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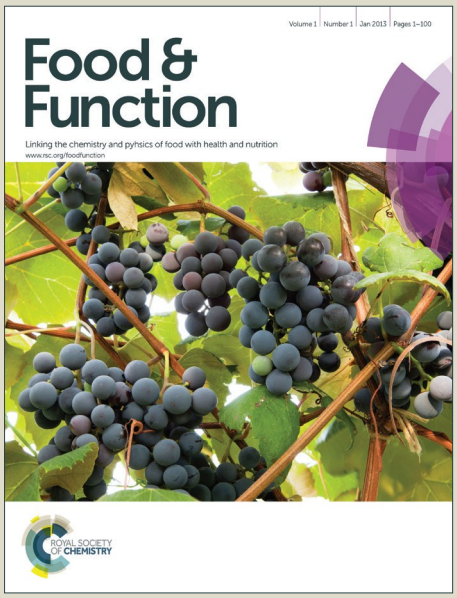

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ARTICLE

Raman Microspectroscopy for Probing the Impact of a Dietary Antioxidant on Human Breast Cancer Cells

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P. S. C. Medeiros[a], A. L. M. Batista de Carvalho[a], C. Ruano[b], J. C. Otero[b] and M. P. M. Marques[a,c]

Breast cancer is the second most common type of cancer worldwide and the most frequent among women, being the fifth cause of death from neoplastic disease. Since this is an oxidative-stress related neoplasia, it is largely preventable. A dietary isoflavone abundant in soybean – daidzein – was currently investigated as to its chemopreventive and/or chemotherapeutic properties towards the human MDA-MB-231 (metastatic, estrogen-unresponsive) and MCF-7 (estrogen-responsive) breast cancer cell lines. Biological assays for evaluation of antitumour and anti-invasive activities were combined with state-of-the-art vibrational microspectroscopy techniques. At 50 and 100 μM concentrations and 48 h incubation time, daidzein was found to induce a marked decrease in cell viability (*ca.* 50%) for MDA-MB-231 and MCF-7 cells (respectively *ca.* 50% and 42%) and 40% inhibition of cell migration 48 h. MicroRaman analysis of fixed cells upon exposure to this isoflavone unveiled its metabolic impact on both cell lines. Multivariate data analysis (unsupervised PCA) led to a clear discrimination between control and DAID-exposed cells, with distinctive effects on their biochemical profile, particularly regarding DNA, lipids and protein components, in a cell-dependent way. This is the first reported study on the impact of dietary antioxidants on cancer cells by microRaman techniques.

A Introduction

Cancer is one of the major causes of morbidity and mortality worldwide, closely following cardiovascular disorders. Breast carcinoma, in particular, is an important public health problem since it is the second most lethal cancer among women worldwide: according to the World Health Organisation (WHO), it affects 28% of the women in Europe[1], being the second most lethal cancer type in Portugal (16%).[2] Epidemiological evidence has demonstrated a clear difference in breast cancer incidence between eastern and western countries, asian women being the least affected, which can be closely related to dietary habits.

Phytochemicals, secondary plant metabolites widespread in fruits, vegetables, cereals and beverages such as tea, coffee and wine, and very abundant in a mediterranean diet, are an abundant source of dietary antioxidants known to significantly decrease the harmful effect of oxidative species, thus being promising chemopreventive agents towards carcinogenesis.[3-6] Actually, several studies have established a direct relationship between the intake of polyphenols and the prevention of oxidative stress-induced diseases, namely neurodegenerative and cardiovascular disorders, and some types of cancer.[7,8]

The isoflavone daidzein (DAID), in particular, is the second most abundant component of soybean (following genistein), a growingly consumed food product, which renders this isoflavone a very suitable target of study regarding its potential health beneficial effects. In addition, it exhibits structural and functional similarities to the endogenous hormone estrogen[9] (Fig. 1). Since some breast cancers are estrogen-dependent (ER^+), DAID may compete with natural estrogen reducing its bioavailability and therefore inhibiting cancer cell growth.[10,11] Also, this phytoestrogen displays a recognised high antioxidant activity and has been shown to trigger cell death in a variety of cancers, in some cases with a higher inhibitory activity relative to clinically used drugs such as tamoxifen.[12,13] Particularly regarding mammary cancer, daidzein was shown to be more effective than genistein towards inhibition of growth of this type of tumours in rodents.[13] Furthermore, DAID has been found to act as a chemopreventive agent, its dietary consumption from soybean being reported to reduce both prostate and breast carcinomas.[14-16] However, the specific mechanism through which polyphenolic antioxidants protect against deleterious oxidative processes has not yet been completely elucidated, being proposed to suppress carcinogenesis mainly during the initiation phase, since they exert

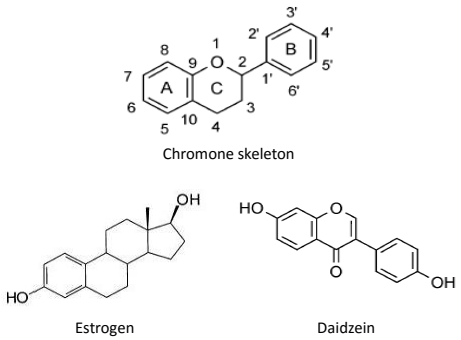

Fig. 1 General chromone skeleton (adapted from [17]), and structures of daidzein and estrogen.
(The atom numbering and ring labeling are included).

