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Abstract

The effects of ground pistachio kernel extracts have been evaluated on cellular viability, intracellular reactive oxygen species (ROS) production and cell death in MCF-7 breast cancer cells. Results showed a significant decrease in cell viability in a dose and time dependent manner. 48h-treatments with different concentrations of the extracts induced intracellular ROS generation and showed that cell death was in an apoptosis-independent manner. It was also found that 48h-treatments led to a dose dependent reduction in both extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) with respect to untreated cells. Therefore, a considerable alteration of cell bioenergetics would occur as consequence of such treatments. Combination of oxidative stress and bioenergetic alterations could be responsible, at least in part, of the cell death observed in the present model. In conclusion, pistachio kernel extract showed promising beneficial effects that could be exploited as a "natural adjuvant" in combination with chemotherapy treatment.

Keywords: pistachio kernel; phenolic compounds; intracellular reactive oxygen species (ROS); apoptosis; energetic metabolism; breast cancer.

1. Introduction

Pistachios (*Pistacia vera* L., Anacardiaceae family) are one of the oldest valuable edible tree nuts and are commonly used all over the world. Although pistachios are native to Asia, particularly in Iran and Iraq, they are being commercially produced in United States, Australia, Turkey, Greece, Italy, Spain and China, among others (Saitta et al., 2014). Pistachio was first planted in Spain in 1996 and in 2016 the cultivation area was over 15,000 ha, with 12,000 ha (80%) corresponding to Castilla-La Mancha (MAGRAMA data, Spanish Ministry of Agriculture).

Pistachios are nuts with a dry, hard, outer egg shaped shell, with a tasty kernel inside that are consumed in food industry (Saitta et al., 2014), as salted and roasted snacks or as an ingredient in bakery and confectionery products, desserts, and ice-creams. Composition of pistachio kernels is complex and exhibit interesting nutritional properties as follows (Bulló et al., 2015; Hernández-Alonso, Bulló & Salas-Salvadó, 2016; Liu at al., 2014; Ojeda-Amador et al., 2018): they are relatively rich in monounsaturated fat comprising 5.6 % saturated fatty acids, 13.7 % polyunsaturated fatty acid and 23.8 % monounsaturated (oleic and linoleic acids represent more than 60% of the total fat); they are a good source of vegetable protein (comprising about 20% of total weight) with approximately 2% L-arginine; their amount of carbohydrate is low to moderate (about 27.5% by weight) but they are rich in fibre containing 10% by weight of insoluble forms and 0.3% of soluble forms; regarding to vitamins and minerals, they are rich in Cu, Ca, Mg, Fe, vitamin A, vitamin C and B vitamins, with high amounts of B_1 and the absence of B_{12}. Among the bioactive compounds, pistachios are rich sources of hydrophilic bioactives such as phenolic compounds (including phenolic acids and flavonoids) (Tomaino et al., 2010; Rodríguez-Bencomo et al., 2015; Martínez et al., 2016) and lipophilic bioactives such as carotenoids (including lutein and zeaxanthin which are responsible for giving colour to pistachio nuts), chlorophylls and phytosterols (including stigmasterol, campesterol and β-sitosterol).

Pistachios are among the top fifty foods with high antioxidant and anti-inflammatory potential by their content of oleic acid, phytosterols, phenolic acids and tocopherols (Bulló et al., 2015). Hydrophilic and lipophilic bioactive content (polyphenols, xanthophylls and tocopherols, in particular) has been demonstrated to be rapidly

bioaccessible into the stomach which maximizes their transformation and absorption in the upper small intestine and promotes health effects (Bulló et al., 2015). For this reason nut consumption is being encouraged into health diets for their nutritional quality and potential contribution to health promotion (Dreher, 2012).

Results from the PREDIMED study (Prevention with Mediterranean Diet) showed that the high intake of nuts like pistachios is associated with a lower incidence of cardiovascular diseases, metabolic syndrome and other inflammatory conditions (Reboredo-Rodríguez et al., 2017; Salas-Salvadó et al., 2008). However, relationship between cancer and nut consumption has been investigated in a lesser extent with some preliminary interesting results from clinical studies. This is the case of EPIC study (European Prospective Investigation into Cancer and Nutrition), a large trial involving ten European countries, nearly 500,000 participants. Among its conclusions highlight a higher consumption of nuts and seeds was associated with a 31% risk reduction of colorectal cancer development for women (http://www.thelancet.com/oncology).

According to Yang and coworkers (2012), the high content of vitamin E and other antioxidants founded in pistachios may reduce the risk of other cancer types.How dietary compounds present in natural products do regulate and induce these beneficial effects against carcinogenesis is a matter which remains to be elucidated (Pan et al., 2017a; Pan et al., 2017b). In this context, the main objective of this study presented here was to assess the potential health effects of pistachio kernel extracts on MCF-7 breast cancer cell line by evaluating the cytotoxic properties by viability assays, intracellular reactive oxygen species (ROS) production and effect on apoptosis. The energetic metabolism has also been studied by measure extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) using the XF24 Extracellular Flux Analyzer. Given the fact that phenolic profile can be very effective in its biological activity, a systematic phenolic characterization of the studied extract was undertaken by HPLC-DAD-MSn.

2. Materials and Methods

2.1. Pistachio and Lyophilized Extracts

Pistachios of the Sirora variety were provided by the regional research centre "Centro de Mejora Agraria El Chaparrillo" (Ciudad Real, Spain) during 2013/14 and 2014/15 harvest seasons. Pistachio extract rich in phenolic compounds has been obtained as followed: 2 g of ground pistachio kernel was extracted by 20 mL of ethanol:H_2O (80:20, v/v) employing ultrasound assisted agitation (10 min, 25°C, 250W; sonicator Q500, Qsonica, Newtown, CT, USA) and then evaporated using a centrifugal vacuum concentrator (miVac Duo, Genevac Ltd., Ipswich, UK) and finally freeze-dried (Cryodos-45, Telstar, Terrassa, Spain). The lyophilized extracts were kept dry until *in vitro* analysis.

2.2. Determination of Total Phenolic Content (TPC)

Total Phenolic Content (TPC) was analyzed following the method described by Vázquez, Janer & Janer (1973) and Gutfinger (1981). Briefly, a double extraction of the 0.4 g of ground pistachio kernel or of its extract was performed in 20 mL MeOH:H_2O:HCOOH (80:20:0.1; 10 + 10 mL), with 2 min vortex, followed by 5 min ultrasound and centrifugation at 2000g for 10 min. The combined extracts were filtered prior to analysis. Suitable aliquots (100-500 μL) of the polar extracts were transferred into a 10-mL volumetric flask. Water (up to 8 mL) and the Folin-Ciocalteu reagent (0.5 mL) were added. After 3 min, the absorbance of the solution was measured at 725 nm against a blank solution with a UV-visible spectrophotometer (Agilent Technologies 8453). A calibration curve was established using gallic acid (GAE) as the external standard.

2.3. Determination of Total Antioxidant Capacity (TAC)

Total Antioxidant Capacity (TAC) (Quiles et al., 2006) has been evaluated for two different methods: 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) radical assay and the oxygen radical absorbance capacity (ORAC).

The radical scavenging effect of the methanolic extract towards the synthetic radical DPPH was performed as reported previously Brand-Williams, Cuvelier & Berset (1995)

and Sánchez-Moreno, Larrauri & Saura-Calixto (1998) and Morita, Naito, Yoshikawa and Niki (2017). Briefly, 100 µL of the polar extract (prepared as described above in point 2.2) was added to a methanolic DPPH solution (2.9 mL, 6×10^{-5} M) and stored in the dark for 30 min. The decrease in absorbance of the resulting solution was then measured at 515 nm using an Agilent 8453 spectrophotometer. A calibration curve was constructed using Trolox as the external standard.

ORAC assay was performed according to Dávalos, Gómez-Cordovés & Bartolome (2004). Briefly, 20 µL of the polar extract (prepared as described above in point 2.2) and 120 µL of fluorescein (70 nM) at 37 ºC for 15 min were preincubated. After that, 60 µL of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, 24 mM) was added and the mixture incubated at 37 ºC (kinetics measured every minute for 80 min). The experiment was carried out in Nunclon black 96-well flat-bottom plates (Sigma-Aldrich, Madrid, Spain) and measurements acquired using a plate reader with emission and excitation filters set at 528 and 485 nm, respectively. The fluorescence curves were normalized with respect to the blank curve (without antioxidant). The calibration curve was prepared using Trolox as the external standard.

2.4. Determination of the Phenolic Compounds by HPLC-DAD-MSn

Individual phenolic compounds were determined by an HPLC-DAD-ESI-MS/MS method adapted from conditions previously described (Castillo-Muñoz et al., 2009). The analysis was performed on an Agilent 1100 series system (Agilent, Waldbronn, Germany) equipped with a photodiode array detector (DAD) and an LC/MSD Trap VL electrospray ionization mass spectrometry (ESI-MS/MS), both coupled with an Agilent ChemStation for data processing.

