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Intracellular Distribution and Biological Effects of Phytochemicals in a Sex Steroid-Sensitive Model of Human Prostate Adenocarcinoma

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Abstract: Prostate function is critical for male fertility and its well-known oncological biomarker, namely Prostate-Specific Antigen (PSA), can be also used to monitor prostate epithelial human cells upon treatment with pharmaceutical drugs or natural bioactive compounds.

The LNCaP human prostate cell line was previously set up as a model system to investigate chemicals affecting prostate epithelium functionality by means of a tiered approach integrating two different toxicological endpoints, cell viability (MTS) and PSA secretion assays. Here, the same approach has been used to characterize the biological effects of phytochemicals on prostate epithelium. The anti-androgenic ability of phytochemicals to inhibit DHT-induced PSA secretion has been investigated also characterizing their intracellular distribution, in the presence or absence of sex steroids. Intracellular distribution allows to verify whether and to which extent each phytochemical is able to enter the cell and to reach the nucleus, the latter being the target of the supposed transcriptional modulatory activity upon phytochemicals' binding to sex steroid receptors.

Some phytochemicals, supposed to have a role in the functionality of the prostate epithelium, have been tested in a dose-dependent manner in both MTS and PSA secretion assays. In parallel, to establish the "effective concentration", in comparison to the "nominal one", the intracellular amount of each phytochemical has been assessed upon cell fractionation of LNCaP-treated cells and subsequent chromatographic measurements.

Keywords: Androgen receptor, biomarker, cytotoxicity, flavonoids, intracellular distribution, nominal *vs* effective concentration, PSA secretion, testing strategy.

INTRODUCTION

Phytochemicals are small molecules, frequently secondary metabolites, such as flavonoids, phenolic acids and glucosinolates and they are present as dietary components of food [1]. Their role in plants is mostly devoted to a defense role against (a)biotic stresses. As suggested by epidemiological studies, phytochemicals became of interest for human well-being as having a potential defense role in human health, in particular against many endocrine-related diseases [1-3].

Flavonoids are a vast group of heterogeneous polyphenols with various health benefits [2-8] which are ubiquitously found in fruits, vegetables, tea and wine. They share a chemical structure formed by two aromatic rings, linked together by 3 carbon atoms which form an oxygenated heterocycle. Flavonoids can be further divided into six main subclasses - flavonols, flavones, flavanones, flavan-3-ols, anthocyanidins and isoflavones - depending on the type of heterocycle involved. Interest in the anti-carcinogenic effects of flavonoids has emerged from *in vitro* and *in vivo* experimental evidence on cancer processes such as proliferation, inflammation, angiogenesis, invasion and metastasis [9-12].

Indeed, many phytochemicals have been suggested as chemo-preventive agents in cancer treatment, with a similar role as androgen inhibitors and/or anti-oxidant and anti-inflammatory drugs. The major epidemiological and clinical studies advocating the role of phytochemicals, such as silibinin, green tea polyphenols, genistein, curcumin, lycopene, selenium and Vitamin E, along with pharmaceutical androgen inhibitors, have been explored showing great promise in preventing prostate cancer (PCa), although many of them (*e.g.*, flavonoids) have limitations due to their poor bioavailability [13]. In any case, a majority of the attempted clinical trials suggested positive outcomes supporting their role as preventive agents despite their incoherency in study designs and data interpretations of neutral or negative results. Hence, a better understanding of the ideal target population, time of intervention, appropriate dosage and extent of intervention is required in order to facilitate their clinical applicability [13].

Prostate function is critical for male fertility since its secreted product, the prostatic fluid, provides essential molecules to ensure the sperm activation and capacitation of the male ejaculates in all mammals [14-16]. The prostatic fluid contains mainly zinc, citrate and kallikreins (KLKs). KLKs are extracellular serine proteases including KLK3, known as Prostate-Specific Antigen/(PSA). PSA secretion is a well-established oncological biomarker that has been shown to act also as a toxicological biomarker when applied to monitor human immortalized prostate epithelium cells treated with environmental and dietary contaminants [17,18], pharmaceutical drugs [18,19] and/or phytochemicals [20].

LNCaP cells have features of normal prostate epithelial cells and are androgen-responsive and PSA-secreting [21,22]. They have been shown to be also responsive to environmental and dietary contaminants with androgen- and estrogen-like activities [18,23]. Hence, such an *in vitro* model system has been previously used to successfully screen, in a double-blind experiment, chemicals affecting male fertility altering the 5α-dihydrotestosterone (DHT)-mediated PSA secretion upon a two-step experimental approach integrating an aspecific toxicological endpoint (cell viability and indirect proliferation by MTS assay) and a cell, prostate-specific toxicological endpoint (the PSA secretion assay) [18].

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Here, the same approach has been used to characterize the biological effects of some relevant phytochemicals, commonly found in human diets [11,12,24-26].

Some phytochemicals, supposed to have a role in the functionality of the prostate epithelium, have been tested in a dose-dependent manner in both MTS and PSA secretion assays. The selected phytochemicals, namely apigenin/API, genistein/GEN, luteolin/LUT, naringenin/NRG, quercetin/QRC and resveratrol/RESV have been implicated in: i) the prevention of DHT-induced PSA secretion [18,27], and ii) the induction of apoptosis of prostate tumor cell lines and the inhibition of prostate cell proliferation [28-31]. Some of them are under investigation in phase II clinical trials [13,32]. Despite this, the *in vitro* studies performed so far usually suggest an effective concentration of phytochemicals in the µM range that are not feasible to be reached by regular consumption of fruits and vegetables. Hence, we decided to assess in LNCaP cells: i) their role in both MTS and PSA secretion assays applying a dose-dependent range of concentrations (between 1pM and 100µM), and ii) their intracellular distribution, by cell fractionation and subsequent chromatographic measurements, to establish if the "nominal concentration" of each tested phytochemical correspond to the "effective concentration" at both intracellular and subcellular level.

The obtained results showed that LNCaP treatments with 17β-estradiol (E2) and flavonoids partially inhibit, in a non-linear dose-response manner, the DHT-induced PSA secretion. Furthermore, the "effective concentration" of both sex steroids and flavonoids was always different from the "nominal concentration" and depended on the co-treatment with either flavonoids or sex steroids. At the subcellular level, finally, it was evidenced that flavonoids and sex steroids localized mainly at the microsomal fraction/MF and, to a minor extent, within the nuclear fraction/N. Among flavonoids, the amount of NRG, RESV, GEN, API and LUT at the nuclear level is similar to DHT, whereas QRC reached the nucleus to an extent similar to E2.

MATERIALS AND METHODS

Chemicals

Chemicals used in this study are: DHT (CAS no. 521-18-6, purity ≥99.0%) (the endogenous androgen and AR-agonist) and the polyphenols resveratrol (CAS no. 501-36-0, purity ≥99.0%), naringenin (CAS no. 67604-48-2, purity ≥95.0%) were purchased from Fluka (Munich, Germany); 17β-estradiol (CAS no. 50-28-2, purity ≥98.0%) (the endogenus estrogen and estrogen receptor/ER-agonist) and the polyphenols apigenin (CAS no. 520-36-5, purity ≥95.0%), genistein (CAS no. 466-72-0, purity ~98.0%) and quercetin (CAS no. 117-39-5, purity ≥95.0%) were purchased from Sigma-Aldrich (Munich, Germany). Luteolin (CAS no. 491-70-3, purity ≥ 99.0%) was purchased from Extrasynthese (Genay, France). All solvents were high-performance liquid chromatography (HPLC) grade, and all water was ultrapure grade.

