Potential chemopreventive activities of a polyphenol rich purified extract from olive mill wastewater on colon cancer cells

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ABSTRACT
Olive oil, a major feature of the Mediterranean diet, is an abundant source of phenolic compounds. Olive oil production is associated with the generation of waste material, termed ‘olive mill wastewater’ (OMWW), that has been reported to be enriched in soluble polyphenols. Given the known beneficial activity of polyphenols, we investigated whether the use of purified extracts from OMWW, termed A009, rich in hydroxytyrosol, might have anticancer activities on colon cancer (CC) cell lines in vitro and in vivo and could represent a chemopreventive preparation for CC. A009 from different batches inhibited proliferation, migration, invasion, adhesion, sprouting of CC cells and release of angiogenic, pro-inflammatory cytokines (VEGF, IL-8). Our data demonstrate that a novel purified, polyphenol enriched extract, obtained from food industry waste material, with similar activity than purified hydroxytyrosol but easier to produce in large quantities and with an environment-sensitive approach, has potential cancer chemopreventive properties for colon cancer cells.

Keywords: Olive mill wastewater
Polyphenols
Hydroxytyrosol
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Abbreviations: 7-AAD, 7-amino-actinomycin D; COD, chemical oxygen demand; CC, colon cancer; DAD, diode array detector; ECM, extra-cellular matrix; FACS, fluorescence-activated cell sorting; FSC, forward scatter; FITC, fluorescein isothiocyanate; 5-FU, 5-fluorouracil; FBS, foetal bovine serum; HPLC, high-performance liquid chromatography; H façon, hydroxytyrosol; LC, liquid chromatography; MS, mass spectrometry; MTT, (5-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide; OMWW, olive mill wastewater; PFA, paraformaldehyde; SSC, side scatter; UV–vis, ultraviolet–visible; VCR, volume concentration ratio
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1. Introduction

Epidemiological studies have shown a significant influence of environment factors, lifestyle and diet in the incidence and in the prevention of pathologies associated with oxidative damage, such as cardiovascular diseases, diabetes, neurodegenerative conditions and cancer (Alarcón de la Lastra, Barranco, Motiña, & Herreries, 2001; Lipworth, Martinez, Angell, Hsieh, & Trichopoulos, 1997; Sporn & Suh, 2000; Stark & Madar, 2002; Stoneham, Goldacre, Seagroatt, & Gill, 2000; Trichopoulos, Lagiou, Kuper, & Trichopoulos, 2000; Tuck & Hayball, 2002). As cancer represents a major cause of death (Jemal et al., 2008; Mozaffarian et al., 2015), there is currently an emphasis on exploring approaches to prevent cancer incidence and mortality.

In the case of colon cancer (CC), lower rate of incidence has been observed amongst populations living within the Mediterranean basin (Stark & Madar, 2002; Stoneham et al., 2000). Several studies have investigated dietary habits playing a relevant role in the prevention of CC. Olive oil represents a basic component of the Mediterranean diet (Escrich, Moral, Grau, Costa, & Solanas, 2007; Rafehi et al., 2012; Widmer, Flammer, Lerman, & Lerman, 2015) and several data from both observational and clinical studies showed that consumption of olive oil is associated with reduced risk for chronic-degenerative diseases (InterAct Consortium, 2014; Prinelli et al., 2015; Schwingshackl & Hoffmann, 2014; Widmer et al., 2015).

Olive oil is a source of at least 30 phenolic compounds (Dais & Hatzakis, 2013). Amongst the phytochemicals present in olive oil, phenols and their secoiridoid derivatives represent the most abundant components that may be responsible for their preventive and protective effects. The concentrations and the relative proportions of olive oil polyphenols depend on several factors, including the cultivars, the soil, the climate, the procedures for oil production and storage, and the degree of drupe matura-tion (Visioli, Bellomo, & Galli, 1998). The chemopreventive effects of olive oil polyphenols have been investigated in in vitro models (Fabiani et al., 2002; Rosignoli, Fuccelli, Sepparta, & Fabiani, 2016). Single phenols have been investigated for their molecular properties; however, most of the studies concerning the benefits associated with olive consumption have been focused on the oil compartment of the fruit.