Aliquots (1.75 mL) of phenolic extracts were evaporated in a rotary evaporator at 35 °C under vacuum, and were eventually dissolved in 200 µL of methanol/water (20:80, v/v) by being sonicated (5 min) and vortexed (2 min) before injecting 20 µL in the system. Before injection, extracts were diluted sixteen times to avoid saturation of the signal. Separation was achieved on a narrow-bore column Zorbax Eclipse XDB-C18 (2.1 x 150 mm; 3.5 µm particle; Agilent) thermostated at 40 ºC and using a ternary gradient and a 0.19 mL/min flow rate. Eluents were (A) acetonitrile/water/formic acid (3:88.5:8.5, v/v/v), (B) acetonitrile/water/formic acid (50:41.5:8.5, v/v/v), and (C)

methanol/water/formic acid (90:1.5:8.5, v/v/v). The linear solvent´s gradient was as follows: zero min, 98 % A 2 % B and 0% C; 8 min, 98 % A 2% B and 0% C; 40 min, 70 % A 17 % B and 13 % C; 54 min, 50 % A 30% B and 20 % C; 54.5 min, 30 % A 40 % B and 30 % C; 59 min, 0 % A 50 % B and 50 % C; 60 min, 0 % A 50 % B and 50 % C; 67 min, 98 % A 2 % B and 0 % C. For identification, an Ion Trap ESI-MS/MS detector was used in negative ion modes, setting the following parameters: dry gas N_2, 8 L/min; drying temperature, 325 ºC; nebulizer, N_2, 50 psi; scan range, 50-1, 200 m/z. Identification was based on spectroscopic data (UV-Vis and MS/MS) obtained from standard or previously reported data. Quantification was made using the DAD chromatograms recorded at 280 nm (flavanols, flavanones and phenolic acids), 360 nm (flavonols) and 520 nm (anthocyanins). Calibration curves for the analyzed compounds were prepared from pure standards (cyanidin-3-*O*-galactoside; cyanidin-3-*O*-glucoside; myricetin-3-galactoside; quercetin; quercetin-3-glucoside; quercetin-3-rutinoside; procyanidin B1; procyanidin B2; catechin; epicatechin; catechin gallate; eriodictyol-7-*O*-glucoside; from Sigma-Aldrich).

2.5. Cell Culture

MCF-7 breast cancer cell line was kindly provided by Prof. Mirco Fanelli of the Department of Biomolecular Sciences (Università degli Studi di Urbino Carlo Bo, Urbino, Italy) and was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum as well as 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were maintained in a CO_2 incubator at 37 °C under a humidified atmosphere (95% air, 5% CO_2).

2.6. Cell Viability by MTT and TALI® Viability Assays

MTT Assay

Cells were seeded into 96-well plates at a density of 5×10^3 cells/well in complete growth medium. Following overnight incubation, MCF-7 cells were treated with different concentrations (0.0, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 mg/mL) of the ground pistachio kernel extract for 24, 48 and 72 h. The extract was directly dissolved in the cell culture medium. After incubation, 30 µL of RPMI medium containing 2

mg/mL of MTT were added to each well, and cells were incubated for another 2 h at 37 °C. MTT solution was then discarded, and 100 µL of DMSO were added to each well to dissolve the formazan crystal. The level of colored formazan derivative was analyzed on a microplate reader at a wavelength of 590 nm (Moongkarndi et al., 1991; Studzinski et al; 1995).

TALI® Viability Assay

Cells were seeded into 6-well plates at a density of 1.5×10^5 cells/well in complete growth medium. Following overnight incubation, MCF-7 cells were treated with different concentrations of the ground pistachio kernel extract (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 24, 48 and 72 h. Cell viability was determined by using the Tali® Viability Kit-Dead Cell Green reagent following the manufacturer's instructions and as previously reported Remple & Stone (2011). The proportions of viable and dead cells were analyzed by the Tali®RFP + Viability assay on the Tali® Image-Based Cytometer (Thermo Fisher Scientific, Milan, Italy).

2.7. Assessment of Intracellular ROS Production by TALI® Image-Based Cytometer

Determination of intracellular ROS levels was performed using the CellROX® Oxidative Stress Kit (Invitrogen®, Life Technologies, Milan, Italy) according to the manufacturer's instructions. Cells were seeded in 6-well plates at a density of 1.5×10^5 cells/well in complete growth medium. Following overnight incubation, MCF-7 cells were treated with different concentrations of the ground pistachio kernel extract (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 48h. After treatment, cells were trypsinized and centrifuged at 1500 rpm for 10 min at room temperature. Then, the supernatant was discarded and the cellular pellet was re-suspending in 0.5 mL of complete medium. Finally, 1 µL of CellROX® Orange Reagent was added and samples were incubated for 30 min at 37 °C. Cells were centrifuged once to remove the medium and re-suspended again in 100 µL PBS. Finally, samples were analyzed with the Tali® Image-Based Cytometer and the results were expressed as the percentage of cells with increased ROS levels compared with the control (Alvarez-Suarez et al., 2016; Giampieri et al., 2017).

2.8. Apoptosis Detection by TALI® Image-Based Cytometer

Apoptosis was measured using the Tali® apoptosis assay kit and propidium iodide (Invitrogen®, Life Technologies, Milan, Italy) as previously reported (Giampieri et al., 2014; Islam et al., 2017). Cells were seeded in 6-well plate at a density of 1.5×10^5 cells/well and treated with different concentrations of the ground pistachio kernel extract (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 48h. At the end of the treatment, cells were centrifuged (1500 rpm for 5 min) and resuspended in100 µL of Annexin V Binding Buffer (ABB). After that, 5 µL of Annexin V Alexa Fluor® 488 were added, mixed again and the solution was incubated in the dark at room temperature for 20 min. Then, cells were centrifuged again at 1000 rpm for 10 min, resuspended in 100 µL of ABB and 1 µL of propidium iodide was added, mixed and incubated in the dark at room temperature for another 5 min. Finally, samples were analyzed using the Tali® Image-Based Cytometer and the percentage of apoptotic, dead cells and live cells was determined on the basis of the corresponding fluorescence histogram compared with an untreated control. The annexin-V positive/propidium iodide negative cells were recognized as apoptotic cells by the cytometer software whereas the annexin V-positive/propidium iodide positive cells were identified as dead cells. Similarly, the annexin V-negative/PI negative cells were identified as viable cells. The results were expressed as fold increase compared with the control.

2.9. Evaluation of Glycolysis and of Mitochondria Functionality

XF24 Extracellular Flux Analyzer from Seahorse Bioscience, Inc. (North Billerica, MA, USA) was utilized to ECAR and OCR, representing glycolysis and oxidative phosphorylation (OXPHOS), respectively.

MCF-7 cells were seeded in 24-well XF cell culture microplates at 3.0×10^4 cells/well for 24 h and then treated with different concentrations of the ground pistachio kernel extract (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 48 h. At the end of the treatment, the medium was replaced with 500 µL/well of XF-24 running media (supplemented with 25mM glucose, 4mM glutamine, 1mM sodium pyruvate, without serum). The plates were pre-incubated at 37 °C for 20 min in the XF Prep Station incubator (Seahorse Bioscience, Billerica MA, USA) in the absence of CO_2 and then run on the XF24 analyzer to obtain ECAR and OCR.

Injections of different compounds that modulate glycolysis or mitochondrial respiration previously optimized, were injected sequentially in each well: for ECAR, rotenone (1 μM), glucose (30 mM) and 2-Deoxy-D-glucose (2-DG) (100 mM), while for OCR, oligomycin A (2 μg/mL), FCCP (2.5 μM) and rotenone/antimycin A (1 μM/10 μM). ECAR and OCR were recorded during specified programmed time periods (three readings each) as the average numbers between the injections of inhibitors mentioned above.

The final data calculation was performed after the readings had been normalized with counting the cell number in each well. ECAR and OCR are expressed as mpH unit change/min per 3.0×10^4 cells and pMoles/min per 3.0×10^4 cells, respectively.

2.10. Statistical Analysis

The results are expressed as the mean values with standard deviations (SD) of three experiments and the statistical analysis was performed using STATISTICA software (Statsoft Inc., Tulsa, OK, USA). The significant differences represented by letters were obtained by a one-way analysis of variance (ANOVA) followed by a Tukey's honestly significant difference (HSD) post hoc test ($p<0.05$).

3. Results and Discussion

3.1. Characterization of the Pistachio Kernel and Lyophilized Extracts

The characterization of the pistachio kernel and the lyophilized phenolic-rich extracts from Sirora variety are shown in **Table 1a**. As can be seen, TPC value was 7.03 g GAE/kg and DPPH and ORAC antioxidant capacity values were 0.02 and 0.15 mol Trolox/kg, respectively, for the pistachio kernel. The lyophilized extracts used in the present study – described in the previous section - showed much higher values of TPC (50.90 g GAE/kg; 7 times higher than the kernel), DPPH (12.60 mol Trolox/kg; 600 times higher) and ORAC (1.07 mol Trolox/kg; 7 times higher). Sirora variety has been chosen among eight different pistachio cultivars (Aegina, Avdat, Kastel, Kerman, Larnaka, Mateur, Napoletana and Sirora) assessed in a previous work by Ojeda-Amador, Fregapane & Salvador (2018). Sirora together with Mateur and Napoletana varieties showed the highest values of TPC (8.12 and 8.05 g GAE/kg, respectively) and revealed also high values of TAC (0.03 and 0.01 mol Trolox/kg for DPPH and 0.28 and 0.19 for ORAC mol Trolox/kg, respectively) in the kernel.

The main families of phenolic compounds determined in the Sirora pistachio kernel and its extract are reported in **Table 1b**. As expected, and similar to TPC results, a much higher concentration in the lyophilized extract (14.19 g/kg) as compared to the kernel (2.61 g/kg) is observed. Almost 90% of the phenolic compounds identified are accounted by the flavanols group, both in kernel and extract; being procyanidins (6.26 g/kg) more abundant than catechins (4.05 g/kg) and epicatechins (2.40 g/kg). Much lower amounts of anthocyanins (0.90 g/kg; mainly cyanidin-3-*O*-galactoside), flavonols (0.28 g/kg; mainly myricetin-3-galactoside, kaempferol-3-galactoside and quercetin-3-galactoside), flavanones (0.11 g/kg; mainly eriodictyol-7-*O*-glucoside) and phenolic acids (0.18 g/kg) were observed.

