European Review for Medical and Pharmacological Sciences 2021; 25: 3074-3082

Protective effects induced by the food supplement Fluxonorm® in the lower urinary tract

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Abstract. – OBJECTIVE: Fluxonorm® is a dietary supplement that includes water-soluble extracts of *Solidago virga-aurea*, *Phyllantus niruri*, *Epilobium angustifolium*, *Peumus boldus* and *Ononis spinosa*. The aim of the present study was to evaluate the tolerability and efficacy of Fluxonorm® in improving lower urinary tract symptoms in patients with benign prostatic hyperplasia (BPH) in combination with standard of care.

PATIENTS AND METHODS: Lower urinary tract symptoms can be improved by a marked anti-inflammatory action on the lower urinary tract (irritative symptoms) and/or by an anti-proliferative action (obstructive symptoms) on the prostate. Thirty patients were enrolled to evaluate the effect of Fluxonorm® on improving lower urinary tract symptoms. All patients complained of lower urinary tract symptoms (LUTS), such as hesitancy, poor flow, intermittent flow, incomplete voiding (obstructive symptoms), as well as increased frequency, nocturia and urgency (storage symptoms). All patients were treated with one tablet of Fluxonorm® (1200 mg) daily for 30 days to corroborate the results of our observation in which the food supplement (800 μg/mL) was also studied on the human prostate cancer PC3 cell line (antiproliferative activity) and on prostaglandin (PG)E2 production (anti-inflammatory activity). In addition, the effect of this compound on cyclooxygenase-2 (COX-2) gene expression was investigated. Finally, a bioinformatic analysis was conducted with the aim of unravelling the mechanism of action underlying the observed bio-pharmacological effects.

RESULTS: As hypothesized in our preclinical research, adding Fluxonorm® to the therapy of enrolled patients improved all studied clinical parameters, including maximum flow (Qmax), after one month of treatment. In the preclinical evaluation, this formulation reduced PC3 cell viability and PGE2 production. The effects were also paralleled by reduced COX-2 gene expression and Fluxonorm®'s partly related content of catechin. While docking studies pointed out to the putative inhibition of matrix metalloproteinse-2 by gallic acid, as a further mechanism underlying the observed anti-proliferative effects, in PC3 cells exposed to Fluxonorm®.

CONCLUSIONS: Fluxonorm® improved the efficacy of standard therapy, in terms of antioxidant/anti-inflammatory effects, for the management of lower urinary tract symptoms (LUTS). This could be related, albeit partially, to the blunting effect of this compound on PGE2 production.

Key Words:
Solidago virga-aurea, *Phyllantus niruri*, *Epilobium angustifolium*, *Peumus boldus*, *Ononis spinosa*, Lower urinary tract, Anti-proliferative, Prostaglandin E2, Matrix metalloproteinase-2, Docking.

Introduction

Benign prostatic hyperplasia and inflammation of the lower urinary tract (LUTS) may be common disorders in men after the age of fifty[1-3]. α-Blockers are first-choice drugs for treating LUTS, especially in patients suffering from prostatitis and benign prostate hyperplasia (BPH)[4]. However, numerous herbal extracts, especially in pharmacological associations, revealed efficacious in reducing the burden of oxidative stress and inflammation that characterizes these disorders[5-12]. The efficacy was also paralleled by a low grade of side effects, especially when the phytotherapy remedies were prepared with biocompatible solvents, namely water and hydroalcoholic solutions[13,14].

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Fluxonorm® is a novel supplement based on water extracts from *Phyllantus niruri, Solidago virga-aurea, Peumus boldus, Ononis spinosa* and *Epilobium angustifolium*. All aforementioned plants have traditional uses in the management of inflammatory and/or infectious disorders, in the urinary tract, although the mechanisms underlying these effects are still matter of debate[15-21]. Additionally, there is still lack in scientific about the clinical efficacy. In a recent paper[22], the phytochemical composition of the water extracts of *P. niruri, S. virga-aurea, P. boldus, O. spinosa* and *E. angustifolium* was explored through HPLC-UV-MS technique. The results showed the catechin as the common prominent phenolic compound, in all tested extract. All single extracts and the formulation revealed effective in reducing prostaglandin production in an *ex vivo* model of prostate inflammation, although with different potency. Additionally, *in silico* components-targets and docking studies confirmed the catechin as the main phenolic compound responsible of the observed anti-inflammatory effects.