Cell Culture and Reagents

Human metastatic prostate adenocarcinoma LNCaP cells (clone FGC, cell line passage number at delivery +15) were purchased from ECACC (European Collection of Cell Cultures)/Sigma-Aldrich (Munich, Germany) and routinely grown in RPMI1640-based complete medium (growth medium/GM with fetal bovine serum/FBS) as previously described [18,20]. All experiments were performed at LNCaP cell passage number from +19 to +21. Before treatments, LNCaP cells were synchronized overnight (GM w/o serum) as previously described [18,20] and, once washed with 1x phosphate buffer solution/PBS, pH 7.2 w/o Ca^{2+} and Mg^{2+}. Treated either with the sex steroids DHT and E2 or with the phytochemicals of interest in Treatment Medium/TM (GM containing 10% charcoal-stripped/CS-FBS instead of usual FBS). All chemicals were dissolved in DMSO to obtain 100mM stock solutions, expect for E2 at 1mM. Aliquots of each stock solution were stored at -20°C and working stock solutions and dilutions were prepared just before use in 1x PBS, pH 7.2 w/o Ca^{2+} and Mg^{2+}.

Cell culture reagents (RPMI1640 w/o phenol red, HEPES, PBS, sodium pyruvate, L-glutamine, penicillin and streptomycin, FBS and CS-FBS) were purchased from PAA Laboratories GmbH (Pasching, Austria) except for amphotericin B (Gibco Invitrogen Life Technologies, San Giuliano Milanese, Italy) and DMSO (Sigma-Aldrich, Munich, Germany).

LNCaP Treatments and MTS Assay

Cell viability and indirect cell proliferation of each chemical was assessed by the "Cell Titer 96® AQueous One Solution Cell Proliferation Assay" kit (Promega Italia, Milan, Italy) as previously described [18,20]. Briefly, LNCaP cells were plated at a density of 5 x 10^3 cells / well in 96-well plates in 200µl TM and incubated 72hrs performing dose-response curves for E2 and each phyto-chemicals either alone or in presence of DHT from 1pM to 100µM. In co-treatment experiments, the endogenous androgen-AR agonist DHT was added 20min later at 10nM, a concentration within the range of physiological functional activity of sex steroid hormones. The vehicle DMSO was also dose-dependently tested (up to 10%). At the end, 100µl of each cell culture supernatant (SN) were collected and stored at -20°C until the PSA secretion assay was performed (see below), while each remaining culture was quenched by adding 20µl (1/5 volume of cell culture) of "Cell Titer 96® Aqueous one Solution Reagent" and, after further incubation at 37°C was measured in the dark, absorbance (490nm) using a 96-well plate reader (Wallac 1420 VICTOR3™ Multilabel reader, Perkin Elmer).

PSA Secretion Assay

Cell culture SNs, obtained and stored as described above (MTS assay), were used to assess simultaneously the amount of human free and total PSA by a dual-label time-resolved fluoroimmunoassay using the commercial kit "ProStatus™ PSA Free/Total kit DELFIA® Reagents" (Perkin Elmer Italia, Monza, Italy) following the manufacturer's instructions. Using a 96-well plate reader (Wallac 1420 VICTOR3™ Multilabel reader, Perkin Elmer), europium (free PSA-labeled antibodies) and samarium (total PSA-labeled antibodies) fluorescence has been measured at 615nm and 642nm, respectively.

Cell Fractionation

LNCaP cells, plated in TM at a density of 4 x 10^6 cells, have been treated with the sex steroids hormones and each plant bioactive, either alone (at 10µM) or in the presence of DHT or E2 (at 10nM or 10µM), and further incubated for 72hrs. In co-treatment experiments, the endogenous androgen-AR agonist DHT or the endogenous estrogen and ER-agonist E2 were added 20min later. Subcellular fractionation has been performed as previously described with some modifications [33]. Briefly, after 72hrs, cell culture media were collected, labelled as medium (M) and stored at -20°C. The cells were detached with 1x tripsin/EDTA solution, resuspended in GM and centrifuged 10min at 0.1g. Once discarded the supernatant/SN, pellet has been transferred in a fresh Eppendorf tube and washed once in 1x PBS (10min at 0.1g). SN has been discarded and pellet resuspended in Lysis Buffer (20mM TrisHCl, pH7.4, 2mM EDTA and a 1% v/v protease inhibitor cocktail from Sigma containing AEBSF, aprotinin, bestatin, E64, leupeptin, pepstatin A). Upon 30min incubation on ice, samples were centrifuged 5min at 1,000g, 4°C: the pellet has been stored at -20°C labelled as the nuclear fraction (N), while the SN was previously transferred in a fresh Eppendorf tube and centrifuged 30min at 30,000g, 4°C in order to separate the cytoplasm (the newly obtained SN, labelled and stored as C), from the microsomal fraction (the newly obtained pellet, labelled and stored as MF).

Intracellular Distribution of Chemicals

All cellular fractions were thawed at room temperature (RT) and extracted with HPLC or GC grade methanol (1:1 v/v) vortexing for 3 min. The samples were centrifuged at 12,000 g for 5min at 4°C (Heraeus Biofuge Primo R Centrifuge, Thermo Scientific-Italy), each supernatant was then filtered through a 0.22µm filter membrane and injected in the respective devices.

Polyphenols Determination

The polyphenol content was analyzed according to Mandalari and coworkers (2006) [34] with some modifications. Briefly, the extracts were analyzed using a Phenomenex Luna C18 reverse-phase column (250 x 4.6mm, 5µm; Phenomenex, Macclesfield, UK) in combination with an Agilent HP1100 system (Agilent Ltd, West Lothian, UK) coupled with a photodiode array detector and a binary pump. A linear gradient was applied using two solvents - solvent A) 0.1% v/v trifluoracetic acid in ultrapure water and solvent B) 0.1% trifluoroacetic acid in HPLC grade MeOH as follows: t=0 (95% A, 5% B), t=11min (75% A, 25% B), t=38min (100% B), t=47min (95% A, 5% B) and t=57min (95% A, 5% B). The flow rate was 1 mL/min, and the thermostatically-controlled autosampler and column oven were set at 10°C and 25°C, respectively. Specific UV-visible data were collected at 220, 270, 325, 370, and 520 nm (with overall data collected between 200 and 600 nm). All known compounds were measured directly using standard curves obtained by running commercial standards.

DHT Determination

The analytical determination was carried out according to Zhang and coworkers (2009) [35]. Briefly, chromatographic separation of DHT was performed by an HP-5MS capillary column (30 m length×0.25 mm I.D.×0.25 µ m film thickness, Agilent Scientific, USA). Column flow was volumetric at 1.0 mL/min using ultra-purified helium (>99.999%) as carrier. Injection was carried out in split-less mode at 260°C with a 12 min solvent delay. The transfer line temperature was set at 300°C. The MS analyzer was set at 70 eV, electron impact source temperature at 230°C, electron-multiplier voltage at 1588 mV. The temperature program was as follows: initially from 100°C (5min) to 250°C at a ramp rate of 25°C/min, 250°C for 7 min, finally from 250°C to 310°C at a ramp rate of 30°C/min. The identification of target anabolic steroid was based on the standard mass spectra of the National Institute of Standards and Technology (NIST-08) MS spectral library, standard compounds and the corresponding references. Ions for monitoring DHT at m/z were the ion fraction groups of (231, 290, 55 and 163) on the basis of results obtained from an initial scan from 40 to 400 AMU. The quantization was based on the Extracted Ionization Chromatograms (EICs), and the corresponding quantitative ions for DHT at m/z were 231 using an external standard calibration curve.

