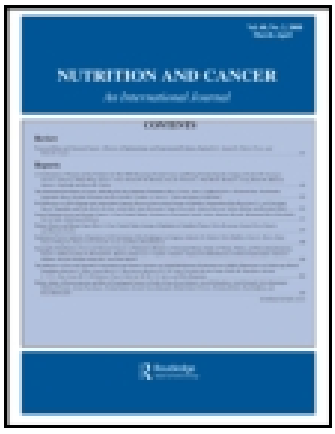

Nutrition and Cancer

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/hnuc20

Natural Grape Extracts Regulate Colon Cancer Cells Malignancy

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Published online: 23 Feb 2015.

To cite this article: Paola Signorelli, Carlotta Fabiani, Andrea Brizzolari, Rita Paroni, Josefina Casas, Gemma Fabriàs, Dario Rossi, Riccardo Ghidoni & Anna Caretti (2015) Natural Grape Extracts Regulate Colon Cancer Cells Malignancy, Nutrition and Cancer, 67:3, 494-503, DOI: 10.1080/01635581.2015.1004591

To link to this article: http://dx.doi.org/10.1080/01635581.2015.1004591

Nutrition and Cancer, 67(3), 494–503

ISSN: 0163-5581 print / 1532-7914 online
DOI: 10.1080/01635581.2015.1004591

Routledge
Taylor & Francis Group

Natural Grape Extracts Regulate Colon Cancer Cells Malignancy

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Natural dietary components are evolutionary-selected molecules able to control inflammation and cancerous transformation and progression. Because many studies assessed the beneficial properties of key molecules extracted from grapes, we aimed at investigating the properties of Liofenol^{TM}, a natural red wine lyophilized extract, devoid of alcohol and composed by a miscellaneous of components (polyphenols, flavonoids, anthocyanins). We proved that the colon cancer cell line HCT116 responded to Liofenol^{TM} treatment by reducing their proliferation, in association with an increase of p53 and p21 cell cycle gate keepers. Liofenol^{TM} increased dihydroceramides, sphingolipid mediators involved in cell cycle arrest and reduced proliferation rate. We observed a strong induction of antioxidant response, with the activation of the transcriptional factor Nrf2, involved in redox homeostasis and differentiation, without altering tumor sensitivity to chemotherapy. Liofenol^{TM} induced an important morphology change in HCT116 cells, migration inhibition, undifferentiated stem/stem-like cells markers downregulation, and E-cadherin downregulation, interested in epithelia to mesenchymal malignant transition. We conclude that lyophilized grape extract, at dose comparable to putative dietary doses, can activate molecular pathways, involving Nrf2 signaling and the modulation of structural and signaling sphingolipid mediators that cooperate in promoting differentiation and reducing proliferation of digestive tract cancer cells.

Submitted 18 May 2014; accepted in final form 18 November 2014.

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INTRODUCTION

"Let food be thy medicine and medicine be thy food" (Hippocrates, ca. 460 BC–370 BC). This belief grounded the history of medicine since its very beginning. It is estimated that the majority of all chronic diseases are lifestyle related (1) and both human epidemiologic and animal studies have drawn an inverse relationship between consumption of vegetables, fruits and risk of carcinogenesis in the whole digestive tract, lung, endometrium, and pancreas (2). As an example, natural compounds provide suppression of the inflammatory processes that are involved in chronic diseases or that favor cell hyperproliferation and transformation (3–5). Notably, they may ultimately suppress the final steps of carcinogenesis, namely angiogenesis and metastasis formation. Dietary agents also synergize with chemotherapeutic drugs, thereby reducing the dose of treatment and their whole body toxicity (6–9). Several dietary compounds such as curcumin (6), genistein (10), dietary isothiocyanates (11), silibinin (8), and the grape fruit contained polyphenol resveratrol (7,12–19) have been shown to block the cell cycle in tumoral cells, counteracting the dysregulation of proliferation that is a hallmark in all types of cancer.

Hyperproliferation is intimately associated with de-differentiation, and undifferentiated cancer stem/stem-like cells (CSCs/CSLCs) are mainly responsible of chemoresistance. Many evidences support the notion that phyto-components and in particular anti-oxidant polyphenols can induce differentiation in tumor cells (9,20–24). Malignant transformation is frequently promoted or accelerated by cell response to stress.

Prolonged and/or intensive oxidative, replicative, metabolic stresses evoke prolonged survival responses that may overcome or elude the normal check points of the cell cycle. The transcription factor Nrf2 (NF-E2-related factor 2) is a master regulator of the antioxidative stress response and positively controls the expression of a battery of antioxidative proteins and enzymes implicated in detoxification and drug metabolism, glutathione generation, and cytoprotection. Accordingly, Nrf2 is activated by many chemopreventive agents and it is essential for their activity in prevention of cancer and other diseases in animal models (25–28). The cell cycle inhibitor p21 stabilizes and activates Nrf2 in response to stress, as a defensive mechanism promoting survival (29,30). On the other side, Nrf2 is also linked to cell differentiation, being found to positively regulate differentiation of neurons (31,32), adipocytes (33) as well as in cancer such as acute myeloid leukemia cells (34) and in digestive tract colon cancer cells (35). Thus Nrf2 has a dual role. It acts as a stress defender by activating detoxification mechanisms, as well as by opposing cancer growth, via cell cycle block and promotion of differentiation. Natural occurring compounds have been shown to activate Nrf2 and to exert protection from oxidative stress (36). Moreover, chemopreventive properties exerted by sulforaphane (broccoli) (37), curcumin (turmeric) (38,39) and resveratrol (grapes) (40,41) have been related to the induction of Nrf2 therefore these compounds are currently in clinical trials for a variety of cancers. Finally, phytochemicals-induced Nrf2 activation has been shown to inhibit tumor invasion and metastatic potential (42), the last being regulated by redox signaling at multiple steps (43). It has been proved that many natural compounds modulate sphingolipids metabolism (6). Sphingolipids are a broad class of membrane structure-related compounds, actively involved in signaling events, such as inflammation or stress, and controlling cell cycle, differentiation and aging, autophagy and apoptosis (44). Our group demonstrated that gastric cancer cell treatment with the polyphenol resveratrol induces the accumulation of dihydroceramide (2), a sphingolipid molecule that has been proposed to inhibit cell cycle via regulation of Rb phosphorylation (45).

