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A carotenoid-enriched extract from pumpkin delays cell proliferation in a human chronic lymphocytic leukemia cell line through the modulation of autophagic flux

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is the most frequent form of leukemia in the adult population. From an asymptomatic state, which can lay for years, CLL rapidly evolves becoming fatal. Apoptosis resistance and induction of a protective form of autophagy are possible explanations of the poor responsivity of CLL to conventional and innovative therapeutic drugs. Carotenoids, including α- and β-carotene, lycopene, and derivatives, such as retinoic acid, have been studied for their significant antiproliferative and differentiating activity in cancer. Here, we report that a carotenoid-enriched extract (CE) obtained from pumpkin by supercritical CO_2 extraction showed an antiproliferative effect on HG3 cell line derived from human B-CLL cells. CE induced a 40% delay in cell proliferation compared to untreated cells, without signs of cytotoxicity. This delay was associated with $p27^{Kip1}$ over-expression, AMPK activation and modulation of autophagy flux. In HG3 cells treated with CE, we detected a 30% autophagosome intracellular increase and changes in the expression of canonical biochemical markers of autophagy (LC3-II, p62). These results suggest the presence in the CE of a pool of bioactive carotenoids acting additively or synergistically in retarding cancer cell growth.

1. Introduction

Chronic lymphocytic leukemia (CLL) is characterized by the accumulation (>5 10^9/l) of neoplastic CD5 B lymphocytes in the blood, bone marrow and lymphoid tissues. The incidence of CLL is approximately 4–5/100,000 people per year in Europe and, at diagnosis; the median age of patients is approximately 70 years. However, the incidence of this form of leukemia increases markedly with age up to >100,000 per year in aged people (>70 years). Therefore, considering the constant rise of the elderly population in the Western world, it is easy to predict that in the next future CLL incidence will further increase.

Despite the discovery of new drugs targeting protein-protein interaction (BH3 mimetics such as ABT 199/venetoclax) (Roberts et al., 2016) and promising new therapeutic combinations (rituximab/venetoclax or ibrutinib/venetoclax) (Seymour et al., 2018; Wiestner, 2019), CLL remains an incurable disease. A further difficulty in the management of CLL is its clinical and biological heterogeneity: monoclonal B-cell lymphocytosis (MBL) refers to the presence of fewer than 5 10^9/l monoclonal B cells in the peripheral blood of an asymptomatic individual with neither lymphadenopathy, nor organomegaly, although immuno-phenotypically identical to CLL (Strati and Shanafelt, 2015). The latter feature of CLL creates the need to develop safe, personalized therapy in frail/old patients at the initial or asymptomatic stages characterized by a MBL at high risk for CLL due to familiar history, genetic polymorphism, p17 deletions or elevated B-cell count (Strati and Shanafelt, 2015).

CLL etiology, like for other neoplasms, is unknown due to the complex interactions of genetic and environmental factors. Diet is a modifiable environmental risk factor, even if epidemiological data on the association between diet and CLL are heterogeneous and mainly associated with a single food or single nutrients (for example, Vitamin D insufficiency) (Shanafelt et al., 2011). A recent population-based multi-case control study evaluated the association between the adherence to a specific dietary pattern, i.e. Western, Prudent and Mediterranean diets, and CLL both in terms of different Rai stage and overall outcomes of the disease (Solans et al., 2018). The study indicated a direct association between high-fat dairy products, processed meat and caloric drinks and CLL, independently from the Rai stage, while no association with the Mediterranean or Prudent diet was reported, suggesting that a proportion of CLL cases could be prevented by modifying dietary habits (Solans et al., 2018).

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Until now, except for Vitamin D, only few and sometimes conflicting results from epidemiological studies have been published where the risk of CLL was negatively associated with the consumption of naturally occurring bioactive compounds or classes of compounds present in edible plants (Chen et al., 2017). On the contrary, results obtained from *in vitro* and *ex vivo* studies, where single molecules or mixtures of phytochemicals widely present in the Mediterranean diet were employed, showed promising anti-leukemic and pro-apoptotic effects against CLL. In general, these phytochemicals belong to class of polyphenols (quercetin, epigallocatechin-gallate) or carotenoids (lycopene) (Kumazoe et al., 2015; Russo et al., 2017b; Salman et al., 2007; Spagnuolo et al., 2012). The use of pre-clinical models confirmed the importance of targeting apoptotic pathways to sensitize B-CLL to cell death (Roberts et al., 2016; Zhang et al., 2002).

A relatively still unexplored aspect of CLL biology regards the implication of autophagy, a genetically regulated, autodigestive cellular process, mainly linked to cell survival in response to endogenous or exogenous stresses (Smith et al., 2020). Only few studies explored the role of autophagy in CLL pathogenesis following exposure to phytochemicals (as a single compound or in association with chemotherapeutic drugs) able to target autophagy pathways and interfere with leukemic B-cells progression (Mahoney et al., 2013; Russo and Russo, 2018).

Among phytochemicals, carotenoids are yellow-orange-red organic pigments, which contribute to the coloring of many plants, or other photosynthetic organisms, such as algae and some species of bacteria. They are chemically lipophilic compounds and are defined as isoprenoid polyenes, with a long chain of carbon atoms conjugated with double bonds, often ending in a ring. Based on their chemical structure, they are divided into two classes: i) carotenes, hydrocarbons without oxygen, which are precursors of retinol, including β-carotene, α-carotene, lycopene and ii) xanthophylls, consisting of chains containing oxygen atoms, including lutein, zeaxanthin and astaxanthin (Milani et al., 2017). They can also be divided into carotenoids possessing provitamin A activity (β-carotene, β-cryptoxanthin, and α-carotene), which are retinoid precursors, and without provitamin A activity (lycopene, lutein, and zeaxanthin) (Eggersdorfer and Wyss, 2018). Beyond their antioxidant capacity, the inhibitory action of carotenoids on cancer growth is related to their ability to interfere with different cell signaling pathways that regulate proliferation, growth, differentiation and cell death (Gao et al., 2019; Jeong et al., 2019). From a therapeutic perspective, the carotenoid derivative, All-Trans Retinoic Acid (ATRA), is a well-known drug acting as a therapeutic agent (alone or in association with Arsenic Trioxide) for the cure of acute promyelocytic leukemia (APL), characterized by a typical translocation t(15;17) and the expression of the oncogenic protein PML/RARα, able to interfere with differentiation pathway of myelocite (Schenk et al., 2014). However, it is known that ATRA pharmacological effects do not rely exclusively on PML/RARα stability to restore myeloid differentiation pathway in APL blasts. The “pleiotropic” effects of ATRA are mediated by nuclear retinoid receptors (RAR) as well as non-genomic, signal transduction pathways, such as MAPK and PKA. Therefore, the future of ATRA may probably be in clinical oncology, as a differentiating agent or as a cytotoxic drug (alone or in association with other chemotherapeutics) in other subtypes of acute myeloid leukemia, and, perhaps, in CLL (Ghnewa et al., 2017) and solid tumors (Ni et al., 2019). Accordingly, recent papers showed pharmacological effects of ATRA or synthetic retinoids derivatives on B-CLL cells in association with fludarabine, a conventional chemotherapeutic drug largely used to treat CLL (Bruno et al., 2012; Ghalamfarsa et al., 2015).