3.2. Cytotoxic Effects of Ground Pistachio Kernel Extracts on MCF-7 cells

In order to investigate the cytotoxic effects of ground pistachio kernel extract on MCF-7 cell line, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay and Tali® viability assay were performed. Cells were treated for 24, 48 and 72 h with different concentrations (0.0, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5,

15.0 mg/mL) of the ground pistachio kernel extract (equivalent to 0.0 to 763.50 µg TPC/mL, considering that the total concentration of phenolic compounds is 50.90 g GAE/kg). This experiment has been repeated for two consecutive weeks and the results suggested that the number of live and dead cells was always the same (after treatment with different doses and times). However, when cells were seen under a microscope or with Tali® Image-Based Cytometer, other trend was observed. These contradictory results make thinking that the spectrophotometric reading of MTT assay was interfered by the matrix and the results obtained by this method were not reliable (data not shown). Therefore, to confirm the number and proportion of viable and dead cells after such treatment, Tali® viability assay was carried out. In this case, cells were treated for 24, 48 and 72 h with different concentrations (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) of the ground pistachio kernel extract (equivalent to 0.0 to 254.50 µg TPC/mL) (**Figure 1**). As depicted in such figure, no significant differences in the level of live and dead cells compared with the control ($p<0.05$) has been found after 24h-treatment. On the other hand, 48 and 72h-treatments provided significant differences in the level of live/dead cells. The results suggested that phenolic compounds present in the treatment could be the responsible for this cytotoxic effect and therefore such compounds decreased cell viability in a dose and time dependent manner. Other studies with different phenolic extracts reported the cytotoxic effects in different cancer cell lines. For example, Fathalizadeh et al. (2015) showed that pistachio rosy hull extract treatment reduced cell viability (the concentration inhibition fifty (IC_{50}) ~ 0.3 mg/mL) in a dose-dependent manner in HepG2 liver cancer cell line. In another study, Hilbig et al. (2018) showed that pecan nut shell extracts presented cytotoxicity against MCF-7 cells (IC_{50} = 74.11 µg/mL) and these cytotoxicity has been attributed to the phenolic constituents of such extracts (gallic acid, 4-hydroxybenzoic acid, catechin, chlorogenic acid, vanillic acid, caffeic acid, epicatechin, epigallocatechin, epicatechin gallate and ellagic acid). It is important to highlight that in the last period interesting results have been obtained using anthocyanins-rich plant extracts on cancer cells. For example, the IC_{50} for black lentil, sorghum and red grape on HT-29 and HCT-116 human colon cancer cell proliferation ranged from 0.9 to 2.0 mg/mL (Mazewski et al., 2018).

Once the cytotoxic effects of such extract have been proved, the subsequent experiments were performed with the concentration previously establish for 48 h to determine the intracellular ROS production, the apoptotic rate, glycolysis and

mitochondrial respiration. 48h-treatments have been selected because it was the minimum incubation time to appreciate significant differences between live/dead cell proportions.

3.3. Intracellular ROS Production by Ground Pistachio Kernel Extracts on MCF-7 cells

ROS are known for their dual effects on cancer. On one hand, increased ROS production leads to a pro-oncogenic situation; on the other hand, once tumor cells have developed, an increase in ROS presence can lead to tumor cell death (Losada-Echeberría et al., 2017). This last fact has been linked to some anticancer drugs such as doxorubicin and paclitaxel. Doxorubicin is an anthracyclin that present a quinine moiety whose metabolic reduction results in one-electron transfer to molecular oxygen, generating molecules that present strong oxidizing potential, including superoxide anion, hydrogen peroxide and hydroxyl radical (Doroshow et al., 1986). Paclitaxel induces oxidative stress while killing breast cancer cells (Schrijvers 2003; Alexandre et al., 2007; Hadzic et al., 2010), with production of hydrogen peroxide and formation of DNA oxidative adducts after 2 h of treatment (Ramanathan et al., 2005). Increasing evidences suggested that phenols can act as prooxidants under certain conditions leading to tumor cell death (Elbling et al., 2005; Hadi et al., 2007). To determine intracellular ROS production, MCF-7 cells were treated with different concentrations of the ground pistachio kernel extracts for 48h and analyzed using the CellROX® Orange assay kit by Tali® Image-based Cytometer (**Figure 2**). The highest percentage of intracellular ROS production was obtained after treatment with 5.0 mg/mL of the ground pistachio kernel extracts (3.75% fold increase compared to the control), showing significant differences ($p<0.05$) with respect to the control and the remaining concentrations. These results suggested that phenolic compounds present in the lyophilized extract induce ROS generation, and therefore could be considered a chemotherapeutic effect in suppressing cancer growth. This effect has been previously demonstrated using single phenolic compounds like luteolin, apigenin, epigallocatechin-3-gallate and resveratrol (Khan et al., 2014). Also the effects of propolis and the phenolic compounds present in propolis sample (caffeic acid, dihydrocinnamic acid, and *p*-coumaric acid) were investigated on human laryngeal epidermoid carcinoma (HEp-2) cells. Results showed that caffeic acid induced late apoptosis or necrosis in HEp-2 cells,

while propolis induced apoptosis, both probably due to ROS generation (da Silva et al., 2017). Another example could be thymol which was evaluated on bladder cancer cells trying to underlying its mechanism. In this case, thymol inhibited bladder cancer cell proliferation in a dose and time-dependent manner and induce the generation of ROS (Li et al., 2017).

3.4. Apoptosis Regulation by Ground Pistachio Kernel Extractson MCF-7 cells

Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death (Elmore, 2007). The ability to modulate the life or death of a cell is recognized for its immense therapeutic potential (Elmore, 2007), so induction of apoptosis represents an efficient strategy for cancer chemotherapy to evaluate and design new chemotherapeutic agents (Taraphdar et al., 2001). In order to determine the level of live, dead cells and those in early apoptotis, MCF-7 breast cancer cells were treated with different concentrations of the ground pistachio kernel extracts for 48h and analyzed by Tali® Image-based Cytometer. As shown in **Figure 3**, the addition of the extracts significantly induced the level of dead cells compared to the control ($p<0.05$) and significantly reduce the proportion of cells in early apoptosisompared to the control ($p<0.05$). A possibility explaining these differences could be that the treatments quickly induces apoptosis. If the apoptotic process runs very fast after the treatments in most of cells, a great number of them would be in later apoptotis state at the moment of the analysis and they would be quantified as dead cells. However, other apoptosis-independent cell death mechanisms could be responsible for the increase in dead cell observed after the different treatments at least in part. In some cases the type of stimuli and/or the degree of stimuli determines if cells die by apoptosis or other cell death mechanisms (Elmore, 2007). In fact, different authors have traditionally addressed the issue of distinguishing apoptosis from necrosis, two processes that can occur independently, sequentially, as well as simultaneously (Hirsch, 1997; Zeiss, 2003). Many phenolic compounds inhibit the proliferation in different cancer cell lines inducing apoptosis. An example could be fraxetin that inhibits the proliferation of the MCF-7 breast cancer cell line and the induction of apoptosis may be achieved by upregulating FasL, Fas and Bax and

443 downregulating Bcl-2 (Liu et al., 2017). Others can directly stimulate other
444 programmed cell death (i.e., necroptosis, autoptosis), such it will be discussed bellow.
445 In absence of apoptosis, cell death has been traditionally considered to occur by necrosis
446 which is unplanned and unregulated form of death, and consequently, it is considered
447 undesirable. It is further thought that necrosis was inherently undesirable in terms of
448 cancer therapy by two facts (Zong & Thompson, 2006). On one hand, it would result in
449 the release of a number of immunostimulants leading to overstimulation of the immune
450 system (Zong & Thompson, 2006). On the other hand, it can trigger the release of
451 growth factors and cytokines leading to increased tumor growth (Hanahan & Weinberg,
452 2011). Other recent studies have provided evidences indicating that the reported effects
453 in the present study about ground pistachio kernel extracts concerning cell death could
454 result beneficial. Firstly, different non-canonical cell death sharing features with
455 necrosis have been currently established and even necrosis was reported to be highly
456 regulated, occurring in a number of physiologic and pathophysiological situations
457 (Vanden Berghe et al., 2014). Moreover, in some *in vivo* studies, chemotherapy-induced
458 necrosis observed in most of cancers usually is not massive and not life-threatening as
459 occurs in tumor lysis syndrome. Finally, stimulation of immune system has also shown
460 to be positive after different treatment against cancer (Frey et al., 2014; Henderson et
461 al., 2004; Brackett & Gollnick, 2011).

462

463 *3.5. Glycolysis and Mitochondrial Respiration of Ground Pistachio Kernel Extracts on*
464 *MCF-7 cells*

465 In this section we have investigated the ECAR and the OCR measured by the Seahorse
466 XF-24 Extracellular Flux Analyzer.

467 To determine the ECAR, cells were exposed to three different chemical reagents
468 (rotenone, glucose and 2-DG). Rotenone is an electron transport chain (ETC) inhibitor
469 that acts at complex I, thus it inhibits mitochondrial respiration; glucose is used to
470 obtain the maximum glycolitic capacity (i.e., when the glycolysis it is not limited by
471 substrate amount); and 2-DG is a competitive inhibitor of the production of glucose-6-P
472 from glucose (step 2 of glycolysis), thus it inhibits glycolysis.