In this context, the aim of the present study was to explore the efficacy of Fluxonorm® in multiple models of lower urinary tract inflammation, with particular regards to BPH, in a clinical trial, and antiproliferative effects on human prostate cancer (PC3) cell line, *in vitro*. Basing on our recent investigation of the phenolic composition of food supplement, a bioinformatics analysis was conducted for unravelling the mechanism of action underlying the observed bio-pharmacological effects, as well. The obtained results support the rationale for this formulation use in the clinical management of LUTS.

Patients and Methods

Plant Material

Water soluble extracts of *P. niruri, O. spinosa*, *S. virga-aurea, P. boldus, E. angustifolium* and the registered trademark formula Fluxonorm® (*O. spinosa*/*S. virga-aurea*/*P. niruri*/*P. boldus*/*E. angustifolium* 12.5:12.5:18.7:25.0:31.2), were kindly provided as dried materials by Omega Pharma S.r.l. (Cantù, Italy). These extracts were rehydrated in bidistilled water, as previously described[22].

Cell Culture and Viability Test

The effects of Fluxonorm® (800 μg/mL) on the human prostate PC3 cancer cell viability were determined through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. The viability was measured in basal conditions and after exposure to serotonin (5-HT) 100 nM. The experimental conditions were fully described in our previous paper[23-25].

PGE2 Radioimmunoassay

As reported in our previous study[26], the level of prostaglandin (PG)E_2 in cell medium were determined by radioimmunoassay.

RNA Extraction, Reverse Transcription and Real-Time Reverse Transcription Polymerase Chain Reaction (Real-Time RT PCR)

Total RNA was extracted from the PC3 cells using TRI Reagent (Sigma-Aldrich, Milan, Italy), according to the manufacturer's protocol. Contaminating DNA was re-moved using 2 units of RNase-free DNase 1 (DNA-free kit, Ambion, Austin, TX, USA). The RNA concentration was quantified at 260 nm by spectrophotometer reading (Bio-Photometer, Eppendorf, Hamburg, Germany), and its purity was assessed by the ratio at 260 and 280 nm readings. The quality of the extracted RNA samples was also determined by electrophoresis through agarose gels and staining with ethidium bromide, under UV light. One microgram of total RNA extracted from each sample in a 20 μL re-action volume was reverse transcribed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA, USA). Reactions were incubated in a 2720 Thermal Cycler (Thermo Fisher Scientific, Waltham, MA, USA) initially at 25°C for 10 min, then, at 37°C for 120 min and finally at 85°C for 5 s. Gene expression of COX-2 was determined by quantitative real-time PCR using TaqMan probe-based chemistry. PCR primers and TaqMan probes, including β-actin used as the housekeeping gene, were purchased from Thermo Fisher Scientific [Assays-on-Demand Gene Expression Products, Mm00478374_m1 for cyclooxygenase-2 (COX-2) gene; Hs99999903_m1 for β-actin gene]. The real-time PCR was carried out in triplicate for each cDNA sample. Data were elaborated with the Sequence Detection System (SDS) software version 2.3 (Thermo Fisher Scientific, Waltham, MA, USA). Gene expression was relatively quantified by the comparative $2^{-\Delta\Delta Ct}$ method.

In Silico Molecular Docking

Docking calculations were conducted through the Autodock Vina of PyRx 0.8 software. The

crystal structures of the target proteins were derived from Protein Data Bank (PDB). The PDB code was: 1CK7 for matrix metalloproteinase-2 (MMP-2). Details about docking calculations are described in our recent paper[27].

