Chemical composition of *Lycium europaeum* fruit oil obtained by supercritical CO_2 extraction and evaluation of its antioxidant activity, cytotoxicity and cell absorption

Running title: Chemical composition and bioactivity of *Lycium europaeum* fruit oil

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ABSTRACT

We studied the total phenols and flavonoids, liposoluble antioxidants, fatty acid and triacylglycerol profiles, and oxidative status of oil obtained from *Lycium europaeum* fruits following supercritical CO_2 extraction (at 30 MPa and 40 °C). Linoleic (52%), palmitic (18%), oleic (13%), and α-linolenic (6%) were the main oil fatty acids, while trilinolein and palmitodilinolein/oleodilinolein represented the main triacylglycerols. The oil was characterized by high levels of all-trans-zeaxanthin and all-trans-β-carotene (755 and 332 μg/g of oil, respectively), α-tocopherol (308 μg/g of oil), total phenols (13.6 mg gallic acid equivalents/g of oil), and total flavonoids (6.8 mg quercetin equivalents/g of oil). The oil showed radical scavenging activities (ABTS and DPPH assays) and inhibited Caco-2 cell growth. Moreover, the incubation of differentiated Caco-2 cells with a non-toxic oil concentration (100 μg/mL) induced a significant intracellular accumulation of essential fatty acids. The results qualify *L. europaeum* oil as a potential source for food/pharmaceutical applications.

Keywords: *Lycium europaeum* oil; Essential fatty acids; Antioxidants; Radical scavenging activity; Cytotoxicity.

1. Introduction

The genus *Lycium* consists of about 70 species of spiny shrubs and small trees (Solanaceae) growing in temperate and subtropical regions (Fukuda, Yokoyama, & Ohashi, 2001). Like other genera in the Solanaceae, the vegetative plant parts are poisonous, though the berries are edible (Hummer et al., 2012). The fruits of two closely related species, *L. barbarum* and *L. chinense*, popularly known as "Goji", have long been used in traditional Chinese medicine and cuisine as functional food (Potterat, 2010). *L. barbarum* fruits have also become popular in western countries for their supposed beneficial effect in the prevention of chronic diseases (cancer, atherosclerosis and diabetes), promotion of weight loss and general longevity (Yu et al., 2005; Inbaraj, Lu, Hung, Wu, Lin, & Chen 2008). Since 2005, the production and sales of these products have increased rapidly, because nutritionists have described the berry (goji berries) as an 'exotic superfood' (Hummer et al., 2012). Now gojiberries are processed for juice and juice combination drinks, dried in tea, and as nutraceutical supplements (Hummer et al., 2012). Dried fruits can be eaten raw and used in confectionary goods or in bakery products, added to trail mix, cereals, muffins, energy bars or soups (Gao, Ali, & Khan, 2008; Hummer et al., 2012). Many functional components in *L. barbarum* fruits, including flavonoids, carotenoids, polysaccharides, glycolipids and their homologues, have been reported to be closely associated with the health-enhancing effect (Wang, Chang, Inbaraj, & Chen, 2010; Potterat, 2010; Yu et al., 2005; Yao, Peng, Xu, Li, Wu, & Xiao, 2011; Gao et al., 2008). Polysaccharides represent quantitatively the most important group of substances in the fruit (23% based on the dried weight) (Potterat, 2010). *L. barbarum* fruits contain large quantity of carotenoids, a group of lipid-soluble compounds with color ranging from yellow to red, that have been demonstrated to be effective in preventing chronic diseases such as cardiovascular disease and skin cancer (Wang et al., 2010; Inbaraj, Lu, Hung, Wu, Lin, & Chen, 2008). The carotenoid pigments of gojiberries mainly include zeaxanthin, β-carotene and β-cryptoxanthin (Potterat, 2010; Wang et al., 2010;

Inbaraj et al., 2008). Moreover, the seeds of *L. barbarum*, that account for 4% of the total fruit mass, contain abundant oil, more than 84-88% of which are unsaturated fatty acids such as linoleic (18:2 n-6), oleic (18:1 n-9), and linolenic (18:3 n-3) acids (Li et al., 2011; Guoliang et al., 2011). *L. barbarum* seed oil exhibited excellent antioxidant activities in DPPH radical scavenging assay and β-carotene bleaching test (Li et al., 2011).

