Exploring the anticancer properties of colonic metabolites derived from Virgin Olive Oil

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Abstract

Colorectal cancer (CRC) is among the leading causes of mortality through the world. The Mediterranean diet has shown protective action against CRC due to the intake of different components, being one of the main ones, the virgin olive oil (VOO). Hydroxytyrosol, (HT) the most important phenolic compound in VOO, is reported to present several biological activities including anticancer effect. However, the molecular mechanisms underlying the protective action of HT on CRC are still unclear as well as its effect on cancer stem cell's subpopulation. The aim of this thesis was to evaluate the effect hydroxytyrosol and its colonic metabolites, namely phenyl (PA and PPA) in inhibiting proliferation and targeting cancer stemness on a 3D cell model of CRC - HT29 cell spheroids.

Results showed that, HT presented the highest antiproliferative effect in both monolayers (EC_{50} = 93.58 µM) and spheroids (EC_{50} = 3938 µM) of HT29 cells. Additionally, HT was the only compound capable of targeting cancer stemness by i) reducing $ALDH^+$ cells (reductions of 16.4% and 39% for 200 µM and 600 µM, respectively) and ii) totally inhibiting colony formation at concentrations above 50 µM. For the same concentrations tested, PA and PPA showed no significant effect. Gene expression analysis revealed that HT at 200 µM slight reduced the expression of markers related to stemness (*NANOG, OCT4*), Epithelial to Mesenchymal Transition (*VIM*, *TGFβ1*), Sonic Hedgehog Pathway (*GLI1, PTCH1*) and proliferation (*CCNA2)*.

This research work shows great promise in developing new and better ways of tackling colorectal cancer treatment, focusing not only on targeting normal cancer cells, but also cancer stem cells resorting to olive oil bioactive compounds.

Keywords: cancer stem cells, colorectal cancer, hydroxytyrosol, virgin olive oil, 3D cell model

Introduction

Colorectal Cancer

Colorectal cancer (CRC) is among the leading causes of mortality and morbidity through the world, representing a major public health problem.[1] CRC represents 10.2% of all cases of cancer worldwide and 9.2% of the total cancer deaths, being the third most common cancer worldwide and the fourth most common cause of oncological death.[1,2] 55% of all the CRC cases occur in developed or developing countries, which may reflect an increased prevalence of risk factors for CRC associated with the western lifestyle. This risk factors include cigarette smoking, excess body weight, diet (including high consumption of alcohol, as well as red and processed meat and low consumption of fruits, vegetables, dietary fiber and calcium) and physical inactivity.[1,3]

Mediterranean Lifestyle and Virgin Olive Oil

Lifestyle modifications are an important tool to reduce the incidence and therefore the mortality rates of this type of cancer. Cancers like CRC present a lower incidence, a lower death rate and a higher life expectancy in Mediterranean countries when compared to Northern Europe. It is estimated that the incidence of cancer could be reduced by 25% if the populations of the Western countries consumed a traditional Mediterranean diet.[4–8]

Olive oil is one of the main components of the traditional Mediterranean diet.[4,5] It contains a vast range of substances such as monounsaturated free fatty acids, hydrocarbon, squalene, tocopherols, and phenolic compounds.[5] Olive oil is a source of, at least, 30 phenolic compounds, being one of the most important ones, hydroxytyrosol (HT).[5] During gastric digestion, complex virgin olive oil phenols (oleuropein, derivatives or secoiroids) are transformed into HT, which is later transformed into its metabolites like phenylacetic acid (PA) and phenylpropionic acid (PPA). [9]

Hydroxytyrosol

HT is a potent antioxidant and has several biological activities: significant anti-inflammatory, control of oxidative stress, inhibition of several tumor cell lines as a chemotherapeutic agent, and apoptosis promoter,

improving endothelial cell function, having protective effect on liver steatosis and presenting neuroprotective effects as well. This compound may exert antitumor activity on human colon adenocarcinoma cells, affecting the transcription of genes involved in programmed cell death and activating caspase-3. HT also enhances carcinogen detoxification and upregulates xenobiotic metabolizing enzymes.[7,9,10]

Cancer Stem Cells

The high ineffectiveness in treating CRC has been attributed to the existence of rare, highly drug-resistant and quiescent or slow proliferating cells with stem-like properties: Cancer Stem Cells (CSC).[11]

A tumor can be viewed as an aberrant organ initiated by a tumorigenic cancer cell (stem cell) that acquired the capacity for indefinite proliferation through accumulated mutations and drives the tumor growth.[12]

CSC model, represented in figure 1, relies on four premises: i) a substantial fraction of cellular heterogeneity observed in tumors results from its hierarchical organization, which is often reminiscent of the hierarchy in the tissue of origin; ii) tumor hierarchies are fueled by rare self-renewing CSCs (normally quiescent), whereas the bulk of the tumor is composed of non-CSCs that do not contribute to long term growth; iii) CSC identity is hardwired, meaning that there Is limited plasticity in the tumor hierarchy; iv) CSCs are resistant to standard chemotherapy and radiation treatments, which target, preferentially, non-CSCs.[13]

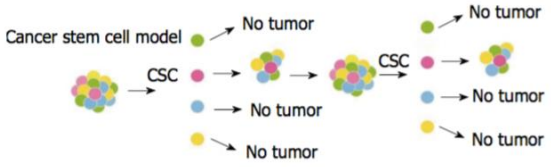

Figure 1 - Cancer Stem Cell model: only a rare subpopulation of undifferentiated cells has the necessary properties for tumor initiation, maintenance and spreading (adapted).[14]

Although there exists a variety of traditional targeting agents for the treatment of patients with CRC, and multiple combination therapies, there is not a curative solution available. This strengthens the model of CSCs and supports the hypothesis that most of the currently available therapies only target the bulk of the tumor mass (mainly constituted by proliferating cells) while sparing the rare, quiescent CSCs which are able to re-initiate tumor growth thus giving origin to both recurrences and metastases.[14]

Food bioactive compounds may represente an attractive alternative to conventional chemotherapeutical drugs. Compounds like curcumin, resveratol, sulforaphane and epigallocatechin-3-gallate are some natural compounds already resported as capable of targeting CSC subpopulations.[11]

Project Motivations

This study was performed in order to assess the action of hydroxytyrosol and its colonic metabolites in modulating cancer stemness using 3D cellular models with CSC traits. For that HT, PA and PPA were tested alone and HT in combination with 5-Fluoracil (5-FU) in HT29 cell spheroids.