Large volumes of waste water (olive mill wastewater; OMWW) are generated during olive oil production, particularly during malaxation process (continuous washing of olive paste with warm water prior to the procedure of separation of the oil from the paste) (Kanakis et al., 2013). OMWW is a problematic and polluting effluent which alters soil and water quality, with a relevant negative impact on ecosystemic functions, reducing chemical oxygen demand and leading to eutrophication (Justino et al., 2012). Altogether these issues represent one of the major concerns for the olive oil industries, leading to an increase in disposal costs. An alternative use for OMWW is therefore highly ausplicable.

Here, we investigated a polyphenol rich purified extract from OMWW, termed A009, whose major phenolic components were determined. One of the most abundant polyphenols in A009 was hydroxytyrosol (HyT). Other studies reported that OMWW could be used to isolate polyphenols (Sedej et al., 2016; Vougogiannopoulou et al., 2015). The biological properties of polyphenols include anti-oxidant, anti-apoptotic, anti-tumour and anti-inflammatory activities.

We tested the potential cancer chemopreventive activity of different batches of A009 as determined by inhibition of cell growth, adhesion, migration, sprouting and invasion and angiogenic, pro-inflammatory cytokine production, VEGF and IL-8, on two human colon-carcinoma cell lines (HT-29, HCT-116) and the murine colon-carcinoma cell line CT-26 in vitro and confirmed in vivo using the CT-26 murine xenograft model.

2. Materials and methods

2.1. Reagents, chemicals and animals

The synthetic hydroxytyrosol (HyT), ≥98% in purity (AgIcon), was purchased from Cayman Chemicals (Ann Arbor, Michigan, USA). RPMI medium supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Euroclone, Milan, Italy) and 1% glutamine (Euroclone, Milan, Italy), defined as complete medium, was used for cell line culturing and to induce migration and invasion in related in vitro assays (Bruno et al., 2013). HPLC reagents, standards and solvents, were LC-MS grade and purchased from Sigma-Aldrich. Standard stock solutions were prepared by dissolving standard in a mixture of methanol:water, 90:10.

2.2. Cell cultures

Human colon cancer (CC) cell lines HT-29, HCT-116 and the murine CT-26 CC cell line where purchased from ATCC and grown in RPMI medium (Sigma Aldrich Milan, Italy), supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Euroclone, Milan, Italy), 1% glutamine (Euroclone, Milan, Italy), 1% penicillin-streptomycin at 37 °C in 5% CO2.

2.3. Preparation of A009 and phenolic quantification

OMWW were provided by Agriturismo La Vialla (Castiglion Fibocchi, Arezzo, Italy) and used to obtain the phenol rich purified extract A009 (Italian Patents n°1420804; n°1420805). The experiments were performed using four different batches of A009 (A–D, Supplementary Table S1). A009 was obtained from OMWW using two sequential cross-flow filtration processes as previously described (Rossi et al., 2015). Briefly, microfiltration (MF) was performed on a pilot plant equipped with tubular ceramic modules membranes in alumina oxide with a MWCO 0.45 micron. The MF permeate was further concentrated by reverse osmosis (RO) in a Polyamide spiral wound module (Microdyn Nadir, Wiesbaden, Germany) with a filtering surface of 7 m². The RO permeate, representing ultrapure water, was discarded. Finally, the RO concentrate, with a volume concentration ratio (VCR) of 3.6, constituted the olive extract (here termed A009).

Phenolic composition of A009 was obtained by HPLC-DAD-MS-MS. Samples were analysed by HPLC (Supplementary Fig. S1) with UV–vis and MS (Supplementary Fig. S2) detection. Sample extraction was performed as described previously in Zhang,
Briefly, OMWW was homogenized and 10 mL was added with 0.25 mL syringic acid as internal standard (1.5 mg/mL). The solution was centrifuged (15 min, 3200 × g), filtered (acetate cellulose filter, 0.45 μm, diameter 30 mm), and diluted with pure Met-OH (methanol) to 25 mL.

The identification of phenolic compounds from samples was carried out as previously reported (Klen, Wondra, Vrhovsek, & Vodopivec, 2015) by interpreting their mass spectra determined via LC-MS-MS and comparing to data reported in literature identified the compounds. The LC-MS-MS system consisted of a Shimadzu LCMS-8030 quadrupole mass spectrometer (Kyoto, Japan) operated in the electrospray ionization (ESI) mode, and a Shimadzu Nexera HPLC system (Kyoto Japan) which consisted of a degasser, two eluent pumps, a column oven and an auto sampler. The separation was performed on a reversed-phase Thermo Scientific Hypersil gold column (ODS 250 × 4.6 mm, 3 micron), at room temperature. The mobile phase consisted of 1% aqueous formic acid (solvent A) and 1% formic acid in acetonitrile/methanol (25/75) (solvent B). Separation was performed using the following elution gradient: 2% B isocratic during 10 min, from 2 to 98% B linear during 30 min, 98% B isocratic during 7 min, then starting condition during 5 min to re-equilibrate the column. The flow rate was 0.6 mL/min, and the injection volume was 10 μL. The column oven was set at 30 °C. The auto-sampler was set at 15 °C and the auto-sampler needle was rinsed before and after aspiration of the sample using methanol.