E2 Determination

The analysis of E2 was carried out according to Zou and coworker (2012) [36] with some modifications. Briefly, the separation was performed on a Phenomenex-Luna column (5µm C18 (2) 100 Å 250 x 4.60 mm 5µm; Phenomenex, Italy). An Agilent ChemStation for LC system was utilized to control the system and for the acquisition and analysis of the chromatographic data. Quantification was done by the evaluation of peak areas of samples with respect to the pure reference standard. Acetonitrile and water (55/45 v/v) were used as mobile phase with an isocratic elution. The flow rate of mobile phase was 1.0 ml/min. The injection volume was 50µl, and the DAD detector was set up at 210nm. The column temperature was set at 25°C.

Validation of Analytical Method

The HPLC method has been validated according to the International Conference on Harmonisation - Harmonised Tripartite Guideline 2005 [37], in terms of selectivity, linearity, limit of detection and quantitation, precision and recovery from biological sample. A good analytical method should be able to measure accurately the analyte in the presence of suspected interferences such as its own degradation products and any co-eluting endogenous compounds that may drive from a biological matrix. The chromatographic separation of polyphenols, sex steroid hormones and its possible degradation products don't show any overlap of each other (base-line separations); also, as highlighted into chromatograms, no interferences are found at the retention times of chemicals or its degradation products from endogenous constituents present into the microsomal fraction, cytoplasm and nucleus samples after their appropriate extraction (see supplemental material).

A test solution with different concentrations of pure reference standards was prepared and analyzed by using the analytical parameters described above. The detection limit was calculated as the amount of chemicals that resulted in a peak three times higher with respect to the baseline noise. The linear calibration range, regression equation, and detection limit of compounds were calculated and the results were listed in Table **1**.

Statistical Analysis

All cytotoxicity and PSA expression data were obtained from three independent experiments performed in triplicate. Data were expressed as mean ± S.D. and statistically analyzed by the non parametric Dunnett test multiple comparison. P-values below 0.05, 0.01 and 0.001 were considered statistically significant as indicated in Figure legends.

Intracellular distribution data were also obtained from three independent experiments performed in triplicate. Data were

Table 1. Parameters for method validation.

Compounds	Parameters					
	Calibration Range (µg/L)	*Coefficient Regression R^2*	*R.S.D* (%), n = 6 within-day*	*R.S.D (%), n = 6 between-day*	*LOD§ (µg/L)*	*LOQ¥ (µg/L)*
5α-dehydrotestosterone	12.5 - 100	0.9991	2.744	3.368	1.049	3.497
17-β-estradiol	6.25 - 100	0.9990	1.992	2.409	2.953	9.843
Apigenin	6.25 - 100	0.9990	0.070	0.148	0.037	0.123
Genistein	6.25 - 100	0.9993	2.229	2.825	3.220	10.732
Luteolin	6.25 - 100	0.9990	0.115	0.246	0.045	0.150
Naringenin	6.25 - 100	0.9991	1.153	1.348	0.366	1.219
Quercetin	6.25 - 100	0.9994	0.283	0.434	0.035	0.117
Resveratrol	6.25 - 100	0.9997	3.410	4.362	0.670	2.235

*Relative standard deviation; § Limit of detection; ¥Limit of quantification.

expressed as mean ± S.D. and statistically analyzed by Student t test using a SigmaPlot 12.0 software. P-values below 0.05, 0.01 and 0.001 were considered statistically significant as indicated in Figure legends.

RESULTS

Cell Viability and Indirect Cell Proliferation by MTS Assay

In order to assess the cytotoxicity of the flavonoid of interest, we used the MTS assay, a cell viability and indirect cell proliferation assay, which is a general and cell aspecific toxicological endpoint. At first, we performed the same MTS assay with the reference compounds, namely the sex steroids DHT and E2 and the vehicle DMSO.

In Figs. **1** and **2** (top panels) the results obtained with the reference compounds DHT, E2 and DMSO are shown. In Fig. **1**, the androgen DHT (range of concentrations 1pM-100μM) showed a mild increase with a maximum of about 20% of LNCaP cell viability (Fig. **1**, top, left panel). Whereas the vehicle DMSO (range of concentrations $1x10^{-6}$ % to 10% v/v) did not affect at all cell viability and indirect cell proliferation up to 0.01% and results cytotoxic in a statistically significant dose-dependent manner at concentrations ≥0.1% (Fig. **1**, top, right panel). In Fig. **2**, the estrogen E2 (range of concentrations 1pM-100μM) showed a dose-dependent increase of LNCaP cell viability and indirect cell proliferation up to 1μM (top, left panel), statistically significant from 10nM to 1μM, whereas it decreased significantly at 10μM (about -40%) and 100μM (about -85%). The same results have also been obtained upon LNCaP co-treatment with a range of E2 concentrations (1pM-100μM) in the presence of 10nM DHT, whereas DHT concentration corresponds to the endogenous androgens showing the best AR activation [38] (Fig. **2**, top, right panel).

Fig. **3** shows the effects on LNCaP cell viability of the selected flavonoids, either alone (Panels A, C, E, G, I, K) or in the presence of 10nM DHT (Panels B, D, F, H, J, L). In the absence of DHT, API, LUT and QRC did not affect cell viability at any tested concentration. However, NRG (-9% at 1μM, -8% at 10μM and -20% at 100μM) and RESV (-10% at 1μM, -14% at 10μM and -62% at 100μM) affected it in a statistically significant manner at concentrations ≥1μM. Finally, GEN (-27% at 10μM and -78% at 100μM) affected LNCaP cell viability in a statistically significant manner at concentrations ≥10μM. In the presence of 10nM DHT, the co-treatment with each tested flavonoid did not show changes in comparison to 10nM DHT-treated LNCaP, except for GEN at concentrations ≥10μM (in comparison to DHT-treated cells: -66% at 10μM and -120% at 100μM, in comparison to CTRL: -22% at 10μM and -77% at 100μM) and RESV at the higher concentration (100μM, -67% in comparison to DHT-treated cells, -57% in comparison to CTRL).

PSA Secretion Assay

In order to assess the role of flavonoids using a cell specific toxicological endpoint, we used a well-known clinical biomarker that reflects a cell functional endpoint, namely the PSA secretion. Using a fluorimetric-based kit, it was possible to estimate both free and total PSA secretions at the same time within the range of concentrations (1pM-100μM) used for the MTS assay. Also in this case the role of the selected flavonoids has been compared to the effects of the same reference molecules.