Clinical Study

A total of 30 patients were enrolled for this study. The study was conducted in the Clinical Center "Vita Salus" (Isernia, Italy), in the period 1-31 August 2019. The informed consent of all 30 patients was collected by Dr. F. Neri, before the beginning of the study. An Internal Review Board approved the study protocol that was designed and conducted according to all good clinical practice criteria. All the patients complained of lower urinary tracts symptoms (LUTS), such as hesitancy, poor stream, intermittent flow, incomplete emptying (obstructive symptoms), as well as increased frequency, nocturia and urgency (storage symptoms). Inclusion criteria included: patients with LUTS requiring α-blocker, patients with LUTS not eligible to therapy with α-blocker or not willing to start α-blocker treatment due to possible adverse events, patients not responsive to α-blockers (as for example patients who required urethral catheterization even when treated with α-blocker). All patients underwent physical examination, with rectal examination in order to exclude prostate cancer. All patients answered the International Prostatic Symptoms Score (IPSS), a validated questionnaire. The eighth question was used to assess quality of life (QoL). Moreover, patients underwent uroflowmetry and the maximum flow (Qmax) was noted. All the patients were treated with one tablet of Fluxonorm® (1200 mg) daily for 30 days. At the end of the treatment, all tests were repeated. Moreover, 20 patients underwent urine analyses including pH and specific urine weight before and after the treatment.

Statistical Analysis

The *in vitro* experimental data were analyzed through the analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test. The GraphPad Prism software was employed for the statistical analysis (La Jolla, CA, USA). Regarding the clinical study, differences before and after treatment with Fluxonorm® in terms of Qmax, IPSS score and QoL were evaluated with the Wilcoxon test for paired values. All tests were two tailed. $p<0.05$ was considered statistically significant.

Results

Fluxonorm® treatment (800 μg/mL) induced a significant reduction of PC3 cell viability, in both basal and proliferative conditions induced by the challenging with serotonin (5-HT) 100 ng/mL (Figure 1).

In PC3 cells, the food supplement (800 μg/mL) was also able to inhibit the release of PGE_2 (Figure 2) and the gene expression of COX-2 (Figure 3). In Figure 4, the putative interactions between gallic acid, one of the most prominent phytocompound in the whole food supplement, and matrix metalloproteinase-2 (MMP-2) are described. The virtual screening experiment demonstrated the micromolar (24.4 μM) affinity of gallic acid with the docked enzyme.

While Table I and Table II report the main characteristics of included patients and the evaluated parameters following Fluxonorm® (1200 mg/die) administration, respectively. Specifically, Table II shows the improvement of IPSS, Qmax, and QoL parameters. More specifically, the median Qmax improved from 12.0 to 14.0 ml/s (Figure 5A). Moreover,

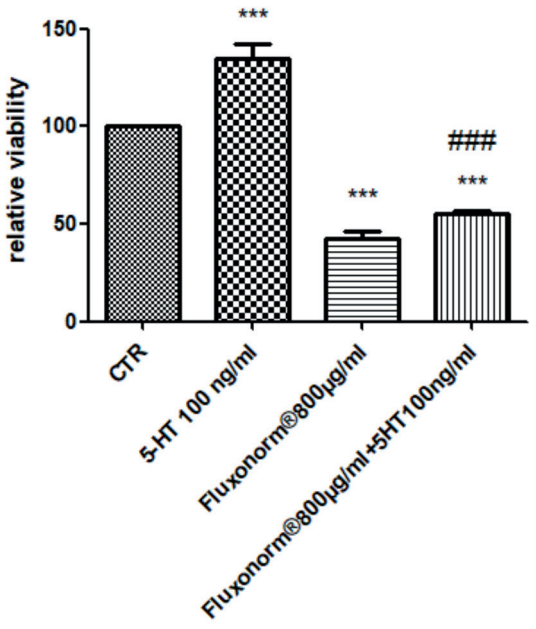

Figure 1. Antiproliferative effects induced by Fluxonorm® 800 μg/mL in human prostate cancer PC3 cell line in basal and serotonin (5-HT: 100 ng/mL)-induced proliferative conditions. ANOVA, $p<0.0001$, ***$p<0.001$ *vs.* CTR, ###$p<0.001$ *vs.* 5-HT 100 ng/mL.

Table I. Main baseline characteristics of included patients (n = 30 patients).