[a] "Química-Física Molecular", Univ. Coimbra, 3004-535 Coimbra, Portugal
[b] Dep. Physical-Chemistry, Fac. Science, Univ. Malaga, Unidad Asociada CSIC, Spain
[c] Dep. Life Sciences, Univ. Coimbra, 3000-456 Coimbra, Portugal

their effect mostly as radical scavengers. Although these bioactive compounds usually act as antioxidants, they are characterised by a dual activity, and may also behave as pro-oxidants and trigger carcinogenesis depending on their concentration, target molecule and specific environment.[18-22] The prevalence of both properties in a single compound can be beneficial in terms of rationale preventive or therapeutic purposes.

The potential application of this kind of compounds against cancer requires a detailed knowledge of their effect on biological matrices such as cells, with a view to better understand their mechanism of action at a molecular level. Raman microspectroscopy is a cutting-edge technique that yields an accurate and non-invasive probing of the cellular chemical fingerprint in the presence of the tested compounds, at different exposure times,[22-26] probing their biodistribution and metabolic impact. In fact, since microRaman is extremely sensitive to the chemical profile of a sample, it allows to distinguish the smallest variations even in heterogeneous biological models. In cells, in particular, it is possible to achieve sub-cellular resolution and identify organelles and biomolecules, as well as to obtain information on the chemical composition of the intracellular medium under different conditions (*e.g.* pH, temperature, ionic strength or exposure to drugs).[22,27,28]

Following previous studies by the authors on dietary polyphenolic compounds – both regarding their structural and conformational preferences[17,29-40] and their chemopreventive and/or chemotherapeutic properties towards several cancer cells – the present work reports the effect of daidzein on the biochemical profile of two human breast cancer cell lines: MCF-7 (estrogen-dependent, ER^+) and MDA-MB-231 (estrogen-independent, ER^-). The results thus obtained are expected to help establishing an accurate balance between DAID´s antioxidant and prooxidant capacities, aiming at a prospective use against breast cancer: either as a chemopreventive agent (sole or in combination with other flavones) or as an adjuvant in chemotherapy (coupled with conventional drugs such as tamoxifen, paclitaxel, docetaxel, methotrexate, gemcitabine or doxorubicin[41,42] and cisplatin.[43-45] A multidisciplinary approach was followed, coupling biological assays (at the "Molecular Physical-Chemistry" R&D Group of the University of Coimbra, Portugal) to cutting-edge Raman microspectroscopy techniques (at the Physical-Chemistry Department of the University of Malaga, Spain). Several parameters were evaluated, for concentrations between 25 and 100 µM and exposure times from 24 h to 72 h: (i) cell proliferation and viability; (ii) cell migration and metastatic activity; (iii) level of reactive oxygen and nitrogen species (ROS/RNS); (iv) cellular biochemical profile. To the best of the authors´ knowledge, this is the first reported study on the impact of dietary antioxidant compounds on cancer cells tackled by microRaman spectroscopy.

B Experimental

B.1 Reagents

Antibiotics (penicillin-streptomycin 100x solution), crystal violet, glacial acetic acid, 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), daidzein (4´,7-dihydroxyisoflavone), dimethylsulfoxide (DMSO), Dulbecco´s modified Eagle´s high glucose (4500 mg/l) medium (DMEM-HG), ethylenediaminetetraacetic acid (EDTA, disodium salt, dihydrate), formalin (10% neutral-buffered formalin, *ca.* 4% formaldehyde), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 0.05% *(w/v)* solution), phosphate buffered saline (PBS), Sulforhodamine B (SRB, monosodium salt), 0.5% *(w/v)* solution), Trypan blue (0.04% *(w/v)* solution), trypsin-EDTA (0.05% *(w/v)* solution) and inorganic salts (analytical grade) were obtained from Sigma-Aldrich Química S.A. (Sintra, Portugal). Fetal bovine serum (FBS) was purchased from Gibco-Life Technologies (Porto, Portugal) and BD Matrigel™ from BD Biosciences (Porto, Portugal).

B.2 Cell Culture

The human breast cancer cell lines MDA-MB-231 (human Caucasian triple-negative, claudin-low, breast carcinoma, (ER-)) and MCF-7 (human Caucasian estrogen-dependent breast carcinoma (ER+)) were purchased from the European Collection of Cell Cultures (ECCAC, Salisbury, UK).

Stock cultures of cells were maintained at 37 °C, under 5% CO_2. They were grown in monolayers, in Dulbecco's modified Eagle's high glucose (4500 mg/L) medium (DMEM-HG), supplemented with 10% *(v/v)* heat-inactivated fetal bovine serum (FBS), 1% *(v/v)* penicillin (100 U/mL)/streptomycin (100 mg/mL) and sodium bicarbonate-20 mM (pH 7.4). The cells were subcultured twice a week, and were harvested at 80% confluence using Trypsin-EDTA (1x). Under these conditions, a duplication time of 26 h was determined. The cells were always in the logarithmic phase of growth when the tested isoflavone was added.

B.3 Evaluation of Cytotoxic and Antiproliferative Effects

Cytotoxicity and cell density evaluation after exposure to daidzein, for concentrations between 25 and 100 µM, were assessed by the MTT[46,47] and SRB[48] assays, respectively.

Cells were plated at a $3x10^4$ cells/cm^2 density and DAID solutions (25, 50, 75 and 100 µM) were added after 24 h. Both untreated and drug-treated cultures were analysed at 48 and 72 h. A 0.01% *(w/v)* DMSO solution was always considered as a control, since DMSO was used as a solvent for daidzein.

B.4 Assessment of Anti-invasive Ability

The invasive ability of the MDA-MB-231 cells was measured using the transwell migration technique (Boyden chamber assay)[49-51]. Each insert from a 24-well plate was coated with 100 µl of Matrigel (250 µg/ml) and incubated at 37 °C for 2 h. 500 µl of serum-free medium containing MDA-MB-231 cells ($5x10^4$ cells/ml) and daidzein (100 µM), or 0.1% *(w/v)* DMSO (for the control), were then added into the upper chamber, and 750 µl of complete medium into the lower one. After 72 h incubation, cells on the inserts were scrubbed out and the migrated cells were fixed with 4%-formalin for 20 min, stained with 0.1%-crystal violet and photographed under an optical microscope (at a 10x amplification). The invasive cells were counted in the several fields, in triplicate.