Mass spectrometry (MS) was performed acquiring spectral data with the following ESI inlet conditions: nebulizing gas and drying gas were nitrogen at a flow rate of 3.0 and 15.0 L/min, respectively; the interface voltage was set to −3.5 kV; desolvation gas were nitrogen at a flow rate of 3.0 L/min, and an auto sampler. The separation was performed on a reversed-phase Thermo Scientific Hypersil gold column (ODS 250 × 4.6 mm, 3 micron), at room temperature. The mobile phase consisted of 1% aqueous formic acid (solvent A) and 1% formic acid in acetonitrile/methanol (25/75) (solvent B). Separation was performed using the following elution gradient: 2% B isocratic during 10 min, from 2 to 98% B linear during 30 min, 98% B isocratic during 7 min, then starting condition during 5 min to re-equilibrate the column. The flow rate was 0.6 mL/min, and the injection volume was 10 μL. The column oven was set at 30 °C. The auto-sampler was set at 15 °C and the auto-sampler needle was rinsed before and after aspiration of the sample using methanol.

Adaptation of the UV–vis spectral shapes and MS fragmentation profiles of known compounds was carried out as previously reported (Klen, Wondra, Vrhovsek, & Vodopivec, 2015). HPLC analysis was performed using a diode-array detector liquid chromatographic system (model 1050), equipped with an auto-sampler (model 1100, Agilent Technologies, Palo Alto, CA, USA). The software used was Agilent Chemstation. A Thermo Scientific Hypersil gold column (ODS 250 × 4.6 mm, 3 μm), equipped with a Thermo Scientific pre-column (C18 ODS 4.0/4.6 mm), was used.

The injection volume was 20 μL. Elution was performed at a flow rate of 0.8 mL min⁻¹ using a methanol (solvent A), acid water (0.2% orthophosphoric acid) (solvent B), acetonitrile (solvent C) mixture as a mobile phase. Quantification revealed that the major phenolic compound of A009 is HyT, along with several other phenolic compounds (Supplementary Table S1).

All these results were complemented with the UV spectra provided by DAD (Diode Array Detector) in terms of the absorbance bands.

### 2.4. In vitro cell proliferation

HT-29, HCT-116, CT-26 (10⁴ cells/well) were plated into 96-well plate in RPMI supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Euroclone), 1% glutamine (Euroclone), 1% penstrept (Euroclone) at 37 °C in 5% CO₂ and left to adhere overnight. Cell proliferation was assessed by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma Aldrich, Milan, Italy) assay. Following cell adhesion, treatments were performed with decreasing dilutions (ranging from 1:50 to 1:10000) of A009, or HyT, prepared in complete RPMI medium. The HyT solution (70% EtOH:30% water) was prepared for each experiment to obtain the same concentration of HyT present in the relative A009 batch, finally diluted in RPMI complete medium. Multiple final dilutions were used.

Reference HyT was 1.75 × 10⁻² M for batch A, 3.71 × 10⁻² M for batch B, 3.57 × 10⁻² M for batch C and 1.63 × 10⁻² M for batch D (Supplementary Table S1). 70% EtOH, further diluted in RPMI complete medium, was used as a negative control (solvent). Cells were treated for 24–96 h and the relative absorbance was measured at 570 nm by a FLUOstar spectrophotometer (FLUOstar Omega BMG LABTECH, Ortenberg, Germany).