473 With respect to the OCR measurements, basal respiration is mainly driven by the
474 concomitant re-entry pathways through the proton leak and ATP synthases. After that,

cells were exposed consecutively to four modulators of oxidative phosphorylation, such as oligomycin A, FCCP and rotenone/antimicyn A. Oligomycin A stops the ATP synthase so that residual respiration is due solely to the proton leak (complex V). The addition of the protonophore FCCP causes an artificial proton conductance into the membrane. This maximal respiration is now regulated by the activity of the ETC and/or the delivery of substrate. At the end, rotenone/antimycin A which are ETC inhibitors were added and they act complex I and III, respectively. In this way, any residual respiration is non mitochondrial and needs to be subtracted from the other rates.

Regarding energetic metabolism, it was found that treatments with different concentrations of the ground pistachio kernel extracts led to a reduction in both ECAR and OCR with respect to untreated cells (**Figure 4a and 4b**). In addition, these differences were substantial in cells treated with higher doses (especially with the concentration of 5.0 mg/mL). These results suggest that phenolic compounds from ground pistachio kernel extracts inhibit not only mitochondrial respiration but also lactate production and glucose consumption (i.e. glycolysis) in a dose-dependent manner. Therefore, a considerable alteration of cell bioenergetics would occur as consequence of ground pistachio kernel extract treatment.

In general, cancer cells show higher reliance on glycolysis for their survival than normal cells. Thus, glycolysis inhibition alone might represent a harmful mechanism selective to cancer cells (Samudio et al., 2009; Koppenol et al., 2011). In fact, different anti-cancer agents have also shown to inhibit glycolysis. Indeed, multiple phenolics (such as, catechin, cyanidin, myricetin and quercetin) (Gao & Chen, 2015) have revealed this pattern. Despite many cancer cells types have exhibited dysfunctional mitochondria and have increased glycolysis (i.e., Warburg effect) (Warburg, 1956), some of them have been reported to produce energy through OXPHOS *in vivo*, whereas the surrounding stromal cells are highly glycolytic and fueling the cancer cells oxidative metabolism (i.e. “reverse-Warburg” effect) (Witkiewicz et al. 2012; Valencia et al., 2014). This phenomenon has been reported in metastasis but also in primary tumors (Bonuccelli et al., 2010). The existence of this phenomenon makes ground pistachio kernel extracts particularly interesting since the inhibition of mitochondrial respiration, evidenced by the decrease in OCR, could contribute to the possible antitumor and antimetastatic effects of pistachio phenolic compounds. In any case, the alterations in both metabolic bioenergetic processes would presumably lead to a severe and rapidly depletion in cell

energy reserves as consequence of the decrease in ATP production. An effect also found in 3-bromopyruvate-treated cells (Valenti et al., 2015).

The accumulation of ROS previously demonstrated with the addition of pistachio extracts could arise from two facts, a higher production of ROS or an inhibition of antioxidant systems **(Figure 5)**. The decrease in OCR can be consequence of mitochondrial ETC impairment which has associated with an increase in ROS generation at mitochondria (Verrax et al., 2011; Forbes et al., 2014). For instance, agents such as arsenic trioxide (As_2O_3) which impair the function of the respiratory chain are known to increase the production of superoxide (Pelicano et al., 2003). Therefore, the observed effect in mitochondrial respiration could be responsible at least in part of the ROS levels associated with ground pistachio kernel extract treatment found in the present model. It has been proposed that cancer cells are particularly sensitive to ROS overproduction since they usually exhibit an abnormal redox status associated with increased basal levels of ROS and frequent alterations of the antioxidant system (Verrax et al., 2011; Trachootham et al., 2009). Actually, ROS overproduction as consequence of mitochondrial ETC impairment has been described as killing mechanism for some chemotherapeutics. This has been exemplified by adaphostin that induces cancer cell death by inhibiting the complex III of the mitochondrial respiratory chain, leading to mitochondrial ROS generation (Le et al., 2007).

Despite of many mechanisms under this effect need to be elucidated, the putative reduction of cellular ATP levels and the increase in oxidative stress rapidly would induce cell death in an apoptosis-independent manner. In support of this, it has been reported that other chemotherapeutics with similar effects on cell metabolism and oxidative stress, such as 3-bromopyruvate increases cell death via necrosis rather than apoptosis (Eguchi et al. 1997, Ko et al. 2012, Qin et al. 2010).

Notwithstanding, it is unclear how ground pistachio kernel extracts exert this metabolic alteration. A possibility is that one or several phenolic compounds present in pistachio extracts directly inhibit some enzymes of glycolytic pathway and mitochondrial ETC complexes, particularly those which activities require oxygen consumption (i.e. Complex III). In fact, some anti-cancer agents have shown to simultaneously inhibit several components of both processes (Valenti et al., 2015). Other possibility is that the extracts directly increase ROS production activating enzymatic systems as NADPH oxidases or promoting inorganic ROS formation. In addition, a decrease in the activity

of endogenous antioxidant defenses including detoxifying enzymes or endogenous ROS scavenger compounds could lead to ROS accumulation in cells. In turn, oxidative stress could be responsible for mitochondrial dysfunction if oxidative damage to ETC complex proteins occurs. This could be also extent to enzymes participating in glycolysis.

4. Conclusions

Nuts and in particular pistachio as known present high amounts of phenolic compounds which can exert beneficial health effects against different common diseases.

In the last years, many research studies were focused on searching natural alternatives to improve current chemotherapy treatments. The remarkable phenolic content and the corresponding antioxidant capacity of the pistachio kernel polar extracts prepared in the current experimental work make them good candidates for evaluate its potential as chemotherapeutic in MCF-7 cells.

In the present study, this lyophilized extract was tested in MCF-7 breast cancer cell line. Different experiments have been performed in order to reveal their effects on cell viability, intracellular ROS level and apoptosis. The results here obtained suggest that phenolic compounds from ground pistachio kernel extracts would result cytotoxic for this model in a dose-(0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) and time-(48 and 72h) dependent manner. They would also induce an increase in cell ROS level and death. With regard to energetic metabolism, it was found that treatments at different concentrations led to a reduction in ECAR and OCR with respect to untreated cells, suggesting that phenolic compounds from pistachio extracts inhibit not only mitochondrial respiration but also lactate production and glucose consumption in a dose-dependent manner. The cell death observed in the present model seems to be responsible, at least in part, as a result of a combination of oxidative stress and bioenergetic alterations.

Pistachio kernel extract showed promising beneficial effects that could be exploited as a "natural adjuvant" in combination with chemotherapy treatment. Likewise, this kind of extracts could be used as an additive in the development of new food products, which would take into account not only particular sensorial features but also health benefits. Therefore, this study represents the starting point to test pistachio kernel extracts on human health. Further studies must be conducted in order to confirm these findings also in humans or animal models.

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Table and Figure Captions

Table 1a. Characterization of TPC and TAC of Sirora kernel and its lyophilized extract.

Table 1b. Main phenolic families determined in Sirora kernel and its lyophilized extract (g/kg FW).

Figure 1. Inhibition of cell proliferation by ground pistachio kernel extracts in MCF-7 cell line. After 24h of cell seeding, MCF-7 cells were treated with different concentrations of ground pistachio kernel extracts (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for (**A)** 24h, **(B)** 48h and **(C)** 72h. Cell viability was measured by using Tali® Image-Based Cytometer and results were expressed as a percentage (%) of live or dead cells compared to control cells. Data are shown as the mean±SD of three independent experiments (n=3). Columns belonging to the same set of data with different superscript letters are significantly different from control ($p<0.05$).

Figure 2. Ground pistachio kernel extracts induces ROS generation in MCF-7 cells. MCF-7 cells were treated with different concentrations of ground pistachio kernel extracts (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 48h. Intracellular ROS levels were calculated by using CellROX® Orange assay kit and the Tali® Image-Based Cytometer was used to quantify ROS induction (% of propidium iodide positive) in MCF-7 cells following the treatment previously described. The blue line of the thumbnail histogram indicated the set threshold. Data are shown as the mean±SD. Columns with different superscript letters are significantly different from control ($p<0.05$).

Figure 3. Live, dead and early apoptosis levels in MCF-7 cells treated with different concentrations of ground pistachio kernel extracts (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 48h. Tali® Image-Based Cytometer was used to quantify live, dead and early apoptosis cells following the treatment previously described. Data are shown as the mean±SD.

853 Columns with different superscript letters are significantly different from control
854 ($p<0.05$).

855 **Figure 4a.** Reduction of the glycolysis by ground pistachio kernel extracts in MCF-7
856 cells. ECAR was observed by using the Seahorse XF24 Extracellular Flux Analyzer. All
857 data are expressed as the mean±SD of three experiments (n=3).

858

859 **Figure 4b.** Regulation of mitochondrial respiration by ground pistachio kernel extracts
860 in MCF-7 cells. OCR was observed by using the Seahorse XF24 Extracellular Flux
861 Analyzer. All data are expressed as the mean±SD of three experiments (n=3).

862

863 **Figure 5.** Schematic representation of the possible mechanism of action of ground
864 pistachio kernel extracts in MCF-7 cells. Abbreviations: **ROS** (Reactive Oxygen
865 Species); **I** (Complex I); **II** (Complex II); **III** (Complex III); **IV** (Complex IV); **V**
866 (Complex V); **cytC** (cytochrome C); **CoQ** (Coenzyme Q).