In recent years, several studies focused on the capacity of carotenoids, such as astaxanthin and fucoxanthin, or carotenoid extracts to modulate autophagic processes in cancer cells (Bae et al., 2020; Hou et al., 2013; Russo et al., 2017c; Shen et al., 2014). We previously reported that a carotenoid enriched extract (CE), obtained by supercritical CO_2 (SC-CO_2) from *Cucurbita moschata* sp., exerted an anti-proliferative effect on different malignant cell line through the activation of a “non-protective” form of autophagy (Russo et al., 2017c). To our knowledge, no studies investigated the effects of a mixture of carotenoids in CLL-derived cell lines or in ex vivo models of B-cells isolated from patients. To verify the capacity of CE to interfere with cell growth in CLL-derived cell lines, we employed in the present study HG3 cells established from a CLL clone by EBV infection (Rosen et al., 2012).

2. Material and methods

2.1. Preparation and vehiculation of carotenoid extract

The carotenoid extract was obtained from pumpkin (*Cucurbita moschata* Duch.), belonging to the cultivar “Long of Naples”, a typical Southern Italy food product, which is a food matrix particularly rich in carotenoids (Durante et al., 2014; Salehi et al., 2019; Toti et al., 2018). SC-CO_2 technology was employed to obtain food grade CE as previously described (Durante et al., 2014). Briefly, flesh (mesocarp and endocarp) and seeds [blended in a ratio of 1:1 by dry weight (dw)] of ripe pumpkin peponides were extracted by SC-CO_2 to obtain an oil, which amount of isoprenoids (carotenoids and tocochromanols) and fatty acids was characterized by HPLC and GC–MS, respectively. To deliver CE to cells, CE was resuspended in a volume of organic solvents DMSO/THF (dimethyl sulfoxide/tetrahydrofuran, 9:1, Sigma-Aldrich, Milan, Italy), added to fetal bovine serum (FBS; 10% in RPMI medium, Gibco. TermoFischer-Scientific, Milan, Italy), to reach the indicated final concentrations in the medium. CE was expressed as μg/ml (w/V). In the control cells (CTRL-CE) the same volume of mixture of DMSO/THF was added to FBS.

2.2. Cell culture and proliferation/viability assay

HG3 cells, a lymphoblastoid cell line with B1 cell characteristics, established from a chronic lymphocytic leukemia clone by EBV infection (Rosen et al., 2012), was employed in the present study. Cells were cultured in RPMI medium supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin (Euroclone, Milan, Italy) at 37 °C in a humidified atmosphere containing 5% CO_2 (Rosen et al., 2012). HG3 cells were stimulated for 96 h with increasing concentrations of CE (50, 100, 200 μg/ml, w/V), with its control (CTRL-CE) or with 0.01% DMSO (Sigma-Aldrich), ATRA (2 μM) (Sigma-Aldrich) or β-carotene (1 and 0.01 μg/ml, w/V) (Sigma-Aldrich). For the cell proliferation curve, HG3 cells were treated for 24, 48, 72 and 96 h with CE (100 μg/ml, w/V) or CTRL-CE. At the end of the incubation, the number of living cells was measured by CyQuant (Invitrogen, TermoFischer-Scientific), a fluorescent dye that selectively binds to nucleic acids. Briefly, at the end of each experimental treatment, HG3 cells were incubated for 1 h at 37 °C with CyQuant mixture, containing the nuclear dye (CyQuant nuclear stain) and background suppressor (able to selectively enter in dead cells). At the end of incubation, fluorescence was measured at the excitation wavelength of 485 nm and 530 nm emission, using a microplate reader (Synergy HT BioTek, Milan, Italy) as previously described (Russo et al., 2017b). Results, expressed as cell number in Fig. 1, were calculated with a calibration curve obtained by interpolation of nuclear fluorescence emitted by different, known number of HG3 cells (in the range 5×10^3–7×10^4).

2.3. Autophagy assay and measurement of autophagic flux

Autophagy was measured by using Cyto-ID Autophagy Detection Kit (Enzo Life Science, Milan, Italy) as reported by Klionsky et al. (2016) and Russo et al. (2014). HG3 cells were treated for 96 h with CE (100 μg/ml, w/V), or ATRA (2 μM) as a positive control. The autophagy detection marker (Cyto-ID) and the nuclear dye (Hoechst 33342) were added after treatments. Subsequently, cells were washed in assay buffer and photographed (400× magnification) using a fluorescence inverted microscope (Axiovert 200 Zeiss, Milan, Italy). The quantification of autophagosomes was performed by normalizing FITC (Cyto-ID) and blue (Hoechst) fluorescence using a microplate fluorescence reader (Synergy HT BioTek) and results were expressed as FICT/DAPI fluorescence ratio. The autophagic flux was monitored by the accumulation of