### 2.5. Detection of apoptosis in vitro

HT-29, HCT-116, CT-26 (2 × 10⁴ cells/well) were plated into 6-well plates and exposed to decreasing dilutions (1:500, 1:250) of A009, HyT or EtOH for 24 h and 48 h in RPMI supplemented with 10% FBS (Euroclone, Milan, Italy), 1% glutamine (Euroclone) and 1% penicillin–streptomycin (Euroclone) at 37 °C in 5% CO₂. Cells were recovered, washed twice with PBS, transferred to 5 mL polystyrene tubes (BD Biosciences Milan, Italy). Cells were resuspended in AnnexinV-binding buffer (BD Biosciences, Milan, Italy) and stained with fluorescein isothiocyanate (FITC)-conjugated AnnexinV and 7-amino-actinomycin D (7-AAD) (BD Biosciences Milan, Italy) for 15 min at 4 °C in the dark. Cells were then washed in PBS, supernatants were discarded and resuspended in 400 μL of PBS. Analysis was performed using a FACScanII flow cytometer (BD Biosciences). Physical parameters FSC/SSC were used to exclude cell debris and the rate of dead cells was determined as AnnexinV+A7AAD⁻ events. Data were analysed using FACSDiva Software 6.1.2.

### 2.6. Cell adhesion assay in vitro

Adhesion assay was performed as previously described (Carrega et al., 2015). Cells were incubated for 24 h as indicated above. After treatment, 3 × 10⁴ cells were seeded on 4-well chamber slides pre-coated for 45 min with 2 μg/mL fibronectin (Sigma Aldrich). Following 90 min incubation, the supernatants were removed and cells were washed with PBS. Cells were fixed with 4% paraformaldehyde (PFA) and stained with DAPI (Sigma Aldrich). Assays were performed in triplicates wells. Five microscope fields were randomly selected from the wells for each treatment to count the number of adherent cells and the data were analysed using analysis of variance (ANOVA).

### 2.7. Migration and invasion assays in vitro

Migration and invasion assays were performed using a modified Boyden chambers, as previously described (Albini & Benelli, 2007; Albini et al., 1987; Albini, 2016). HT-29, HCT-116, CT-26 cells (5 × 10⁴), treated with A009 or CTRL for 24 h, were washed with PBS, resuspended in serum-free medium and placed in the
upper compartment of the Boyden chamber. RPMI medium, supplemented as described in the Materials and Methods section was used as a chemoattractant and added in the lower compartment. 10 μm pore-size polycarbonate filters, pre-coated with Matrigel (1 mg/mL, BD) for chemoinvasion assay and with collagen IV (50 μg/mL, Sigma Aldrich) for chemotaxis assay were used as the interface between the two chamber compartments. Following 6 h (chemotaxis) or 24 h (chemoinvasion) of incubation at 37 °C in 5% CO₂, the filters were recovered, cells on the upper surface were mechanically removed and migrated or invaded cells on the lower filter surface were fixed with absolute ethanol and stained with DAPI. Cells were counted in a double-blind manner in 5 consecutive fields with a Zeiss Microscope associated with a Nikon camera.

2.8. In vitro sprouting assay

The sprouting assay was performed using 48 well plates coated with Matrigel. HCT-116 and CT-26 (4 × 10⁴) cells were seeded into the Matrigel and immediately treated with A009 or CTRL for 24 h, as described above. The formation of sprouts was observed at 24 h using an inverted microscope at 10× magnification. Assays were performed in triplicate wells. Fifteen microscope fields were randomly selected from three wells for each treatment to count the number of sprouts/cell and the data were analysed using analysis of variance (ANOVA).

2.9. Analysis of cytokine production

The effect of cytokine production by A009 treated CC cell lines was determined by Flow Cytometry. HCT-116 cells were treated with increasing dilution (1:250, 1:500) of A009 or CTRL for 24 h. Golgi stop agent Monensin (2 mg/L, Sigma) was added during the treatment. Cells were detached, washed in PBS, permeabilized using the Cytofix/Cytoperm reagent and finally stained for intracellular cytokines using PE-conjugated monoclonal antibodies against VEGF and IL-8 (R&D System, Minneapolis, MN). Cytokines were measured in a FACSCantoII flow cytometer (BD), as already described (Solinas et al., 2012).

2.10. Tumour cell growth in vivo

The effect of A009 or HyT on tumour cell growth was assessed using the CT-26 colon carcinoma cell line in syngenic BalbC mice. The use of animals was in accordance with the Italian and European Community guidelines (D.L. 2711/92 No.116; 86/ 609/EEC Directive). The procedure was approved by the local animal experimentation ethics committee (Univ. Insubria ID#05/13) and by the Italian Health Ministry. 10⁵ CT-26 cells per animal, suspended in 100 μL of liquid pre-chilled Matrigel (10 mg/mL, BD) were subcutaneously injected into the right flank of each mouse. Treated animals (8 per group) received intraperitoneally (i.p.) injection of A009 or HyT at 1:500 or 1:250 dilutions. PBS and 5-fluorouracil (5-FU; 10 mg/kg every 2 days, 1 mg/kg every day) were used as negative and positive controls, respectively. We also tested the effects of A009 administered orally (per os) using A009 or HyT at 1:100 or 1:50 in drinking water. Two days prior and two days following tumour cell injection, treatments were administered via i.p. or per os. Tumour xenograft growth rates were monitored every 2 days with a caliper and determined using the rational ellipse formula:

\[
\text{Tumour volume} = (\text{width}^2 \times \text{length}) \div 0.5.
\]