	Median (IQR), N (%)
Age (years)	67.0 (55.0-72.5)
Prostate volume (mL)	40.0 (29.0-62.5)
Medical therapies at baseline	
α-blocker	9 (75.0%)
Combination	2 (16.7%)
5-α-reductase	1 (8.3%)
Medical therapies associated with Fluxonorm®	
α-blocker	10 (66.7%)
Combination	5 (33.3 %)
Bladder catheter history	2 (8.0%)

the median IPSS reduced from 19.5 to 9.5 points (Figure 5B). Finally, the median QoL score improved from 5.0 to 2.0 points (Figure 5C). One patient reported hypotension for the use of the α-blocker whereas one of the patients that was receiving 5α-reductase inhibitor suspended it because not-tolerant to the drug and willing to suspend it. For the assessment of adverse events, we relied on "Common Terminology Criteria for Adverse Events methods". Any abnormal clinical finding temporally associated with the use of Fluxonorm® was considered as an adverse event. No statistically significant variation in the urinary pH and urinary specific weight was recorded (data not shown).

Discussion

In the present research, Fluxonorm® 800 µg/mL was tested in PC3 cells in both basal and after challenging the cells with 5-HT 100 ng/mL. Besides, being a well-known neurotransmitter in the brain, 5-HT acts as pro-inflammatory agent in the periphery[28]. Additionally, several investigations suggest the capability of 5-HT to increase the viability of tumoral cells[29]. Recently, we demonstrated[24,25] the mitogen effect of 5-HT in colon cancer HCT116 cells, whereas herbal extracts proved efficacious in preventing the cancer proliferation induced by the 5-HT. The antiproliferative effects induced by Fluxonorm® suggest protective effects against prostate cancer onset. Considering our previous findings of reduced PGE_2 production in isolated prostate specimens challenged with the pro-inflammatory lipopolysaccharide stimulus, we suggest that

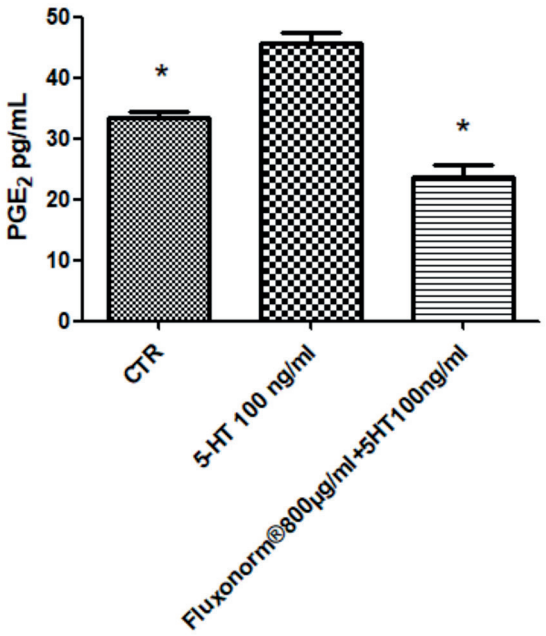

Figure 2. Inhibitory effects on PGE2 synthesis induced by Fluxonorm® 800 µg/mL in human prostate cancer PC3 cell line challenged with serotonin (5-HT: 100 ng/mL). ANOVA, $p<0.01$, *$p<0.05$ *vs.* 5-HT 100 ng/mL.

Table II. Main results after treatment. Wilcoxon ranked test for paired data; Quality of life is referred to question n° 8 of the International prostatic symptoms score.

	Before Fluxonorm® Median (IQR)	After Fluxonorm® Median (IQR)	*p*-value
Qmax (mL/s)	12.0 (6.5, 14)	14 (22.7, 16.1)	<0.001
International Prostatic Symptoms Score (IPSS)	19.5 (16.1, 25)	9.5 (4.8, 12.3)	<0.001
Quality of life (QoL)	5.0 (4.0, 5.8)	2.0 (1.0, 3.0)	<0.001
Combination	5 (33.3 %)		
Bladder catheter history	2 (8.0%)		