B.5 ROS/RNS Measurement

In order to determine ROS/RNS levels (reactive oxygen and nitrogen species)[52], cells were exposed to DAID for 48 h, after which they were washed and further incubated with the fluorigenic probe dichlorodihydrofluorescein diacetate (DCFH-DA) for 30 min. Upon

removal of the medium, the cells were washed with PBS, trypsinised, ressuspended in PBS and fluorescence was measured at 533 nm with an excitation radiation of 480 nm.

B.6 Sample Preparation for Spectroscopic Analysis

Cells were seeded at a concentration of 3×10^4 cells/cm^2, on an optical substrate suitable for Raman data collection[24]: MgF_2 (2x20 mm) disks, previously cleaned with ethanol-70% (*v/v*). Upon incubation for 24 h, cells were treated with different concentrations of DAID (50 and 100 μM) and left to culture for further 48 h. The growth medium was then removed, cells were washed twice with NaCl-0.9%, fixed in 4% formalin (diluted in NaCl-0.9% from the commercial 10% neutral-buffered formalin solution) for 10 min[53] and washed several times with pure water (to remove any residual salt). The disks were allowed to air-dry prior to spectroscopic analysis. All samples were prepared in triplicate, in a single experiment.

B.7 Raman Microspectroscopy

The Raman spectra were acquired at room temperature, in a Renishaw Reflex inVia Raman microspectrometer equipped with a 100x Leica objective (NA=0.9 fd=0.27mm) and a RenCam CCD (Charge Coupled Device) detector (at the Physical-Chemistry Department of the University of Malaga). The 785 nm line from a diode laser was used as the exciting radiation, yielding 55 mW and 0.10 mW at the sample position, respectively for solid daidzein and the cell samples. Data were recorded for the spectral ranges 50 to 2000 cm^{-1} for the solid isoflavone, and 500 to 1800 cm^{-1} for the cells. 35 to 40 spectra were collected *per* sample (from both the cytoplasm and the nucleus), with 10 s of exposure and 5 co-added scans *per* chosen point, sampling approximately 40 cells *per* sample, at a 1 cm^{-1} spectral resolution.

Apart from the control (untreated cells), samples containing daidzein-50 and 100 μM (upon a 48 h exposure) were analysed.

B.8 Data Preprocessing and Analysis

All biological assays were performed in triplicate. The results were expressed as the mean ±SD value. Statistical analysis was carried out using One-Way ANOVA, followed by the post hoc Turkey's and Dunnett's multi-comparison tests for the SRB and MTT results, respectively. The differences were considered significant for p<0,05 p<0,01 or p<0,001.

The WiRE 2.0 software was used for Raman spectra collection. Data pre-processing was performed with the MatLab software (version R2015b, The MathWorks Inc., Natik, MA). Spurious peaks caused by cosmic rays were removed and the spectra were selected for treatment by discarding data with very poor S/N ratio. The spectra were baseline corrected by detrending (2nd order polynomial[54], thus minimising the effects of variable thickness within and among the cell samples), smoothed by the Savitsky-Golay filter (11 points, 1st order polynomial, which enabled reduction of noise without band distortion), and unit vector normalised.

Interpretation of the spectral data was carried out after multivariate analysis of the results – unsupervised principal components analysis (PCA) – which was carried out in Matlab. The order of the PCs denotes their importance to the dataset, PC1 corresponding to the highest amount of variation.

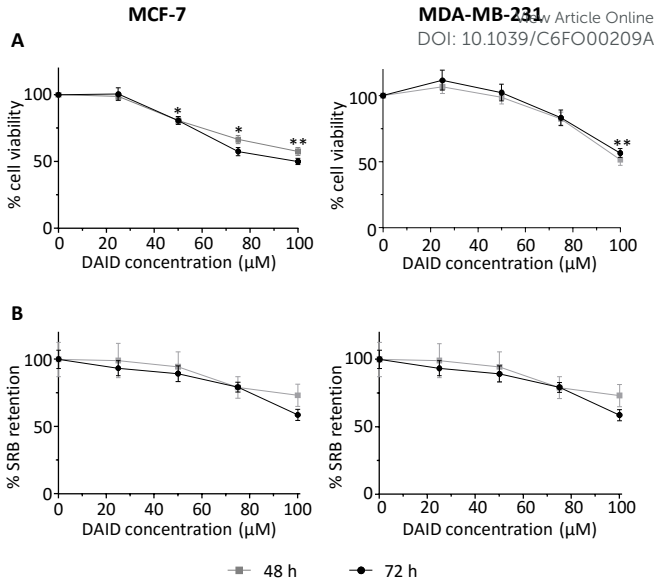

Fig. 2 Dose and time-dependent response plots for the effect of daidzein against the MCF-7 and MDA-MB-231 cell lines: (A) Cytotoxic activity (MTT assay). (B) Antiproliferative capacity (SRB assay).
The results are represented as a percentage of the control (cells treated with the DMSO vehicle). The data are the mean ± SEM of the values obtained for 3 independent experiments, carried out in triplicate (n=3). The one-way ANOVA statistical analysis was used, the Dunnett's (A) and Tukey's (B) post-test was carried out: *p < 0.01, **p < 0.001.