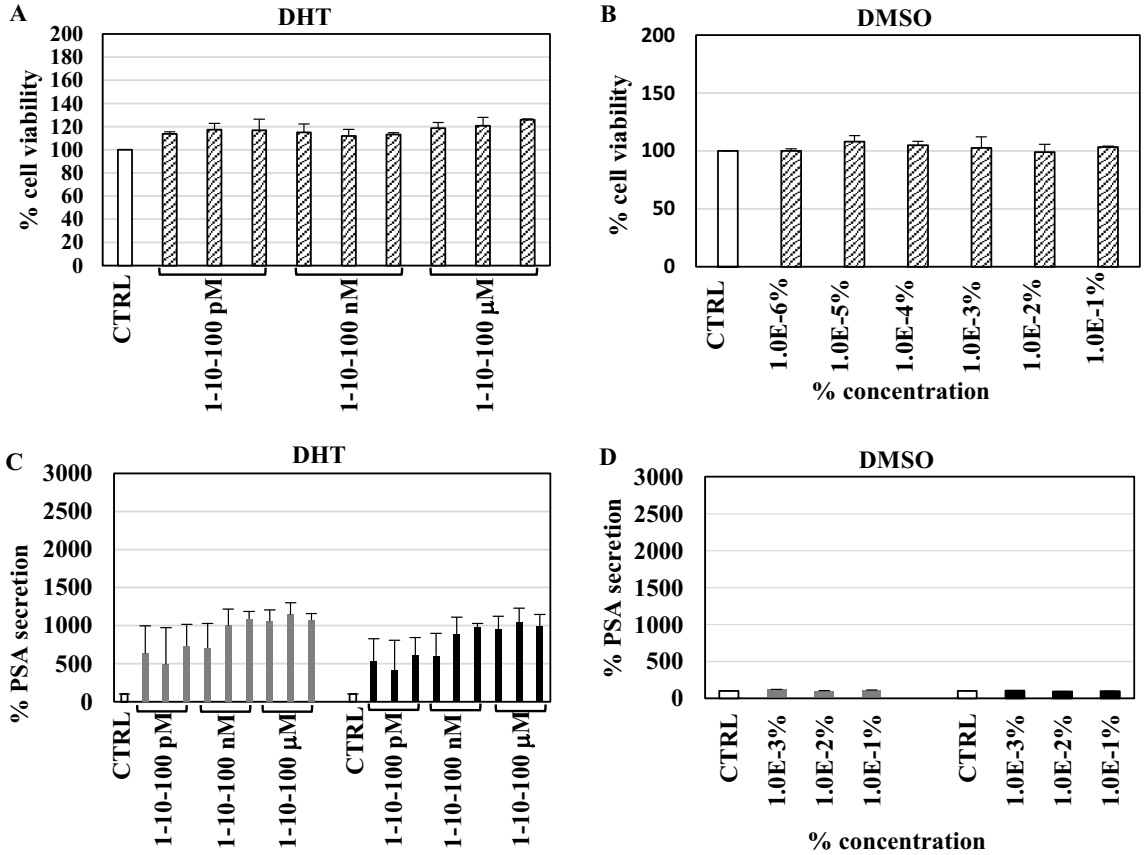

Fig. (1). LNCaP cell viability and PSA secretion after treatment with DHT and DMSO. (**A** and **B**) MTS assay (cell viability and indirect cell proliferation). (**C** and **D**) free and total PSA secretion assay. Data expressed as percentage values (±S.D.) in comparison to CTRL values corresponding to 100% and represent mean value three independent experiments performed in triplicate. P-values (Dunnett test): $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ *vs* CTRL.

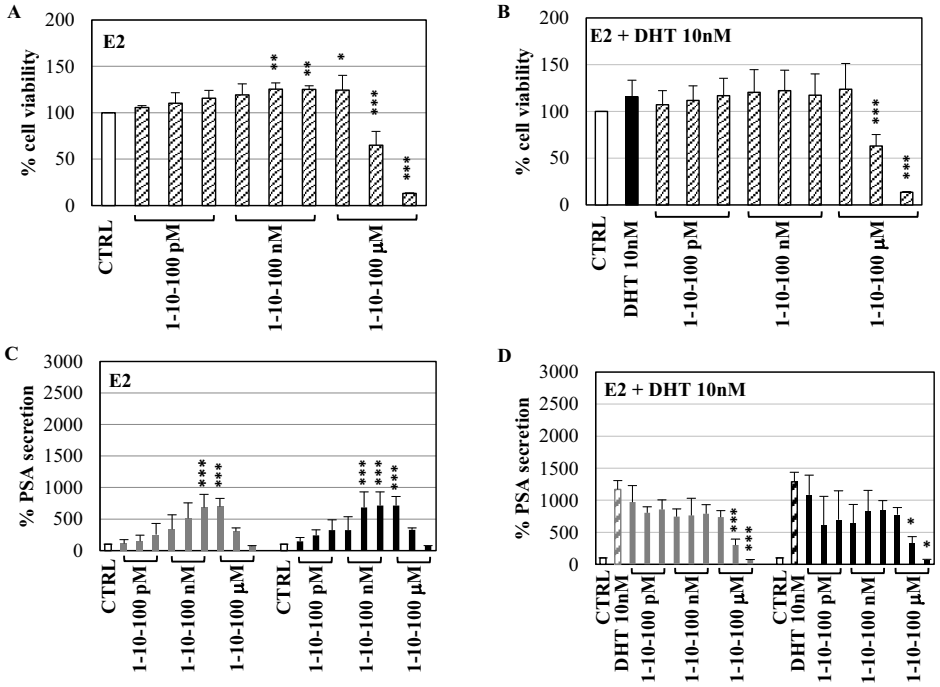

Fig. (2). LNCaP cell viability and PSA secretion after treatment with E2 ± DHT 10nM. (**A** and **B**) MTS assay (cell viability and indirect cell proliferation). (**C** and **D**) free and total PSA secretion assay. Data expressed as percentage values (±S.D.) in comparison to CTRL values corresponding to 100% and represent mean value three independent experiments performed in triplicate. P-values (Dunnett test): *p<0.05, **p<0.01, ***p<0.001 *vs* CTRL.

In Figs. **1** and **2** (bottom panels) the results obtained with DHT, E2 and DMSO are shown. In Fig. **1** (left, bottom panel), DHT showed a statistically significant non-linear increase of LNCaP free and total PSA secretions.

In Fig. **2** (bottom panels), the role of E2 on PSA secretion has been assessed in the absence or the presence of 10nM DHT. E2 alone induced free and total PSA secretions up to 1µM (free PSA secretion was statistically significant at 100nM and 1µM whereas total PSA secretion was statistically significant between 10nM and 1µM). At the higher concentrations (≥10µM) the reduced free and total PSA secretions were directly associated to the number of viable cells (see Fig. **2**, top, left panel). DHT-induced PSA secretion, on the other side, was decreased by E2 at all concentrations (Fig. **2**, bottom, right panel): a different pattern of dose-response was evidenced comparing free PSA secretion (decrease of about 37%) to total PSA secretion (decrease of about 40%) was observed at 1µM, when cells were still viable.