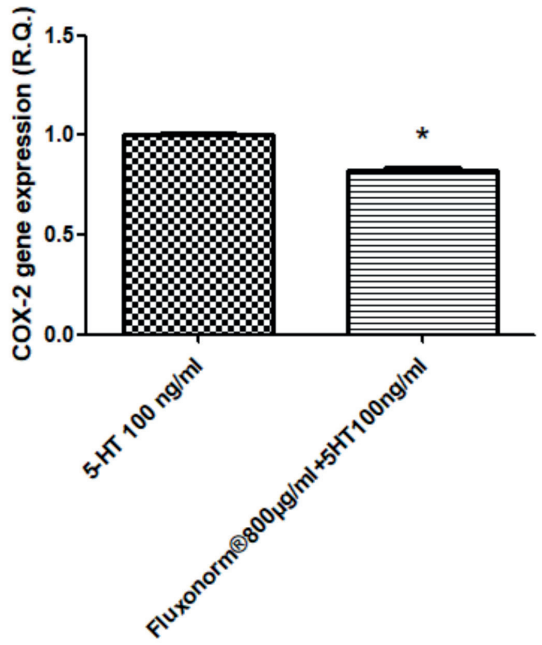

Figure 3. Inhibitory effects on COX-2 gene expression induced by Fluxonorm® 800 µg/mL in human prostate cancer PC3 cell line challenged serotonin (5-HT: 100 ng/mL). *$p<0.05$ *vs.* 5-HT 100 ng/mL.

the reduction of PGE_2 production could be one of the main factors influencing the observed antiproliferative effects, in PC3 cells. PGE_2 is the main COX-2-deriving prostanoind[30], whose levels were increased in prostate and kidney, in both inflammatory conditions and cancer[31,32]. This is consistent with the antiapoptotic properties of this prostanoid. In this regard, in the present study we also measured the Fluxonorm® effects on both PGE_2 production and COX-2 gene expression in PC3 cells exposed to 5-HT. The 5-HT stimulus increased both PGE_2 level and COX-2 gene expression, in PC3 cells, compared to the untreated control group. This is consistent, albeit partially, with the aforementioned increase of cell proliferation. By contrast, the Fluxonorm® treatment blunted both 5-HT-induced PGE_2 production and COX-2 gene expression, thus partly corroborating the PGE_2 as a putative antiapoptotic factor underlying the PC3 cell proliferation[33]. Currently, literature data[22,34] suggest that the content in catechin could be responsible of the reduced PGE_2 production, in the prostate. Additionally, catechins were found effective in reducing PC3 cell viability, possibly through the stimulation of apoptotic pathways[35,36]. Our experimental results suggest the PGE_2 as a reliable biomarker to predict anti-inflammatory and pro-apoptotic effects exerted by Fluxonorm® administration, *in vitro*. However, considering the phenolic composition of the herbal extracts included in the Fluxonorm® formula, we cannot exclude further mechanisms involved in the observed anti-inflammatory and anti-proliferative effects. In our recent study[22], the targets-components analysis conducted *via* the bioinformatics platform STITCH (http://stitch.embl.de/) highlighted, besides the capability of catechin to interact with COX-2, also putative interactions of gallic acid, with matrix metalloproteinase-2 (MMP-2). Recent studies[37,38] also pointed out the inhibition of MMP-2 as one of the mechanisms underlying the reduced PC3 cell viability induced by natural compounds. The present docking results showed that gallic acid could form hydrogen bonding interactions with the catalytic site of the MMP-2, with a putative affinity in the micromolar scale. This further suggests the cooperation of multiple mechanisms underlying the observed bio-pharmacological effects. Nevertheless, the evaluation of PGE_2 level in PC3 cells remains a cornerstone, in the present

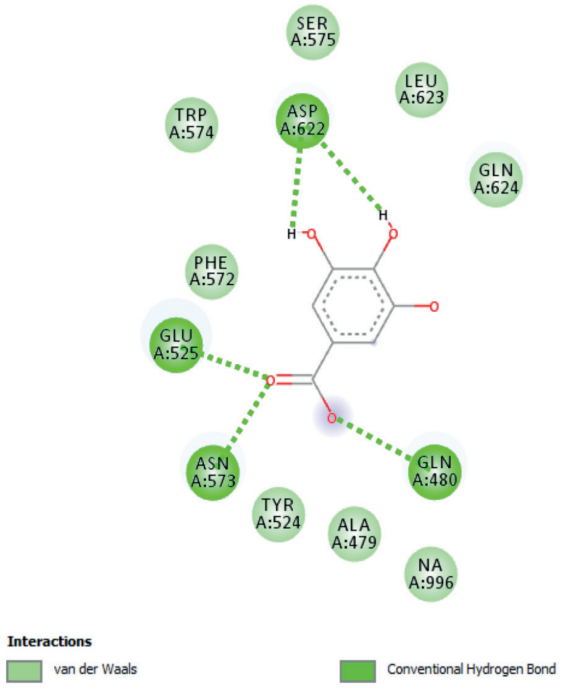

Figure 4. Putative interactions between gallic acid and matrix metalloproteinase-2 (MMP-2; PDB: 1CK7). Free energy of binding (ΔG) and affinity (Ki) are −6.3 kcal/mol and 24.4 µM, respectively.