C Results and Discussion

The health-beneficial properties of isoflavones rely on their hydroxyl groups, as well as on the substitution pattern of their chromone core. Although this chromone moiety seems to have an essential role for biological activity, other structural parameters are determinant such as the nature, number and position of the different substituent groups in rings A, B and C[17] (Fig. 1). Additionally, these structural characteristics affect water solubility which is an important parameter if isoflavones are to be used as chemopreventive or chemotherapeutic agents *in vivo*.[55] Furthermore, the capacity of these compounds to conjugate with proteins, enzymes and diverse biological receptors within a biological matrix (cell or tissue) is strongly dependent on their electronic distribution and three-dimensional conformation. In general, the sites of highest electronegativity within an isoflavone are the 5-hydroxyl and 7-hydroxyl positions at the A-ring, and the 3´ and 4´ hydroxylation sites at the B-ring[17] (Fig. 1).

C.1 Cytotoxic and Antiproliferative Effects

Analysis of the mitochondrial desidrogenase activity in the presence of daidzein for both breast cancer cell lines under study evidenced a cytotoxic effect for a 48 h exposure, that was maintained at identical levels at 72 h (Fig. 2(A)). While for the MDA-MB-231 cells the viability was affected only for DAID doses of 75 and 100 μM (up to a maximum of 50%), a daidzein-induced viability decrease was detected for the MCF-7 cell line at 50, 75 and 100 μM (reaching 42% for the highest concentration).

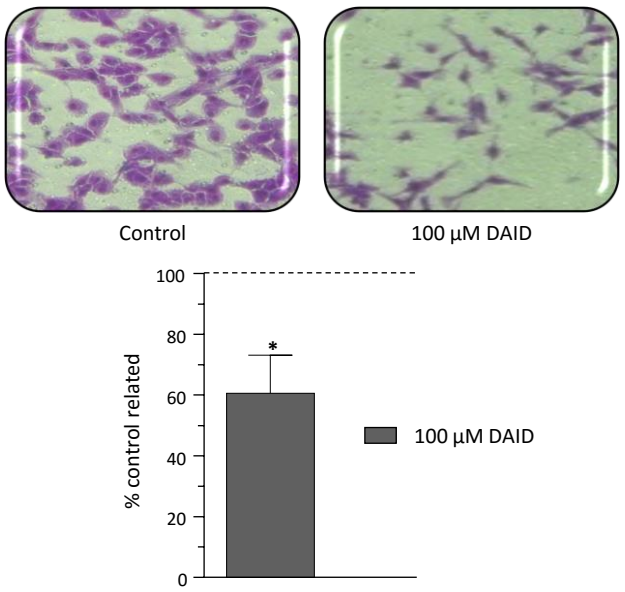

Fig. 3 Inhibition of cell migration by daidzein (at 72 h) for the MDA-MB-231 cell line.

The results were obtained by the transwell migration assay (Boyden chamber assay), and are represented as a percentage of the control (cells treated with the DMSO vehicle) considered as 100% (horizontal dashed line). The data are the mean ± SEM of the values obtained for 3 independent experiments (n=3). The one-way ANOVA statistical analysis was used, and the Dunnett's post-test was carried out: *$p < 0.05$.

Regarding antiproliferative activity, the results obtained by the colorimetric SRB method did not show significant differences between the two tested cell lines (Fig. 2(B)). The slight cell growth inhibition detected for DAID dosages above 50 µM may suggest that this isoflavone interferes with protein synthesis, in agreement with reported studies on other flavonoids.[56] In this case, the effect of the antioxidant appears to be higher on the MDA-MB-231 cells, for which there is an enhanced growth inhibition for longer exposure times (72 h *vs* 48 h) as opposed to the MCF-7 cell line.

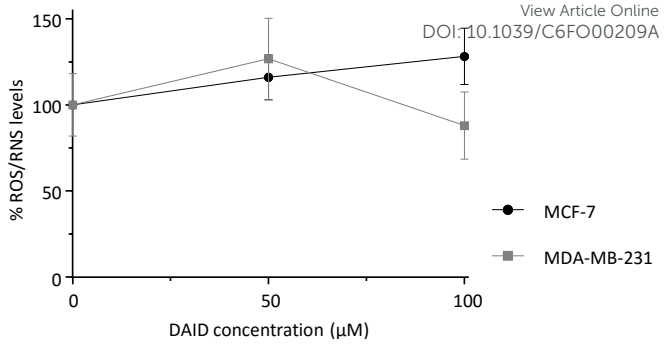

Fig. 4 ROS/RNS levels in MCF-7 and MDA-MB-231 cell lines, upon 48 h exposure to daidzein.

The results, obtained using the fluorigenic probe DCFH-DA, are represented as a percentage of the control (untreated cells) considered as 100%. The data are the mean ± SEM of the values obtained for three independent measurements, carried out in triplicate (n=3). The one-way ANOVA statistical analysis was used, and the Dunnett's post-test was carried out.

These results, reflecting a more significant impact of the isoflavone towards the estrogen-dependent MCF-7 cell line relative to the estrogen-unresponsive MDA-MB-231 cells, were foreseen in view of the estrogen mimetic properties of daidzein (Fig. 1), which is expected to interact effectively with estrogen receptors therefore inhibiting estrogen synthesis[17,57,58] and leading to a growth inhibition of MCF-7 cancer cells. In turn, for the MDA-MB-231 cells lacking estrogen receptors, daidzein is suggested to affect cell proliferation and viability by interference with different metabolic pathways, namely through cell cycle arrest (changes in regulatory proteins) and induction of apoptosis (*via* activation of caspases).