The effect of flavonoids in DHT-induced free and total PSA secretions of LNCaP cells is shown in Fig. **4**: left panels (A, C, E, G, I, K) display LNCaP treatment with each flavonoid alone, whereas right panels (B, D, F, H, J, L) display the LNCaP co-treatment in the presence of 10nM DHT. As expected, flavonoids alone did not induce PSA secretion except for LUT (Fig. **4**, panel E) which showed an increase of both free and total PSA secretions at ≥1µM. In the presence of DHT, all flavonoids showed only a mild capability to inhibit the DHT-induced free and total PSA secretions. API (Fig. **4**, panel A) and LUT (Fig. **4**, panel F) showed an effect at all tested concentrations, without a linear dose-response, and with a maximum effect at 100pM (API: -30% free PSA secretion, -33% total PSA secretion; LUT: -40% free PSA secretion, -44% total PSA secretion) and 100µM (API: -50% free PSA secretion, -51% total PSA secretion; LUT: -39% free PSA secretion, -42% total PSA secretion). GEN (Fig. **4**, panel D) showed also a non-linear dose-response with only a mild decrease of DHT-induced free and total PSA secretions at 100pM and 10nM, whereas the strong decrease at concentrations ≥10µM was due to the decreased cell viability (see Fig. **3**, panel D). NRG (Fig. **4**, panel H) showed a non-linear dose-response but the maximum effect of the inhibition of DHT-induced free and total PSA secretions occurred at 100pM (-10% free PSA secretion, -17% total PSA secretion) and at 100µM (-45% free PSA secretion, -50% total PSA secretion). Interestingly, QRC (panel J) did not only show a non-linear dose-response decrease at all concentration of the DHT-induced free and total PSA secretions but the maximum effect was reached at the lowest tested concentration with an inhibition at 1pM corresponding to about 41% free PSA secretion and 43% total PSA secretion. Finally, RESV (Fig. **4**, panel L), appeared to inhibit DHT-induced free and total PSA secretions up to 10µM, whereas at the highest concentration the reduction was clearly due to the reduced cell viability (see Fig. **3**, panel L).

INTRACELLULAR DISTRIBUTION OF CHEMICALS

To establish if the "nominal concentration" of each tested flavonoid corresponds to the "effective concentration" at both intracellular and subcellular level, we performed a cell fractionation (as described in Materials and Methods) in order to obtain three intracellular fractions, namely the cytoplasm/C, the microsomal fraction/MF and the nuclear fraction/N. The amount of each chemical entering into the cells and its intracellular localization had been determined, along with the LNCaP-treated medium/M to measure also the amount of chemical remaining outside the cell. Such procedure allowed us to determine the "effective intracellular" concentration of each tested chemical.

At first, we assessed the intracellular distribution of the sex steroids DHT and E2, each at 10µM. As shown in Fig. **5A**, only about 55% DHT entered into LNCaP, of which most of the DHT distributed in the MF (about 48%) and in a minimal percentage within N (about 5%) and C (about 2%). About 90% E2 entered into LNCaP (Fig. **5B**), mostly within the MF (about 60%) and less extensively in N (about 20%) and C (about 10%). Hence, only about 10% E2 appeared to remain outside LNCaP, whereas about 45% DHT did not entered in LNCaP.

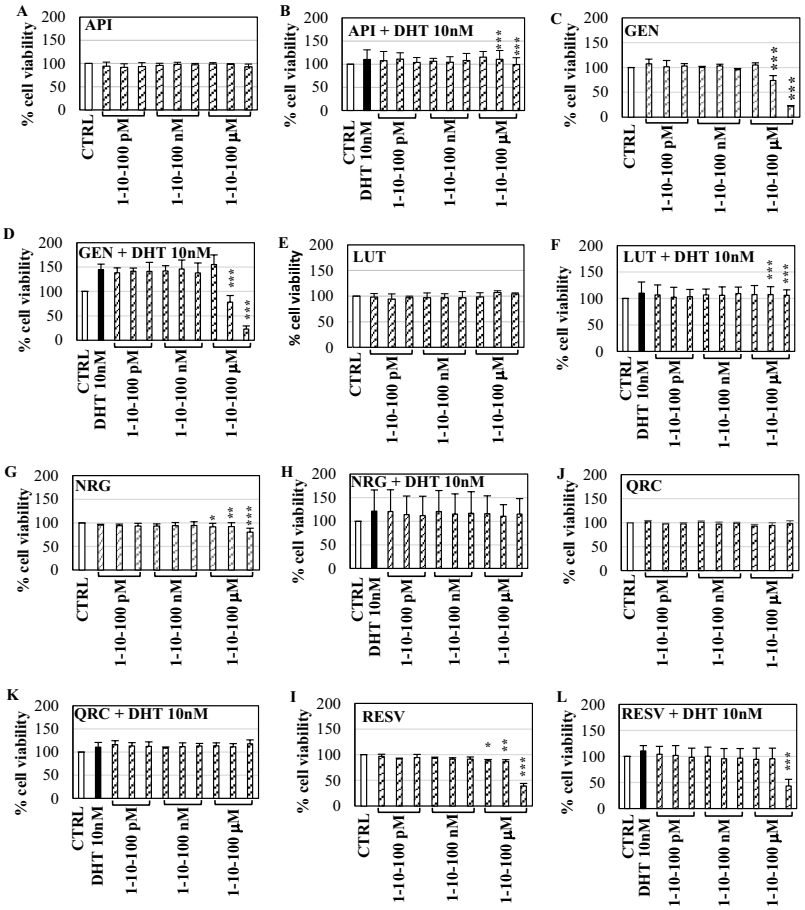

Fig. (3). LNCaP cell viability and indirect cell proliferation (MTS assay) upon treatment with plant bioactives ± 10nM DHT. (**A** and **B**) apigenin/API; (**C** and **D**) genistein/GEN; (**E** and **F**) luteolin/LUT; (**G** and **H**) naringenin/NRG; (**J** and **K**) quercetin/QRC; (**I** and **L**) resveratrol/RESV. Data are expressed as percentage values (± S.D.) in comparison to CTRL values corresponding to 100% and represent mean values of three independent experiments performed in triplicate. P-values (Dunnett test): *p<0.05, **p<0.01, ***p<0.001 *vs* CTRL.

Afterward, we assessed the intracellular distribution of each flavonoid alone (10µM), in the presence of either 10nM DHT or 10nM E2 (Fig. **6**), in order to assess whether sex steroids were able to modulate the ability of each flavonoid to enter into LNCaP. In almost all cases, the flavonoid-sex steroid co-treatment changed the amount of chemical remaining outside LNCaP hence entering into LNCaP.

In particular, Fig. (**6A**) API alone remained outside LNCaP corresponded to about 22.6% of the total nominal concentration, whereas API within LNCaP corresponded to about 80% (MF=61.6%, N=8.2%, C=7.2%). Upon DHT co-treatment, an increased amount of API within LNCaP was observed and, at the same time, its intracellular distribution changed with a decrease at the MF level and an increase in both N and C. Upon E2 co-treatment, the intracellular amount of API is almost corresponding to 100% and also in this case the intracellular distribution changed but, oppositely to DHT, increased at the MF level and decreased in N and C.

When GEN was tested alone (Fig. **6B**), about 47.5% of its nominal concentration remained outside LNCaP, whereas about 51.8% (MF=40.6%, N=8%, C=3.2%) entered into LNCaP. Upon DHT co-treatment, no significant changes were observed in GEN intracellular localization. Upon E2 co-treatment, the intracellular amount of GEN decreased to 34.7%, mainly at the MF level, and the amount outside LNCaP increased to 64.7%.

Fig. **6C** shows that almost all (94.6%) LUT entered into LNCaP (MF=81%, N=9%, C=4.5%). Upon DHT-treatment, a small increased intracellular amount of LUT was observed concomitantly to a decrease at the MF level and an increase in both N and C. Upon E2 co-treatment, no significant changes were observed in LUT intracellular localization.