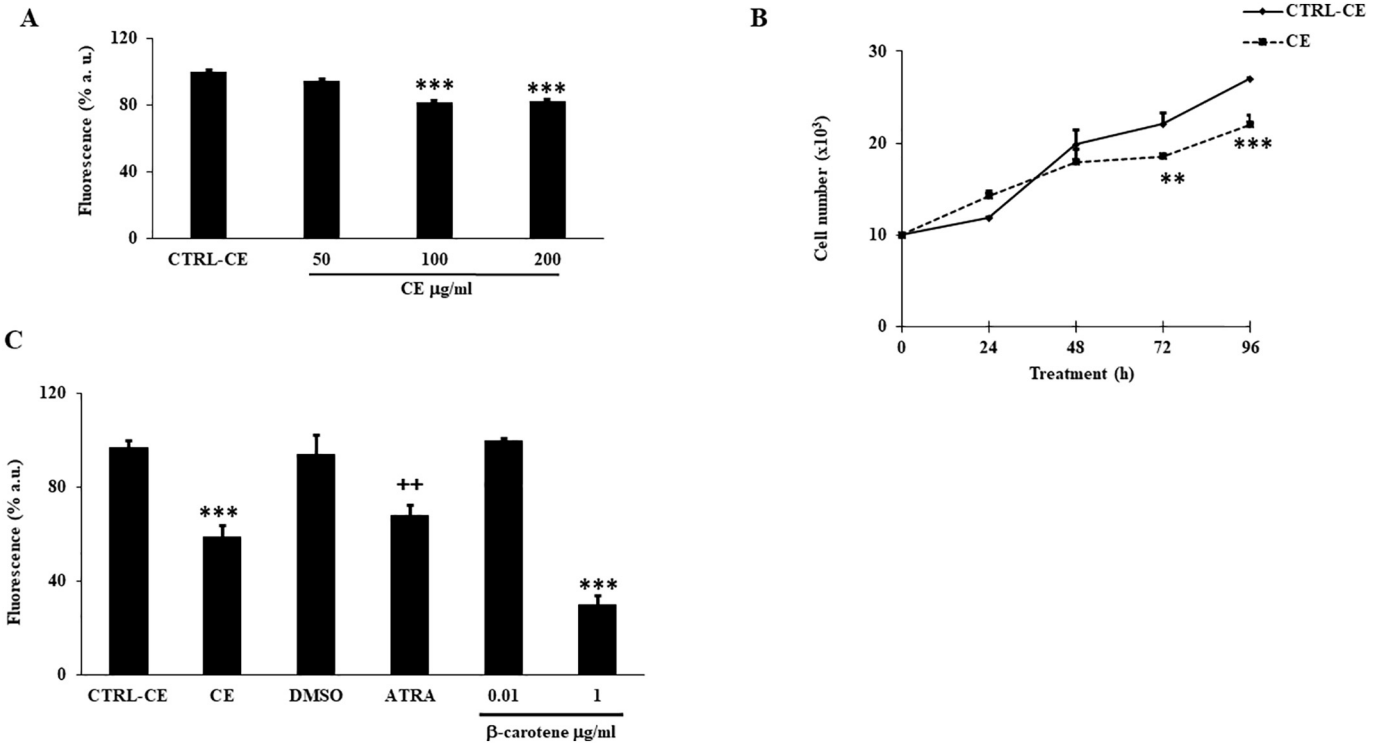

Fig. 1. CE affects HG3 cell proliferation. (A) HG3 cells were treated for 96 h with increasing concentrations of CE (50–200 µg/ml, w/V) and with its respective controls (CTRL-CE). Viable cells were quantified using the Cy-Quant protocol, as reported in Material and methods. Bars represent the mean of two experiments (±SD). Symbols indicate significance: ***$p < 0.001$ vs CTRL-CE. (B) HG3 cells were treated for 24, 48, 72 and 96 h with CE (100 µg/ml, w/V) or its respective control. At the end of incubation, viable cell number was quantified by the Cy-Quant method. Line graphs represent the mean of two experiments (±SD). Symbols indicate significance: ***$p < 0.001$, **$p < 0.01$ vs CTRL-CE. (C) HG3 cells were treated for 96 h with CE (100 µg/ml, w/V), ATRA (2 µM), β-carotene (0.01 and 1 µg/ml, w/V) and their relative controls (CTRL-CE and DMSO). At the end of incubation, cell viability was measured by Cy-Quant method. The results are expressed as % fluorescence. Bars represent the mean of three experiments (±SD). Symbols indicate significance: $^{++}p < 0.01$ vs DMSO, ***$p < 0.001$ vs CTRL-CE.

autophagosomes induced by chloroquine (Sigma-Aldrich), an FDA approved drug and well known inhibitor of autophagic flux able to decrease the formation of autophagosome/lysosomes fusion (Mauthe et al., 2018). HG3 cells were incubated for 96 h with CE (100 µg/ml, w/V) in the presence/absence of 20 µM chloroquine added in the culture medium during the last 2 h of incubation. At the end of treatments, cells were lysed by using lysis buffer containing protease and phosphatase inhibitors (Russo et al., 2013b).

2.4. Immunoblotting analysis

The cell lysates (20–30 µg of total proteins) derived from the treatments described above were loaded on a 4%–12% precast gel (Novex Bis-Tris precast gel 4%–12%; Life Technologies) using MES (2-(N-morpholino)ethanesulfonic acid) or MOPS [(3-(N-morpholino) propanesulfonic acid)] (50 mM MOPS; 50 mM Tris, pH 7; 1% SDS; 1 mM EDTA) buffer. The immunoblots were performed following standard procedures, using as primary antibodies: anti $p27^{kip1}$, anti-LC3, anti-p62/SQSTM1, anti-$pAMPK^{Thr172}$, anti AMPK (Cell Signaling Technology, Milan, Italy), and anti-α-tubulin antibodies (Sigma-Aldrich). Gels were transferred on PVDF membranes (Bio-Rad Laboratories, Milan, Italy) using a Trans Blot Turbo System (Bio-Rad). Following incubation with the primary antibodies, membranes were incubated with horseradish peroxidase-linked secondary antibody raised against mouse or rabbit and, subsequently, the ECL Plus Western blotting detection system kit (GE Healthcare, Milan, Italy) was used to reveal the bands. Signal intensities were quantified measuring optical density on a Gel Doc 2000 Apparatus (Bio-Rad Laboratories) and Multi-Analyst software (Bio-Rad Laboratories). The densitometric analysis was expressed as the ratio with respect to α-tubulin band intensities.

2.5. Statistical analysis

Data are presented as mean values ± standard deviation (SD), and the significance between the treated group (indicated as CE) and the control groups (CTRL-CE) was measured using the Student's test of at least four determinations.