The genus *Lycium* is represented in Italy by 5 taxa (Conti, Abbate, Alessandrini, & Blasi, 2005), and *L. europaeum* is the only one spontaneous species in some regions. *L. europaeum*, a phanerophyte shrub, is a Mediterranean medicinal plant (Atzei, 2009; Pieroni, Nebel, Quave, Münz, & Heinrich, 2002; Touati, Chliyeh, Ouazzani Touhami, Benkirane, & Douira, 2013; Turker, Kizilkaya, Cevik, & Gonuz, 2012). The berries of *L. europaeum* (boxthom) are traditionally consumed in Mediterranean regions for its health benefiting properties in the prevention of several diseases (Touati et al., 2013; Turker et al., 2012) and as food in Turkey during autumn (Turker et al., 2012). Extracts obtained from *L. europaeum* fresh fruits exhibited free radical scavenging capacities, showed the ability to protect lipids, proteins and DNA against H_2O_2-induced oxidative damage, and were found to contain appreciable amount of beneficial health phytochemicals, like phenolic compounds and carotenoids (Turker et al., 2012; Ghali, Vaudry, Jouenne, & Marzouki, 2015). Moreover, *L. europaeum* hydro-alcoholic fruit extract displayed cytotoxic activity on cancer cells (Ghali et al., 2015).

To the best of our knowledge, there is no prior report on lipid composition, nutritional properties and biological activity of *L. europaeum* fruit oil. There is actually a great attention to assess the functional lipid characteristics and potential health properties of nonconventional vegetable oils obtained from traditional medicinal/food plants and herbs for the unique chemical properties of their constituents and nutraceutical potential (Alasalvar, Amaral, & Shahidi, 2006; Uluata & Ozdemir, 2012).

The aim of the present work was to study the chemical composition, the antioxidant profile, and the biological/nutritional properties of the fixed oil extracted from whole fruits of

L. europaeum collected in the island of Sardinia (Italy). The oil was obtained by supercritical fluid extraction with CO_2 (SFE-CO_2), an alternative, environmentally benign, separation technique, amply used to obtain essential and fixed oils from spices/plants (Piras, Rosa, Falconieri, Porcedda, Dessì, & Marongiu 2009; Piras et al., 2013). Total flavonoids, total phenols, liposoluble antioxidants (carotenoids and α-tocopherol), fatty acid and triacylglycerol profiles, and oxidative status were determined in the extracted *L. europaeum* fixed oil. Total free radical-scavenging capacity of the oil was determined by ABTS and DPPH assays. The oil was also tested in Caco-2 cells to evaluate the effect on cell viability and in differentiated Caco-2 cell monolayers (intestinal epithelium model) for the lipid profile modulation, as a preliminary study of its bioavailability and potential health benefits.

2. Materials and methods

2.1. Chemicals

Standards of fatty acids and fatty acid methyl esters, carotenoids (all-trans-zeaxanthin, all-trans-β-carotene, β-cryptoxanthin), α-tocopherol, cholesterol, trilinolein (LLL), triolein (OOO), tripalmitin (PPP), trilinolenin (LnLnLn), 1,2-dilinoleoyl-3-palmitoyl-rac-glycerol (LLP), 1,2-dilinoleoyl-3-oleoylrac-glycerol (LLO), 1,2-dioleoyl-3-linoleoy-rac-glycerol (OOL), of purity greater than 98%, Desferal (deferoxamine mesylate salt), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Folin-Ciocalteau's phenol reagent, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox, and all solvents used, of the highest available purity, were purchased from Sigma–Aldrich (Milan, Italy). The abbreviations used for the fatty acids in triacylglycerols (TAGs) are: L, linoleic; Ln, linolenic; O, oleic; P, palmitic. The methanolic HCl (3 N) was purchased from Supelco (Bellefonte, US). *cis,trans*-13-Hydroperoxyoctadecadienoic acid (*c,t*-13-HPODE) and *cis,trans*-9-hydroperoxyoctadecadienoic acid (*c,t*-9-HPODE) were obtained from Cascade (Cascade Biochem. Ltd., London, UK). Cell culture materials were purchased from Invitrogen (Milan, Italy). All the chemicals used in this study were of analytical grade.