Fig. **6D** shows that around 27.5% of the nominal concentration of NRG remained outside LNCaP, whereas the amount within LNCaP corresponded to about 71.6% (MF=65.9%, N=3.6%, C=2.2%). Upon DHT co-treatment, an increased amount of NRG remained outside LNCaP and the corresponding decrease within LNCaP was reflected by intracellular distribution changes with a clear decrease at the MF level and an increase at the N level. Upon E2 co-treatment, the increased intracellular amount of NRG was almost completely at the MF level.

QRC alone (Fig. **6E**) entered almost completely into LNCaP (about 90% of the nominal concentration) and only about 8% remained outside LNCaP. The QRC intracellular distribution was as follows: MF = about 62.3%, N = about 20.5%, C = about 6.8%. Upon DHT co-treatment, the decreased intracellular amount of QRC was almost completely at the MF level. Upon E2 co-treatment, the intracellular amount of QRC increased mainly at the MF level concomitantly to a reduction at N and C.

RESV entered almost completely into LNCaP (about 95% of the nominal concentration) and only about 4.6% remained outside LNCaP (Fig. **6F**). The intracellular distribution of RESV alone was as follows: MF = about 85.2%, N = about 6.5%, C = about 3.4%. Upon DHT co-treatment, the intracellular amount of RESV

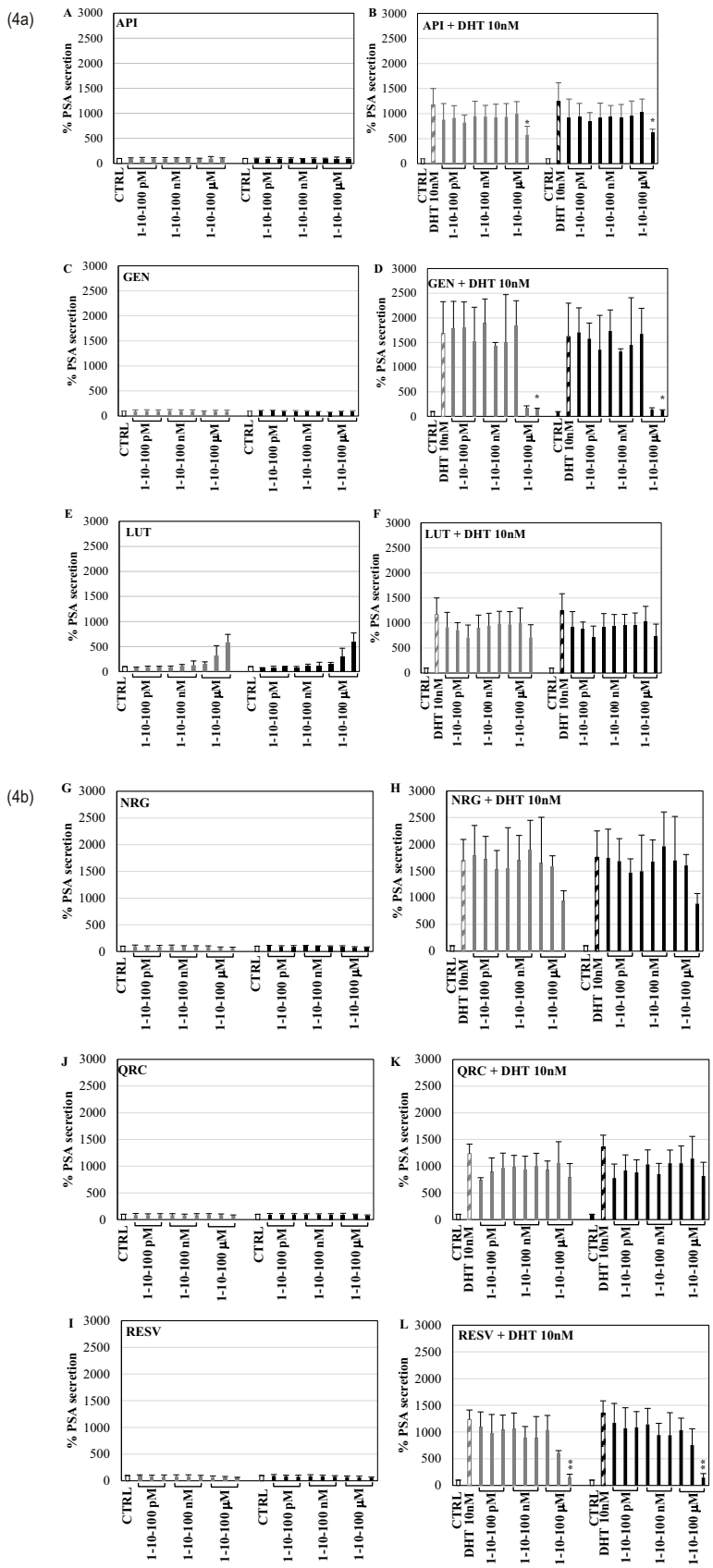

Fig. (4). LNCaP free and total PSA secretion upon treatment with plant bioactives ± 10nM DHT. (**A** and **B**) apigenin/API; (**C** and **D**) genistein/GEN; (**E** and **F**) luteolin/LUT; (**G** and **H**) naringenin/NRG; (**J** and **K**) quercetin/QRC; (**I** and **L**) resveratrol/RESV. Data are expressed as percentage values (± S.D.) in comparison to CTRL values corresponding to 100% and represent mean values of three independent experiments performed in triplicate. P-values (Dunnett test): *p<0.05, **p<0.01, ***p<0.001 *vs* CTRL.

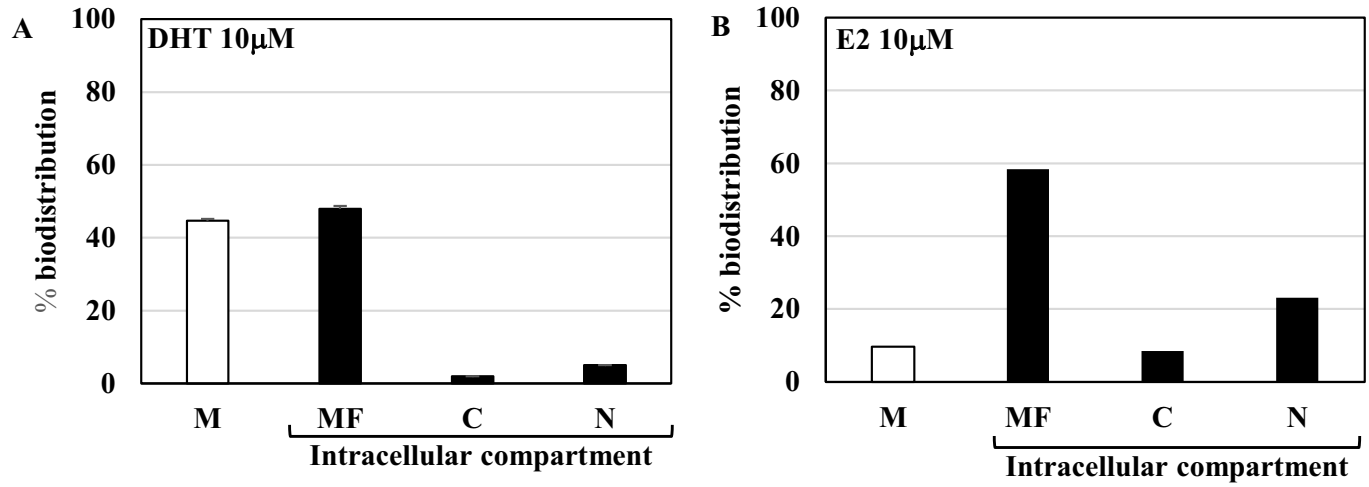

Fig. (5). Sex steroids intracellular distribution in LNCaP cells. (**A**) Intracellular distribution of 10mM DHT (**B**) Intracellular distribution of 10mM E2. Data are expressed as percentage values (± S.D.) in comparison to CTRL values corresponding to 100% and represent mean values of three independent experiments performed in triplicate. P-values (Student t-test ?): *p<0.05, **p<0.01, ***p<0.001 *vs* CTRL.