3. Results

3.1. Antiproliferative effect of CE on HG3 cell line

To verify the biological effects of CE in CLL, we employed the lymphoblastoid cell line, HG3, derived from B-cells isolated from a CLL patient and immortalized by EBV (Rosen et al., 2012). This cell line has been previously employed in our laboratory to demonstrate the ability of the flavonoid quercetin to enhance the pro-apoptotic effects of chemotherapeutics in CLL resistant to treatment (Russo et al., 2017b). The pumpkin carotenoid extract, obtained by SC-CO_2, as reported in the Material and methods, was delivered to HG3 cells following a protocol that favored its bioavailability and the transport through the cell culture medium. Shortly, we developed a method to solubilize CE in a combination of solvents (DMSO/THF, 9:1) following the pre-mixing with FBS before addition to RPMI to obtain the final concentration of FBS (10%) and the desired amount of CE (50–200 µg/ml). Fig. 1A shows the results obtained on cell viability when CE was added to HG3 cells in a dose-dependent treatment (50–200 µg/ml, w/V). Since we did not detect any significant difference between 100 and 200 µg/ml, we employed the lower active dose in the following experiments.

Based on the results shown in Fig. 1A, we measured the effect of CE (100 µg/ml, w/V) on cell proliferation at different time points using

CyQuant dye, which associates the FITC nuclear fluorescence to the number of viable cells, as described in Material and methods. As clearly reported in Fig. 1B, a significant delay in cell growth was evidenced from 72 to 96 h of treatment compared to controls (**$p < 0.02$, ***$p < 0.001$, respectively). The absence of cell death/cytotoxicity in HG3 after 72 and 96 h was further confirmed using Trypan Blue exclusion dye (Fig. S1). The antiproliferative effect of CE on HG3 cells was demonstrated in a different experiment where we observed a reduction in cell number of about 40% after 96 h of treatment with CE (100 μg/ml, w/V) (Fig. 1C). As a positive control, ATRA was tested in the assay since, in a previous study, we reported its antiproliferative effect on HG3 cells (Russo et al., 2016). To verify if the delay in cell proliferation caused by CE was simply attributable to the presence of β-carotene in the CE, one of the most abundant carotenoids in the extract (Durante et al., 2014) (Table S1) and well-known for its effects on cell growth regulation (Raju et al., 2005), we employed it in mono-treatment and in pure form. The concentration tested in our experiment was comparable to the one present in CE and determined by HPLC analysis (Durante et al., 2014) (Table S1). At this concentration (0.01 μg/ml), β-carotene did not affect cell proliferation (Fig. 1C). When β-carotene was applied at a concentration 100-times higher (1 μg/ml; Fig. 1C), it resulted even more effective than CE in reducing cell viability in HG3, although, at this concentration, the presence of cytotoxicity was detected (data not shown). These results confirm previous data on leukemia cells (Upadhyaya et al., 2007) and suggest the presence in CE of a pool of bioactive carotenoids. The possibility that they can act synergistically to delay HG3 cell growth represents an attractive hypothesis that, at the current state of knowledge, requires a rigorous demonstration to be pursued in future works.

3.2. CE modulates autophagic flux in HG3 cell line

The delay in cell proliferation after treatment with CE could be related to the activation of the autophagic process. The rationale of this hypothesis resided in three circumstantial evidence: i. CE did not induce a clear cell cycle arrest, but a delay in cell growth; ii. CE treatment was not associated with any sign of cytotoxicity, e.g., apoptosis or necrosis (data not shown); iii. previous studies demonstrated the capacity of CE to modulate autophagy (Russo et al., 2017c).

To verify the activation of autophagy by CE, the Cyto-ID autophagy detection kit was employed (Klionsky et al., 2016). An increased number of autophagosomes (mature vesicles) after CE treatment for 96 h was detected and quantified as reported in Fig. 2A. The number of autophagosomes was significantly higher (30%; $p < 0.001$) respect to CTRL-CE, after CE treatment. ATRA was also used as an autophagy inducer in HG3 (Russo et al., 2016). In Fig. 2B, the autophagosomes (FITC stained, versus nuclear blue stain, obtained using Hoechst dye) were visible in the micrographs obtained following the Cyto-ID protocol. These data confirm that autophagy can play a role in delaying cancer cell proliferation induced by CE. As established by current methods largely accepted by the scientific community, the activation of