Materials and Methods

Cell Culture and Cell Lines

CRC cell line HT29 (ATCC, Manassas, VA, USA) was maintained in RPMI-1640 medium (GIBCO, MA, USA) supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS). Caco-2 cell line was purchased from Deutsche *Sammlung von Microorganismen und Zellkulturen* (Barunshweig, Germany) was maintained RPMI-1640 medium supplemented with 10% (v/v) heat inactivated fetal bovine serum and 1% (v/v) penicillin-streptomycin. Both cell lines were kept at 37ºC in a humidified atmosphere of 5% CO_2 in air. All the culture medium and supplements were obtained from GIBCO® (Thermo Fisher Scientific, MA, USA).

VOO Compounds and Metabolites

Hydroxytyrosol was purchased from Extrasythese® (Genay, France). PA, PPA and 5-FU were purchased from Sigma-Aldrich® (Sigma-Aldrich, St. Louis, MO, USA). All the stock solutions were prepared with miliQ water, filtered and kept at -20ºC.

Antiproliferative Assay Using HT29 Cell Line Cultured in Monolayer (2D Cell Model)

Briefly, HT29 cells were inoculated in 96-well plates with a density of 1×10^4 cells/well. After 24h of incubation at 37ºC and 5%CO_2, the medium of each well was replaced by the compounds diluted in RPMI + 0.5% (v/v) FBS. The concentration of HT, PA and PPA ranged between 25 µM and 400 µM. Controls were included with culture medium alone and the maximum % (v/v) of solvent used (water). After 72h of incubation, the cell viability was assayed by MTT method. Results are shown as percentage of living cells relative to the control and are a mean of triplicates from three independent experiments GraphPad Prism 6 (GraphPad Software, Inc., CA) was used for plot the data, as well as for the calculation of the inhibitory concentration required to inhibit 50% of cell viability (EC_{50} value).

Cytotoxicity Assay Using Caco-2 Cell Line

This assay was performed as described by Serra et al. [15] Briefly, cells were seeded on 96-well plates at a density of 2×10^4 cells/well and the medium was replaced every 48h (RPMI + 10% (v/v) FBS + 1% (v/v) PEN). After confluency (more or less 7 days), Caco-2 cells were incubated with the compounds diluted in low-serum culture medium (RPMI + 0.5% (v/v) FBS) for 72h. The concentration of the compounds ranged between 125 µM and 2000 µM. Controls were included with culture medium alone and the maximum % (v/v) of solvent used (water). After this period of 72h, cells were washed twice with 100 μL of warm PBS per well, and 100 µL of colorimetric reagent MTT was added to each well with a concentration of 0.5 mg/ml with RPMI 0.5% (v/v) FBS and incubated at 37ºC and 5% CO_2 for 3 hours. After 3 hours of incubation, the cell medium was removed and DMSO was added to each well (150 μL/well). Cell viability was quantified by measuring the absorbance of each well at 570nm in a microplate reader Epoch 2 from Bioteck Instruments, (Winooski, VT, USA). 5 independent experiments were performed with triplicates for each condition and the results are shown as percentage of living cells relative to the control. GraphPad Prism 6 (GraphPad Software, Inc., CA) was used for plot the data, as well as for the calculation of the inhibitory concentration required to reduce 50% of cell viability (IC_{50} value).

3D Cell Culture Using a Stirred-Tank Culture System

CRC spheroids were generated using a stirred tank bioreactor, as described elsewhere.[16,17] Briefly, HT29 single cells were inoculated in a 125 mL spinner flask (Corning, NY, USA) at a density of 2.5 x 10^5 cell/ml with culture medium (RPMI-1640 supplemented with 10% (v/v) FBS). The culture started with 60% of the final volume and the remainder was added after 6h (VT = 100ml). The initial stirring rate was 40 rpm and it was increased for 50 and 60 rpm after 8 and 28h. The culture was maintained for 8 days at 37ºC with 5% CO_2 in a humidified atmosphere. From the 4th day of culture onwards, half of the culture medium was replaced on a daily basis.

Antiproliferative Assay Using HT29 Spheroids (3D cell model)

The antiproliferative effect of the HT, PA and PPA and the chemotherapeutic 5-fluororacil was assessed using spheroids collected at day 8 of culture, as described elsewhere.[11,16–19] Briefly, spheroids were seeded at a density of 5 spheroids/well on 96-well plates with 100 μL of cell culture medium (RPMI +10% (v/v) FBS). At time-point 0h, the viability of the cells was evaluated using PrestoBlue Cell Viability Reagent (Life Technologies, Carlsbad, USA). This procedure was done according to the manufacturer's instructions. The spheroids were then incubated with the compounds with concentrations ranging from 125 µM to 4000 µM and with 5-FU with concentrations ranging from 0.01 mg/mL to 0.32 mg/mL for 72h in humidified atmosphere at 5%CO_2 and 37ºC. Controls were included with culture medium alone and the maximum % (v/v) of solvent used (water).

After the 72h period of incubation, the plates were centrifuged (200g, 5min.) and the supernatant removed, the spheroids were washed with PBS twice and the step of centrifugation repeated. After the removal of the PBS, cell viability was measured using MTT viability assay. This assay allows for the comparison of the viability at time-point 0h and 72h.

The results were expressed in % of cell viability in relation to the control cells using the following equation:

$$Cell\ viability\ (\%) = \frac{Abs\ (\ treated\ spheroids)_{72h} / FI\ (treated\ spheroids)_{0h}}{Abs\ (control\ )_{72h} / FI\ ratio\ (control\ cells)_{0h}} \times 100 \quad (1)$$

Where FI is the fluorescence intensity obtained at time-point 0h and Abs is the absorbance of the spheroids at 72h.