Liofenol^{TM} is a natural extract of red wine, devoid of alcohol and composed by a mixture of natural polyphenols, flavonoids, anthocyanins. In this article, we focused on the anticancer activity of Liofenol^{TM} by investigating its role in reducing tumor progression and invasion in HCT116 cells, a colorectal carcinoma cell line. We proved that chemotherapeutics properties of Liofenol^{TM} are mediated by sphingolipids metabolic profile modulation, namely by an increase of dihydroceramides, and by Nrf2 activation.

METHODS AND MATERIALS

Reagents and Antibodies

Liofenol^{TM}, a Gocciorosso red wine extract from organic agriculture, was kindly provided by Immobiliare Ca'Novella srl (Alessandria, Italy), SRB, bovine serum albumin, and Tamoxifen (Tam) were from Sigma-Aldrich (St. Louis, MO). Etoposide (Eto) was from Teva (Milan, Italy). Penicillin and streptomycin (Invitrogen) were purchased from Life Technologies Italia (Monza, Italy). DMEM culture media, fetal bovine serum (FBS), and the chemoluminescence system LiteAbLot were purchased from EuroClone Life Science Division (Milan, Italy). The following primary antibodies and dilutions were used: anti-p53, 1:500 (#9282, Cell Signaling, Beverly, MA); anti-p21, 1:500 (sc-397, Santa Cruz, Dallas, TX); anti-Nrf2, 1:1,000 (sc-722, Santa Cruz, Dallas, TX); anti-E-cadherin, 1:1,000 (#CM1681, ECM Biosciences, Versailles, KY); anti-β-actin, 1:5,000 (A5316, Sigma). The secondary antibody (1:10,000) was from Jackson Laboratories (Bar Harbor, ME). The synthetic oligonucleotides used in this study were purchased from M-Medical (Milan, Italy). All reagents were of the maximal available purity degree.

Liofenol^{TM} Production Methods

Liofenol^{TM} is extracted from "Gocciorosso" organic, unfiltered red wine coming from red grapes (average age of vineyards: 40 yr) cultured in an organic farm located in Monferrato (Piemonte, Italy). The vineyards are sun exposed in a southwesterly direction, on a 30–40% inclinated land. The grapes have been manually detached and placed in plastic crates, each of 15 kg, avoiding the breaking of the grape skins. The wine production needs 10 to 15 days grapes fermentation after de-stemming and no sulphites or any other substance have been added. No mechanical, chemical, and physical accelerated filtration or precipitation have been performed, only periodic decanting with the natural aging process (12 mo). Liofenol^{TM} is then obtained by vaporization of red wine Gocciorosso both under vacuum and low temperature conditions without any other physical/chemical handling or alteration and no other substances addition. It's conserved in glass containers and the wine:Liofenol^{TM} ratio is about 20:1. As for any natural phyto-complex, Liofenol^{TM} therefore maintains the special features of the antioxidant matrix of elements of the original Gocciorosso wine.

Liofenol^{TM} Composition Analysis

Liofenol^{TM} composition analysis was performed at Consiglio per la Ricerca e Sperimentazione in Agricoltura–Centro di Ricerca per l'Enologia di Asti, Asti, Italy.

Liofenol^{TM} Stock Solution Preparation

Liofenol^{TM} was weighted and dissolved in phosphate buffered saline (PBS) to obtain a stock solution (300 μg/μl), sterile filtered (0.22 μm pore diameter, Nalgene) and stored at -20°C. The stock solution was diluted in medium for cell treatment to a final treatment dose of 100–300–600–1200 μg/ml.

Cell Line and Treatments

HCT116 cells, a human colorectal carcinoma cell line, were obtained from American Type Culture Collection (Rockville, MD). Cells were grown in DMEM media supplemented with 10% FBS. For experiments, cells were seeded in 6 wells-plate at 5×10^5 cells/well. Twenty-four hours after seeding, when cells reached about 60% confluence, the medium was replaced with a fresh one, containing Liofenol™ at different treatment doses and cells were allowed to grown for additional 24–48–72 h.

Sulforhodamine B Proliferation Assay

Cells were seeded in 96-wells tissue culture plates and allowed to adhere for 24 h before Liofenol™ treatment. Cell proliferation was assessed at various time points, by sulforhodamine B (SRB) monosodium salt assay. Data are expressed as the proliferation index (fold increase of proliferation vs. vehicle-treated cells at Time 0).

Wound-Healing Assay

Cells were seeded in 6-well plates and grown overnight in culture medium containing 10% FBS to reach 90% confluence. Cell monolayer was wounded by scratching with a 200-μl pipette tip out of the edge, followed by washing 3 times with serum-free medium. Succesively, cells were grown in complete medium for 24 and 48 h. For each well, images of the scratch were taken at 0, 24, and 48 h with a phase contrast microscope at 10× magnification, to monitor migration progress.

Ferric Reducing Antioxidant Power Test

The ferric reducing antioxidant power (FRAP) assay was performed according to previously published method (46), with minor modifications. Briefly, to prepare the FRAP solution, 100 mL of acetate buffer 300 mM, adjusted to pH 3.6 with acetic acid, were mixed with 10 mL of ferric chloride hexahydrate 20 mM (in distilled water) and 10 mL of 2,4,6-tris(2-pyridyl)-s-triazine 10 mM (in HCl 40 mM). One hundred μl of sample were added to 3 mL of a freshly prepared FRAP solution in glass test tubes in triplicate and the absorbance measured at 593 nm after 5 min of incubation at 37°C against a blank of acetate buffer. Aqueous solutions of FeSO4•7H2O (100–1000 μM) were used for the calibration and the results were expressed as FRAP value [μM Fe (II)] of the samples (47).