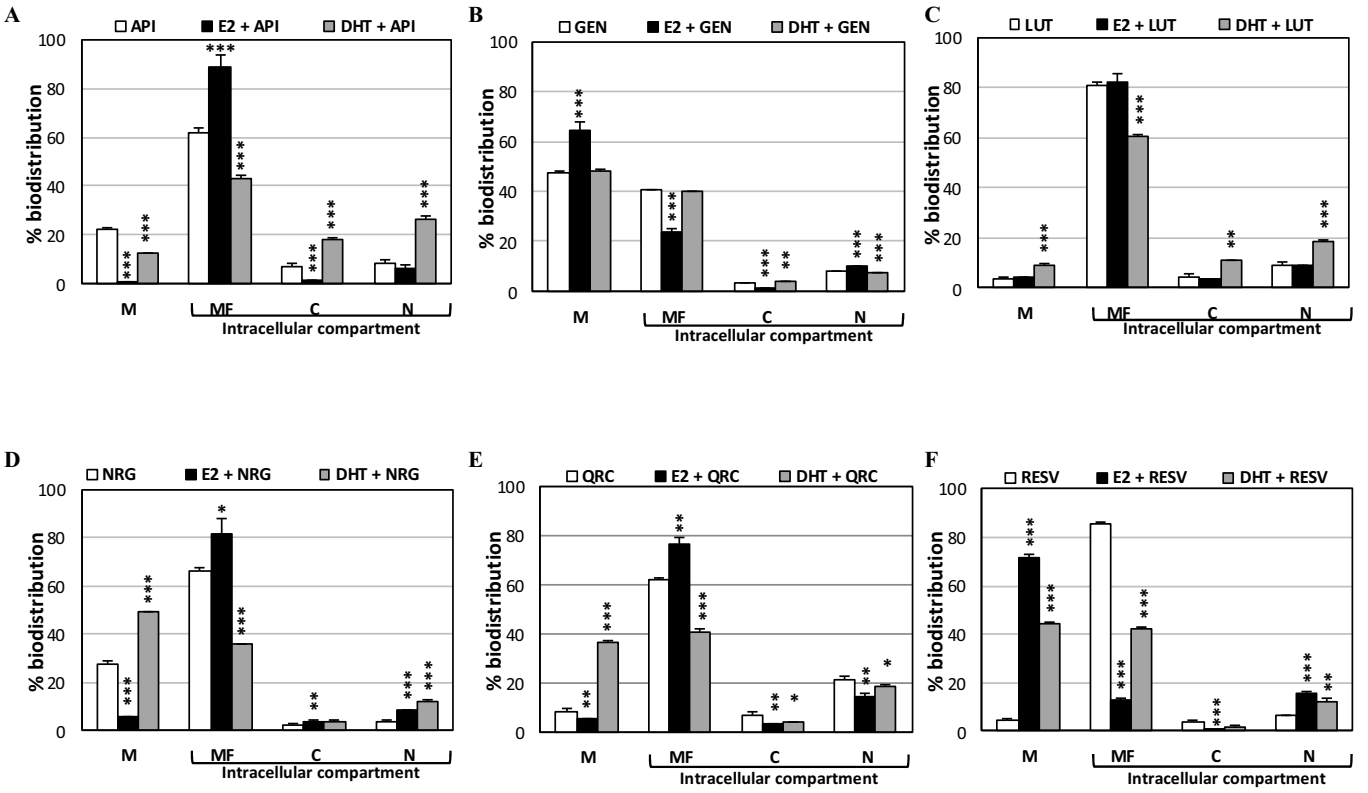

Fig. (6). Plant bioactive intracellular distribution in LNCaP cells. (**A**) Intracellular distribution of apigenin/API alone (white bars), in presence of E2 (black bars), in presence of DHT (grey bars). (**B**) Intracellular distribution of genistein/GEN alone (white bars), in presence of E2 (black bars), in presence of DHT (grey bars). (**C**) Intracellular distribution of luteolin/LUT alone (white bars), in presence of E2 (black bars), in presence of DHT (grey bars). (**D**) Intracellular distribution of naringenin/NRG alone (white bars), in presence of E2 (black bars), in presence of DHT (grey bars). (**E**) Intracellular distribution of quercetin/QRC alone (white bars), in presence of E2 (black bars), in presence of DHT (grey bars). (**F**) Intracellular distribution of resveratrol/RESV alone (white bars), in presence of E2 (black bars), in presence of DHT (grey bars). Data are expressed as percentage values (± S.D.) in comparison to CTRL values corresponding to 100% and represent mean values of three independent experiments performed in triplicate. P-values (Student t-test): *p<0.05, **p<0.01, ***p<0.001 *vs* CTRL.

Table 2. Sex steroids intracellular distribution in LNCaP cells upon co-treatment with plant bioactives.

Treatment	Medium/M	Microsomal Fraction/MF	Cytoplasm/C	Nuclear fraction/N
E2	9.74 ± 1.02	58.17 ± 0.91	8.61 ± 0.13	23.17 ± 0.62
E2 + API	9.81 ± 0.78	78.43 ± 1.65	0.17 ± 0.002	10.78 ± 0.58
E2 + GEN	7.02 ± 0.49	68.50 ± 1.19	11.30 ± 0.82	13.08 ± 0.86
E2 + LUT	11.46 ± 0.86	73.96 ± 1.58	0.19 ± 0.003	14.06 ± 0.57
E2 + NRG	9.36 ± 0.98	79.64 ± 1.85	0.14 ± 0.006	10.16 ± 0.77
E2 + QRC	10.97 ± 0.85	78.73 ± 1.21	8.61 ± 0.005	23.17 ± 0.19
E2 + RESV	9.97 ± 0.63	81.77 ± 1.27	0.26 ± 0.01	7.48 ± 0.49

Treatment	Medium/M	Microsomal Fraction/MF	Cytoplasm/C	Nuclear fraction/N
DHT	44.66 ± 1.12	47.96 ± 0.90	1.95 ± 0.03	5.01 ± 0.12
DHT + API	15.20 ± 0.88	80.92 ± 1.65	0.00 ± 0.00	3.59 ± 0.50
DHT + GEN	22.58 ± 0.69	62.18 ± 1.09	0.00 ± 0.00	14.52 ± 0.66
DHT + LUT	16.11 ± 0.56	80.24 ± 1.98	0.00 ± 0.00	3.12 ± 0.07
DHT + NRG	28.76 ±0.98	60.35 ± 1.75	0.00 ± 0.00	10.74 ± 0.67
DHT + QRC	9.27 ± 0.75	89.17 ± 1.35	0.00 ± 0.00	0.38 ± 0.01
DHT + RESV	8.38 ± 0.43	85.13 ± 1.17	0.00 ± 0.00	6.13 ± 0.43

decreased almost completely at the MF level and with an increased amount at the N level. Upon E2 co-treatment, RESV capability to enter into LNCaP was strongly reduced and the amount remained outside corresponded to about 70%. The RESV decreased intracellular amount was mostly at the MF level but at the same time it increased at the N level.