2.2. Plant materials and SFE extraction

Mature fruits of *L. europaeum* were collected in September 2013 in the Santa Gilla pond area (Cagliari, Sardinia Island, Italy); the latitude and longitude of the collection site were 39° 13' 27,69" N and 9° 01' 29,69" E, respectively. The berries, collected from plants of the same population, were air dried in the absence of light for 20 days. A voucher specimen was deposited at the General Herbarium of the Department of Life and Environmental Sciences, University of Cagliari (Herbarium CAG 993). Before use, the vegetable matter was ground with a Malavasi mill (Bologna, Italy) to achieve particles sizes in the range 250–425 μm.

Supercritical CO_2 extraction of dried and ground *L. europaeum* (190 g) whole fruits was performed in a laboratory apparatus, equipped with a 320 cm^3 extraction vessel and a 200 cm^3 separator vessel connected in series. Extraction was carried out in a semi batch mode: batch charging of vegetable matter and continuous flow solvent. Extraction of *L. europaeum* oil was performed at 30 MPa and 40 °C for 4 h in the extraction vessel, and at 2 MPa and 15 °C in the separator to recover the extract.

2.3. Total polyphenol and flavonoid contents

Total phenolic content (TPC) of the fixed oil was evaluated using the Folin–Ciocalteu reagent (Singleton & Rossi, 1965). TPC value was evaluated by measuring the absorbance at 750 nm in a Cary 50 UV-VIS spectrophotometer (Varian, Palo Alto, USA). Gallic acid was used as standard, and the results were calculated as gallic acid equivalents (GAE)/g of oil extract. The total flavonoid content (TFC) in the oil was determined by aluminium chloride colorimetric method (Zhishen, Mengcheng, & Jianming, 1999). The absorbance of the resulting reaction mixture was measured at 415 nm. Quantification of flavonoids was done on the basis of standard curve of quercetin prepared in 80% ethanol (EtOH) and the results were expressed in mg of quercetin equivalents (QE)/g of oil extract.

2.4. DPPH scavenging capacity assay

The $DPPH^{\bullet}$ radical scavenging activity of the oil was analyzed according to the procedure previously described (Matos et al., 2015). The oil, in dimethyl sulfoxide (DMSO) solution, was added to a mixture of 100 mM acetate buffer (pH 6.5) and 0.3 mM DPPH in EtOH in a cuvette, and left at room temperature, in the dark, for 15 min. The absorbance of the resulting solutions was measured at 515 nm. The results were expressed as the concentration of the oil needed to scavenge the 50% of initial $DPPH^{\bullet}$ radicals (EC_{50}). Trolox was considered as a positive control.

2.5. ABTS radical scavenging assay

The ABTS method is based on the capacity of an antioxidant to scavenge the free $ABTS^{\bullet+}$ and was performed as previously reported (Re, Pellegrini, Proteggente, Pannala, Yang, & Rice-Evans, 1999). Absorbance at 734 nm was measured 1 min after mixing different concentrations of *L. europaeum* fixed oil (0.5-2.5 mg/mL) with $ABTS^{+}$ solution. The $ABTS^{\bullet+}$ scavenging capacity was expressed as the concentration of oil necessary to give a 50% reduction in the original absorbance (EC_{50}). Trolox was used as a positive control.