Effect of pistachio kernel extracts in MCF-7 breast cancer cells: inhibition of cell proliferation, induction of ROS production, modulation of glycolysis and of mitochondrial respiration

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Abstract

The effects of ground pistachio kernel extracts have been evaluated on cellular viability, intracellular reactive oxygen species (ROS) production and cell death were assessed in MCF-7 breast cancer cells. Results showed a significant decrease in cell viability in a dose and time dependent manner. 48h-treatments with different concentrations of the extracts induced intracellular ROS generation and showed that cell death was in an apoptosis-independent manner. It was also found that 48h-treatments led to a dose dependent reduction in both extracellular acidification rate (ECAR) and oxygen consumption rate (OCR)with respect to untreated cells. Therefore, a considerable alteration of cell bioenergetics would occur as consequence of such treatments. Combination of oxidative stress and bioenergetic alterations could be responsible, at least in part, of the cell death observed in the present model. In conclusion, pistachio kernel extract showed promising beneficial effects that could be exploited as a "natural adjuvant" in combination with chemotherapy treatment.

Keywords: pistachio kernel; phenolic compounds; intracellular reactive oxygen species (ROS); apoptosis; energetic metabolism; breast cancer.

1. Introduction

Pistachios (*Pistacia vera* L., Anacardiaceae family) are one of the oldest valuable edible tree nuts and are commonly used all over the world. Although pistachios are native to Asia, particularly in Iran and Iraq, they are being commercially produced in United States, Australia, Turkey, Greece, Italy, Spain and China, among others (Saitta et al., 2014). Pistachio was first planted in Spain in 1996 and in 2016 the cultivation area was over 15,000 ha, with 12,000 ha (80%) corresponding to Castilla-La Mancha (MAGRAMA data, Spanish Ministry of Agriculture).

Pistachios are nuts with a dry, hard, outer egg shaped shell, with a tasty kernel inside that are consumed in food industry (Saitta et al., 2014), as salted and roasted snacks or as an ingredient in bakery and confectionery products, desserts, and ice-creams. Composition of pistachio kernels is complex and exhibit interesting nutritional properties as follows (Bulló et al., 2015; Hernández-Alonso, Bulló & Salas-Salvadó, 2016; Liu at al., 2014; Ojeda-Amador et al., 2018): they are relatively rich in monounsaturated fat comprising 5.6 % saturated fatty acids, 13.7 % polyunsaturated fatty acid and 23.8 % monounsaturated (oleic and linoleic acids represent more than 60% of the total fat); they are a good source of vegetable protein (comprising about 20% of total weight) with approximately 2% L-arginine; their amount of carbohydrate is low to moderate (about 27.5% by weight) but they are rich in fibre containing 10% by weight of insoluble forms and 0.3% of soluble forms; regarding to vitamins and minerals, they are rich in Cu, Ca, Mg, Fe, vitamin A, vitamin C and B vitamins, with high amounts of B_1 and the absence of B_{12}. Among the bioactive compounds, pistachios are rich sources of hydrophilic bioactives such as phenolic compounds (including phenolic acids and flavonoids) (Tomaino et al., 2010; Rodríguez-Bencomo et al., 2015; Martínez et al., 2016) and lipophilic bioactives such as carotenoids (including lutein and zeaxanthin which are responsible for giving colour to pistachio nuts), chlorophylls and phytosterols (including stigmasterol, campesterol and β-sitosterol).

Pistachios are among the top fifty foods with high antioxidant and anti-inflammatory potential by their content of oleic acid, phytosterols, phenolic acids and tocopherols (Bulló et al., 2015). Hydrophilic and lipophilic bioactive content (polyphenols,

971 xanthophylls and tocopherols, in particular) has been demonstrated to be rapidly
972 bioaccessible into the stomach which maximizes their transformation and absorption in
973 the upper small intestine and promotes health effects (Bulló et al., 2015). For this reason
974 nut consumption is being encouraged into health diets for their nutritional quality and
975 potential contribution to health promotion (Dreher, 2012).
976
977 Results from the PREDIMED study (Prevention with Mediterranean Diet) showed that
978 the high intake of nuts like pistachios is associated with a lower incidence of
979 cardiovascular diseases, metabolic syndrome and other inflammatory conditions
980 (Reboredo-Rodríguez et al., 2017; Salas-Salvadó et al., 2008). However, relationship
981 between cancer and nut consumption has been investigated in a lesser extent with some
982 preliminary interesting results from clinical studies. This is the case of EPIC study
983 (European Prospective Investigation into Cancer and Nutrition), a large trial involving
984 ten European countries, nearly 500,000 participants. Among its conclusions highlight a
985 higher consumption of nuts and seeds was associated with a 31% risk reduction of
986 colorectal cancer development for women (http://www.thelancet.com/oncology).
987
988 According to Yang and coworkers (2012), the high content of vitamin E and other
989 antioxidants founded in pistachios may reduce the risk of other cancer types.How
990 dietary compounds present in natural products do regulate and induce these beneficial
991 effects against carcinogenesis is a matter which remains to be elucidated (Pan et al.,
992 2017a; Pan et al., 2017b). In this context, the main objective of this study presented here
993 was to assess the potential health effects of pistachio kernel extracts on MCF-7 breast
994 cancer cell line by evaluating the cytotoxic properties by viability assays, intracellular
995 reactive oxygen species (ROS) production and effect on apoptosis. The energetic
996 metabolism has also been studied by measure extracellular acidification rate (ECAR)
997 and oxygen consumption rate (OCR) using the XF24 Extracellular Flux Analyzer.
998 Given the fact that phenolic profile can be very effective in its biological activity, a
999 systematic phenolic characterization of the studied extract was undertaken by HPLC-
1000 DAD-MSn.
1001

2. Materials and Methods

2.1. Pistachio and Lyophilized Extracts

Pistachios of the Sirora variety were provided by the regional research centre "Centro de Mejora Agraria El Chaparrillo" (Ciudad Real, Spain) during 2013/14 and 2014/15 harvest seasons. Pistachio extract rich in phenolic compounds has been obtained as followed: 2 g of ground pistachio kernel was extracted by 20 mL of ethanol:H_2O (80:20, v/v) employing ultrasound assisted agitation (10 min, 25°C, 250W; sonicator Q500, Qsonica, Newtown, CT, USA) and then evaporated using a centrifugal vacuum concentrator (miVac Duo, Genevac Ltd., Ipswich, UK) and finally freeze-dried (Cryodos-45, Telstar, Terrassa, Spain). The lyophilized extracts were kept dry until *in vitro* analysis.

2.2. Determination of Total Phenolic Content (TPC)

Total Phenolic Content (TPC) was analyzed following the method described by Vázquez, Janer & Janer (1973) and Gutfinger (1981). Briefly, a double extraction of the 0.4 g of ground pistachio kernel or of its extract was performed in 20 mL MeOH:H_2O:HCOOH (80:20:0.1; 10 + 10 mL), with 2 min vortex, followed by 5 min ultrasound and centrifugation at 2000g for 10 min. The combined extracts were filtered prior to analysis. Suitable aliquots (100-500 μL) of the polar extracts were transferred into a 10-mL volumetric flask. Water (up to 8 mL) and the Folin-Ciocalteu reagent (0.5 mL) were added. After 3 min, the absorbance of the solution was measured at 725 nm against a blank solution with a UV-visible spectrophotometer (Agilent Technologies 8453). A calibration curve was established using gallic acid (GAE) as the external standard.

2.3. Determination of Total Antioxidant Capacity (TAC)

Total Antioxidant Capacity (TAC) (Quiles et al., 2006) has been evaluated for two different methods: 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH) radical assay and the oxygen radical absorbance capacity (ORAC).

The radical scavenging effect of the methanolic extract towards the synthetic radical DPPH was performed as reported previously Brand-Williams, Cuvelier & Berset (1995) and Sánchez-Moreno, Larrauri & Saura-Calixto (1998) and Morita, Naito, Yoshikawa and Niki (2017). Briefly, 100 µL of the polar extract (prepared as described above in point 2.2) was added to a methanolic DPPH solution (2.9 mL, 6×10^{-5} M) and stored in the dark for 30 min. The decrease in absorbance of the resulting solution was then measured at 515 nm using an Agilent 8453 spectrophotometer. A calibration curve was constructed using Trolox as the external standard.

ORAC assay was performed according to Dávalos, Gómez-Cordovés & Bartolome (2004). Briefly, 20 µL of the polar extract (prepared as described above in point 2.2) and 120 µL of fluorescein (70 nM) at 37 ºC for 15 min were preincubated. After that, 60 µL of 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH, 24 mM) was added and the mixture incubated at 37 ºC (kinetics measured every minute for 80 min). The experiment was carried out in Nunclon black 96-well flat-bottom plates (Sigma-Aldrich, Madrid, Spain) and measurements acquired using a plate reader with emission and excitation filters set at 528 and 485 nm, respectively. The fluorescence curves were normalized with respect to the blank curve (without antioxidant). The calibration curve was prepared using Trolox as the external standard.

2.4. Determination of the Phenolic Compounds by HPLC-DAD-MSn

Individual phenolic compounds were determined by an HPLC-DAD-ESI-MS/MS method adapted from conditions previously described (Castillo-Muñoz et al., 2009). The analysis was performed on an Agilent 1100 series system (Agilent, Waldbronn, Germany) equipped with a photodiode array detector (DAD) and an LC/MSD Trap VL electrospray ionization mass spectrometry (ESI-MS/MS), both coupled with an Agilent ChemStation for data processing.