EC_{50} values were obtained through dose-response curves using GraphPad Prism 6 (GraphPad Software, Inc., Ca). The assay was performed with 6 replicates of each sample concentration in, at least, 2 independent studies.

Aldefluor Assay (ALDH) by Fluorescence-Activated Cell Sorting (FACS)

The ALDH assay was performed according with the methodology described elsewhere.[11,16] Briefly, spheroids collected at day 8 of culture were placed, at a density of 50 spheroids/well in a 6-well plate. Spheroids were inoculated with several concentrations of the compounds (HT – 200, 400, 600 µM; PA – 600 and 1400 µM; PPA – 600 and 1400 µM; 5-FU - 0.01mg/ml) previously diluted in RPMI + 0.5% (v/v) FBS). Controls were included with culture medium alone and the maximum % (v/v) of solvent used (water). After 72 hours of incubation, the spheroids were collected to eppendorfs and centrifuged 5 min at 200g. Then the supernatant was removed and the spheroids were washed with PBS and dissociated with trypsin. ALDEFLUOR™ Assay kit (STEMCELL Technologies, Vancouver, Canada) was used according to manufacturer's instructions.[20] A negative control using diethylaminobenzaldeyde (DEAB) was prepared for each sample to correct fluorescence background.

Cells were sorted using the flow cytometer CyFlow® Space (Partec, Germany) and the data measured by the FloMax 3.0 software (Partec, Germany). The collected

data was further analyzed with Flowing 2.5 software (Turko Centre for Biotechnology, Finland).

Soft Agar Colony Forming Unit (CFU) Assay

This assay was performed as described by Pereira et al.[11] Briefly, the bottom layer of the semi-solid support was obtained from mixing in a 1:1 ratio 1.2% of low-melting agarose (Lonza, Rockland ME, USA) with 2x RPMI with 20% (v/v) FBS resulting in a 0.6% agarose with complete medium solution. 2 mL of this solution was pipetted into each well of a 6-well plate carefully to avoid the formation of bubbles. Plates were left at room temperature inside the laminar flow chamber for 30 min to 1 hour. For the upper layer, 0.6% low-melting agarose (Lonza, Rockland ME, USA) was mixed in a 1:1 ratio with warm PBS to a final volume of 6 ml/falcon (duplicates of 2 wells for each concentration) forming a solution of 0.3% agarose with PBS.

HT29 spheroids collected at day 7 of culture were dissociated as described previously (see Aldefluor Assay (ALDH) by Fluorescence-activated cell sorting (FACS)) and the cellular suspension adjusted to 1 x 10^3 cell/ml in 0.3% low-melting agarose diluted in PBS. The compounds to be tested (HT – 5, 10, 20 and 50 µM; PA – 20, 200 and 500 µM; PPA - 20, 200 and 500 µM and 5-FU – 0.001, 0.0001 and 0.00001 mg/ml) were added to this solution and 2 ml was transferred to each well. Controls were included with culture medium alone and the maximum % (v/v) of solvent used (water).

The plates were cultured in a humidified atmosphere at 5%CO_2 and 37ºC for 21 days. Twice a week, 200 µL of RPMI-1640 culture medium supplemented with 10% (v/v) was added to each well. After this period the number of visible colonies was counted visually.

The results were expressed in plate efficiency, which accounts for the ratio of number of colonies counted and the total cell number inoculated in each well:

$$Plate\ efficiency\ (\%) = \frac{number\ of\ colonies}{total\ cell\ number} \times 100 \qquad (2)$$

This result was normalized relative to the control. This assay was performed with duplicates for each condition in, at least, 5 independent experiments.

RNA Extraction and Reverse Transcription

Spheroids were seeded at a density of 50 spheroids /well in 6-well plates. They were treated with HT at concentrations of 200 µM to 600 µM. Controls with water at the maximum % (v/v) were considered controls. After a 72h incubation period, the spheroids were transferred to an eppendorf and centrifuged for 5 min at 200g. the cellular lysis was accomplished by adding 600 µL of lysis buffer RTL (QIAGEN, Hilden, Germany) with 1% (v/v) of β-mercaptoetanol and with further mechanical dissociation. RNA extraction was performed using the RNeasy® Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. Total RNA samples were reverse transcribed into cDNA using Supercript II Renverse Transcriptase 10.000 U (200U/µL) in a T3 Thermocycler (Biometra, Gena, Germany).

Real-Time Polymerase Chain Reaction (qPCR)

qPCR reactions were performed in 96-well plates. Briefly, 2 µL of the previously obtained cDNA was amplified with Power SYBR® Green Quantitative PCR MasterMix (Applied Biosystems, CA, USA) or Kapa SYBR® Fast Universal qPCR kit (Kapa Byosistems, MA, USA) according to the optimal conditions for each primer. All the PCR reactions were carried out in PCR 96-Microwell plates (Axygen Scientific) in a final volume of 15 µL and the experiment was performed in triplicates. For each sample, the master mix was prepared to a final volume of 13 µL. Afterwards, 2 µL of cDNA were added to the respective well and the plate was sealed with PlateMax ultra-clear sealing film (Corning Axygen). Afterwards the plate was centrifuged for 1 minute at 1200rpm. Finally, the qPCR reactions were carried out in ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and monitored in SDS Software (Applied Biosystems). The thermal cycling comprised the following steps: initial denaturation at 50ºC for 2 min followed by 10 minutes at 95 ºC, then the PCR stage takes place with 40 cycles of denaturation and annealing at 95ºC for 15 seconds followed by 1 minute at 60ºC, and lastly a dissociation stage at 95ºC for 15 seconds followed by 60ºC for 1 minute. All the data collected was normalized to the expression levels detected for HRPT1, subsequently normalized relatively to the solvent control.

Statistical analysis

Statistical analysis of the results was performed using GraphPad Prism 6 software (GraphPad Software, Inc., CA). Comparisons between samples were made by One-way ANOVA analysis. Comparisons with more than two variables were performed by a Two-way ANOVA analysis. Both types of statistical analysis followed Tukey's multiple comparison tests. Values of $p<0.05$ were considered statistically insignificant.