2.6. Fixed oil saponification

Separation of fatty acids, α-tocopherol, and carotenoids was obtained by mild saponification as follows: 100 μL of Desferal solution (25 mg/mL of H_2O), 1 mL of a water solution of ascorbic acid (25% w/v), and 0.5 mL of 10 N KOH were added to aliquots (3 mg) of *L. europaeum* fixed oil in EtOH solution (Rosa et al., 2012). The mixtures were left in the dark at room temperature for 14 h. After the addition of n-hexane (10 mL) and H_2O (7 mL), samples were centrifuged for 1 h at 900g. The hexane phase, containing the unsaponifiable fraction (with carotenoids and α-tocopherol), was collected and the solvent was evaporated. A portion of the dried residue was dissolved in methanol (MeOH) (300 μL) and injected into the high-performance liquid chromatography (HPLC) system. Further n-hexane (10 mL) was added to the mixtures, samples were acidified with 37% HCl to pH 3–4 and then centrifuged for 1 h at 900g. The hexane phase (saponifiable fraction, with free fatty acids and conjugated diene fatty acid hydroperoxides, HP) was collected, the solvent was evaporated and the dried residues was dissolved in acetonitrile (CH_3CN) with 0.14% acetic acid (CH_3COOH) (v/v) (Rosa et al., 2012). Aliquots of the samples were injected into the HPLC system. An aliquot of dried fatty acids was methylated with methanolic HCl (3 N) for 30 min at room temperature (Christie, 1993; Rosa et al., 2012). Fatty acid methyl esters, in n-hexane solution, were injected into the GC system. All solvent evaporation was performed under vacuum.

2.7. Analyses of oil unsaturated fatty acids, carotenoids, α-tocopherol, and hydroperoxides

Analyses were carried out with an Agilent Technologies 1100 liquid chromatograph equipped with a diode array detector (DAD) and an Infinity 1260 evaporative light scattering detector (ELSD) (Agilent Technologies, Palo Alto, USA). Carotenoids and α-tocopherol were measured with the use of an Inertsil ODS-2 column, 150 × 4.6 mm, 5 μm particle size (Superchrom, Milan, Italy), and MeOH as the mobile phase, at a flow rate of 0.7 mL/min. Carotenoids were detected at 450 nm and α-tocopherol at 292 nm (Rosa, Atzeri, Putzu, & Scano, 2016). The column temperature was maintained at 37 °C. The identification of carotenoids (all-trans-zeaxanthin, all-trans-β-carotene, and β-cryptoxanthin) and α-tocopherol was performed by comparing retention times and conventional UV spectra of unknown peaks with reference standard compounds and data reported in the literature. Using the chromatography conditions developed, the retention times RT were: 5.0 min, 10.2 min, 14.1 min, and 41.8 min for all-trans-zeaxanthin, α-tocopherol, β-cryptoxanthin, and all-trans-β-carotene, respectively. Quantification of compounds was made from peak area ratio, which was based on a calibration curve (in the amount range of 100-1000 ng) generated from standard compounds in MeOH solution. Analyses of fatty acids (unsaturated were detected at 200 nm, saturated with ELSD) and HP (detected at 234 nm), were carried out with a XDB-C_{18} Eclipse column equipped with a Zorbax XDB-C_{18} Eclipse guard column (Agilent Technologies), with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (75/25/0.12, v/v/v), at a flow rate of 2.3 mL/min, as previously described (Rosa et al., 2012; Rosa, Murgia, Putzu, Meli, & Falchi, 2015). Recording and integration of the chromatogram data were carried out through an Agilent OpenLAB Chromatography data system. Calibration curves of all of the compounds (correlation coefficients > 0.995) were found to be linear for DAD detector and exponential for ELSD.

Fatty acid methyl esters were measured on a gas chromatograph Hewlett-Packard HP-6890 (Hewlett-Packard, Palo Alto, USA) with a flame ionization detector and equipped with a cyanopropyl methyl-polysiloxane HP-23 FAME column (Rosa et al., 2012). The composition

of individual fatty acid was calculated as a percentage of the total fatty acid amount (g %), using the Hewlett-Packard A.05.02 software.

2.8. Oil triacylglycerol composition

Aliquots of fixed oil were dissolved in EtOH (2 mg/mL). Analyses of triacylglycerol (TAGs) were carried out with an HPLC 1100 equipped with 1260 Infinity ELSD; separation was performed with an Inertsil ODS-2 column, and MeOH as the mobile phase, at a flow rate of 2 mL/min (Rosa et al., 2015). Standard solutions of TAGs (LnLnLn, LLL, LLO, LLP, OOL, OOO, and PPP) were prepared in MeOH. Quantification was based on the internal normalization method, assuming that the detector response was the same for all compounds (Amaral, Cunha, Seabra, Alves, Oliveira, & Pereira, 2004). Moreover, the use of equivalent carbon number (ECN) allowed the attribution of compounds of which no analytical standards were found. ECN was calculated according to the equation ECN = CN – 2DB, where CN is the number of carbon atoms and DB is the number of double bonds (Lísa & Holcapek, 2008).