Aliquots (1.75 mL) of phenolic extracts were evaporated in a rotary evaporator at 35 °C under vacuum, and were eventually dissolved in 200 µL of methanol/water (20:80, v/v) by being sonicated (5 min) and vortexed (2 min) before injecting 20 µL in the system. Before injection, extracts were diluted sixteen times to avoid saturation of the signal. Separation was achieved on a narrow-bore column Zorbax Eclipse XDB-C18 (2.1 x 150

mm; 3.5 µm particle; Agilent) thermostated at 40 ºC and using a ternary gradient and a 0.19 mL/min flow rate. Eluents were (A) acetonitrile/water/formic acid (3:88.5:8.5, v/v/v), (B) acetonitrile/water/formic acid (50:41.5:8.5, v/v/v), and (C) methanol/water/formic acid (90:1.5:8.5, v/v/v). The linear solvent´s gradient was as follows: zero min, 98 % A 2 % B and 0% C; 8 min, 98 % A 2% B and 0% C; 40 min, 70 % A 17 % B and 13 % C; 54 min, 50 % A 30% B and 20 % C; 54.5 min, 30 % A 40 % B and 30 % C; 59 min, 0 % A 50 % B and 50 % C; 60 min, 0 % A 50 % B and 50 % C; 67 min, 98 % A 2 % B and 0 % C. For identification, an Ion Trap ESI-MS/MS detector was used in negative ion modes, setting the following parameters: dry gas N_2, 8 L/min; drying temperature, 325 ºC; nebulizer, N_2, 50 psi; scan range, 50-1, 200 m/z. Identification was based on spectroscopic data (UV-Vis and MS/MS) obtained from standard or previously reported data. Quantification was made using the DAD chromatograms recorded at 280 nm (flavanols, flavanones and phenolic acids), 360 nm (flavonols) and 520 nm (anthocyanins). Calibration curves for the analyzed compounds were prepared from pure standards (cyanidin-3-*O*-galactoside; cyanidin-3-*O*-glucoside; myricetin-3-galactoside; quercetin; quercetin-3-glucoside; quercetin-3-rutinoside; procyanidin B1; procyanidin B2; catechin; epicatechin; catechin gallate; eriodictyol-7-*O*-glucoside; from Sigma-Aldrich).

2.5. Cell Culture

MCF-7 breast cancer cell line was kindly provided by Prof. Mirco Fanelli of the Department of Biomolecular Sciences (Università degli Studi di Urbino Carlo Bo, Urbino, Italy) and was grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum as well as 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were maintained in a CO_2 incubator at 37 °C under a humidified atmosphere (95% air, 5% CO_2).

2.6. Cell Viability by MTT and TALI® Viability Assays

MTT Assay

Cells were seeded into 96-well plates at a density of 5×10^3 cells/well in complete growth medium. Following overnight incubation, MCF-7 cells were treated with different concentrations (0.0, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 mg/mL) of the ground pistachio kernel extract for 24, 48 and 72 h. The extract was directly dissolved in the cell culture medium. After incubation, 30 µL of RPMI medium containing 2 mg/mL of MTT were added to each well, and cells were incubated for another 2 h at 37 °C. MTT solution was then discarded, and 100 µL of DMSO were added to each well to dissolve the formazan crystal. The level of colored formazan derivative was analyzed on a microplate reader at a wavelength of 590 nm (Moongkarndi et al., 1991; Studzinski et al; 1995).

TALI® Viability Assay

Cells were seeded into 6-well plates at a density of 1.5×10^5 cells/well in complete growth medium. Following overnight incubation, MCF-7 cells were treated with different concentrations of the ground pistachio kernel extract (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 24, 48 and 72 h. Cell viability was determined by using the Tali® Viability Kit-Dead Cell Green reagent following the manufacturer's instructions and as previously reported Remple & Stone (2011). The proportions of viable and dead cells were analyzed by the Tali®RFP + Viability assay on the Tali® Image-Based Cytometer (Thermo Fisher Scientific, Milan, Italy).

2.7. Assessment of Intracellular ROS Production by TALI® Image-Based Cytometer

Determination of intracellular ROS levels was performed using the CellROX® Oxidative Stress Kit (Invitrogen®, Life Technologies, Milan, Italy) according to the manufacturer's instructions. Cells were seeded in 6-well plates at a density of 1.5×10^5 cells/well in complete growth medium. Following overnight incubation, MCF-7 cells were treated with different concentrations of the ground pistachio kernel extract (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 48h. After treatment, cells were trypsinized and centrifuged at 1500 rpm for 10 min at room temperature. Then, the supernatant was discarded and the cellular pellet was re-suspending in 0.5 mL of complete medium. Finally, 1 µL of CellROX® Orange Reagent was added and samples were incubated for 30 min at 37 °C. Cells were centrifuged once to remove the medium and re-suspended

again in 100 µL PBS. Finally, samples were analyzed with the Tali® Image-Based Cytometer and the results were expressed as the percentage of cells with increased ROS levels compared with the control (Alvarez-Suarez et al., 2016; Giampieri et al., 2017).

2.8. Apoptosis Detection by TALI® Image-Based Cytometer

Apoptosis was measured using the Tali® apoptosis assay kit and propidium iodide (Invitrogen®, Life Technologies, Milan, Italy) as previously reported (Giampieri et al., 2014; Islam et al., 2017). Cells were seeded in 6-well plate at a density of 1.5×10^5 cells/well and treated with different concentrations of the ground pistachio kernel extract (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 48h. At the end of the treatment, cells were centrifuged (1500 rpm for 5 min) and resuspended in100 µL of Annexin V Binding Buffer (ABB). After that, 5 µL of Annexin V Alexa Fluor® 488 were added, mixed again and the solution was incubated in the dark at room temperature for 20 min. Then, cells were centrifuged again at 1000 rpm for 10 min, resuspended in 100 µL of ABB and 1 µL of propidium iodide was added, mixed and incubated in the dark at room temperature for another 5 min. Finally, samples were analyzed using the Tali® Image-Based Cytometer and the percentage of apoptotic, dead cells and live cells was determined on the basis of the corresponding fluorescence histogram compared with an untreated control. The annexin-V positive/propidium iodide negative cells were recognized as apoptotic cells by the cytometer software whereas the annexin V-positive/propidium iodide positive cells were identified as dead cells. Similarly, the annexin V-negative/PI negative cells were identified as viable cells. The results were expressed as fold increase compared with the control.

2.9. Evaluation of Glycolysis and of Mitochondria Functionality

XF24 Extracellular Flux Analyzer from Seahorse Bioscience, Inc. (North Billerica, MA, USA) was utilized to ECAR and OCR, representing glycolysis and oxidative phosphorylation (OXPHOS), respectively.

MCF-7 cells were seeded in 24-well XF cell culture microplates at 3.0×10^4 cells/well for 24 h and then treated with different concentrations of the ground pistachio kernel extract (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 48 h. At the end of the treatment, the medium

was replaced with 500 μL/well of XF-24 running media (supplemented with 25mM glucose, 4mM glutamine, 1mM sodium pyruvate, without serum). The plates were pre-incubated at 37 °C for 20 min in the XF Prep Station incubator (Seahorse Bioscience, Billerica MA, USA) in the absence of CO_2 and then run on the XF24 analyzer to obtain ECAR and OCR.

Injections of different compounds that modulate glycolysis or mitochondrial respiration previously optimized, were injected sequentially in each well: for ECAR, rotenone (1 μM), glucose (30 mM) and 2-Deoxy-D-glucose (2-DG) (100 mM), while for OCR, oligomycin A (2 μg/mL), FCCP (2.5 μM) and rotenone/antimycin A (1 μM/10 μM). ECAR and OCR were recorded during specified programmed time periods (three readings each) as the average numbers between the injections of inhibitors mentioned above.

The final data calculation was performed after the readings had been normalized with counting the cell number in each well. ECAR and OCR are expressed as mpH unit change/min per 3.0×10^4 cells and pMoles/min per 3.0×10^4 cells, respectively.

2.10. Statistical Analysis

The results are expressed as the mean values with standard deviations (SD) of three experiments and the statistical analysis was performed using STATISTICA software (Statsoft Inc., Tulsa, OK, USA). The significant differences represented by letters were obtained by a one-way analysis of variance (ANOVA) followed by a Tukey's honestly significant difference (HSD) post hoc test ($p<0.05$).

3. Results and Discussion

3.1. Characterization of the Pistachio Kernel and Lyophilized Extracts

The characterization of the pistachio kernel and the lyophilized phenolic-rich extracts from Sirora variety are shown in **Table 1a**. As can be seen, TPC value was 7.03 g GAE/kg and DPPH and ORAC antioxidant capacity values were 0.02 and 0.15 mol Trolox/kg, respectively, for the pistachio kernel. The lyophilized extracts used in the present study – described in the previous section - showed much higher values of TPC (50.90 g GAE/kg; 7 times higher than the kernel), DPPH (12.60 mol Trolox/kg; 600 times higher) and ORAC (1.07 mol Trolox/kg; 7 times higher). Sirora variety has been chosen among eight different pistachio cultivars (Aegina, Avdat, Kastel, Kerman, Larnaka, Mateur, Napoletana and Sirora) assessed in a previous work by Ojeda-Amador, Fregapane & Salvador (2018). Sirora together with Mateur and Napoletana varieties showed the highest values of TPC (8.12 and 8.05 g GAE/kg, respectively) and revealed also high values of TAC (0.03 and 0.01 mol Trolox/kg for DPPH and 0.28 and 0.19 for ORAC mol Trolox/kg, respectively) in the kernel.