Results and Discussion

Antiproliferative effect and cytotoxicity evaluation of Hydroxytyrosol and its colonic metabolites - 2D cell models

Aiming at assessing the antiproliferative effect of HT, PA and PPA on colorectal cancer cells, the HT29 cell line was used as a 2D cell model (in monolayer).

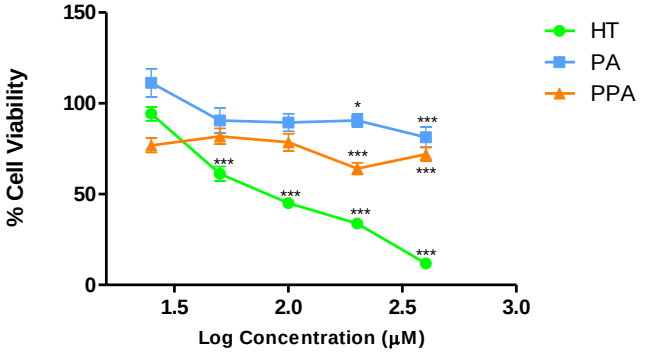

Figure 2 - Dose response profiles of the antiproliferative effect of HT, PA and PPA after a 72h period of incubation on HT29 cell monolayer. The results are expressed as mean ± standard deviation of 3 independent assays, each of them performed in triplicates. *p-value < 0.05, **p-value <0.01 and ***p-value < 0.0001 are relative to the control.

Results showed significant differences for the three compounds, which were tested under the same conditions and for equal concentrations. It is clearly noticeable that HT presents an antiproliferative effect in a dose dependent manner with an EC_{50} of 93.58 µM. The same effect was not observed for the PA and PPA compounds. As shown in Figure 2, PA and PPA presented a lower antiproliferative effect. PPA causes a decrease in cell viability for almost all the concentrations tested, being the cell viability around 70%. However, no dose-response dependency was observed for this compound. Regarding PA, a slightly dose-response tendency was obtained, as cell viability drops to around 75%-80% for concentrations above 50 µM.

The antiproliferative effects of HT are already reported in the literature, not only in colon cancer cell lines, but also in hepatoma, breast and prostate cancer cell lines. [9,10,21–25]

In order to evaluate if the tested concentrations were toxic to the normal epithelium, cytotoxicity studies were performed on confluent Caco-2 cell line.

As it can be seen in figure 3, PA and PPA did not cause any cytotoxic effect for any of the concentrations tested. On the other hand, HT significantly decreased cell viability relative to the control for the higher concentrations tested (1000 and 2000 µM). The calculated EC_{50} value for HT was 1971.0 µM. Importantly, since the EC_{50} of the antiproliferative assay is lower than the EC_{50} determined in Caco-2 cells it can be suggested that HT inhibits colorectal cancer cell growth without compromising the viability of normal colon cells.

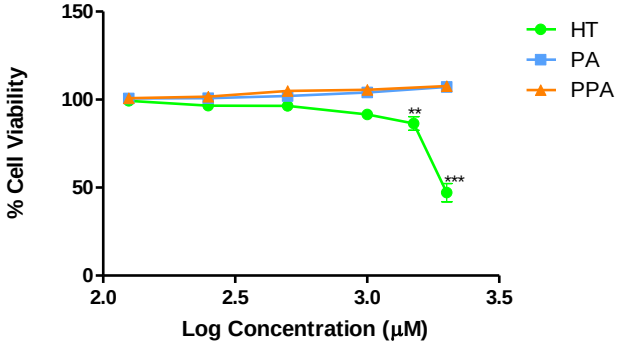

Figure 3 - Cytotoxicity effect of HT, PA and PPA on a 2D cell model of intestinal barrier. Dose-response profiles of cytotoxic effect provoked by HT, PA and PPA compounds after a period of incubation of 72 hours in Caco-2 cell line. Green represents hydroxytyrosol, blue represents Phenylacetic acid and orange represents Phenylpropionic acid. The results shown here are the mean of 5 independent experiments, each one performed with triplicates ± SD. **p-value < 0.01 and ***p-value < 0.0001 are relative to the control.

Antiproliferative effect of hydroxytyrosol and its colonic metabolites using a 3D model for colorectal cancer

After screening for the potential effect of HT, PA and PPA on HT29 monolayer culture, this ability was assessed in a 3D cell model of CRC. Spheroids collected on day 7/8 of culture better mimic the real tumor complexity. In figure 4 is presented the dose-response profiles of HT and its colonic metabolites.

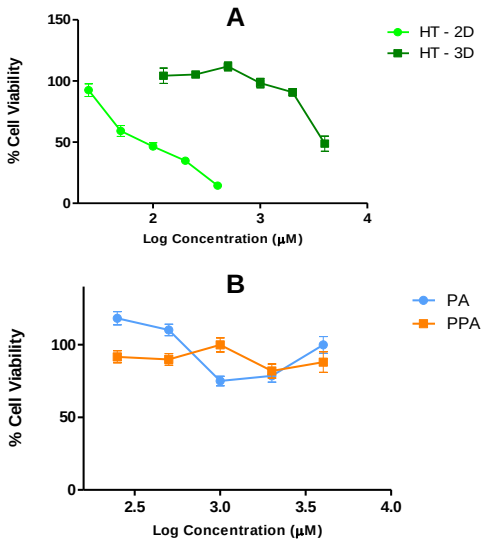

Figure 4 – (A) Antiproliferative effect of Hydroxtyrosol on the 2D and 3D cell models of colorectal cancer using the HT29 cell line and with an incubation period of 72 hours. In light green is represented the curve response for the HT using the 2D cell model and in dark green the curve for the 3D cell model; (B) **Antiproliferative effect of PA and PPA on the 3D cell model of colorectal cancer using the HT29 cell line and with an incubation period of 72 hours.** In blue is represented the curve response for the PA and in orange the curve response for the PPA. Results are expressed in mean of, at least

2 independent experiments performed in triplicates for the 2D and with six replicates for the 3D ± SD;

Figure 4 (A) shows an antiproliferative effect of HT on the 3D cell model, being clearly visible by the profile of the curve that this compound inhibited cell proliferation in a dose-dependent manner. The calculated EC_{50} for the 3D cell model was 3938 µM.