The health state of the animals (food and water consumption, dejections, skin conditions, reactivity to stimuli) was monitored during the experimental procedures. At the end of the experiment, tumours were excised and weighed.

2.11. Statistical analyses

The statistical significance between multiple data sets was determined by one-way ANOVA using Graph-Pad PRISM. Tumour growth curves were determined by two-way ANOVA. FACS data were analysed by FACSDiva Software 6.1.2. Data are expressed as means ± SEM.

3. Results

3.1. A009 inhibits the proliferation of colon cancer cells

A009 (dilution range 1:50 to 1:10,000) from 4 different batches made from different OMWW were tested for its ability to interfere with tumour cell growth by MTT assay on two human (HT-29, HCT-116) and one murine (CT-26) colon-cancer cell lines. Total phenol composition of the four diverse batches of A009 was determined by HPLC analysis, showing similar composition in phenols (Supplementary Table S1) with some differences. As shown in Supplementary Table S1, the most abundant polyphenol is the HyT, which, for this reason, was chosen as reference compound, at the same concentration contained in the relative batch.

The effect of A009 on cell proliferation was compared with the HyT alone. A009 (Fig. 1, Batch A) was able to inhibit HT-29 cell proliferation from 48 h following treatment, showing a slower growth rate, starting from the 1:500 dilution (Fig. 1A). A009 inhibited growth also of human HCT-116 (Fig. 1B) and murine CT-26 (Fig. 1C) cell lines. Batches B, C, D behaved in the same way as shown in Supplementary Fig. S1. The four batches showed comparable effects in reducing cell proliferation (batches B, C and D, shown in Supplementary Fig. S3 a, b, c respectively for HT-29, HCT-116 and CT-26 cells). We found that the effects of A009 on cell proliferation were similar to those exhibited by HyT alone at the same dilution relative to the batch used. The lowest (1:50) dilution of A009 that arrested cell proliferation in the three cell lines was further investigated. This was not due to toxicity. No significant pro-apoptotic effect was observed in HT-29, HCT-116, CT-26 cell lines, treated with A009 batch A (Fig. 2) as well as with batches B, C, D (Supplementary Fig. S4A, B, C, respectively for HT-29, HCT-116 and CT-26 cells). Given the comparable activities, one of the A009 batches (batch C) and the lowest active dilutions (1:500 and 1:250) were selected for the remaining experiments.

3.2. A009 impairs the adhesion of colon cancer cells in vitro

Tumour cell adhesion to ECM represents a key step in the metastatic cascade (Albini, 2016; Seyfried & Huysentruyt, 2013). Using
Fig. 1 – Anti-proliferative properties of A009 on human and murine CC cells. Cell proliferation was evaluated at sequential time points (24, 48, 72, 96 h) using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. A009 (batch A) and HyT (hydroxytyrosol) inhibit proliferation of both human (A, B) and murine (C) CC cells in a concentration-dependent manner. Results are shown as mean ± SEM. The experiment was performed also with a negative control. Solvent (70% ethanol) is shown as a negative control. Batches B, C and D behaved similarly (Supplementary figure S1).
Fig. 2 – Induction of apoptosis in human and murine CC cell lines treated with A009. Effect of A009 (batch A) and HyT (hydroxytyrosol) on human (A and B) and murine (C) CC cell survival. Cell apoptosis was assessed by flow cytometry and viable cells are described as Annexin V-/7-AAD- cells. No apoptosis induction was observed after 24 h and 48 h of treatment with A009. Untreated (NT) and solvent (70% ethanol) were used as negative controls. Mean ± SEM of 3 independent experiments is shown.
fibronectin as an ECM layer, we evaluated the ability of A009 (batch C) to interfere with the attachment of HT-29, HCT-116 and CT-26 cells that were pre-treated for 24 h with A009 (1:500, 1:250) or solvent (EtOH 70%/30% water diluted in RPMI). A009 and HyT significantly impaired HCT-116 (Fig. 3A), HT-29 (Fig. 3B) and CT-26 (Fig. 3C) adhesion.