The main families of phenolic compounds determined in the Sirora pistachio kernel and its extract are reported in **Table 1b**. As expected, and similar to TPC results, a much higher concentration in the lyophilized extract (14.19 g/kg) as compared to the kernel (2.61 g/kg) is observed. Almost 90% of the phenolic compounds identified are accounted by the flavanols group, both in kernel and extract; being procyanidins (6.26 g/kg) more abundant than catechins (4.05 g/kg) and epicatechins (2.40 g/kg). Much lower amounts of anthocyanins (0.90 g/kg; mainly cyanidin-3-*O*-galactoside), flavonols (0.28 g/kg; mainly myricetin-3-galactoside, kaempferol-3-galactoside and quercetin-3-galactoside), flavanones (0.11 g/kg; mainly eriodictyol-7-*O*-glucoside) and phenolic acids (0.18 g/kg) were observed.

3.2. Cytotoxic Effects of Ground Pistachio Kernel Extracts on MCF-7 cells

In order to investigate the cytotoxic effects of ground pistachio kernel extract on MCF-7 cell line, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay and Tali® viability assay were performed. Cells were treated

for 24, 48 and 72 h with different concentrations (0.0, 0.5, 1.0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0 mg/mL) of the ground pistachio kernel extract (equivalent to 0.0 to 763.50 μg TPC/mL, considering that the total concentration of phenolic compounds is 50.90 g GAE/kg). This experiment has been repeated for two consecutive weeks and the results suggested that the number of live and dead cells was always the same (after treatment with different doses and times). However, when cells were seen under a microscope or with Tali® Image-Based Cytometer, other trend was observed. These contradictory results make thinking that the spectrophotometric reading of MTT assay was interfered by the matrix and the results obtained by this method were not reliable (data not shown). Therefore, to confirm the number and proportion of viable and dead cells after such treatment, Tali® viability assay was carried out. In this case, cells were treated for 24, 48 and 72 h with different concentrations (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) of the ground pistachio kernel extract (equivalent to 0.0 to 254.50 μg TPC/mL) (**Figure 1**). As depicted in such figure, no significant differences in the level of live and dead cells compared with the control ($p<0.05$) has been found after 24h-treatment. On the other hand, 48 and 72h-treatments provided significant differences in the level of live/dead cells. The results suggested that phenolic compounds present in the treatment could be the responsible for this cytotoxic effect and therefore such compounds decreased cell viability in a dose and time dependent manner. Other studies with different phenolic extracts reported the cytotoxic effects in different cancer cell lines. For example, Fathalizadeh et al. (2015) showed that pistachio rosy hull extract treatment reduced cell viability (the concentration inhibition fifty (IC_{50}) ~ 0.3 mg/mL) in a dose-dependent manner in HepG2 liver cancer cell line. In another study, Hilbig et al. (2018) showed that pecan nut shell extracts presented cytotoxicity against MCF-7 cells (IC_{50} = 74.11 μg/mL) and these cytotoxicity has been attributed to the phenolic constituents of such extracts (gallic acid, 4-hydroxybenzoic acid, catechin, chlorogenic acid, vanillic acid, caffeic acid, epicatechin, epigallocatechin, epicatechin gallate and ellagic acid). It is important to highlight that in the last period interesting results have been obtained using anthocyanins-rich plant extracts on cancer cells. For example, the IC_{50} for black lentil, sorghum and red grape on HT-29 and HCT-116 human colon cancer cell proliferation ranged from 0.9 to 2.0 mg/mL (Mazewski et al., 2018).

Once the cytotoxic effects of such extract have been proved, the subsequent experiments were performed with the concentration previously establish for 48 h to

determine the intracellular ROS production, the apoptotic rate, glycolysis and mitochondrial respiration. 48h-treatments have been selected because it was the minimum incubation time to appreciate significant differences between live/dead cell proportions.

3.3. Intracellular ROS Production by Ground Pistachio Kernel Extracts on MCF-7 cells

ROS are known for their dual effects on cancer. On one hand, increased ROS production leads to a pro-oncogenic situation; on the other hand, once tumor cells have developed, an increase in ROS presence can lead to tumor cell death (Losada-Echeberría et al., 2017). This last fact has been linked to some anticancer drugs such as doxorubicin and paclitaxel. Doxorubicin is an anthracyclin that present a quinine moiety whose metabolic reduction results in one-electron transfer to molecular oxygen, generating molecules that present strong oxidizing potential, including superoxide anion, hydrogen peroxide and hydroxyl radical (Doroshow et al., 1986). Paclitaxel induces oxidative stress while killing breast cancer cells (Schrijvers 2003; Alexandre et al., 2007; Hadzic et al., 2010), with production of hydrogen peroxide and formation of DNA oxidative adducts after 2 h of treatment (Ramanathan et al., 2005). Increasing evidences suggested that phenols can act as prooxidants under certain conditions leading to tumor cell death (Elbling et al., 2005; Hadi et al., 2007). To determine intracellular ROS production, MCF-7 cells were treated with different concentrations of the ground pistachio kernel extracts for 48h and analyzed using the CellROX® Orange assay kit by Tali® Image-based Cytometer (**Figure 2**). The highest percentage of intracellular ROS production was obtained after treatment with 5.0 mg/mL of the ground pistachio kernel extracts (3.75% fold increase compared to the control), showing significant differences ($p<0.05$) with respect to the control and the remaining concentrations. These results suggested that phenolic compounds present in the lyophilized extract induce ROS generation, and therefore could be considered a chemotherapeutic effect in suppressing cancer growth. This effect has been previously demonstrated using single phenolic compounds like luteolin, apigenin, epigallocatechin-3-gallate and resveratrol (Khan et al., 2014). Also the effects of propolis and the phenolic compounds present in propolis sample (caffeic acid, dihydrocinnamic acid, and *p*-coumaric acid) were investigated on human laryngeal epidermoid carcinoma (HEp-2)

cells. Results showed that caffeic acid induced late apoptosis or necrosis in HEp-2 cells, while propolis induced apoptosis, both probably due to ROS generation (da Silva et al., 2017). Another example could be thymol which was evaluated on bladder cancer cells trying to underlying its mechanism. In this case, thymol inhibited bladder cancer cell proliferation in a dose and time-dependent manner and induce the generation of ROS (Li et al., 2017).

3.4. Apoptosis Regulation by Ground Pistachio Kernel Extractson MCF-7 cells

Apoptosis is considered a vital component of various processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, embryonic development and chemical-induced cell death (Elmore, 2007). The ability to modulate the life or death of a cell is recognized for its immense therapeutic potential (Elmore, 2007), so induction of apoptosis represents an efficient strategy for cancer chemotherapy to evaluate and design new chemotherapeutic agents (Taraphdar et al., 2001). In order to determine the level of live, dead cells and those in early apoptotis, MCF-7 breast cancer cells were treated with different concentrations of the ground pistachio kernel extracts for 48h and analyzed by Tali® Image-based Cytometer. As shown in **Figure 3**, the addition of the extracts significantly induced the level of dead cells compared to the control ($p<0.05$) and significantly reduce the proportion of cells in early apoptosisompared to the control ($p<0.05$). A possibility explaining these differences could be that the treatments quickly induces apoptosis. If the apoptotic process runs very fast after the treatments in most of cells, a great number of them would be in later apoptotis state at the moment of the analysis and they would be quantified as dead cells. However, other apoptosis-independent cell death mechanisms could be responsible for the increase in dead cell observed after the different treatments at least in part. In some cases the type of stimuli and/or the degree of stimuli determines if cells die by apoptosis or other cell death mechanisms (Elmore, 2007). In fact, different authors have traditionally addressed the issue of distinguishing apoptosis from necrosis, two processes that can occur independently, sequentially, as well as simultaneously (Hirsch, 1997; Zeiss, 2003). Many phenolic compounds inhibit the proliferation in different cancer cell lines inducing apoptosis. An example could be fraxetin that inhibits the proliferation of the MCF-7 breast cancer cell line and the

induction of apoptosis may be achieved by upregulating FasL, Fas and Bax and downregulating Bcl-2 (Liu et al., 2017). Others can directly stimulate other programmed cell death (i.e., necroptosis, autoptosis), such it will be discussed bellow. In absence of apoptosis, cell death has been traditionally considered to occur by necrosis which is unplanned and unregulated form of death, and consequently, it is considered undesirable. It is further thought that necrosis was inherently undesirable in terms of cancer therapy by two facts (Zong & Thompson, 2006). On one hand, it would result in the release of a number of immunostimulants leading to overstimulation of the immune system (Zong & Thompson, 2006). On the other hand, it can trigger the release of growth factors and cytokines leading to increased tumor growth (Hanahan & Weinberg, 2011). Other recent studies have provided evidences indicating that the reported effects in the present study about ground pistachio kernel extracts concerning cell death could result beneficial. Firstly, different non-canonical cell death sharing features with necrosis have been currently established and even necrosis was reported to be highly regulated, occurring in a number of physiologic and pathophysiological situations (Vanden Berghe et al., 2014). Moreover, in some *in vivo* studies, chemotherapy-induced necrosis observed in most of cancers usually is not massive and not life-threatening as occurs in tumor lysis syndrome. Finally, stimulation of immune system has also shown to be positive after different treatment against cancer (Frey et al., 2014; Henderson et al., 2004; Brackett & Gollnick, 2011).

3.5. Glycolysis and Mitochondrial Respiration of Ground Pistachio Kernel Extracts on MCF-7 cells

In this section we have investigated the ECAR and the OCR measured by the Seahorse XF-24 Extracellular Flux Analyzer.