Hydroxyl Radical Scavenging Activity

The hydroxyl radical scavenging activity of Liofenol™ was evaluated in supernatants of HCT116 cells treated with the 2-deoxyribose degradation assay (48), with minor modifications. After incubation for 40 min at 25°C, the reaction was stopped by addition of 1 ml of 4 % (v/v) phosphoric acid. After addition of 1 ml of 1 % (p/v) of thiobarbituric acid, the tubes were warmed (90–95°C) for 15 min to develop a pink chromogen, cooled at room temperature, and 3 ml of 1-butanol was added to eliminate the turbidity of the solutions. After centrifugation at 12,000 × g for 5 min, the upper layers were taken and the absorbance at 532 nm recorded, using a spectrophotometer UV/VIS Beckman DU 640. The percentage of hydroxyl radical scavenging activity of the samples was calculated from the formula: % Scavenging Activity: [(Ab – As)/Ab]* 100 where Ab is the absorbance of the blank (cell culture medium) and As is the absorbance of the sample.

LC–MS Analysis

Sphingolipid extracts from untreated and treated cells, fortified with internal standards [Ndodecanoylsphingosine, Ndodecanoylglucosylsphingosine, Ndodecanoylsphingosyl phosphorylcholine, C17sphinganine (0.2 nmol each) and C17sphinganine1phosphate (0.1 nmol)], were prepared and analyzed as reported (49).

Western Blotting

Cells were scraped in ice-cold PBS containing proteases inhibitors (Roche Italia, Milan, Italy) and spun at 1,200 × g for 5 min at 4°C. An aliquot was used for protein quantification; the remaining cells were resuspended in Laemmli buffer, boiled for 8 min and stored at 20°C. Equal amount of proteins (10 μg) were separated on 10% acrylamide gels by SDS-electrophoresis and transferred onto nitrocellulose membranes. After blocking unspecific binding sites with 5% dry skimmed milk in TBS-Tween 0.1% (TBST), the membranes were incubated (4°C/overnight) with primary antibodies diluted in TBST, followed by incubation (room temperature/1 h) with the appropriate HRP-secondary antibody diluted in TBST-5% dry skimmed milk. The same membranes were immunoblotted against β-actin for data normalization. Proteins were detected by chemoluminescence and bands intensity was quantified by Gel Doc 2000, using Quantity One Software (BioRad Life Science, Hercules, CA).

RNA Extraction and Quantitative RT-PCR

Total RNA was extracted and reverse transcribed according to the manufacturer's instructions (miRNeasy Mini Kit and miSCRIPT II RT kit, QIAGEN [Milan, Italy], respectively). The amplification of target genes (CD44 and CD133, primer sequences as previously reported) (50) was performed using Syber Green system (QIAGEN). Relative mRNA expression of target genes was normalized to the endogenous GAPDH control gene and results calculated by 2-DeltaDeltaCt method (51), as treated vs. the control untreated cells.

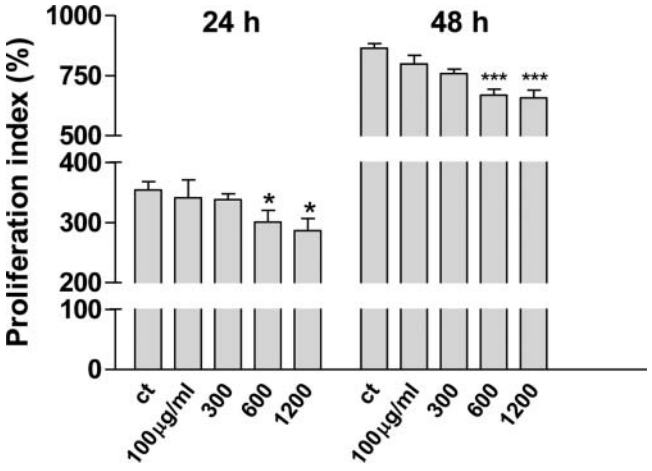

FIG. 1. Liofenol™ induces cell proliferation reduction in HCT116 cell line. Liofenol™ effect on HCT116 cells proliferation at 24 and 48 h after treatment, expressed as percentage of proliferation over the control at the starting time (Time 0). The X axis reports increasing doses of Liofenol™ expressed as μg/ml; ct = the control untreated cells. Medium value of 3 independent experiments. Significance was evaluated by one-way analysis of variance: $P = 0.04$ at 24 h; $P = 0.0001$ at 48 h. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. ct (Bonferroni post-test).

Intracellular ROS Analysis

Intracellular ROS analysis was performed by the d-ROMs test kit according to the manufacturer's instructions (Diacron International srl, Grosseto, Italy).

Statistical Analysis

Any single data was obtained by reproducing the related experiments for a minimum of 3 times. The reported results are the medium value obtained by repetitive results (in case of Western blotting, the most representative images). Data significance was evaluated by one-way analysis of variance followed by the Bonferroni multiple comparisons test when significant ($P < 0.05$) or paired 2-tailed Student t-test. Data are expressed as mean $\pm$ SEM.

RESULTS

Liofenol™ Reduces Cell Proliferation in HCT116 Cell Line

HCT116 cells were cultured in 96-well plate and allowed to grow for 24 and 48 h at 100, 300, 600, and 1200 μg/ml Liofenol™ treatment. Cell proliferation was significantly reduced at the same extent with 600 and 1200 μg/ml, starting from 24 h ($^{*}P < 0.05$ vs. the control) by about 20% (Fig. 1).