Comparing the results obtained in both antiproliferative assays it is possible to verify that the EC_{50} obtained for the 3D assay is 42 times higher than the 2D one. This fact can be explained by the physiological differences between the 2 cell models. The higher concentration of HT necessary to reduce 50% of the cell viability in 3D spheroids can be explained by several factors: i) Diffusional limitations - there is a higher cell concentration within the spheroid, hampering the diffusion of the compound to all the HT29 cells of the spheroid; ii) structural complexity of HT29 cell spheroids: the population of cells in the spheroid is not homogeneous, existing, proliferating cells, quiescent cells, necrotic cells and cancer stem cells within the tumor, all of whom with different characteristics; iii) chemoresistant phenotype of HT29 cell spheroids: the colon cancers are fundamentally resilient to treatments because of radio and chemotherapy resistance by several mechanisms- the stochastic selection of resistant genetic subclones, microenvironmental factors (hypoxia, acidosis) and other extrinsic cellular factors, as well as the existence of CSC that are highly resistant. [26,27] All of this factors are better mimicked in a 3D cell model than in a monolayer (2D) one.[26,27]

The colonic metabolites of HT – PA and PPA – were also tested on the 3D cell model as shown in figure 4 (B). Neither PA nor PPA inhibited cell proliferation in HT29 cell line spheroids. From the results obtained for all the compounds tested, the only compound that exerted some antiproliferative effect was HT. This compound demonstrated the ability to inhibit the growth of the HT29 cells using the model of the disease that better mimics human tumors. However, it is important to mention that the EC_{50} value is within the cytotoxic range obtained in Caco-2 cell line assays. Future studies should be performed in order to validate the safety of HT in normal colon call line.

Inhibition of aldehyde dehydrogenase activity by Aldefluor assay

As shown in figure 5 (A), there was an effect of HT causing the decrease of ALDH⁺ cells in a dose-dependent way: for the concentration of 200 µM the mean percentage of ALDH⁺ cells is 83.6% (reduction of 16.4% relative to the control), for the 400 µM the mean percentage is 63.6% (reduction of 36.4% relative to the control) and for 600 µM the percentage of ALDH⁺ cells is 61% (reduction of 39% relative to control). Between these two highest dosages, there was not a statistically significant difference, suggesting that this increase on the concentration is not enough to cause a decrease on the population of ALDH⁺.

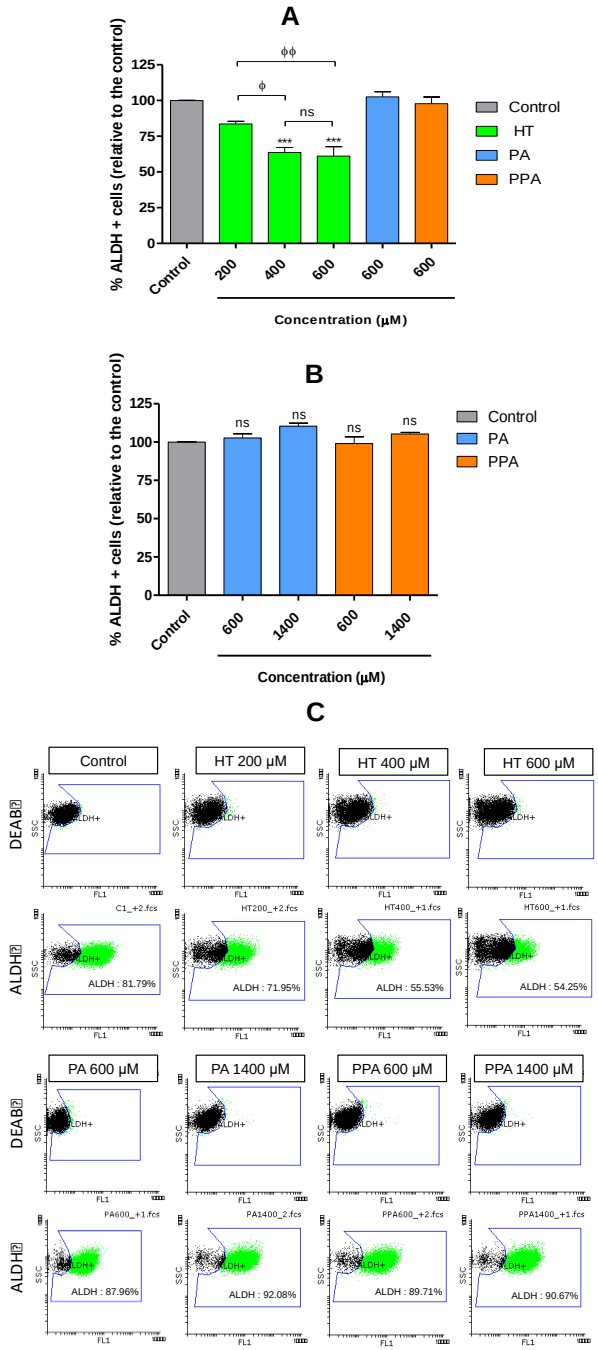

Figure 5 - (A) Effect of HT, PA and PPA on the population of ALDH⁺ cells. The results are shown in mean of 4 independent experiments, each one performed with duplicates, ± SD. (B) **Effect of higher dosages of PA and PPA on the population of ALDH⁺ cells.** The results are shown in mean of 3 independent experiments, each one performed with duplicates, ± SD. (C) **Representative dot plots of ALDH1 subpopulation in HT29 spheroids from flow cytometry analysis using ALDEFLUOR™ assay.** . ***p-value < 0.0001 is relative to the control and ns (p-value>0.05), ϕp-value<0.05 and ϕϕp-value<0.01 are relative to the same compound.