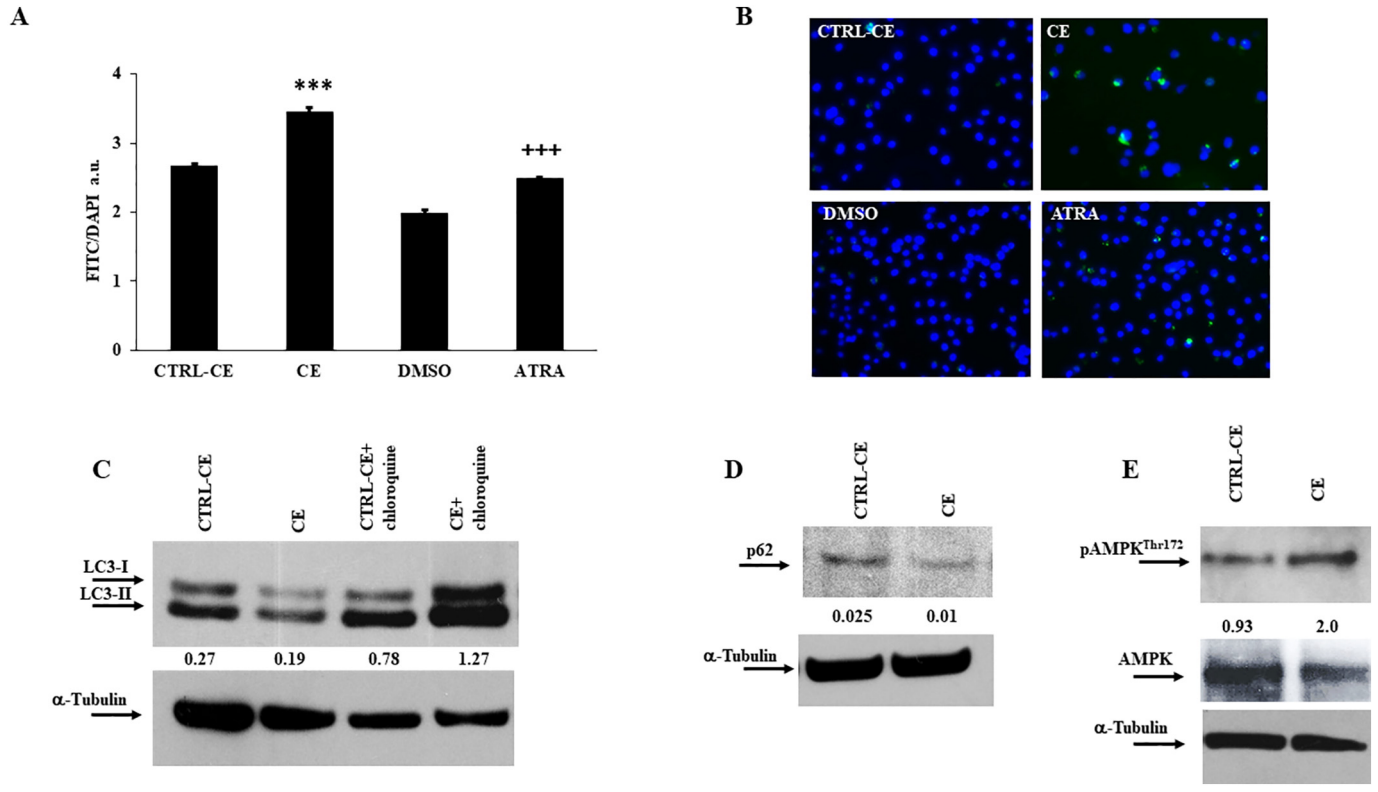

Fig. 2. CE enhances autophagic flux in HG3 cells. (A) Cells were treated for 96 h with CE (100 μg/ml, w/V), or ATRA (2 μM) and their respective controls (CTRL-CE and DMSO). At the end of incubation, autophagosome quantification was measured by Cyto-ID detection kit and expressed as FITC/DAPI fluorescence ratio, as described in Material and methods. Bars represent the mean of two experiments (±SD). Symbols indicate significance: $^{++}p < 0.01$ vs DMSO, ***$p < 0.001$ vs CTRL-CE. (B) Representative images of autophagic vacuoles (green) in HG3 cells treated as indicated in (A). The images were taken with a microscope Axiovert 200 Zeiss (400× magnification). (C) HG3 cells were treated for 96 h with CE (100 μg/ml, w/V) in the presence/absence of 20 μM chloroquine, which was added 2 h before the end of treatment. Bands indicating the expression of LC3-I/II autophagic marker are representative of one out of three separate experiments performed. Densitometric analysis (numbers between panels) was expressed by calculating the ratio between the LC3-II/α-tubulin band intensities. (D) HG3 cells were treated for 96 h with CE (100 μg/ml, w/V). Bands indicating the expression of the p62 autophagic marker are representative of one out of three separate experiments performed. Densitometric analysis (numbers between panels) was expressed by calculating the ratio between the p62/α-tubulin band intensities. (E) HG3 cells were treated for 24 h with CE (100 μg/ml, w/V). Bands indicating the expression of phosphorylated ($pAMPK^{Thr172}$) and active form of AMPK are representative of one out of three separate experiments performed. Densitometric analysis (numbers between panels) was expressed by calculating the ratio between the $pAMPK^{Thr172}$/AMPK/α-tubulin band intensities. The significant differences among bands in the immunoblots in panels C, D, E was set at 5%.

an autophagic flux must be confirmed by several, independent markers. To this aim, we measured the level of expression of LC3-II, the lipidated form of LC3/ATG8 protein (microtubule-associated protein 1 light chain 3), the protein deputed to decor autophagosomes in the final stage of the process. In addition, being autophagy a dynamic process, it is necessary to assess how it is modulated by an external factor that blocks or enhances the autophagic flux. Generally, this analysis requires the presence of a pharmacological inhibitor of autophagy, such as chloroquine, able to block autophagic degradation modifying lysosomal pH (Klionsky et al., 2016; Puri and Chandra, 2014). As reported in the immunoblot shown in Fig. 2C, LC3-II levels were clearly increased in co-treatment CE + chloroquine compared to CE and chloroquine in mono-treatments. Therefore, we can bona fide conclude that CE treatment increased the rate of autophagic flux in HG3 cells after 96 h of treatment.

The result shown in Fig. 2C was further confirmed by the analysis of the expression levels of SQSTM1/p62, known as ubiquitin-binding protein p62, which is included in mature autophagosomes and degraded in lysosomes at the final stages of the autophagy process. The expression of SQSTM1/p62 protein decreases with the activation of autophagic flux, while increased levels are associated with the inhibition/block of the process (Klionsky et al., 2016). As reported in Fig. 2D, CE treatment significantly reduced the expression of SQSTM1/p62, confirming the activation of the autophagic flux in HG3 cells. Finally, we measured the capacity of CE to activate AMP-activated protein kinase (AMPK). This kinase is involved in the early stages of autophagosome formation and is activated by a decrease in ATP and nutrients, resulting a key factor in the biochemical pathway of autophagy and cellular energy metabolism (Russo et al., 2013a). In addition, previous works demonstrated that carotenoid-induced autophagy implies the activation of AMPK (Chang et al., 2017; Jang et al., 2018). Therefore, we verify if this mechanism was also common to the CE mode of action. As shown in Fig. 2E, we detected a clear and significant activation of AMPK in HG3 following CE treatment as demonstrated by the increased expression of the active form of AMPK phosphorylated in Thr-172. This effect paralleled with the reduced intracellular level of ATP measured at the same time-point (Fig. S2).