Liofenol™ Effect Correlates with Upregulation of p53 and p21 Protein Expression

To understand the mechanism causing the reduced proliferation rate, we analyzed the protein expression of p21 and of its transcription factor p53 by Western blotting (Fig. 2), both known to play a key role in these cellular processes. Upon Liofenol™ treatment, p53 expression was dose-dependently

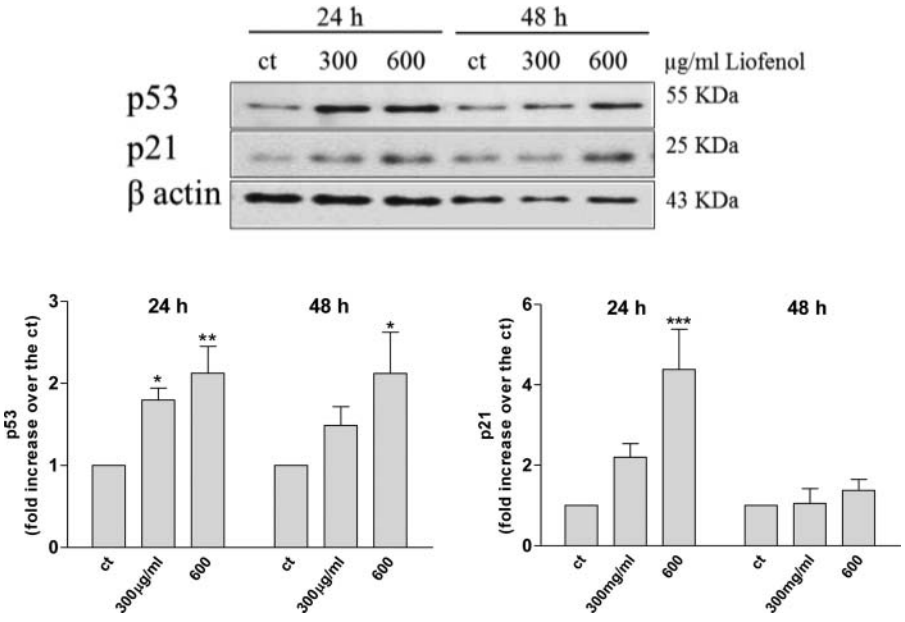

FIG. 2. Liofenol™ effects correlate with upregulation of p53 and p21 protein expression. Liofenol™ effect on p53 and p21 expression level 24 and 48 h after treatment at different doses. The graphs represent the densitometric analysis of p53 and p21 protein bands normalized on the corresponding β actin value. The X axis reports increasing doses of Liofenol™ expressed as μg/ml; ct = the control untreated cells. The Western blot images are the most representative of three independent experiments. ct represents the control untreated cells. Significance was evaluated by one-way analysis of variance: $P = 0.01$ and $P = 0.02$ for p53 at 24 and 48 h, respectively; $P = 0.0001$ for p21 at 24; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. ct (Bonferroni posttest).

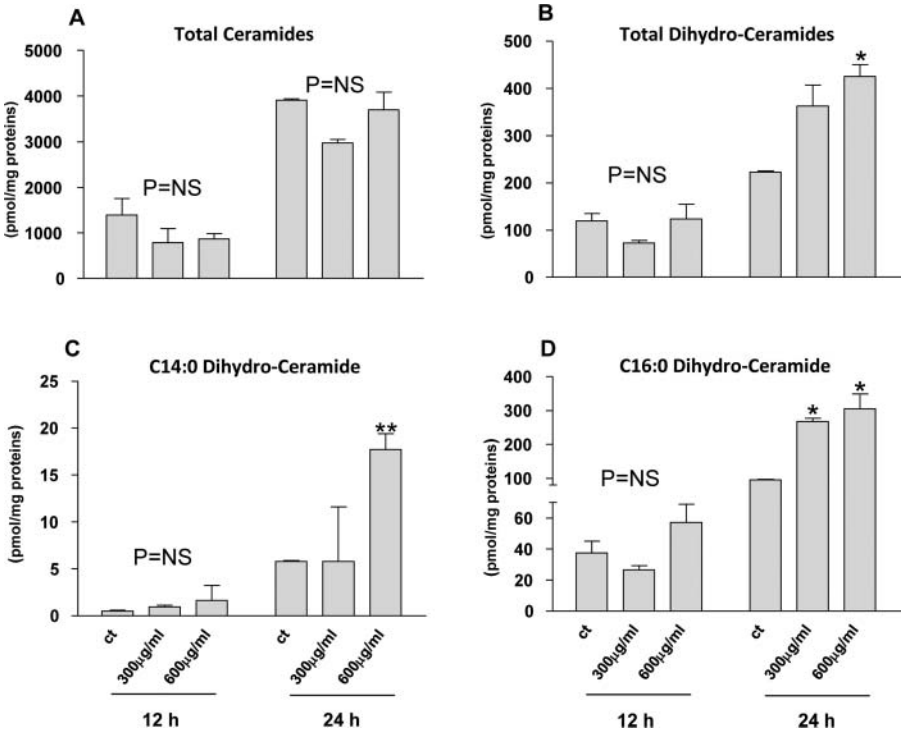

FIG. 3. Liofenol™ induces dihydroceramide increase. Liofenol™ effect on sphingolipid metabolites profile, 12 and 24 h after Liofenol™ treatment at 300 and 600 μg/ml. The graph represents the LC-MS analysis of ceramides and dihydroceramides with different acyl chains, expressed as picomoles normalized onto total mg protein content. A: total ceramides content; B: total dihydroceramides content; C: dihydroceramide C14:0-chain acyl specie; D: dihydroceramide C16:0-chain acyl specie. Significance was evaluated by one-way analysis of variance: $P = 0.03$, $P = 0.02$, and $P = 0.005$ at 24 h for B, C, D and, respectively. $^{*}P < 0.05$. $^{**}P < 0.01$ vs. control untreated cells (ct) (Bonferroni posttest).

increased in HCT116 whole cell lysates, as early as 24 h (two-fold increased expression at 600 μg/ml Liofenol™, 24 h: $^{**}P < 0.01$ vs. the control untreated cells) and remained upregulated at later times (48 h: $^{*}P < 0.05$). p21 expression well correlated with p53 induced upregulation, showing more than fourfold increase as compared to the control when HCT116 cells were treated with 600 μg/ml Liofenol™, 24 h ($^{***}P < 0.001$).