### 3.3. A009 inhibits migration and invasion of colon cancer cells in vitro

The migratory and invasive abilities of cancer cells are critical processes of the metastatic cascade (Albini, 2016). Most malignant tumour cells show a chronically invasive phenotype on the basis of activation of specific cellular and molecular signalling pathways. We investigated the capability of A009 to prevent cell migration (Fig. 4) and invasion (Fig. 5) at 24 h. As shown in Figs. 4 and 5, A009 (batch C) and HyT significantly inhibited the migration and invasion capabilities of HCT-116 (Figs. 4 and 5A), HT-29 (Figs. 4 and 5B) and CT-26 (Figs. 4 and 5C) CC cells.

### 3.4. A009 reduces sprout formation of colon cancer cells in vitro

Sprouting capabilities on matrix have been described for cancer cells and are associated with their invasive properties (Terzuoli et al., 2010). Here, we evaluated the ability of A009 (batch C) to prevent sprouting formation of HCT-116 and CT-26 CC cells on a basement membrane matrix (Matrigel). A009 and HyT significantly reduced sprout formation in HCT-116 (Fig. 6A) and CT-26 (Fig. 6B) cells.

### 3.5. A009 inhibits VEGF and IL-8 levels in vitro

Previous findings reported that HyT had strong anti-inflammatory/anti-angiogenic activities and blocks the expression of VEGF (Lamy, Ouanouki, Beliveau, & Desrosiers, 2014; Terzuoli et al., 2010). Production of VEGF is associated with the capability of cancer cells to stimulate tumour angiogenesis (Albini et al., 2015; Arjaans et al., 2016; Albini, Tosetti, Li, Noonan, & Li, 2012). Here, we examined the effects of A009 on inhibition of VEGF and IL-8 expression by HCT-116 cells. We found that A009 (batch C) and HyT significantly reduced the production of VEGF (Fig. 7A) and IL-8 (Fig. 7B) protein. This is consistent with an anti-angiogenic activity of A009 and HyT.

### 3.6. A009 interferes with tumour cell growth in vivo

We investigated in vivo the ability of A009 to interfere with subcutaneously injected murine CT-26 colon cancer cell growth using the syngenic BalbC murine model. In one experiment, the mice were treated i.p. either with A009 (batch C) or HyT (1:500, 1:250). In another experiment, the mice were treated with A009 (batch A) or HyT (1:100, 1:50) per os in the drinking water. The treatment started two days prior tumour cell injection and the mice were treated every two days during the experiment. As positive control, we used i.p. 10 mg/mL 5-FU, a commonly employed chemotherapeutic agent for colon cancer (Albini et al., 2010; Goldstein, Zeichner, Bartnik, Neustadter, & Flowers, 2016; Sinicrope, Okamoto, Kasi, & Kawakami, 2016), administered every 2 days following tumour challenge. PBS was used as a negative control. In the i.p. setting, the lowest dilution of HyT did not show a significant effect, while both concentrations of A009 showed significant (p < 0.001 and p < 0.0001, respectively) for A009 1:500 and A009 1:250) reduction of tumour growth (Fig. 8A). In the oral treatment setting (per os), we found that A009 (1:100, 1:50) inhibited CT-26 tumour growth (p < 0.0001 for both A009 1:500 and A009 1:250) while the same effect was observed only for the less diluted HyT (1:50) preparation (Fig. 8B).

Tumour weight at sacrifice was also assessed. Tumours excised from mice treated with A009 exhibited a smaller size (Supplementary Fig. S5A), compared to PBS-treated mice and similar to those excised from HyT-treated mice. Assessment of tumour weight revealed that A009 (1:500) and HyT (1:250 at the same concentration of the related A009 batch), limited tumour weight in the per os schedule (Supplementary Fig. S5B and C).

### 4. Discussion

Chemoprevention, as first defined by Michael Sporn in 1976, uses natural or synthetic agents to reverse, suppress, or prevent carcinogenesis, delaying progression to invasive cancer (Sporn & Suh, 2000). Chemoprevention by dietary phytochemicals is particularly attractive for their potential low toxicity and for their ability to modulate a plethora of signal transduction pathways in several biological processes associated with cancer, inflammation and angiogenesis (Albini, Tosetti, Li, Noonan, & Li, 2012; Landis-Piwowar & Iyer, 2014; Ferrari et al., 2011; Sogno et al., 2009; Sporn & Suh, 2000).