2.9. Cell cultures

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC) (Salisbury, UK). Caco-2 cells were obtained from a human colon adenocarcinoma; after confluence, these cells spontaneously differentiated into enterocytes (Travelin, Gråsjö, Taipalensuu, Ocklind, & Artursson, 2002). Subcultures of the Caco-2 cells were grown in T-75 culture flasks and passaged with a trypsin-EDTA solution. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and penicillin (100 units/mL)–streptomycin (100 μg/mL), at 37 °C in 5% CO_2 incubator. For this study, cells on days 0-4 after plating were considered undifferentiated and fully differentiated on days 21.

2.10. Cytotoxic activity: AlamarBlue assay

The cytotoxic effect of fixed oil was evaluated in cancer (undifferentiated) Caco-2 cells and differentiated Caco-2 cells (small intestine enterocytes) by the AlamarBlue assay (O'Brien, Wilson, Orton, & Pognan). Caco-2 were seeded, for experiments in cancer cells, in 96-well plates at a density of 5×10^4 cells/mL in 100 μL of medium and cultured overnight. Caco-2 were seeded, for experiments in differentiated cells, in 24-well plates at a density of 5×10^4 cells/mL, and culture medium was replaced 3 times a week for 21 days until complete differentiation of the cells. Cells were subsequently exposed to various concentrations of the oil (25-500 μg/mL, from a 20 mg/mL solution in EtOH) in complete culture medium and incubated for 24 h. An equivalent volume of EtOH was added to the controls, the maximal final concentration of EtOH was 2.5%. After washing, cells were subjected to the AlamarBlue test (Rosa et al., 2012). After 24 h of incubation, the absorbance was measured at 570 and 600 nm with an Infinite 200 auto microplate reader (Tecan, Austria). The percentage of AlamarBlue reduction was calculated and compared to the controls.

2.11. Cytotoxic activity: MTT assay

The cytotoxic effect of *L. europaeum* fixed oil was also evaluated in cancer Caco-2 cells by the MTT assay (Schiller, Klainz, Mynett, & Gescher, 1992; Rosa et al., 2012). Caco-2 cells were seeded in 96-well plates at a density of 5×10^4 cells/mL in 100 μL of medium and cultured overnight. Cells were subsequently incubated (for 24 h) with aliquots of the oil (25-500 μg/mL, in EtOH solution) in complete culture medium; an equivalent volume of EtOH was added to the controls, the maximal final concentration of EtOH was 2.5%. After washing, cells were subjected to MTT test (Rosa et al., 2012). After incubation (4 h), color development was measured at 570 nm with an Infinite 200 auto microplate reader. The absorbance was proportional to the number of viable cells.

2.12. Fatty acid profile modulation in differentiated Caco-2 cells

Caco-2 cells were plated in Petri dishes at a density of about 10^6 cells/10 mL of complete medium and were used for fatty acid profile modulation experiments at 21 days post-seeding; culture medium was replaced 3 times a week until complete differentiation of cells. The differentiated Caco-2 cells were treated with fixed oil (100 μ*g*/mL, in EtOH solution) for 24 h. An equivalent volume of EtOH was added as a control to cells (the maximal final concentration was 0.5%). After treatment, the cells were scraped and centrifuged at 1200*g* at 4 °C for 5 min; the pellets were then separated from supernatants and used for lipid extraction and analyses (Rosa et al., 2012). Total lipids were extracted from differentiated Caco-2 cell pellets using the $CHCl_3$/MeOH 2:1 mixture (Folch, Lees, & Sloane-Stanley, 1957). Separation of lipid components (cholesterol and fatty acids) in differentiated Caco-2 cells was obtained by mild saponification of the $CHCl_3$ fraction (Rosa et al., 2012). The unsaponifiable (cholesterol) and saponifiable (fatty acids) fractions were collected, the solvent was evaporated, and the dried residues were dissolved in MeOH and CH_3CN with 0.14% CH_3COOH (v/v), respectively. Aliquots of these solutions were injected into the HPLC system. Cholesterol, detected at 203 nm, was measured with a Superchrom Inertsil ODS-2 column, and MeOH as the mobile phase, at a flow rate of 0.7 mL/min. Analysis of cell fatty acids was carried out as described for fixed oil fatty acids (Rosa et al., 2012; Rosa, Murgia, Putzu, Meli, & Falchi, 2015).