Liofenol™ Modulates Sphingolipid Metabolism in HCT116 Cell Line

We previously demonstrated that modulation of sphingolipid biosynthesis and accumulation of dihydroceramides induces a cell cycle delay at the G0 phase (52). We here investigated the effects of Liofenol™ on sphingolipid metabolites profile in HCT116 cells (Fig. 3). In spite of unaltered

TABLE 1
Comparative analysis of the major phytocomponents content of "Gocciorosso 3" red wine and Liofenol™, the liophylized extract

	Goccio Rosso 3 (red wine)		Liofenol™	L/W
	mg/L	mg/g	mg/g	
Total polyphenols index	2227 ± 8.58	2.230 ± 0.01	49.52 ± 0.11	22.16
Total antocyanins index	57 ± 0.74	0.057 ± 0.0007	1.03 ± 0.05	17.88
Total flavonoids index	2086 ± 13.1	2.092 ± 0.013	43.35 ± 4.42	20.72
Pro-antocyanidins index	2596 ± 23.84	2.610 ± 0.02	58.02 ± 3.53	22.28
Vanilla-reactive flavonoids index	1576 ± 5.24	1.580 ± 0.01	32.32 ± 0.17	20.44
Antioxidant power (Ascorbic acid eq.)	1616 ± 10.00	1.621 ± 0.01	37.00 ± 2.40	22.81
Glycerin	1401 ± 0.34	0.014 ± 0.0003	0.297 ± 0.026	21.11
Moisture (% p/p)			25.02	

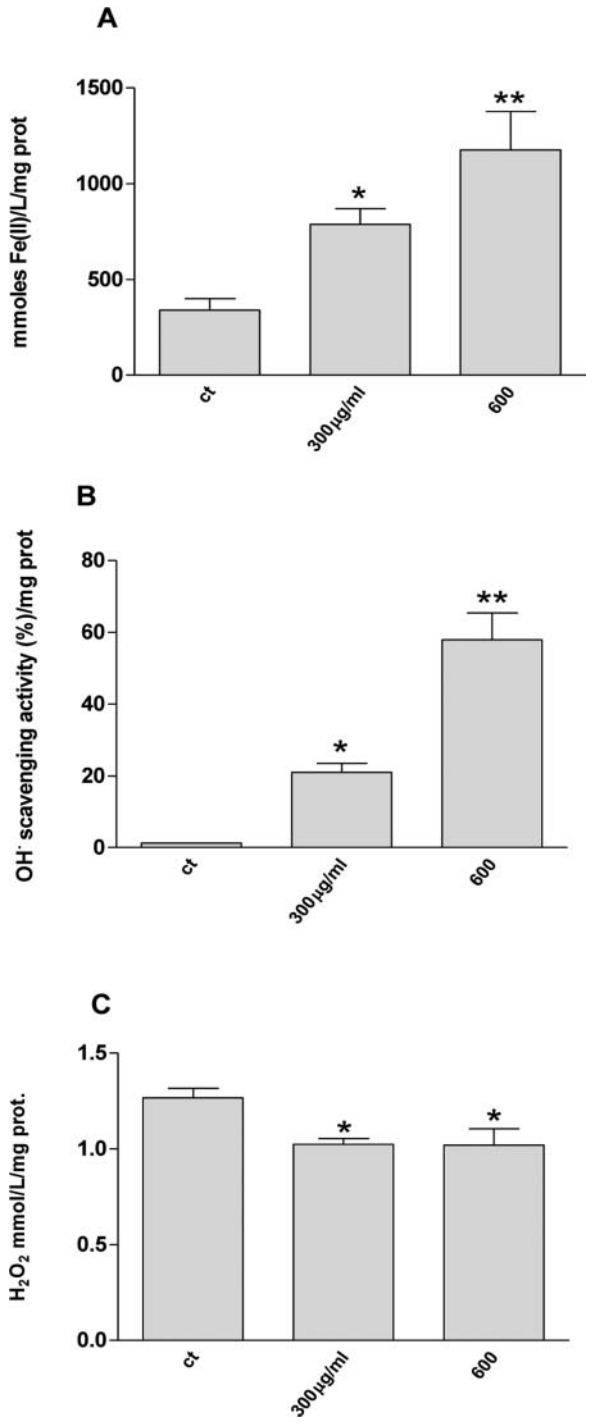

FIG. 4. Liofenol^{TM} exhibits antioxidant activity on HCT116 cell line. Liofenol^{TM} antioxidant activity on HCT116 cells, 24 h after 300 and 600 μg/ml treatment. The antioxidant compounds release in supernatant was analysed by the FRAP (ferric reducing antioxidant power) test (A) and the hydroxyl radical scavenging activity test (B). The intracellular ROS level, expressed as H_2O_2 mmol/L and normalized on sample protein content, was evaluated by the d-ROMs test kit (C). ct = the control untreated cells. Significance was evaluated by one-way analysis of variance: $P = 0.01$, $P = 0.0003$, and $P = 0.04$ h for A, B, C, respectively. $^*P < 0.05$, $^{**}P < 0.01$ vs. ct (Bonferroni posttest).

levels of total ceramides (Fig. 3A), we observed an increase of the total dihydroceramides content (Fig. 3B; 24 h: $^*P < 0.05$ vs. the control untreated cells) with a significant increase of the C14:0- (Fig. 3C) and C16:0- (Fig. 3D) acyl chain species, as expected during cell cycle arrest.