Olive oil, a major feature of the Mediterranean diet, is an abundant source of chemopreventive phenolic compounds. Olive oil production is associated with the generation of waste material, termed ‘olive mill wastewaters’ (OMWW), that has been reported to be enriched in soluble polyphenols (Justino et al., 2012), thus representing an extremely attractive source of nutraceutical products. Currently, there is a growing interest in studying phenolic compounds in OMWW representing an aqueous phase enriched in soluble phenols. However, few studies have focused on the biological effects of this waste product, especially in the context of cancer prevention. One of the most abundant polyphenol in OMWW is HyT (Vougogiannopoulou et al., 2015). The biological properties of polyphenols include anti-oxidant, anti-apoptotic, anti-tumour and anti-inflammatory activities. HyT is able to inhibit both initiation and promotion/progression phases of carcinogenesis by preventing DNA damage induced by different genotoxic molecules and by inhibiting proliferation and inducing apoptosis in different tumour cell lines (Acquaviva et al., 2012; Fabiani et al., 2008; Goulas et al., 2009; Owen et al., 2004; Rosignoli et al., 2016). Chemopreventive effects of HyT have also been observed in HL60 human promyelocytic leukaemia cells, HT-29 and DLD1 colon adenocarcinoma cells, reducing cell proliferation due to induction of apoptosis (Achmon & Fishman, 2015; Corona et al., 2009; Sun, Luo, & Liu, 2014; Terzuoli et al., 2010). Oleuropein is also a component of A009. Recent data demonstrated that oleuropein can induce apoptosis through the downregulation of pAkt, suggesting an inhibitory effect on Akt.
Fig. 3 – Effects of A009 on human and murine CC cell line adhesion. A009 (batch C) and HyT (hydroxytyrosol) were able to inhibit CC adhesion compared to untreated cells NT. Representative pictures show adherent HT-29 (A), HCT-116 (B) and CT-26 (C) cells (magnification 10×). Results are shown as mean ± SEM of 5 different fields captured in three experiments. ANOVA statistical analysis was used to determine p-value (***p < 0.001). Untreated (NT) and solvent (70% ethanol) were used as negative controls.

Fig. 4 – Effects of A009 on human and murine CC cell line migration. The ability of A009 (batch C) and HyT (hydroxytyrosol) to interfere with human and murine cell line migration was assessed using a modified Boyden chamber assay compared to untreated cells (NT). Representative pictures show migrated HT-29 (A), HCT-116 (B) and CT-26 (C) cells (magnification 10×). Results are shown as mean ± SEM of 5 different fields captured in three experiments. ANOVA statistical analysis was used to determine p-value (***p < 0.001). Untreated (NT) and solvent (70% ethanol) were used as negative controls.
signalling (Acquaviva et al., 2012). Oleuropein can increase trastuzumab efficacy in breast cancer cells (Fayyaz et al., 2016). Amongst the other soluble polyphenols, verbascoside has been reported to exert anti-proliferative activities towards tumour cells in vitro (Wartenberg et al., 2003), to induce apoptosis by telomere–telomerase cell cycle-dependent modulation, and to enhance repair of DNA damage due to oxidative stress. Verbascoside and natural plant-derived polyphenols (phenylethanoids, resveratrol) have been reported to

Fig. 5 – Effects of A009 on human and murine CC cell line invasion. The ability of A009 (batch C) and HyT (hydroxytyrosol) to interfere with human and murine cell line invasion was assessed using a modified Boyden chamber assay compared to untreated cells NT. Representative pictures show invaded HT-29 (A), HCT-116 (B) and CT-26 (C) cells (magnification 10×). Results are shown as mean ± SEM of 5 different fields captured in three experiments. ANOVA statistical analysis was used to determine p-value ("p < 0.01, ***p < 0.001). Untreated (NT) and solvent (70% ethanol) were used as negative controls.

Fig. 6 – Effects of A009 on human and murine CC cell sprouting. The ability of A009 (batch C) and HyT (hydroxytyrosol) to interfere with human HCT-116 (A) and murine CT-26 (B) cell sprouting on Matrigel was evaluated compared to untreated cells (NT). Results are shown as mean ± SEM of 5 different fields captured in three experiments. ANOVA statistical analysis was used to determine p-value ("p < 0.01, ***p < 0.001). Untreated (NT) and solvent (70% ethanol) were used as negative controls.
synergize with conventional anti-cancer therapies (Alipieva, Korkina, Orhan, & Georgiev, 2014). Anti-tumour effects of olive derivatives have been reported for cancers affecting different organs, including pancreas, oral cavity, oesophagus, prostate and lung (Rafehi, Ververis, & Karagiannis, 2012).