As for PA and PPA, even for higher concentrations, these did not show any effect on the reduction of the population of ALDH⁺ cells, as shown in figure 5 (B). Aldehyde

dehydrogenase activity has been associated with the stemness characteristics of tumors. Colorectal cancer exhibits ALDH activity, being its activity significantly higher in cancer tissues than in healthy ones (about 27-29%).[28] Hydroxytyrosol, as well as other phenols present in VOO, have shown to be able to reduce the $ALDH^+$ population in breast cancer cell lines [29,30]. In this work, it is clear a reduction in the $ALDH^+$ subpopulation of cells caused by HT. This result is of great importance since targeting and reducing the population of cancer stem cells is a big hallmark in the fight against CRC and in inhibiting the progression of CRC to metastasization. Further work would be of great importance to test other CSC markers to attest for the results here obtained with the ALDH.

Assessment of anchorage-independent cell growth

In order to evaluate the effect of HT and its colonic metabolites in preventing anchorage-independent cell growth, soft agar assay was performed. This assay allowed exploring the ability of cells to form colonies from a single cell in a semi-solid support, given by agarose.

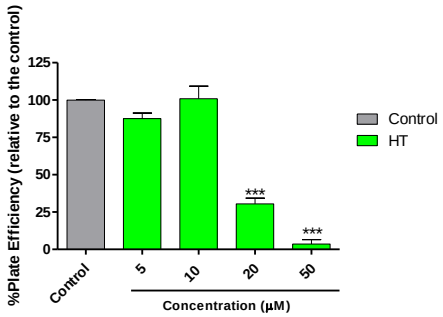

Figure 6 - Plate efficiency for the anchorage-independent cell growth using cells derived from HT29 spheroids, treated with HT for a timespan of 3 weeks. The value of the plate efficiency was calculated recurring to equation 3, which expresses the percentage of single cells that gave rise to a colony unit. Therefore, the lower plate efficiency translates into a higher inhibitory effect by the compound. Results are mean of 5 independent experiments performed in duplicates ± SD. ***p-value<0.001 is relative to the control.

As it can be seen in figure 6, it is clear that HT promoted an inhibition of colony formation in a dose dependent-manner, with an almost total inhibition for the highest concentration tested (96.4% for 50 µM) and a mean reduction on the plate efficiency of 70% for HT 20 µM. These results demonstrate that with a higher concentration, the anti-tumorigenic potential of HT compound becomes more accentuated. It is important to refer that, although not statistically significant, the concentration of 10 µM appears to lead to a slight increase of the plate efficiency, however, the observation of the colonies of this concentration after the 3 weeks of culture, revealed that they were considerably smaller and less compact than the ones formed in the control and 5 µM HT wells.

The colonic metabolites of HT (PA and PPA) were also tested and the results are shown on figure 7. These results revealed that PA and PPA did not have the same inhibitory effect as HT. In fact, PA only showed some a significant inhibition of colony formation for the higher concentration (500 µM), where the mean reduction was 35%. Even though this result is statistically different from the control, it is not statistically different from other concentrations tested of the same compound, namely 20 and 200µM. As for PPA, all of the concentrations tested, from 20, 200 and 500 µM, showed significant reductions in colony formation, approximately between ranging from 27% to 44% relative to the control.

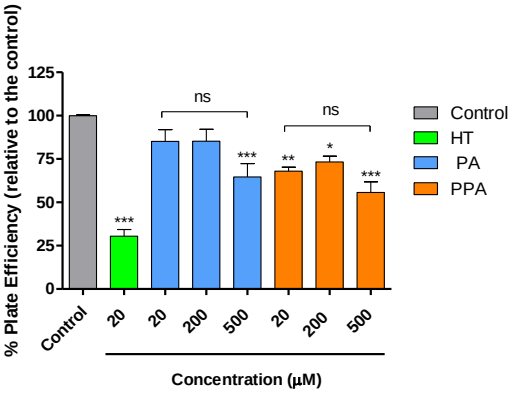

Figure 7 - Plate efficiency for the anchorage-independent cell growth using cells derived from HT29 spheroids, treated with HT, PA and PPA for a timespan of 3 weeks. The value of the plate efficiency was calculated recurring to equation 3, which expresses the percentage of single cells that gave rise to a colony unit. Therefore, the lower plate efficiency translates into a higher inhibitory effect by the compound. Results are mean of 5 independent experiments performed in duplicates ± SD. *p-value < 0.05, **p-value <0.01 and ***p-value < 0.0001 are relative to the control and ns (p-value > 0.05) is relative to the same compound.

Up to date, there are no studies reporting the effect of HT and its colonic metabolites in reducing colony formation of CRC cells. Similar studies with other extracts had already been performed and the range of concentrations tested and showed to be efficient in colony formation inhibition are similar to the ones tested for HT, PA and PPA. [11,16]

Taken together, the results obtained from this assay suggest that among all compounds, hydroxytyrosol might have a potential role in targeting colorectal CSCs.

Combination of HT and 5-fluororacil

5-Fluororacil (5-FU) is a fluorinated pyrimidine, one of the most widely used agents for the treatment of colon cancer.[31] In this study, the effect of the combination of HT with 5-FU was studied in order to assess the potential

effect of this natural compound in improving the efficacy of the drug.

From the antiproliferative assay, whose results are shown in figure 8 (A), it is possible to observe that the EC_{50} value of the combination of the compounds, HT and 5-FU (0.3 mg/ml of 5-FU) is singly higher than the EC_{50} of 5-FU alone (0.13 mg/ml). Therefore, HT did not improve the antiproliferative effect of 5-FU in HT29-cell spheroids.

