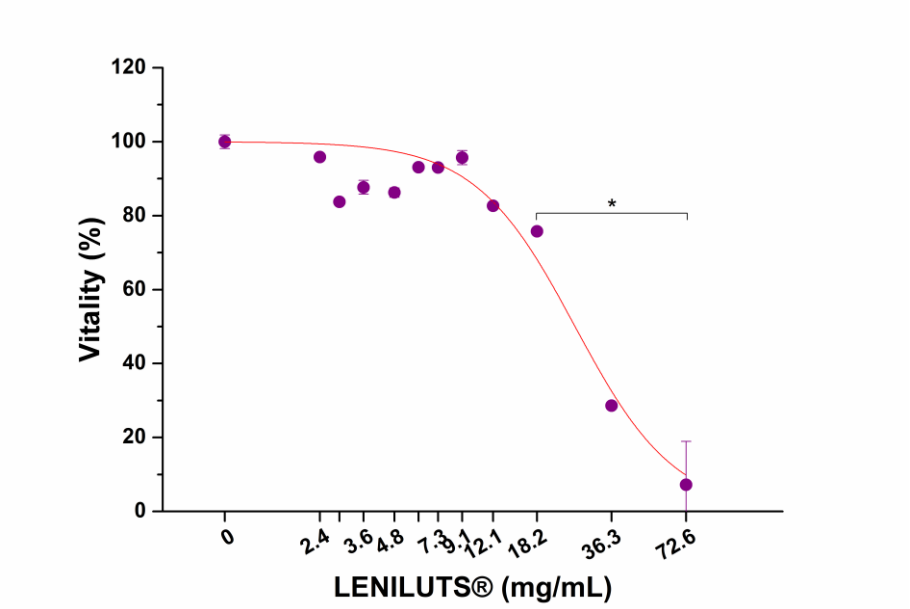


Figure 5. Impact of increasing concentration of digested LENILUTS® on intestinal mucosa viability evaluated by MTS assay. * p5

3.9 Active principles bioavailability

Based on the impact of digested LENILUTS® on intestinal epithelium viability and posology, we set experiments for determining beta-sitosterol, curcuminoids and OPCs bioavailability.

3.9.1 Curcumin

In Table 3 are resumed the curcumin bioavailability data, obtained following intestinal epithelium exposure to digested LENILUTS® for 1 and 3 h. After 1 h exposure, no absorption of curcumin was detected while a 1.7 % absorption, corresponding to 2.8 ± 0.3 ng/mL, was observed in the basolateral (serosal) compartment following 3 h treatment. Since one of the factors limiting curcumin bioavailability is its tendency to accumulate intracellularly before being released in the bloodstream [40], we measured the intracellular accumulation of curcumin at the intestinal epithelium level. As expected, a time-dependent accumulation of curcumin was observed, with a 4 time increase from 1 to 3 h of treatment (9.8 % to 32.2 % respectively) (Table 3).

Table 3. Curcumin bioavailability and cellular accumulation following intestinal epithelium exposure to LENILUTS® for 1 and 3 h. Results are expressed as percentage of absorption and concentration (mean ± standard deviation). N.d.: not determined

	1 h	**3 h**

Curcumin	Absorption (%)	Concentration (ng/mL)	Absorption (%)	Concentration (ng/mL)
Serosal	n.d.	n.d.	1.7 ± 0.1	2.8 ± 0.3
Intracellular	9.8 ± 3.8	1.9 ± 0.7	32.2 ± 4.0	35.0 ± 3.4
Absorbed		1.9		37.8