3.3. Cell growth delay by CE was $p27^{Kip1}$ dependent

To further confirm the capacity of CE to delay cell proliferation, the expression levels of $p27^{Kip1}$, a proliferation marker, regulating cell cycle progression was assessed. More recently, new roles $p27^{Kip1}$ have been described resulting in an intrinsically unstructured protein, possessing CDK-dependent and -independent functions, but also involved in multiple cellular processes, such as cytoskeleton dynamics and cell motility control, apoptosis and autophagy activation (Bencivenga et al., 2017). An increased level of $p27^{Kip1}$ (>40% compared to CTRL-CE) induced by CE treatment was detected after 96 h of stimulation, as shown in Fig. 3, suggesting that CE interferes with the cell cycle machinery regulating cell growth in cancer cells. ATRA, a well-known post-transcriptional up-regulator of $p27^{Kip1}$ (Dimberg et al., 2002), was employed as a positive control in this experiment and, as expected, its treatment was also associated with a >50% increased expression of $p27^{Kip1}$ (Fig. 3).

4. Discussion

The beneficial effects of carotenoids have been largely demonstrated by multiple clinical and in vitro studies, which focused on their ability to reduce the incidence of chronic degenerative diseases, such as cardiovascular disease (CVD), obesity, diabetes mellitus, osteoporosis and cancer (Bae et al., 2020; Costea et al., 2018; Milani et al., 2017; Rowles and Erdman, 2020). However, the therapeutic use of carotenoids (β-carotene) or carotenoid derivatives (retinoids, vitamin A) has been considered a controversial issue in cancer research for many years. Starting from the Peto's hypothesis that β-carotene might reduce the incidence of cancer, especially lung cancer, and the observation that people having higher serum β-carotene concentrations presented a lower rate of lung cancer (Peto et al., 1981), in the middle ninety, the CARET study (β-Carotene and Retinol Efficacy Trial) was designed. It aimed to test the efficacy of a combination of 30 mg β-carotene and 25,000 IU retinyl palmitate (vitamin A) taken daily against placebo in 18,314 men and women at high risk of developing lung cancer. This intervention study was stopped 21 months early “because of no benefit and substantial evidence of possible harm” (Omenn et al., 1996). Almost in parallel, the ATBC (Alpha-Tocopherol, Beta-Carotene) Lung Cancer Prevention Study was planned, based on the hypothesis that α-tocopherol (50 mg/day) and β-carotene (20 mg/day) supplements might reduce the incidence of lung cancer and possibly other tumors. Unexpectedly, also in this case a higher incidence of lung cancer among the men who received β-carotene than among those who did not was observed (Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group, 1994). The unpredictable negative results of these two trials demonstrated the

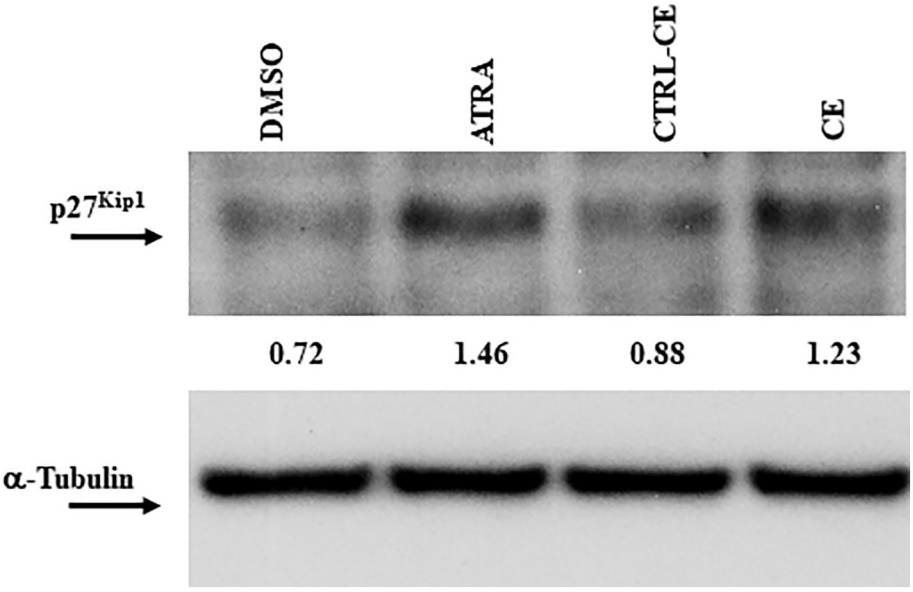

Fig. 3. Immunoblot of $p27^{Kip1}$ following CE induction. HG3 cells were treated for 96 h with CE (100 μg/ml, w/V), ATRA (2 μM) and their respective controls (CTRL-CE and DMSO). Bands are representative of one out of three separate experiments performed. Densitometric analysis (numbers between panels) is expressed as the ratio between the band intensities of $p27^{Kip1}$ over α-tubulin. The significant differences among bands in the immunoblot were set at 5%.

difficulty to translate pre-clinical data and epidemiological indications deriving from observational studies into clinical practice. However, β-carotene supplementation was also associated with beneficial effects. In fact, in the Women's Health Study, involving about 20,000 women supplemented with 50 mg of β-carotene/daily for 2 years, no significant differences in the incidence of cancer, CVD, or total mortality was reported in the cohort including smokers (Lee et al., 1999). Apart from the negative effects on lung cancer, the ATBC study registered a lower rate of prostatic hyperplasia whether or not the participants were smokers or drank alcohol (Heinonen et al., 1998). In the Linxian General Population Trial a combination of 50 μg selenium, 30 mg vitamin E, and 15 mg β-carotene, led to decreased mortality from all causes, cancer overall, and gastric cancer (Qiao et al., 2009). After more than 25 years of intense studies, solid hypotheses have been formulated to explain the reasons why the CARET and ATBC trials failed. Since the effect of β-carotene may decrease when the total quantity of oxidants increases, β-carotene may be less efficient in neutralizing cigarette smoke (Hu and Cassano, 2000). A possible explanation relies on the carcinogenicity of carotenoid oxidative products formed when β-carotene is exposed to smoke β-carotene in smokers (Yeum et al., 1995). The broad benefits deriving from β-carotene supplementation have been largely reviewed in excellent works (Bendich, 2004; Eggersdorfer and Wyss, 2018).