Colon cancer (CC) is the second most common cancer in women and third most common cancer in men worldwide (El Zoghbi & Cummings, 2016). Lifestyle and dietary habits have been reported to significantly impact on colon cancer prevention (Vipperla & O’Keefe, 2016; Vulcan et al., 2015), suggesting the importance of nutrition in cancer (chemo)prevention.

Here we investigated the effects of a novel purified polyphenol-enriched extract of OMWW, A009, abundant in HyT but also containing other polyphenols (Supplementary Table S1), on human and murine colon cancer cells. Chemical characterization of the extracts confirmed the presence of polyphenols, as demonstrated by the HPLC analysis, and the abundance of HyT, shown by HPLC and MS data. In the initial assessment of the ability of A009 to inhibit cancer cell growth in vitro, we tested four different batches of the extract and compared them to HyT at similar concentrations in a dose and time-dependant manner. We found that all batches of A009 inhibited colon cancer cell proliferation in a similar manner, and these effects were not associated to induction of apoptosis.

Since tumour cell motility, invasion, and metastasis to distant sites (Albini, 2016) represent crucial hallmarks of cancer (Hanahan & Weinberg, 2011), we evaluated the effects of A009 on colon cancer cell ability to adhere, migrate and invade. Given that all the batches behaved similarly, we chose batch C for investigation of these parameters. We found that incubation of cells with A009 for 24 h significantly inhibited HCT-116, HT-29 and CT-26 adhesion, invasion and migration. These data were further supported by the ability of A009 to inhibit CT-26 sprouting on a basement membrane matrix (Matrigel). Given the previously reported anti-inflammatory effects of HyT (Lamy et al., 2014; Terzuoli et al., 2010), we examined the ability of A009 to prevent the production of pro-inflammatory and angiogenic cytokines, VEGF and IL-8. Only viable cells, according to FSC/SSC parameters, have been considered. A009 (batch C) significantly (p < 0.01 and p < 0.001, respectively for A009 1:500 and 1:250) reduced VEGF and IL-8 protein expression in HCT-116 cells. All these effects were similar to those exerted by comparable concentrations of HyT. A009, which is easier to produce and less costly than HyT and is a waste product of the food industry, could be therefore a valid and environmentally friendly way to administer polyphenol, HyT-rich preparations.

Finally, the effect of A009 on tumour cell growth was assessed using the CT-26 colon-carcinoma cell lines in 6–8 weeks old syngenic BalbC mice, and compared to the effects of HyT and a well-known colon cancer chemotherapeutic drug, the 5-FU. A009 was able to inhibit tumour cell growth in a dose-dependent manner, exerting an inhibitory effect starting from the lowest dilution whereas purified HyT reduced tumour growth only at the highest dose. These data were further corroborated by the evaluation of weight of tumours excised from treated mice. These results suggest that the A009 extract has additional active components relative to commercially available HyT.

5. Conclusions

Taken together, our results suggest that isolating a purified fraction of the waste material OMWW (A009) represents a promising strategy to both limit environment pollution and obtain a potential water soluble polyphenol rich nutraceutical product with
beneficial effects associated with potential chemopreventive properties for colon cancer.

Authors’ contributions

Conceived and designed the experiments: AA, DMN, AB. Performed the in vitro experiments: BB, DDS, AB, TR, NM. Performed the in vivo experiments: AB, BB. Analysed the data: AA, DMN, AB, DDS, BB, NM, PC. Wrote the paper: AA, DMN, AB, DDS, BB. Purified the A009 extracts and provided their characterization: DP.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.jff.2016.09.009.

REFERENCES


Fig. 8 – Anti-tumour effects of A009 in vivo. CT-26 (10⁶) per animal, suspended in 100 µL of liquid pre-chilled Matrigel (10 mg/mL, BD) was subcutaneously injected into the right flank of each mice. Tumour dimension was measured with a caliper for 12 days after injection. (A) Tumour growth in mice treated i.p. with A009 (Batch C), HyT (hydroxytyrosol) or the reference drug 5-fluorouracil (5-FU). (B) Tumour growth in mice treated per os (drinking water-Batch A) with A009 or HyT. The mean ± SD is shown (**p < 0.001; ****p < 0.0001).