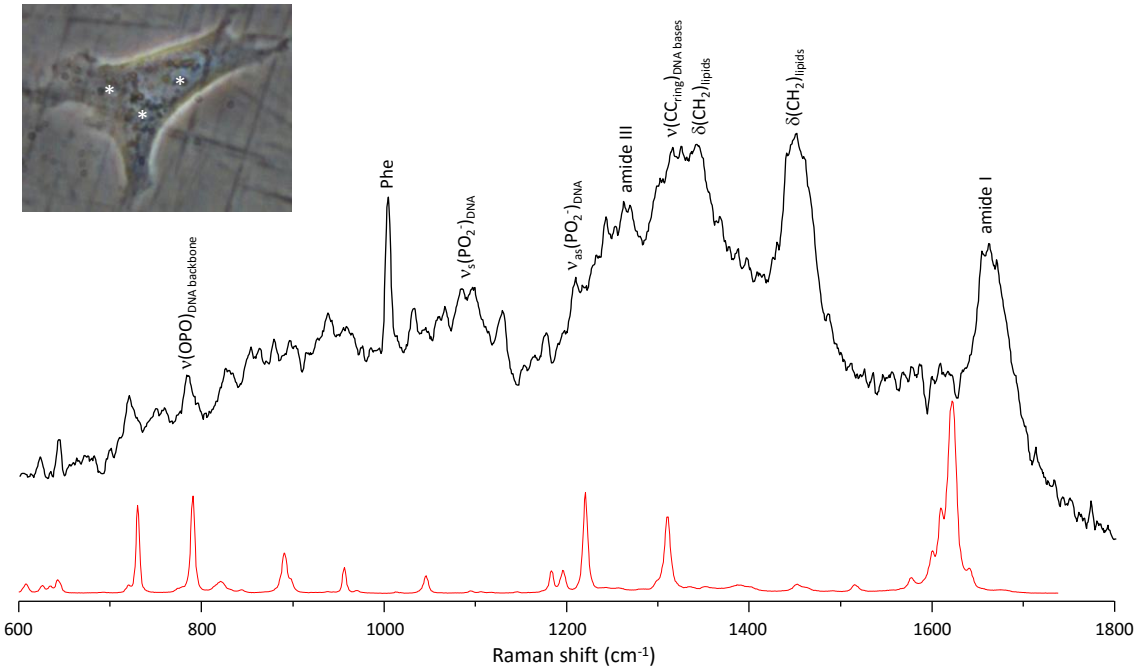

Fig. 5 Mean Raman spectra for untreated MCF-7 cells, and microscopic image of a cell (x100 magnification) showing some of the points at which data were captured. The spectrum of daidzein (from[39]) is shown in red.

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The data presently gathered is in good agreement with that previously reported for daidzein´s effect on human hepatocellular carcinoma cells.[59]

C.2 Anti-invasive and Anti-metastatic Abilities

Cancer metastasis is due to the cell´s aptitude to migrate and invade tissues different (and often distant) from the the primary tumour location, through a three-step process: (i) cell-to-extracellular matrix (ECM) adhesion; (ii) proteolytic cleavage; (iii) and cell migration.[60] ECM degradation (promoting epithelial-to-mesenchymal transition) occurs in the presence of matrix metalloproteinases (MMPs), which are known to be activated by an increase in ROS levels.[60-63] The transwell migration experiments currently performed allowed to evaluate the cells´ migration and invasive abilities in the absence and presence of daidzein, for a 72 h incubation period, as a function of concentration.

This assay was performed only for the MDA-MB-231 cell line (since the MCF-7 cells do not have an invasive potential), cell migration being reduced by *ca.* 40% for 100 µM-DAID (Fig. 3). Hence, at this concentration daidzein showed to have a twofold effect on these triple-negative breast cancer cells, affecting both their viability (Fig. 2(A)) and invasive capacity. These are independent activities, however, triggered by an impact of the flavonoid on distinct biological pathways. Since the transwell migration assay currently used for this analysis evaluates exclusively the migrating ability of the cells by counting the invasive ones, and these are much less than those remaining in the upper compartment of the Boyden chamber (still at a very high density), the results currently reported are considered not to be affected by DAID-induced cell killing or growth inhibition at 100 µM (which occurs concomitantly in the upper compartment).

The data presently obtained is in agreement with the known ability of this phytoestrogen to decrease cell migration through downregulation of MMPs[16] and inhibition of urokinase-type plasminogen activator (uPA), as well as of expression of NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) and AP-1 (activator protein 1).[64]