From the studies cited above clearly emerges how the “naive” hypothesis that cancer risk can be reduced simply modifying single components of a diet, such as carotenoids, administered at not “physiological” doses, must be taken with high caution. A more recent meta-analysis aimed to evaluate the association between carotenoid intake and non-Hodgkin lymphoma (NHL) risk in 10 epidemiological studies (3 cohort and 7 case-control), found that the intake of α-carotene and β-carotene was associated with a significant decreased risk of diffuse large B-cell lymphoma, but not follicular lymphoma or small lymphocytic lymphoma/CLL. The difficulty to characterize the *in vivo* biological effects of single carotenoids reflects the complexity of many pathologies, such as solid tumors and leukemia, where the molecular interactions between carotenoids and their cellular targets are mediated by environmental factors (pollutants, diet, life style) in addition to the own genetic background (Chen et al., 2017).

The “simplistic” assumption that a diet supplemented with high doses of antioxidants could prevent cancer and explain at the molecular level the effects of phytochemicals in cancer biology is clearly risky. It is mandatory to select the most appropriate preclinical models, i.e., cell lines and mice with specific genetic backgrounds, that make “realistic” the chemopreventive/chemotherapeutic activity of single phytochemicals when they are tested in the following interventional studies (Russo et al., 2017a; Russo et al., 2010). A key aspect regards the low/high doses applied. As an example, results emerging from the use of a selective retinoid X receptor (RXR) agonist, bexacarotene, are encouraging. In fact, bexacarotene was effective when used at low doses and in association with raloxifene as a chemopreventive agent, against a specific form of cancer (colon cancer) (Janakiram et al., 2013). Other studies, based on lung cancer, showed that β-carotene and/or lutein at high doses were carcinogenic in smoke-exposed animals due to the production of transient oxidative metabolites, destruction of retinoids signaling and enhanced cell proliferation (Russell, 2004). On the opposite, protection from squamous metaplasia or colon adenocarcinoma was observed in mice treated with low doses of β-carotene (Raju et al., 2005; Russell, 2004).

Starting from these considerations, the present study discloses several novelties. CE consists of a mixture of different carotenoids mimicking the condition potentially present in a human diet enriched in carotenoid-containing foods. The lack of activity of the most representative carotenoid, β-carotene, used at a concentration comparable to the one present in the CE (Fig. 1C) is in agreement with the hypothesis that β-carotene alone is not responsible for the antiproliferative effect of CE observed in HG3 cells. Probably, CE effects are due to a “synergistic interaction” of bioactive carotenoids as also suggested by others (Linnewiel-Hermoni et al., 2015; Sansone et al., 2019). It is worthwhile to note that the composition of CE has been finely defined (Durante et al., 2016; Durante et al., 2014); this represents an advantage that will be further improved in the near future by measuring the biological activities of different combinations of carotenoids present in CE in order to characterize their capacity to synergize and reduce proliferation in cancer cells.

The effects of naturally occurring carotenoids in CLL have been only barely investigated. Data exist on the pro-apoptotic capacity of single carotenoids, such as lycopene, ATRA (a carotenoid derivative), or fenretinide (a synthetic retinoid) in experimental models mimicking CLL (Bruno et al., 2012; Ghalamfarsa et al., 2015; Salman et al., 2007; Zhang et al., 2002) or related to chemotherapy/radiation resistance (Russo et al., 2016). For the first time, we showed the capacity of a carotenoid extract to induce autophagy in a CLL-derived cell line. In the “multifaceted” cross-talk between autophagy and cancer (Gewirtz, 2014), only recently the role of autophagy (protective or cytotoxic) has been explored in leukemia and CLL (Russo and Russo, 2018).

A growing body of evidence revealed that autophagy pathways could be an alternative translational strategy, different from apoptosis, to control CLL physiopathology. A recent paper correlated autophagy markers (LC3-II, ATG5 expression) to an alteration of B Cell Receptor (BCR) signaling in CLL patients (Smith et al., 2020). In a different study, authors measured the autophagic flux in the peripheral blood mononuclear cells from CLL patients (levels of the proteins p62 and lipidated LC3) and explored its correlation with the classical clinical/analytical parameters (Romero-Macias et al., 2019), showing that higher lymphocytosis, lower patients survival and worst prognosis are probably associated to a block of autophagic flux. These evidences found a strong validation in our study. In particular, we observed that CE induced a form of autophagy that we hypothesized to be “not protective” against cell death for the following reasons: i. a similar “not protective” autophagy, was observed in a previous report (Russo et al., 2017c; ii. in HG3, we observed an increase in autophagy flux, a feature associated with a positive effect in CLL patients with lower lymphocytosis and percentage of cancer cells (Romero-Macias et al., 2019). This observation represents a novelty in CLL physiopathology and future studies are necessary to identify the key molecule(s) responsible for the observed “not protective” autophagy and establish if their effects can ameliorate the efficacy of chemotherapy in combined treatments against CLL. In fact, the ability of CE to alter the “basal” autophagy process by increasing the autophagy flux (Fig. 2) appears here for the first time in an in vitro model of CLL and resulted in a significant delay in malignant cell growth (Fig. 1). This effect is probably mediated by a “homeostatic” mechanism involving cellular metabolic alteration (e.g., ATP depletion), which mediates the increase of $AMPK^{Thr172}$, the active form of AMPK, a key step in the biochemical pathway of autophagy (Herzig and Shaw, 2018) (mechanism summarized in Fig. 4). From a mechanistic point of view, an important novelty regards the observation that the autophagy flux activated by CE correlated with the up-regulation of $p27^{Kip1}$, which follows AMPK activation (Fig. 4). In normal tissues, the $p27^{Kip1}$-AMPK axis regulates the balance between autophagy, apoptosis, and senescence (White et al., 2018). A possible explanation suggests that, when AMPK is activated at the initial stages of the autophagic process (following an ADP/ATP imbalance), it phosphorylates $p27^{Kip1}$ on residue Thr-198, stabilizing it and contributing to inhibit cyclin-CDK holoenzymes, resulting in cell cycle arrest (Liang et al., 2007) and, perhaps, cell growth delay, as in the present work. In support of this hypothesis, a broadening of $p27^{Kip1}$ functions, depending on the intracellular context, is emerging with new regulatory roles in cell motility, survival and differentiation beyond the capacity to regulate the cell cycle (Bencivenga et al., 2017; Dembitz et al., 2017). The present data reinforce the conclusion that carotenoids hit the $p27^{Kip1}$-AMPK axis leading to differentiation and cell growth delay in solid tumors and leukemia (Liang et al., 2007; Russo et al., 2017c).