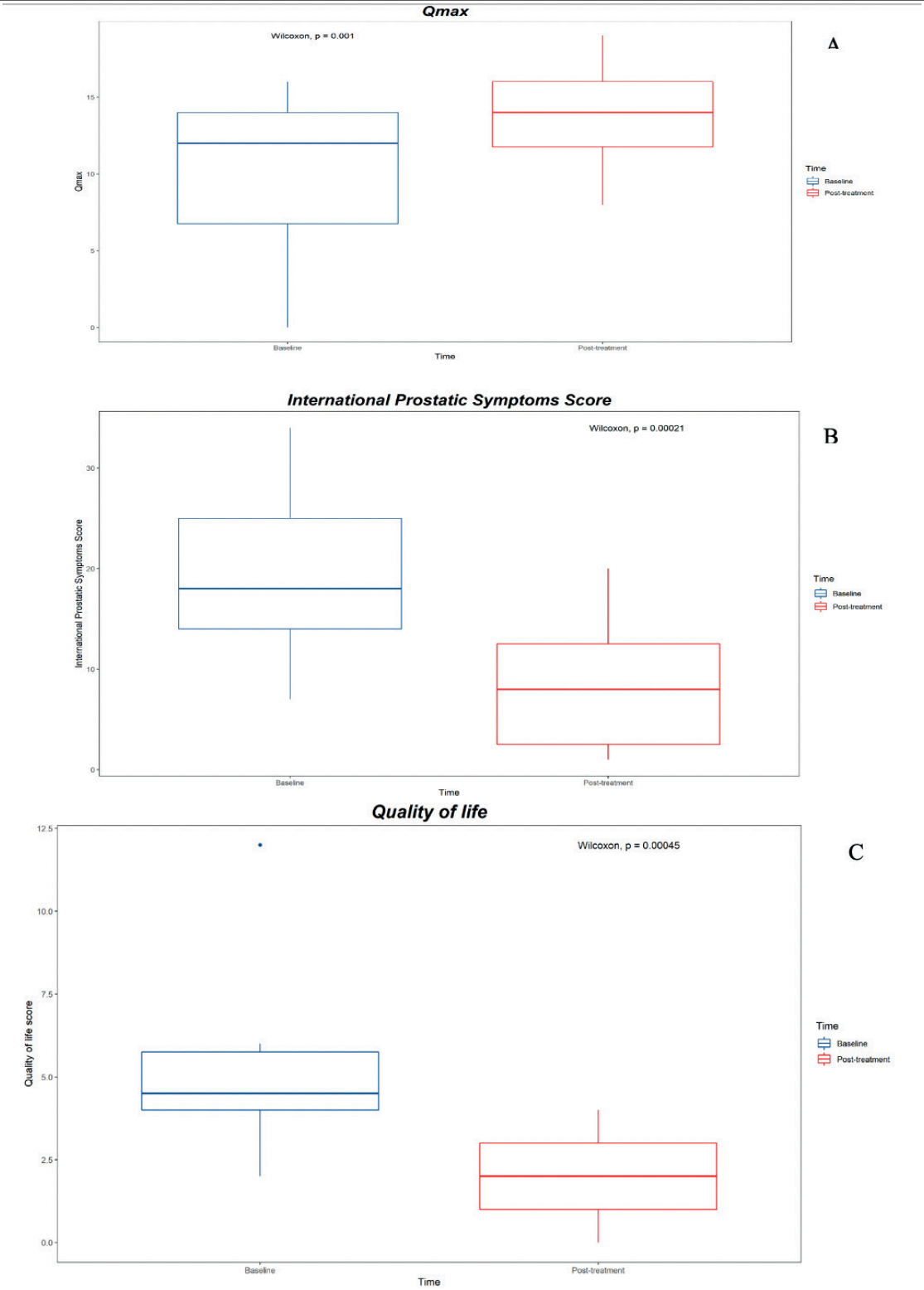

Figure 5. (A-C). All patients underwent physical examination, with rectal examination in order to exclude prostate cancer. All patients answered to the International Prostatic Symptoms Score (IPSS), a validated questionnaire. The eighth question was used to assess quality of life (QoL). Moreover, patients underwent uroflowmetry and the maximum flow (Qmax) was noted. All patients were treated with one tablet of Fluxonorm® daily for 30 days. At the end of the treatment, all tests were repeated. Descriptive statistics relied on median and interquartile range (IQR) for continuously coded covariates and on absolute and relative frequencies for categorical covariates. Differences before and after treatment with Fluxonorm® in terms of Qmax **(A)**, IPSS score **(B)**, and QoL **(C)** were evaluated with the Wilcoxon test for paired values. All tests were two tailed. $p<0.05$ was considered statistically significant.

study, also considering the clinical relevance of this biomarker, whose urinary levels were increased in patients suffering from LUTS[39].

Considering the efficacy as antiproliferative and anti-inflammatory agent, in PC3 cells, Fluxonorm® was clinically tested on a cohort of 30 patients suffering from LUTS. The median age was 62.0 (IQR 52.5-70.5) years. The median prostate volume was 40.0 (IQR 29.0-60.0) mL. Of all, two patients (8.0%) had an history of urethral catherization. Moreover, 12 patients (40%) were already treated with α-blocker, 5α-reductase inhibitor or both. In the selected cohort, 4 patients (10.0%) not receiving any treatment were treated with Fluxonorm® in association to the standard of care. Two patients started a triple treatment with combinations of 5α-reductase inhibitor, α-blocker, and Fluxonorm® and other two patients received the sole food supplement in association to the α-blocker. After one month of treatment, all studied parameters improved, and overall Fluxonorm® had a good safety profile. Indeed, in patients treated with the sole food supplement no adverse event was reported. Moreover, the study suggests that this formulation could be an effective adjuvant treatment for LUTS. In particular, the association of the extracts was effective on the dynamic component of the obstruction improving the α-blocker effect of the standard therapy when used in association or exercising a putative