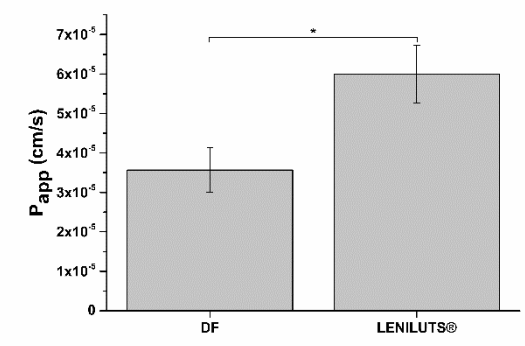
The overall absorption of curcumin from the bioaccessible fraction, considering both serosal compartment and intracellular concentration is 1.9 ng/mL and 37.8 ng/mL after 1 and 3 h exposure respectively (Table 3). As highlighted in Table 3, both beta-sitosterol and OPCs degraded during the digestive process (4.6 % and 3.5 % recovery). However, despite its low bioaccessible fraction, absorption at the basolateral (serosal) compartment was observed for beta –sitosterol following 3 h exposure of the intestinal epithelium to the digested LENILUTS® formulation (Table 4). Conversely, the absorbed concentration of OPCs was lower than the HPLC limit of detection (LOD). As a consequence, no OPCs were detected at the basolateral (serosal) compartment at both exposure time (i.e. 1 and 3 h).

Table 4. Beta-sitosterol bioavailability and cellular accumulation following intestinal epithelium exposure to LENILUTS® for 1 and 3 h. results are expressed as percentage of absorption and concentration (mean ± standard deviation). N.d.: not determined

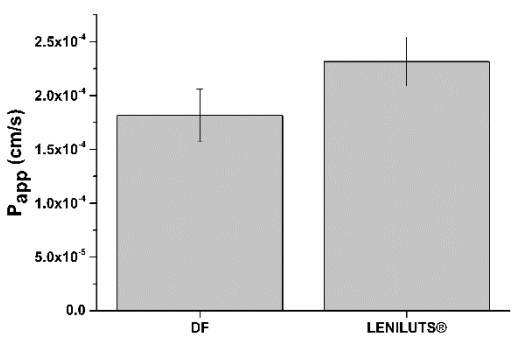
	1 h		3 h	
Beta-sitosterol	**Absorption (%)**	**Concentration (ng/mL)**	**Absorption (%)**	**Concentration (ng/mL)**
Serosal	n.d.	n.d.	62.0 ± 3.1	440.8 ± 66.2

3.10 Impact of digested formulations on intestinal mucosa viability and integrity

After exposure of intestinal epithelia to digested formulations, Caco-2 monolayer viability and barrier integrity were analysed. As expected from the performed dose-response curve, no significant viability reduction was observed following treatment at both considered time (i.e. 1 and 3 h) with tested formulations. Following 1 h exposure, LENILUTS® slightly increases intestinal epithelium apparent permeability (Papp) (Figure 7A), while no significant effect was observed after 3 h of treatment (Figure 7B).



(**A**)



(**B**)

Figure 7. Apparent permeability (Papp) of intestinal epithelium exposed to digestive fluids (DF; control) and diluted digested formulation for 1 (A) and 3 h (B). *p<0.05

As expected from its limited effect on the intestinal epithelium apparent permeability, digested LENILUTS® reduced TEER (trans-epithelial electrical potential) only temporarily, and its values fully recover within 24 h (Figure 8).