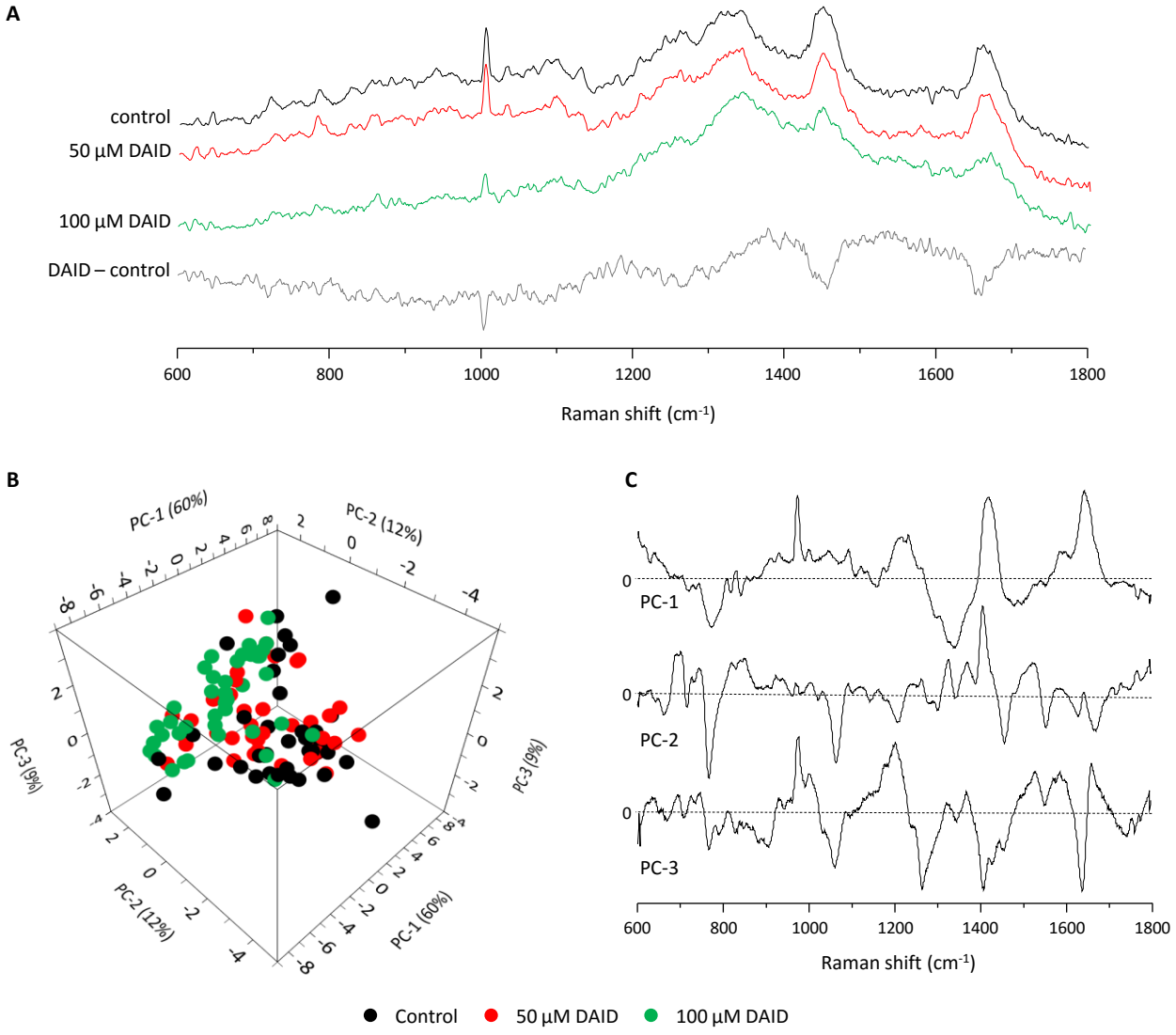

Fig. 6 Mean Raman (600 – 1800 cm^{-1}) and difference spectra to the control (A), and PCA score (B) and loading (C) plots for DAID-treated MCF-7 cells *vs* the control (50 and 100 µM, 48 h exposure).
(For clarity, the spectra and loadings are offset, the dashed horizontal line indicating zero loading).

C.3 ROS/RNS Measurement

Reactive oxygen and nitrogen species are generated in mitochondria as products of natural oxidative processes within the different cellular locations. Data were captured as single point spectra (Fig. 5), within a cell population encompassing randomly distributed cells with a high spectral heterogeneity (also due to cells being in different points of the cell cycle). This procedure, as