To verify whether, in turn, each flavonoid (10nM) was also able to modulate sex steroids (10μM), we performed the same co-treatments performed in Fig. **6** in order to detect either DHT or E2 intracellular localization. The obtained results were summarized in Table **2**. As evidenced, all flavonoids increased the intracellular amount of DHT of which RESV and QRC being the most effective (in particular, almost 2-fold at the MF fraction). In particular, the flavonoids GEN and NRG increased the N localization of DHT 2-fold and almost 3-fold respectively. On the contrary, the effect of each flavonoid on E2 intracellular amount was less significant and only LUT and QRC had a small decreasing effect whereas GEN increased it. However, it was evident that each flavonoid affected E2 intracellular distribution and that the E2 increased at the MF level, decreased at N and C levels. The only exception has been GEN co-treatment which increased the E2 localization at C level.

DISCUSSION

Beneficial effects of phytochemicals on prostate health have been suggested by epidemiological and experimental model studies and in a few cases also by phase II clinical trials [13,32]. In particular, flavonoids and phytochemical-rich plant extracts have been studied either for their anti-inflammatory or anti-proliferative capabilities, since prostate diseases (*i.e.*, prostatitis, benign prostate hyperplasia/BPH and PCa) are associated to different extent to inflammatory and/or proliferative status.

In our study, we selected some of the most studied and/or widely present flavonoids in the Mediterranean diet in order to highlight their direct role on the main human oncological biomarker, PSA secretion. Here, PSA secretion has been used as a toxicological biomarker of effect in an *in vitro* LNCaP model system which maintains the molecular hallmark of human prostate adenocarcinoma, namely the mutated AR^{T877A}. Indeed, some of the selected phyto-chemicals have been previously shown: i) to inhibit PSA secretion only at μM concentrations [27,31], and ii) to be effective upon AR overexpression [27]. Hence, the *in vitro* studies so far performed usually suggest an effective concentration in the μM range that are not feasible to be reached by regular consumption of fruits and vegetables containing such phytochemicals.

Here we investigated, at the same time, the role of some selected phytochemicals on LNCaP cytotoxicity (and indirectly on cell proliferation) and the free and total PSA secretions, both in the absence or presence of DHT. The obtained results highlighted that the selected flavonoids did not show cytotoxicity or eventually it occurred only at high concentrations as in the case of GEN (≥10μM) and RESV (100μM).

In the absence of DHT, the selected flavonoids showed no effects on PSA secretion in comparison to vehicle-treated cells. The only exception was LUT that showed a dose-dependent increase only in the μM range of concentrations, suggesting a possible slight agonistic effect on the mutated AR^{T877A}. E2, in the absence of DHT, showed also a dose-dependent increase up to 1μM of free and total PSA secretions but it was probably a non-specific effect since it occurred along with the increase in cell viability and proliferation.

Most interestingly, in the presence of DHT, the selected polyphenols had, to a different extent and in a non-linear manner,

the ability to partially reduce the DHT-induced free and total PSA secretions. As shown in Fig. **6**, a statistically significant inhibition of free and total PSA secretions was observed by GEN and RESV treatments at concentrations which also caused cytotoxicity. In all cases a non-linear behavior of each polyphenol treatment was observed. Such no-linear responses to each dose-dependent treatment showed that in our experimental model the selected polyphenols can reduce DHT-induced PSA secretion only to a limited extent (generally between 20%-40%). Some of them were more effective at relatively lower concentrations (in the pM range): QRC showed an inhibition peak at 1pM, API and LUT at 100pM, GEN at 10nM, RESV at 100nM and NRG at 100μM.

The intracellular distribution of the studied phytochemicals may partly explain the differences in the DHT-induced PSA secretion. PSA secretion is under control of the transcriptional regulation of the AR-mediated signaling since PSA is an AR-target gene. To exert their transcriptional regulation of PSA, phytochemicals have to induce the nuclear translocation of the AR where it acts as a transcription factor. As shown in Fig. **6**, only a minimal amount of each phytochemical was found within the nucleus. Interestingly, each phytochemical was most present in the MF, which contains mainly plasma membrane, where it has been suggested to localize the receptor fraction regulating the AR-mediated non genomic signaling [39,40].

Indeed, the results obtained from each phytochemical intracellular distribution (Table **2** and Fig. **6**) highlighted that: i) the "nominal concentration" is always different from the "real" amount of each chemical effectively entering the cell, ii) their ability to enter within the cell and to distribute in the different cellular compartments depends also by the presence of DHT or E2, iii) QRC, LUT and API are the phytochemicals which reduce the nuclear localization of DHT the most. Interestingly, the three selected phytochemicals mostly reducing the DHT nuclear localization (namely, the intracellular site where DHT effectively induces PSA gene transcription and eventually lead to PSA production and secretion) are also the ones inhibiting the DHT-induced PSA secretion in the pM range of concentrations.

CONCLUSION

Measurement of PSA secretion is meant to provide useful information on the potential beneficial role of phytochemicals on both PCa and male fertility, since PSA has a pivotal physiological role in allowing sperm activation. Our approach to the role of phytochemicals in prostate epithelium indeed has two main features: i) it assessed the ability of the selected flavonoids to inhibit both free and total PSA secretions in a wide range of concentrations from 1pM to 100μM in order to evaluate their role in physiological and non-physiological concentrations (such as in food supplements) taking into account also low-dose effects, ii) it used a model system in which no exogenous AR protein has been added and in which the endogenous androgen receptor is the mutated AR^{T877A}, representing the main mutated form present in PCa, thus providing insights of the real beneficial effects of flavonoids in an *in vitro* disease-like status.

A third and significant improvement of the reported approach has been the establishment of the "effective concentration" in comparison to the "nominal one": the intracellular amount of each selected phytochemical has been assessed for the first time upon cell fractionation of LNCaP-treated cells and subsequent chromatographic measurements.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's web site along with the published article.

LIST OF ABBREVIATIONS

API	=	Apigenin
AR	=	Androgen receptor
C	=	Cytoplasm
CS-FBS	=	Charcoal-stripped-fetal bovine serum
CTRL	=	Control
DELFIA	=	Dissociation-enhanced lanthanide fluorescence immunoassay
DHT	=	5α-dehydrotestosterone
DMSO	=	Dimethylsulfoxide
E2	=	17β-estradiol
ECACC	=	European Collection of Cell Cultures
ECM	=	Extracellular matrix
FBS	=	Fetal bovine serum
GEN	=	Genistein
GM	=	Complete growth medium
ICCVAM	=	US Interagency Coordinating Committee on the Validation of Alternative Methods
KLK3	=	Kallikrein 3
LUT	=	Luteolin
M	=	Cell culture medium
MF	=	Microsomal fraction
N	=	Nucleus
NRG	=	Naringenin
PBS	=	Phosphate buffer solution
PSA	=	Prostate-specific antigen
QRC	=	Quercetin
RESV	=	Resveratrol
SM	=	Starvation medium
SN	=	Supernatant
TM	=	Treatment medium

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