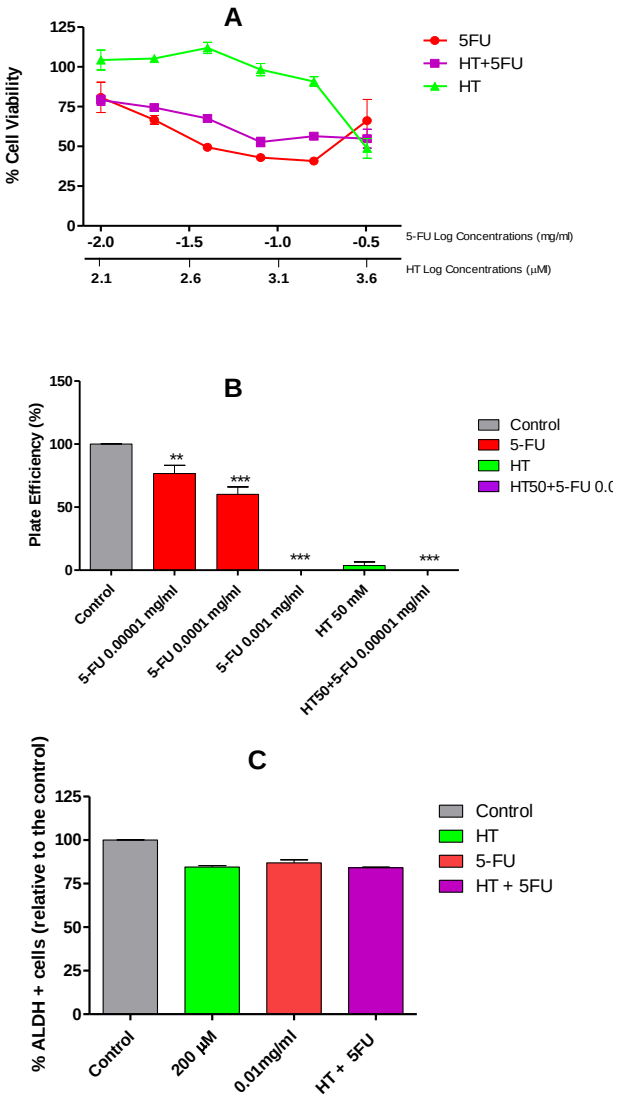

Figure 8 - (A) Antiproliferative effect of Hydroxtyrosol, 5-fluororacil and the combination of the two compounds on the 3D cell model of colorectal cancer using the HT29 cell line and with an incubation period of 72 hours. Results are expressed in mean of, at least, 2 independent experiments performed in triplicates. (B) **– Plate efficiency for the anchorage-independent cell growth using cells derived from HT29 spheroids, treated with 5-fluororacil and a combination of 5-FU and HT for a timespan of 3 weeks.** The value of the plate efficiency was calculated recurring to equation 3, which expresses the percentage of single cells that gave rise to a colony unit. Therefore, the lower plate efficiency translates into a higher inhibitory effect by the compound. (C) **Effect of HT, 5-fluororacil and the combination of both compounds on the population of ALDH⁺ cells.**

As for the colony forming units assay (figure 8(B)), 3 concentrations of 5-FU were tested: 0.001, 0.0001 and 0.00001 mg/ml. The highest concentration completely inhibited the formation of colonies and the less concentrated showed a plate efficiency of over 60%, clearly presenting a dose-dependent response. A combination study was performed for this assay with the less concentrated dose of 5-FU and with HT 50 µM. It was observed a complete inhibition of colony formation for this combination. This result may indicate a synergetic effect between these two compounds, since HT 50 µM presented a mean of 4% plate efficiency. To further assess this possibility, less concentrated dosages of HT should be tested in combination with 5-FU.

Finally, for the ALDH assay, showed in figure 8 (C), it is clear that the percentage or $ALDH^+$ cells did not suffer a decrease for the combination of HT and 5-FU when compared to .the compounds alone, the percentages of the three conditions ranged between 84% and 86%. Therefore, it is possible to conclude that no synergetic effect was observed.

As mentioned before, combination studies were already performed using HT, using for example cetuximab (a monoclonal antibody), α-lipoic acid (a natural non-phenolic compound), as well as combining 5-FU with natural other natural products like hexahydrocurcumin. All this studies showed some type of synergetic effect between the compounds tested. [32–34]

All this data suggest that combination therapies are a promising chemotherapeutic tool. Regarding HT, future studies should focus on combining this compound with other polyphenols or with other chemotherapeutic drugs that could possibly enhance its activity.[35] Multiple *in vivo* and *in vitro* studies have shown that polyphenols more effectively inhibit tumor growth than the compounds employed singularly. Additionally, numerous in vitro and in vivo studies have also shown that polyphenols potentiate effects of conventional therapies and may help to reduce the effective dose of chemotherapeutic drugs, overcome drug resistance and reduce toxicities.[35] The development of nanotechnology to increase bioavailability and antitumoral activities of polyphenols can be a promising solution to overcome issues like the metabolism, stability, interference with other drugs and side effects of these natural compounds' derivatives.[35]

Influence of HT on the expression markers of cancer stemness, EMT, cell cycle and key signaling pathways

The influence of the treatments with HT on gene expression of key markers of cancer cell processes in the HT29 spheroids, collected at day 8, was evaluated by RT-qPCR.

Analyzing the results obtained and shown on figure 9, the highest concentration tested (600 µM) does not appear to induce significant changes in the expression of cancer

markers, with exception of the increase of the stemness markers (*LGR5, NANOG* and *OCT4*), as well as an increase of the *CDKN1A* and reduction of *CCNA2*, associated with cell cycle arrest.

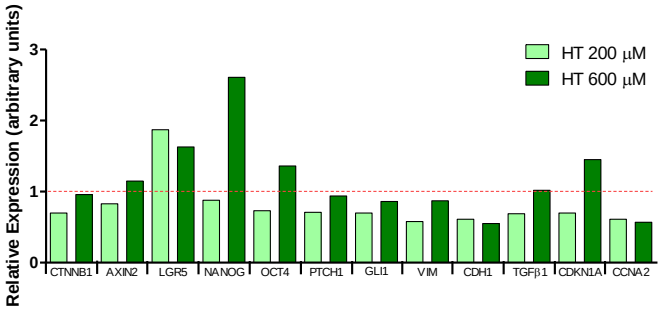

Figure 9 - Relative expression of specific markers using RT-qPCR for Wnt signaling (*CTNNB1, AXIN2*), stemness (*LGR5, NANOG, OCT4*), Sonic hedgehog pathway (*PTCH1, GLI1*), EMT (*VIM, CDH1*), and cell cycle (*TGFB1, CDKN1A, CCNA2*). The results were obtained using day 7 HT29 cell spheroids treated with HT at 2 concentrations for 72 hours. Each expression level was normalized relatively to the solvent control and the endogenous gene control used was HRPT1.