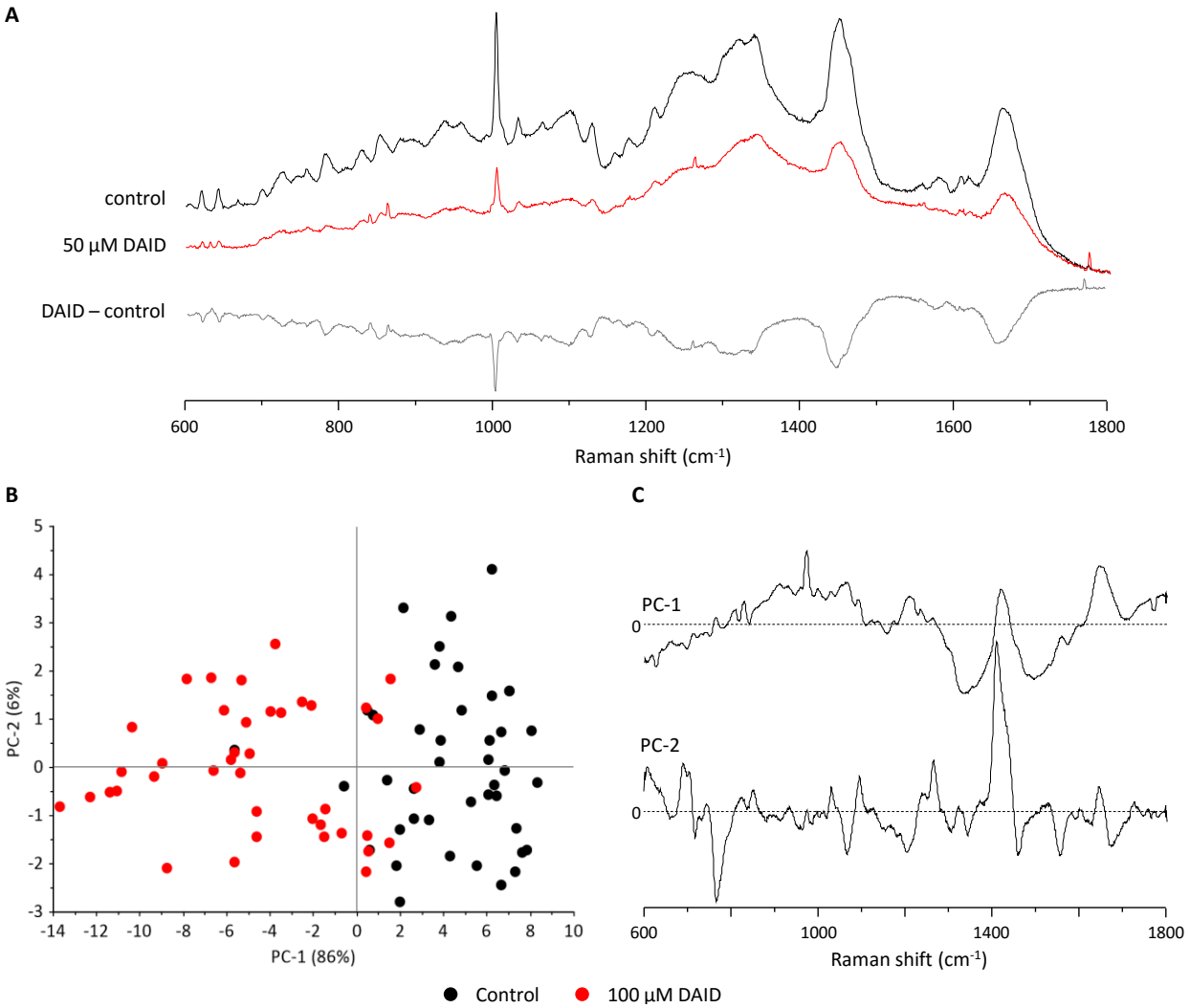

Fig. 7 Mean Raman (600 – 1800 cm^{-1}) and difference spectra to the control (A), and PCA score (B) and loading (C) plots for DAID-treated MDA-MB-231 cells *vs* the control (100 µM, 48 h exposure).
(For clarity, the spectra and loadings are offset, the dashed horizontal line indicating zero loading).

cell, and may regulate apoptotic cell death.[12] In the present study, evaluation of the ROS/RNS levels present in the intracellular medium was performed, for both the MCF-7 and MDA-MB-231 cells, after a 48 h exposure to daidzein (Fig. 4).

The preliminary results currently obtained evidence a cell-selective effect for daidzein, in the concentration range tested, ROS/RNS levels clearly increasing with isoflavone concentration for the estrogen-dependent line (MCF-7), in accordance with reported data for other cell lines.[65] For MDA-MB-231, in turn, the effect of the isoflavone is less marked, and does not appear to change significantly for concentrations above 50 µM.[52]

C.4 Metabolic Profiling by Raman Microspectroscopy

Spectral biochemical signatures of the cell, for each type of cell line at the different conditions tested (DAID concentration and exposure time), were obtained by averaging the Raman spectra acquired from compared to single cell spectral acquisition, reduces the effect of the cell cycle profile. Despite the intense vibrational bands characteristic of daidzein in the presently probed spectral window (Fig. 5), they were untraceable intracellularly.

Upon unsupervised principal components analysis (PCA), a detailed picture of the daidzein-impact on the cellular biochemical profile was achieved. A dose-dependent response was found, as depicted in Figs. 6 and 7 that comprise the mean Raman spectra measured for MCF-7 and MDA-MB-231 cells, respectively, upon incubation with daidzein at different concentrations, as well as the corresponding PCA loading and score plots. From the loading plots corresponding to each principal component it was possible to derive chemical information based on the differentiation between the tested conditions, and to evidence inter-group variance. Additionally, loading plots could be matched with the corresponding experimental spectra, and the

positive and negative peaks in the loadings were ascribed to the different groups observed in the score plots. The fact that discrimination was attained according to PC´s dominated by Raman features characteristic of the cellular components (and not of daidzein) ensures that the detected spectral variances are not due to the presence of the externally added isoflavone but instead to its effect on the cell´s biochemical fingerprint. Hence, the results thus gathered solely reflect the impact of DAID on these cancer cells, followed by the cellular physiological response to this external perturbation.

For the MCF-7 cells, discrimination of the data was quite good, but only for the highest daidzein concentration tested (100 µM) (Fig. 6). It occurred along PC2, the main cellular constituents affected by the isoflavone being DNA (bands at *ca.* 800, 1090 and 1480 cm^{-1} ascribed to $\nu(OPO)_{backbone}$, $\nu_s(PO^2)$ and $\nu(CC)_{ring}$ from adenine and guanine) and lipids (1450 cm^{-1}).

Concerning the estrogen-unresponsive cell line MDA-MB-231, the spectra from the control and DAID-treated cells were clearly distinct and well differentiated by PCA analysis, according to PC1 (Fig. 7). The loading scores reflected a predominant daidzein interference with aminoacids (phenylalanine, Phe, at 1003 cm^{-1}) and proteins ($\delta(CH_3)_{glycoproteins}$ at 1375 cm^{-1} and amide I band at *ca.* 1660 cm^{-1}), apart from DNA phosphates (1090 cm^{-1}) and lipids (1450 cm^{-1}).

These results are in agreement with the DAID-triggered decrease in cell proliferation and viability already evidenced by the MTT and SRB assays, which unveiled a striking cell-selectivity for the daidzein impact on these breast cancer cells – either estrogen-responsive or unresponsive.