Liofenol^{TM} Exhibits Antioxidant Activity on HCT116 Cell Line

Liofenol^{TM} is a red wine lyophilized extracts comprising a miscellaneous of components as reported in Table 1. We first performed the FRAP test investigating the antioxidant activity of Liofenol^{TM} in culture media of HCT116 cells treated with increasing doses (300, 600 μg/ml) of Liofenol^{TM} for 24 h. We evaluated the total concentration of reduced Fe (II) and normalized on the total protein content of each sample, and observed a dose-response effect in reducing potency of the treatment (Fig. 4A; $^*P < 0.05$, $^{**}P < 0.01$ vs. the control untreated cells). Moreover, the hydroxyl radical scavenging activity of Liofenol^{TM}, measured by the 2-deoxyribose degradation assay in culture media of HCT116 cells treated with increasing doses (300, 600 μg/ml) of Liofenol^{TM} for 24 h, was similarly increased (Fig. 4B; $^*P < 0.05$, $^{**}P < 0.01$ vs. the control untreated cells). In agreement with the previous results, the intracellular ROS level of HCT116 treated as above described, expressed as H_2O_2mmol/L and normalized on sample protein content, was significantly decreased by Liofenol^{TM}, in a dose-response manner with respect to the untreated cells at 24 h (Fig. 4C; $^*P < 0.05$).

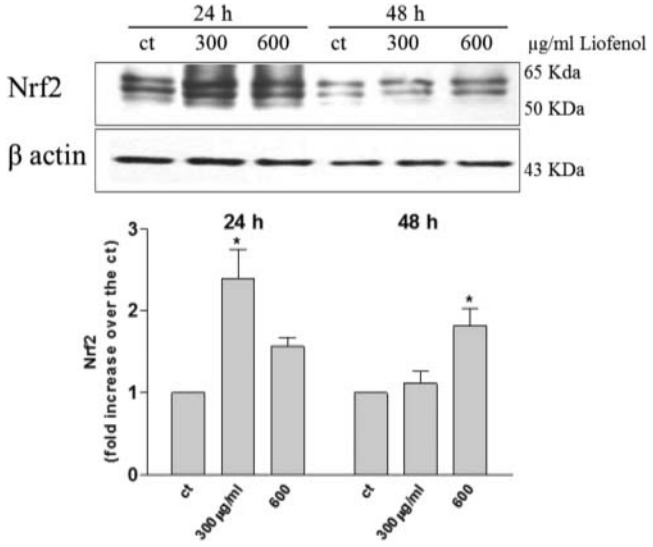

FIG. 5. Liofenol^{TM} induces Nrf2 expression. Liofenol^{TM} effect on Nrf2 expression level 24 and 48 h after treatment at different doses. The graph represents the densitometric analysis of Nrf2 protein bands normalized on the corresponding β actin value. The X axis reports increasing doses of Liofenol^{TM} expressed as μg/ml; ct = the control untreated cells. The Western blot images are the most representative of 3 independent experiments. Significance was evaluated by one-way analysis of variance: $P = 0.01$ at both 24 and 48 h; $^*P < 0.05$ vs. ct (Bonferroni posttest).

We concluded that Liofenol™ was able to significantly favor reduction processes and to reduce oxidant radicals.

Liofenol™ Induces Nrf2 Expression

Nrf2 is a transcription factor, whose ubiquitin-dependent proteolysis is blocked by p21 activation (29,30) allowing its intracellular increase and its transcription activation of genes involved in survival, and in cell redox homeostasis. We thus evaluated Nrf2 protein expression to better understand the mechanism of action of Liofenol™ (Fig. 5). Liofenol™ (300 and 600 μg/ml) increased whole cell lysates Nrf2 protein levels within 24 h of treatment (more than twofold induction with respect to the control; $^*P < 0.05$). Nrf2 increased expression lasted for 48 h when HCT116 cells were treated with 600 μg/ml Liofenol™.

Liofenol™ Regulates the Expression of Markers Involved in Differentiation and Metastasis Formation in HCT116 Cell Line

It is noteworthy that Nrf2 was lately demonstrated to promote differentiation (34,35). At 24 h after 600 μg/ml Liofenol™ administration, we observed a striking phenotypic changes in HCT116, acquiring flattened cell shape accompanied by decreased saturation density (Fig. 6A). Therefore we analyzed the early (12 and 24 h) expression of different markers known to control colon cancer malignancy. First, we analyzed by RT-PCR the mRNA expression of CD44 and CD133, 2 markers of undifferentiated stem/stem-like cells (CSCs/CSLCs) (Fig. 6B). After 12 and 24 h of 600 μg/ml Liofenol™ treatment, the mRNA's level was reduced from 40 to 50% with respect to the control untreated cells (t-test: $^*P < 0.05$ for CD44; $P < 0.01$ for CD133). Second, we