To determine the ECAR, cells were exposed to three different chemical reagents (rotenone, glucose and 2-DG). Rotenone is an electron transport chain (ETC) inhibitor that acts at complex I, thus it inhibits mitochondrial respiration; glucose is used to obtain the maximum glycolitic capacity (i.e., when the glycolysis it is not limited by substrate amount); and 2-DG is a competitive inhibitor of the production of glucose-6-P from glucose (step 2 of glycolysis), thus it inhibits glycolysis.

With respect to the OCR measurements, basal respiration is mainly driven by the concomitant re-entry pathways through the proton leak and ATP synthases. After that, cells were exposed consecutively to four modulators of oxidative phosphorylation, such as oligomycin A, FCCP and rotenone/antimicyn A. Oligomycin A stops the ATP synthase so that residual respiration is due solely to the proton leak (complex V). The addition of the protonophore FCCP causes an artificial proton conductance into the membrane. This maximal respiration is now regulated by the activity of the ETC and/or the delivery of substrate. At the end, rotenone/antimycin A which are ETC inhibitors were added and they act complex I and III, respectively. In this way, any residual respiration is non mitochondrial and needs to be subtracted from the other rates.

Regarding energetic metabolism, it was found that treatments with different concentrations of the ground pistachio kernel extracts led to a reduction in both ECAR and OCR with respect to untreated cells (**Figure 4a and 4b**). In addition, these differences were substantial in cells treated with higher doses (especially with the concentration of 5.0 mg/mL). These results suggest that phenolic compounds from ground pistachio kernel extracts inhibit not only mitochondrial respiration but also lactate production and glucose consumption (i.e. glycolysis) in a dose-dependent manner. Therefore, a considerable alteration of cell bioenergetics would occur as consequence of ground pistachio kernel extract treatment.

In general, cancer cells show higher reliance on glycolysis for their survival than normal cells. Thus, glycolysis inhibition alone might represent a harmful mechanism selective to cancer cells (Samudio et al., 2009; Koppenol et al., 2011). In fact, different anti-cancer agents have also shown to inhibit glycolysis. Indeed, multiple phenolics (such as, catechin, cyanidin, myricetin and quercetin) (Gao & Chen, 2015) have revealed this pattern. Despite many cancer cells types have exhibited dysfunctional mitochondria and have increased glycolysis (i.e., Warburg effect) (Warburg, 1956), some of them have been reported to produce energy through OXPHOS *in vivo*, whereas the surrounding stromal cells are highly glycolytic and fueling the cancer cells oxidative metabolism (i.e. “reverse-Warburg” effect) (Witkiewicz et al. 2012; Valencia et al., 2014). This phenomenon has been reported in metastasis but also in primary tumors (Bonuccelli et al., 2010). The existence of this phenomenon makes ground pistachio kernel extracts particularly interesting since the inhibition of mitochondrial respiration, evidenced by

the decrease in OCR, could contribute to the possible antitumor and antimetastatic effects of pistachio phenolic compounds. In any case, the alterations in both metabolic bioenergetic processes would presumably lead to a severe and rapidly depletion in cell energy reserves as consequence of the decrease in ATP production. An effect also found in 3-bromopyruvate-treated cells (Valenti et al., 2015).

The accumulation of ROS previously demonstrated with the addition of pistachio extracts could arise from two facts, a higher production of ROS or an inhibition of antioxidant systems **(Figure 5)**. The decrease in OCR can be consequence of mitochondrial ETC impairment which has associated with an increase in ROS generation at mitochondria (Verrax et al., 2011; Forbes et al., 2014). For instance, agents such as arsenic trioxide (As_2O_3) which impair the function of the respiratory chain are known to increase the production of superoxide (Pelicano et al., 2003). Therefore, the observed effect in mitochondrial respiration could be responsible at least in part of the ROS levels associated with ground pistachio kernel extract treatment found in the present model. It has been proposed that cancer cells are particularly sensitive to ROS overproduction since they usually exhibit an abnormal redox status associated with increased basal levels of ROS and frequent alterations of the antioxidant system (Verrax et al., 2011; Trachootham et al., 2009). Actually, ROS overproduction as consequence of mitochondrial ETC impairment has been described as killing mechanism for some chemotherapeutics. This has been exemplified by adaphostin that induces cancer cell death by inhibiting the complex III of the mitochondrial respiratory chain, leading to mitochondrial ROS generation (Le et al., 2007).

Despite of many mechanisms under this effect need to be elucidated, the putative reduction of cellular ATP levels and the increase in oxidative stress rapidly would induce cell death in an apoptosis-independent manner. In support of this, it has been reported that other chemotherapeutics with similar effects on cell metabolism and oxidative stress, such as 3-bromopyruvate increases cell death via necrosis rather than apoptosis (Eguchi et al. 1997, Ko et al. 2012, Qin et al. 2010).

Notwithstanding, it is unclear how ground pistachio kernel extracts exert this metabolic alteration. A possibility is that one or several phenolic compounds present in pistachio extracts directly inhibit some enzymes of glycolytic pathway and mitochondrial ETC complexes, particularly those which activities require oxygen consumption (i.e.

Complex III). In fact, some anti-cancer agents have shown to simultaneously inhibit several components of both processes (Valenti et al., 2015). Other possibility is that the extracts directly increase ROS production activating enzymatic systems as NADPH oxidases or promoting inorganic ROS formation. In addition, a decrease in the activity of endogenous antioxidant defenses including detoxifying enzymes or endogenous ROS scavenger compounds could lead to ROS accumulation in cells. In turn, oxidative stress could be responsible for mitochondrial dysfunction if oxidative damage to ETC complex proteins occurs. This could be also extent to enzymes participating in glycolysis.

4. Conclusions

Nuts and in particular pistachio as known present high amounts of phenolic compounds which can exert beneficial health effects against different common diseases.

In the last years, many research studies were focused on searching natural alternatives to improve current chemotherapy treatments. The remarkable phenolic content and the corresponding antioxidant capacity of the pistachio kernel polar extracts prepared in the current experimental work make them good candidates for evaluate its potential as chemotherapeutic in MCF-7 cells.

In the present study, this lyophilized extract was tested in MCF-7 breast cancer cell line. Different experiments have been performed in order to reveal their effects on cell viability, intracellular ROS level and apoptosis. The results here obtained suggest that phenolic compounds from ground pistachio kernel extracts would result cytotoxic for this model in a dose-(0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) and time-(48 and 72h) dependent manner. They would also induce an increase in cell ROS level and death. With regard to energetic metabolism, it was found that treatments at different concentrations led to a reduction in ECAR and OCR with respect to untreated cells, suggesting that phenolic compounds from pistachio extracts inhibit not only mitochondrial respiration but also lactate production and glucose consumption in a dose-dependent manner. The cell death observed in the present model seems to be responsible, at least in part, as a result of a combination of oxidative stress and bioenergetic alterations.

Pistachio kernel extract showed promising beneficial effects that could be exploited as a "natural adjuvant" in combination with chemotherapy treatment. Likewise, this kind of extracts could be used as an additive in the development of new food products, which would take into account not only particular sensorial features but also health benefits. Therefore, this study represents the starting point to test pistachio kernel extracts on human health. Further studies must be conducted in order to confirm these findings also in humans or animal models.

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Table and Figure Captions

Table 1a. Characterization of TPC and TAC of Sirora kernel and its lyophilized extract.

Table 1b. Main phenolic families determined in Sirora kernel and its lyophilized extract (g/kg FW).

Figure 1. Inhibition of cell proliferation by ground pistachio kernel extracts in MCF-7 cell line. After 24h of cell seeding, MCF-7 cells were treated with different concentrations of ground pistachio kernel extracts (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for (**A)** 24h, **(B)** 48h and **(C)** 72h. Cell viability was measured by using Tali® Image-Based Cytometer and results were expressed as a percentage (%) of live or dead cells compared to control cells. Data are shown as the mean±SD of three independent experiments (n=3). Columns belonging to the same set of data with different superscript letters are significantly different from control ($p<0.05$).

Figure 2. Ground pistachio kernel extracts induces ROS generation in MCF-7 cells. MCF-7 cells were treated with different concentrations of ground pistachio kernel extracts (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 48h. Intracellular ROS levels were calculated by using CellROX® Orange assay kit and the Tali® Image-Based Cytometer was used to quantify ROS induction (% of propidium iodide positive) in MCF-7 cells following the treatment previously described. The blue line of the thumbnail histogram indicated the set threshold. Data are shown as the mean±SD. Columns with different superscript letters are significantly different from control ($p<0.05$).

Figure 3. Live, dead and early apoptosis levels in MCF-7 cells treated with different concentrations of ground pistachio kernel extracts (0.0, 0.5, 1.0, 2.0, 5.0 mg/mL) for 48h. Tali® Image-Based Cytometer was used to quantify live, dead and early apoptosis cells following the treatment previously described. Data are shown as the mean±SD.

Columns with different superscript letters are significantly different from control ($p<0.05$).

Figure 4a. Reduction of the glycolysis by ground pistachio kernel extracts in MCF-7 cells. ECAR was observed by using the Seahorse XF24 Extracellular Flux Analyzer. All data are expressed as the mean±SD of three experiments (n=3).

Figure 4b. Regulation of mitochondrial respiration by ground pistachio kernel extracts in MCF-7 cells. OCR was observed by using the Seahorse XF24 Extracellular Flux Analyzer. All data are expressed as the mean±SD of three experiments (n=3).

Figure 5. Schematic representation of the possible mechanism of action of ground pistachio kernel extracts in MCF-7 cells. Abbreviations: **ROS** (Reactive Oxygen Species); **I** (Complex I); **II** (Complex II); **III** (Complex III); **IV** (Complex IV); **V** (Complex V); **cytC** (cytochrome C); **CoQ** (Coenzyme Q).