Actually, the mechanism of action of daidzein appears to be different for each of the two cell lines studied. Since MCF-7 are estrogen-dependent cells, the main mechanism expected to be responsible for the isoflavone´s effect is the modulation of estrogen synthesis by interference with the endogenous estrogen receptors. This estrogen-like activity thus regulates cancer cell growth and differentiation. Reported studies on DAID-treated MCF-7 cells[66,67] exposed an induced cell cycle arrest in the G1 and G2/M phases, coupled to an accumulation of cells in the sub-G0 phase, which are in accordance with the currently observed effect on DNA. Additionally, in this cell line DAID may induce an overexpression of manganese superoxide dismutase (MnSOD), leading to a reduced cell proliferation.[68]

In the light of previous studies in MCF-7 which revealed a DAID-induced apoptosis *via* a mitochondrial pathway,[69] a similar mechanism is presently suggested for the impact of daidzein on the MDA-MB-231 cells. Also, its visible effect on typical aminoacid (*e.g.* Phe) and protein bands in these estrogen-independent cells (Fig. 7(C), PC1 loading plot) is proposed to be associated to an interference with protein synthesis (*e.g.* regulatory proteins and cyclin-dependent kinases), leading to cell cycle arrest and apoptosis and hence to reduced cell viability and migration ability, as evidenced in the cytotoxicity evaluation assays and transwell cell migration experiments.[70-73] In addition, MDA-MB-231 and MCF-7 cells have different glycolipid compositions (*e.g.* neutral glycolipids and gangliosides),[74] which may also justify the presently detected cell line selectivity (MDA-MB-231 *vs* MCF-7) regarding the impact of the tested antioxidant on cellular lipids.

D Conclusions

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The impact of the dietary isoflavone daidzein on the biochemical fingerprint of two human breast cancer cell lines – estrogen-responsive (MCF-7) and estrogen-unresponsive (MDA-MB-231) – was probed by cutting-edge vibrational microspectroscopy techniques, while its main biological activities (antiproliferative, cytotoxic and anti-invasive) were evaluated by standard biological assays. DAID was found to induce a noticeable decrease in cell growth and viability (*ca.* 50%) for both types of cells, upon a 48 h exposure to concentrations of 50 to 100 µM. Apart from being concentration-dependent, the effect exerted by this phytochemical on the cellular metabolic profile was found to be strongly cell-dependent, being more obvious for the estrogen receptor positive cells as compared to the triple negative ones, in accordance with the estrogen-like activity of this isoflavone. Daidzein was shown to impact more noticeably on the cellular protein content for the MDA-MB-231 cell line as compared to the MCF-7 cells, for which the most significant effect was on DNA and lipids. Still, despite the Structure-Activity Relationships (SARs) established *in vitro* for flavonoids[75], the well recognised diversity of their biological activity, affecting multiple cellular pathways, is a limitation when trying to reliably identify specific biochemical mechanisms within the cell that are affected by exposure to these phytochemicals.

Since several human breast adenocarcinomas are heterogeneous, comprising both ER-positive and -negative cells, this dual activity currently verified for daidzein (similar to other flavones previously investigated) is of significant interest for its potential use as a chemopreventive/therapeutic agent. Moreover, the high sensitivity of the Raman microspectroscopy technique (coupled to a dedicated multivariate analysis of the data) allowed to unveil with high accuracy the impact of daidzein at the cellular level, by monitoring the chemical profile of the cell upon incubation with different concentrations of this compound, different effects having been detected for each type of breast cancer cell line: while lipids and DNA were found to be affected in both ER^+ and ER^- cells, for the latter (MDA-MB-231) daidzein also impacted on aminoacids and proteins.

The role of antioxidants in cancer, namely in therapy, is still controversial: acting as pro-oxidants (at high dosages) they may sensitize diseased cells and enhance chemotherapeutic intervention, while their antioxidant capacity helps to minimise deleterious side-effects of anticancer drugs. Therefore, phytochemical compounds are the subject of an increasing research effort towards the development of natural-based agents for cancer prevention and treatment, as well as of alternatives to chemically designed antineoplastic agents – either as constituents of drug combinations or as adjuvants (allowing lower dosages of the conventional drugs and therefore decreased associated toxicity). Indeed, the costs related to the extraction and purification of phytochemicals from their natural sources (fruits and vegetables) might well be lower than those involving the *de novo* rational design of new chemical entities aimed as chemopreventives and/or chemotherapeutics. It is therefore crucial to gain a detailed knowledge, at the molecular level, of the effect of these compounds on the cell´s biochemical profile, which was successfully achieved in the present study for dadzein towards human breast cancer cells. This isoflavone was therefore shown to be a promising chemoprotective agent against human

breast adenocarcinoma,[76] particularly the ER+ type, and can be envisaged as a promising agent in adjuvant chemotherapy.[75]

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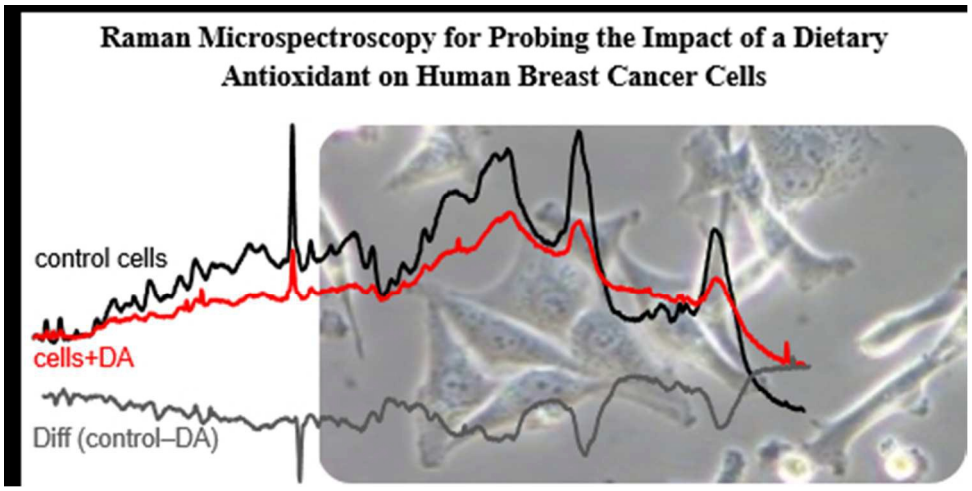

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