The results here obtained raise important questions linked to the potential chemopreventive/chemotherapeutic efficacy of CE in CLL. In fact, to extend and verify its biological activities in *in vivo* models and, possibly, in humans, it is necessary to prepare a stable CE powder as potential active ingredients to be used in prototypes of nutraceutical preparations. Recently, our group described the encapsulation in α-cyclodextrins (α-CDs) of

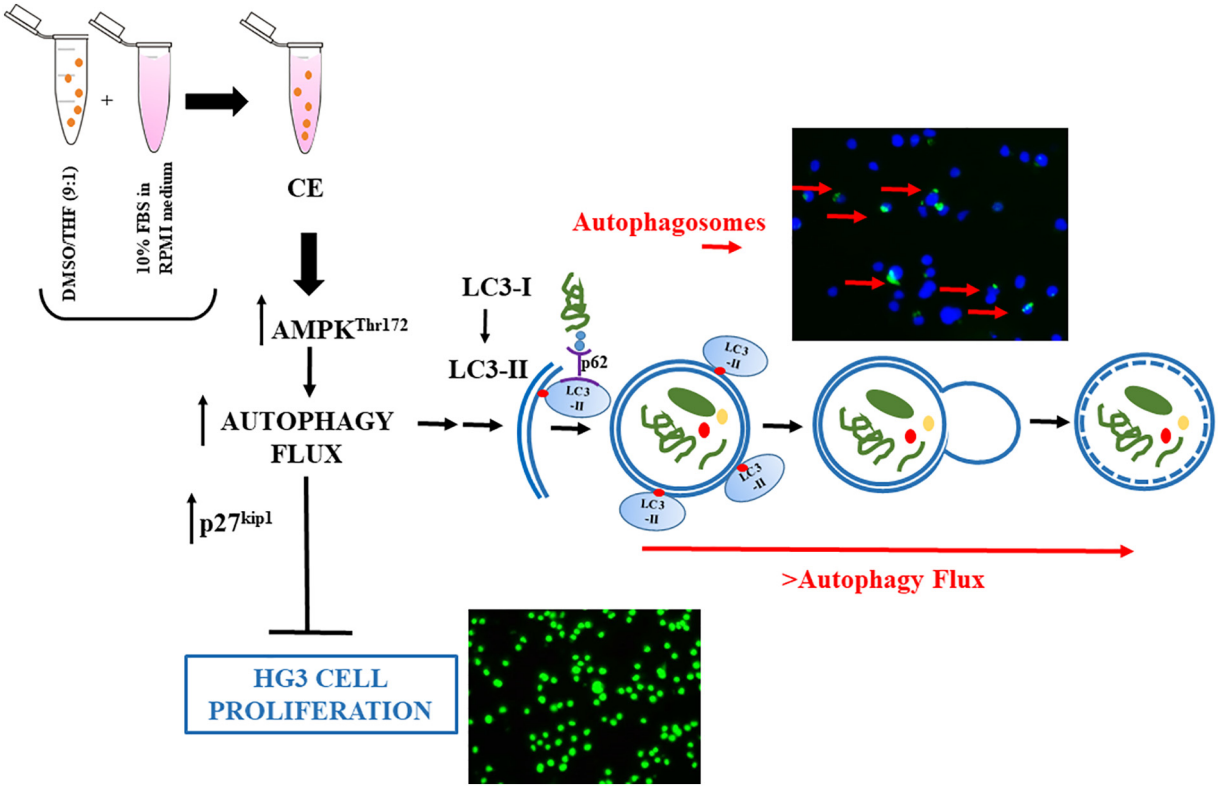

Fig. 4. Cartoon showing how CE increases autophagy flux through a mechanism involving AMPK activation and resulting in a $p27^{Kip1}$-dependent delay in cell proliferation (see text for details).

pumpkin oil for obtaining a freeze-dried powder useful as ready-to-mix ingredients for the formulation of an innovative supplemented pasta (Durante et al., 2019). Furthermore, preliminary data obtained from our laboratory led to the design of an innovative formulation, using a technological approach based on "spray drying" microencapsulation (data in progress). A peculiar aspect in handling carotenoids (as single molecules or complex extracts) regards their highly conjugated structure responsible for the elevated instability, which strongly limits their applications on a large scale. The exposure to physical-chemical factors, such as light, temperature and oxygen, during the processing and handling phase of carotenoids can lead to degradation/oxidation reactions, resulting in isomerization products, also related to a lower biological activity (Eun et al., 2019). The encapsulation in wall/coating polymers represents an important formulation strategy to increase the stability and shelf life of these compounds, which also preserve their biological activity and effectiveness (Eun et al., 2019; Sansone et al., 2019). If supported by adequate preclinical studies, this technology can be applied in the next future in prototypes of CE supplements in clinical studies aimed to extend the CLL asymptomatic phase in old/frail patients (primary chemoprevention) and avoid collateral cytotoxicity always associated with chemotherapy.

In conclusion, the results obtained in the present study confirm that CE is a "phytocomplex" enriched in bioactive compounds such as carotenoids, acting probably "synergistically" to alter the homeostatic level of autophagic flux in the leukemic cell line. Considering the ability of CE to induce a cell growth delay by interfering in well-known "tumor-suppressive" pathways, it can be suggested as a potential new chemopreventive/chemotherapeutic agent.

CRediT authorship contribution statement

Stefania Moccia: Conceptualization, Investigation, Visualization, Writing - original draft. **Maria Russo:** Conceptualization, Investigation, Visualization, Writing - original draft. **Miriana Durante:** Investigation, Writing - review & editing. **Marcello S. Lenucci:** Investigation. **Giovanni Mita:** Investigation, Writing - review & editing. **Gian Luigi Russo:** Supervision, Visualization, Conceptualization, Writing - review & editing, Funding acquisition.

Declaration of competing interest

The authors declare the absence of any commercial or financial relationships. Therefore, no potential conflict of interest is present.

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We are currently collaborating with Profs. R.P. Aquino and F. Sansone from the Department of Pharmacy, University of Salerno, Fisciano (SA), Italy to design innovative CE formulations using "spray drying" microencapsulation. The present work has been partially supported by CNR project NUTR-AGE (FOE-2019, DSB.AD004.271).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crbiot.2020.05.001.

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