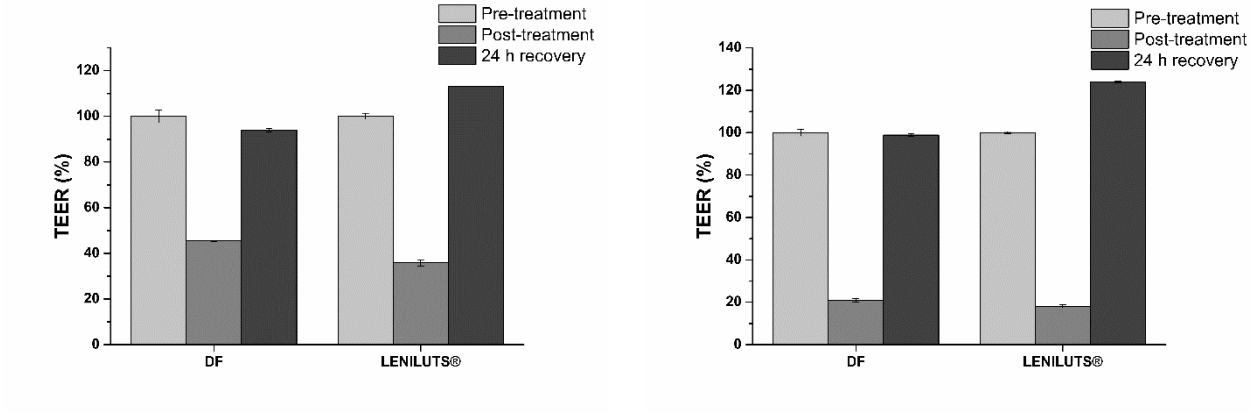


Figure 8. Trans-epithelial electrical resistance (TEER) trend following 1 and 3 h exposure to digested LENILUTS®.

4. Conclusions

Benign prostatic hyperplasia (BPH) is a term used to indicate benign growth of the prostate and it is histologically observed as new glandular or stromal growth. BPH is also used to describe a pathological condition associated with lower urinary tract symptoms (LUTS). The incidence of BPH-associated LUTS increases with each decade of life (beyond 40 years of age) and it represents a significant burden in aging men and they may impair quality of life [1]. BPH etiology is complex, underlying different mechanisms such as persistent and chronic inflammation, circulating hormonal level deregulation, aberrant wound repair processes, and steroid-mediated cell proliferation [4,7–9]. The use of plants and herbs extract for medicinal purposes (phytotherapy) including treatment of BPH has been growing steadily in most countries. The most widely used phytotherapic agent for the treatment of BPH is the extract of the American saw palmetto or dwarf palm plant, *Serenoa repens.* Several studies demonstrated that *Serenoa repens* exerts its biological activity through several mechanisms of actions including antiestrogenic and antiandrogenic effects, anti-inflammatory effects and a decrease in available sex hormone-binding globulin. Despite these plethora of cellular effects, *Serenoa repens* extract is able to mitigate only parts of BPH-related symptoms [14–16]. To improve BPH-related LUTS treatment, a new multi active principle-based formulation, LENILUTS®, have been proposed. Our *in vitro* approach, demonstrated, that the presence of multiple active principle improved the overall efficacy of LENILUTS® formulation, by enhancing its anti-inflammatory, anti-androgenic and pro-apoptotic activity. Indeed, our results clearly show that the association of multiple active principle decreases more efficiently the release of pro-inflammatory cytokine IL-1β and TNF-α, compared to *Serenoa repens* only-based formulations. Furthermore, compared to the *Serenoa repens*-based commercial formulation, LENILUTS® is more effective in reducing some BPH-connected symptoms, such as PSA release, while retaining a better safety towards the prostate. However, at present, LENILUTS® effectiveness is limited by its active principles' poor bioaccessibility and bioavailability. Consequently, further improvements in LENILUT® delivery technology are needed. In conclusion, LENILUTS® formulation, once the delivery technology will be improved and perfectioned,

might be useful in BPH and LUTs treatment, in particular compared to *Serenoa repens* only-based formulations.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

Author Contributions: Conceptualization, Federico Benetti and Andrea Fratter; Data curation, Erik Tedesco, Federico Benetti and Andrea Fratter; Formal analysis, Erik Tedesco and Federico Benetti; Investigation, Erik Tedesco and Simone Castelli; Methodology, Erik Tedesco, Federico Benetti and Simone Castelli; Project administration, Andrea Fratter; Supervision, Federico Benetti and Andrea Fratter; Writing – original draft, Erik Tedesco and Federico Benetti; Writing – review & editing, Erik Tedesco, Federico Benetti, Simone Castelli and Andrea Fratter. All authors have read and agreed to the published version of the manuscript.

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