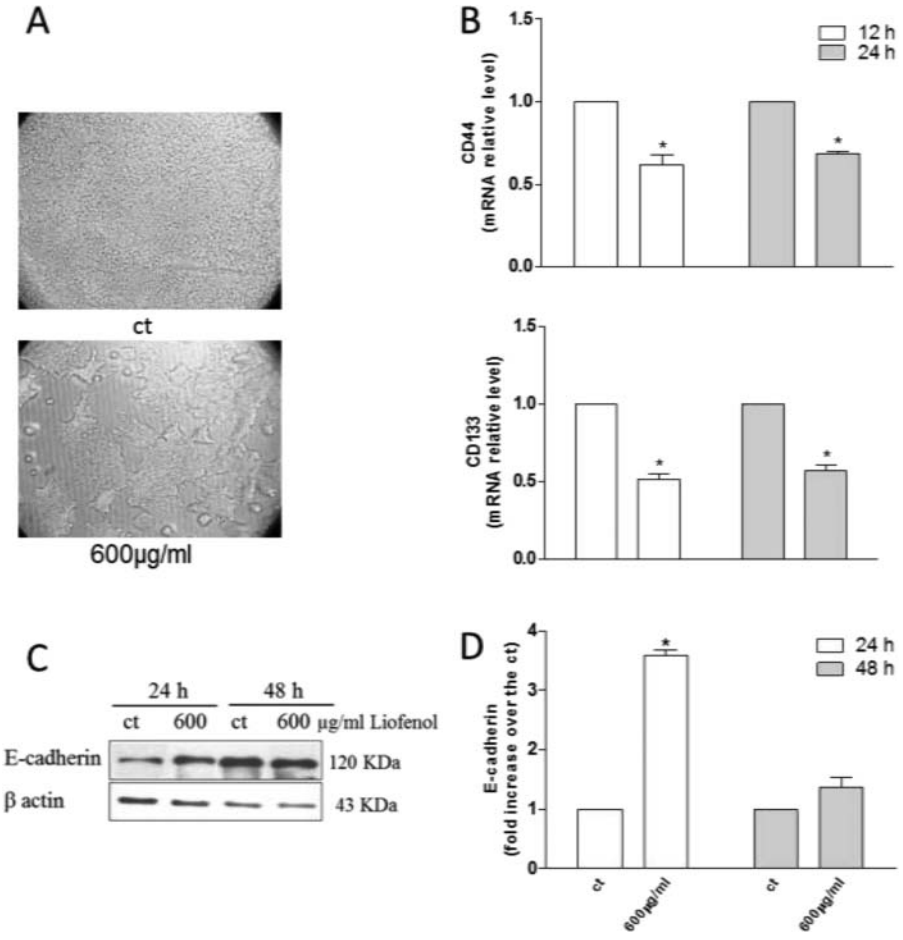

FIG. 6. Liofenol™ modulates the expression of differentiation and migration markers in HCT116 cell line. A: Liofenol™ effect on HCT116 cells differentiation 24 h after treatment at 600 μg/ml. ct = the control untreated cells. Images were shot by inverted microscope (20× magnification) Axiovert 25 CFL (Zeiss, Göttingen, Germany) equipped with a digital camera (DS-2MV; Nikon, Tokyo, Japan). B: mRNA expression level of CD44 and CD133 analyzed by RT-PCR at 12 and 24 h after Liofenol™ treatment at 600 μg/ml. Medium value of 3 independent experiments. Data were normalized to the endogenous GAPDH control gene and expressed as fold change vs. the control untreated cells (ct). Significance was evaluated by paired 2-tailed Student *t*-test: $^*P < 0.01$ for CD44; $^*P < 0.05$ for CD133. C: Liofenol™ effect on E-cadherin expression, 24 and 48 h after Liofenol™ treatment at 600 μg/ml. D: The graph represents the densitometric analysis of the E-cadherin protein bands normalized on the corresponding β actin value. ct = the control untreated cells. The Western blot image is the most representative of 3 independent experiments. Significance was evaluated by paired 2-tailed Student *t*-test: $P < 0.01$ at 24 h; P = NS at 48 h.

analyzed by Western blotting the expression of E-cadherin, whose loss is a hallmark of malignant epithelial-to-mesenchymal transition (EMT), hence of metastatic potential. Liofenol^{TM} 600 μg/ml induced a significant induction of E-cadherin expression within 24 h of treatment, with more than threefold increase (*$P < 0.05$ vs. the control untreated cells) (Fig. 6C and 6D).

Liofenol^{TM} Doesn't Exhibit Drug-Resistance Activity and Reduces Migration Ability

To exclude a possible role of Nrf2 in promoting survival or in promoting chemoresistance, as previously hypothesized (53), we analyzed the effect of Liofenol^{TM} in cotreatment with Tam and Etoposide, two known chemotherapy drugs used in the treatment of cancers from different origins. We treated HCT116 cells with Tam (10 μM) or Eto (5 μg/ml) w/wo Liofenol^{TM} at 600 μg/ml for 24 h. As shown in Fig. 7, proliferation of Tam- and Eto-treated HCT116 was reduced by 75% and 50% in respect with control untreated cells, respectively. Coadministration of Liofenol^{TM} didn't interfere with the efficacy of the 2 chemotherapy drugs, being the proliferation index unaltered (P = NS, Tam vs. Tam/Liofenol^{TM} and Eto vs. Eto/Liofenol^{TM}).

To evaluate the antitumoral/diffentiation activity of Liofenol^{TM}, we investigated the changes in cell migration ability in a wound migration assay in vitro. We compared the wound line between Liofenol^{TM} treated and untreated cells at 24 and 48 hs (Fig. 8) from a qualitative point of view. After cell monolayer scratching by a pipette tip, untreated HCT116 migrated in the wound line, partially recovering it since the first 24 h after scratching. On the contrary, even at the lower dose tested (300 μg/ml), Liofenol^{TM} inhibited HCT116 migration activity since we didn't observe any change in the wound line up to 48 h after scratching.

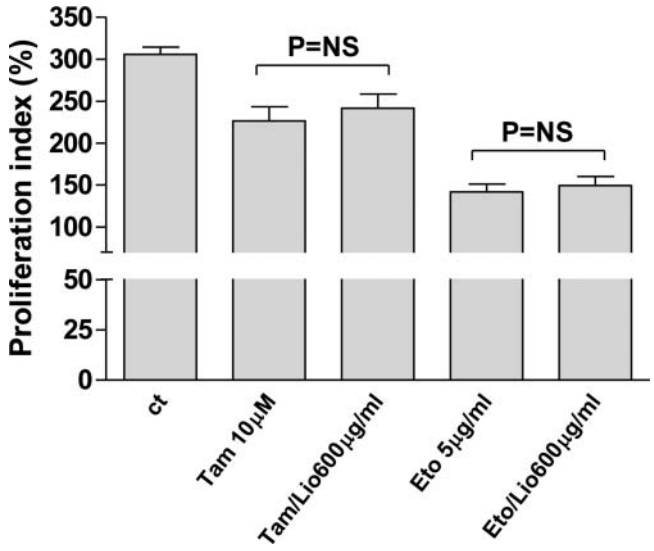

FIG. 7. Liofenol^{TM} doesn't exhibit drug-resistance activity. Liofenol^{TM} (Lio) effect on HCT116 cells proliferation at 24 h after co-treatment with Tamoxifen (Tam, 10 μM) or Etoposide (Eto, 5 μg/ml), expressed as percentage of proliferation *vs* the control at To. ct = the control untreated cells. Medium value of 3 independent experiments. Significance was evaluated by one-way analysis of variance.