α-blocker action by itself. As a concluding remark to highlight the protective effects displayed by the food supplement assayed in the present research, it is of noteworthy interest to mention the clinical study carried out by Micali et al[40], that evidenced a significant reduction in prostate-specific antigen, following green tea catechin administration. Another analogy with the present study was the good tolerability, as well, that further supports the use of catechin-rich food supplements, such as Fluxonorm®, in order to improve the efficacy of the standard pharmacological therapy, in terms of antioxidant/anti-inflammatory response, in the LUTS.

Conclusions

Shortly, Fluxonorm® improved the efficacy of standard therapy, in terms of antioxidant/anti-inflammatory effects, for the management of lower urinary tract symptoms (LUTS). This could be related, albeit partially, to the blunting effect of this supplement on PGE_2 production.

Authors' Contributions

Conceptualization, M.P., G.O. and F.N.; methodology, C.F., L.M., F.C.; software, L.M.; validation, C.F., L.M., F.C., G.Z.; formal analysis, C.F., G.P.; investigation, A.C., S.L., M.Po., F.C., F.N.; resources, G.O.; data curation, C.F., G.O.; writing–original draft preparation, C.F., G.O.; writing–review and editing, L.M., C.F., G.O – this paper was also read by an English language mother-tongue individual; visualization, L.B., G.Z.; supervision, L.B.; project administration, M.P., L.M., G.O., C.F., F.N.; funding acquisition, M.P.

Funding

This work was supported by a grant from Omega Pharma S.r.l. (Cantù, Italy) within the project entitled "Preclinical and clinical investigations on the food supplement Fluxonorm®" (Coordinators: Luigi Menghini, Giustino Orlando, Claudio Ferrante, Massimiliano Petrucci, Fulvio Cesaroni).

Conflict of Interest

Dr. Massimiliano Petrucci, belonging to the company "Omega Pharma S.r.l.", is also co-author of the manuscript. We state that Dr. Petrucci designed the Fluxonorm® formula, although he did not exert any active role in the experimental procedures, that were conducted by the sole experimenters, independently. Before submission, all co-authors approved the manuscript.

Acknowledgements

The authors would like to express their gratitude to Dr. Renato C. Barbacane, for his careful and professional English language editing.

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