Hence, these results hinder the antiproliferative ability of HT. However, considering the increase of the stemness markers, 600 μM is not a potentially promising concentration to pursue further studies. Nevertheless, dosages over 600 μM should be tested in further studies, since an increase of the stemness expression markers for this concentration, does not necessarily correspond to an increase of the same markers for higher concentrations.

Comparing the results obtained between the lowest (200 μM) and highest (600 μM) concentrations, generally there is an increase of the expression levels, more evident on the stemness markers, but also observed on the Wnt, Sonic Hedgehog pathway (SHH) and EMT, with an increase of *VIM*, *TGFβ1* and *CDKN1A*. However, for the lowest concentration tested (200 μM), there seems to exist a slight reduction of the expression of almost all these markers, namely those related to stemness (*NANOG, OCT4*), EMT (*VIM*, *TGFβ1*) and SHH (*GLI1, PTCH1*),with the exception of the stemness marker and Wnt target gene *LGR5*. In terms of antiproliferative ability, this lowest concentration seems to have the opposite effect on the expression of *CDKN1A*, although a favorable effect in the expression of *CCNA2*.Therefore, a potential study of interest would be to combine HT 200 μM with a cytostatic drug.

Although there is the need to repeat this assay in order to validate these results, as well as testing other dosages (below than 200 μM or even above 600 μM, closer to the EC_{50}, as long as it remains under the cytotoxic concentrations), it is possible to hypothesize that for smaller dosages, HT might have an anti-metastastatic effect (suggested by the reduction of *VIM, SHH, TGFβ1*, all involved in tumor invasion and metastasis, and also taken together with the inhibition effect obtained for the anchorage-independent assay). Even though the conclusions withdrawn can be seen as somewhat speculative, since only one independent study was performed, taken together with the results from already published studies, HT presents itself as a promising compound in inhibiting several mechanisms essential to cancer survival and metastasization.

From all the results obtained and described above it is possible to suggest that HT can be considered as a promising bioactive compound with potential application in CRC prevention and therapy. In fact, there are already some clinical studies performed to assess the effect of this VOO compound against diseases like cancer and cardiovascular diseases. Currently there is an ongoing clinical trial for high-risk breast cancer prevention in women that consists in the ingestion of 25 mg of HT orally, everyday for one year.[36] The outcomes of this trial will be measured in two ways: mammographic breast density and the number of participants with adverse events. Other outcome measures will be the expression of Ki67 in tumor tissue and MRI breast density. [36]Importantly, some of the concentrations of HT tested in this work can be attained with a diet enrich in olive oil and olives. As reported previously the total mean daily intake provided by habitual consumption of olives and virgin olive oil is 31 mg of HT. [10] According to Juan et al, if this phenol would reach the colon in a distribution volume of around 250ml, that would result in a concentration of approximately 72 μM a value that induced a 50% inhibition of cell proliferation. [10] Nevertheless it is important to mention that bioavailability studies have shown that HT is dose dependently absorbed in animals and humans after olive oil ingestion, while other phenolic components undergo metabolism in the small and large intestine.[37] In a study performed in humans, the absorption of pure hydroxytyrosol (99.5%) was investigated in the plasma and urine of healthy volunteers. HT was administered as a supplement in aqueous solution (2.5mg/kg body weight) and the absorption of HT was very low, and produced a large number of metabolites.[38] In the present study the colonic metabolites derived from HT, namely PA and PPA, showed lower inhibition of cancer cell proliferation and negligible effect on targeting cancer stemness than the native compounds. In future, other HT metabolites should be tested aiming at evaluating their potential anticancer activities.

As mentioned previously, the development of nanotechnology is a potent tool to increase bioavailability and antitumoral activities of polyphenols and can be a

promising solution to overcome the abovementioned issues.[35]

Conclusions and Future Prospective

In the present thesis, it was investigated for the first time the effect of the VOO compound hydroxytyrosol and its colonic metabolites phenylacetic acid and phenylpropionic acid in inhibiting proliferation and targeting cancer stemness on a 3D cell model of HT29 cells that better mimics and recapitulates the tumor microenvironment.

HT was the only compound able to reduce cell proliferation in this model of CRC with an EC_{50} value of 3938 μM. Importantly, concentrations of 50 μM and 600 μM of HT showed to be effective in inhibiting anchorage-independent cell growth and decreasing in 39% the population of stem cells (ALDH+ cells), respectively. Furthermore, it showed anti-metastasization potential by targeting specific gene expression markers, namely stemness markers (*NANOG, OCT4*), Epithelial to Mesenchymal Transition markers (*VIM*, *TGFβ1*), Sonic Hedgehog Pathway markers (*GLI1, PTCH1*) and proliferation markers (*CCNA2)*.

Altogether, the results obtained in this work suggest that HT presents a great potential as a chemotherapeutic adjuvant in order to more efficiently treat Colorectal Cancer, targeting and modulating CSC. Despite the combination studies with 5-FU revealed that HT does not significantly improve the antiproliferative response and the reduction of CSCs, the effect on inhibiting colony formation was improved indicating that this VOO compound can have a positive impact in chemotherapeutic impact of this conventional drug. More combination studies can also be performed with different chemotherapeutic drugs like oxaliplatin and irinocetan in order to assess the synergetic effect of HT.

In contrast, colonic metabolites derived from HT did not show antiproliferative effect and reduction of CSC population. These results indicate that the metabolization process of HT during gastrointestinal digestion process compromised the bioactivity towards CRC.

To better understand the beneficial effect of HT on CRC and cancer stem cells population, future studies can focus on evaluating the effect of different concentrations of HT on the Aldefluor assay as well as exploring other expression markers. Another future approach will rely on the understanding the molecular mechanisms by which this compound inhibits CRC cell growth through omics platforms, such as metabolomics.

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