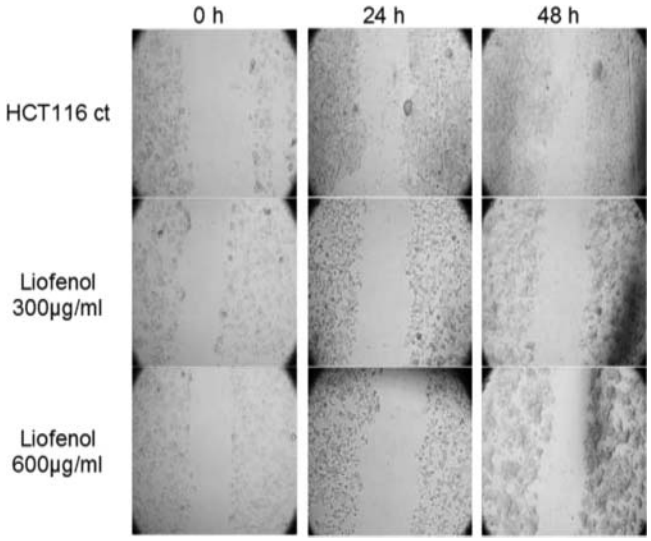

FIG. 8. Liofenol^{TM} inhibits HCT116 migration activity. Liofenol^{TM} effect on HCT116 migration activity at 24 and 48 h after treatment at increasing doses. ct = the control untreated cells. Photos were taken with an inverted microscope (20× magnification) Axiovert 25 CFL (Zeiss, Göttingen, Germany) equipped with a digital camera (DS-2MV; Nikon, Tokyo, Japan).

DISCUSSION

Liofenol^{TM} is a natural red wine lyophilized extracts, devoid of alcohol and composed by a miscellaneous of components such as polyphenols, flavonoids, anthocyanins. We explored the effect of this preparation on digestive tract tumor cells and we observed a strong antiproliferative, differentiation-inducing and antiinvasion potential of a micromolar range treatment of Liofenol^{TM} on colon cancer cells. Liofenol^{TM} was able to drastically reduce colorectal carcinoma cell line HCT116 proliferation and we observed significant morphological changes, with flattened cells, forming islets of clusters and showing decreased confluency, as other authors reported occurring upon diffentiation processes (7,12–19). Perletti et al. (35) reported that PKCδ-overexpression in HCT116 cells induced growth inhibition and phenotypic alterations, with clusters of flattened enlarged cells, similar to those observed under Liofenol^{TM} treatment. They concluded that the antitumor effects was depending on p53 activation of p21 and on the induction of differentiation markers. We similarly found that p21 and p53 were activated in response to Liofenol^{TM} in HCT116 cells, together with the modulation of sphingolipid mediators. In particular we observed an increase of dihydroceramides: a minor specie, containing different acyl chains, whose increase over the steady state intracellular

concentration was shown to arrest or delay cell cycle by Bielawska's group and by our group (45,52). Parallel to reduced proliferation, we demonstrated a significant reduction of CD44 and CD133 expression, 2 colon cancer cells markers of undifferentiated stem/stem-like cancer cells (CSCs/CSLCs) (54). EMT is a developmental process playing an important role in solid tumor progression and metastasis (1, 55), and clinical studies evidenced that the increase of E-cadherin expression correlates with improved patient's survival (56). Liofenol^{TM} increased E-cadherin expression in HCT116 cell line, in agreement with the reduced cell migration activity observed in the wound migration assay. Given its nature of antioxidant agent, similar to many phytochemicals, we investigated Liofenol^{TM} effect on the activity of Nrf2, a key transcriptional factor involved in oxidative stress response. In spite of a cytoprotective role for Nrf2 activation of ARE-containing genes involved in oxidative stress response (53), recent findings assess that redox-sensitive signaling pathways control the basic processes required for metastasis (57) and that Nrf2 plays an important role in the cooperative induction of myeloid leukemia cell differentiation by plant polyphenols (34). To exclude a possible induction of chemoresistance, we demonstrated that Liofenol^{TM} co-treatment of HCT116 cells with chemotherapy drugs, such as Tam and Eto, was not affecting the therapeutic potency of the drugs. Chen et al. indicated that p21, a p53 target gene, stabilizes Nrf2 by interfering with its proteasomal degradation (29). Liofenol^{TM} induced p21 upregulation in HCT116 cells and this may account for Nrf2 increase, proliferation arrest, differentiation, and reduction in migration activity thus highlighting Liofenol^{TM} inhibitory role in cancer progression.

We hypothesize that antioxidant activity triggered by Liofenol^{TM} may involve sphingolipid signaling as effector arm to regulate cell cycle and differentiation in cancer cells. Further study is required to understand how dihydroceramide signaling and Nrf2 transcriptional activity can be related. These findings have implications in cancer therapy, because Liofenol^{TM} treatment is beneficial by entailing the switch to low growth/low metastatic phenotypes without negatively interfering with the conventional chemotherapy. Nrf2 pathway could play a central role in colon cancer chemoprevention.

ACKNOWLEDGMENTS

We thank Dr. Daniela Borsa and Dr. Federico Piano for Liofenol^{TM} composition analysis, performed at Consiglio per la Ricerca e Sperimentazione in Agricoltura-Centro di Ricerca per l'Enologia di Asti (CRA-ENO), Via Pietro Micca 35, 14100 ASTI, Italy.

FUNDING

Financial support from the Institutional Grants of University of Milan and the PhD program in Molecular Medicine, Università di Milano, Italy is acknowledged. Financial support from Generalitat de Catalunya (grant SGR2009 1072), Ministerio de Economía y Competitividad (grant SAF2011 22444) and Fundació La Marató de TV3 (grant 112130) is acknowledged. Financial support from Immobiliare Ca' Novella srl, Alessandria, Italy is acknowledged.

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