2.13. Statistical analyses

Evaluation of statistically significant differences was performed by one-way analysis of variance (One-way ANOVA), followed by the Bonferroni Multiple Comparisons Test, using Graph Pad INSTAT software (GraphPad software, San Diego, USA).

3. Results and discussion

3.1 Fatty acids, triacylglycerols, and liposoluble antioxidants of L. europeaum oil

The oil extract was obtained from *L. europaeum* whole fruits by SFE-CO_2 extraction (at 30 MPa and 40 °C) and the yield, expressed as the percentage by weight of the oil with respect to the weight of the material charged in the extractor, was 1.2% (g/100 g of dried berries). This SFE condition has been previously used for the extraction of fixed oil from different vegetable matrices (Piras et al., 2009; Piras et al., 2013). Moreover, similar SFE condition (30 MPa and 45 °C) represented the optimum extraction condition to obtain *L. barbarum* seed oil (Gouliang et al., 2011). Quali-quantitative information on the individual fatty acids that compose the lipid classes of *L. europaeum* fixed oils was obtained by GC (chromatographic profile, Fig. S1A) and HPLC analyses with DAD and ELSD detection (chromatographic profiles, Fig. S1B). Fatty acid composition (expressed as % of total fatty acids, g/100 g) of *L. europaeum* fixed oil obtained by GC analysis is reported in Table 1. The oil showed a concentration of approximately 21% of saturated fatty acids (mainly palmitic acid 16:0, and stearic acid 18:0, 18 and 2%, respectively), 17% of monounsaturated (mainly oleic acid 18:1 n-9 and palmitoleic acid 16:1 n-7, 13 and 2%, respectively), and 60% of polyunsaturated, mainly constituted by linoleic acid 18:2 n-6 and linolenic acid 18:3 n-3, 52 and 6%, respectively. *L. europaeum* fixed oil showed a composition characterized by a high ratio of unsaturated (77%) to saturated fatty acids (21%), with a high content of polyunsaturated fatty acids, similar to that of wheat germ and black cumin seed oils, obtained by CO_2-SFE in previous experiments at the same experimental condition and reported in Table 1 for comparison (Piras et al., 2009; Piras et al., 2013). *L. europaeum* fixed oil showed a peculiar composition, different from that of olive and rape seed oils characterized by a high ratio of monounsaturated (79% and 71%, respectively) to polyunsaturated fatty acids (8% and 24%, respectively) (Ong and Goh, 2002). The fatty acid profile of *L. europaeum* fruit oil was

306 comparable to that previously reported for *L. barbarum* seed oil (Guoliang et al., 2011)
307 obtained by different extractive procedures. *L. europaeum* oil exhibited a significantly high
308 content of the essential fatty acids 18:2 n-6 and 18:3 n-3 (total value 58%), compounds that
309 cannot be synthesized *de novo* by humans, indispensable for human development and health
310 (Hornstra 2000).

311 Furthermore, the absolute content of the main unsaturated fatty acids in the *Lycium* oil
312 extract was detected by HPLC, as follows: approximately 208 mg/g of oil extract for 18:2 n-6,
313 and minor amount for 18:1 n-9, 16:0, and 18:3 n-3 (58, 35, and 30 mg/g of oil, respectively)
314 as reported in Table 2. The oil oxidative status was evaluated by HPLC determination of the
315 HP level. The oil showed an average HP content of 13.50 ± 0.43 μmol/g of fixed oil. Table 2
316 also shows the content of the liposoluble antioxidants, carotenoids and α-tocopherol, in
317 saponified *L. europaeum* oil extract. All-trans-zeaxanthin was found to be present in largest
318 amount (754.62 μg/g), followed by all-trans-β-carotene (332.15 μg/g). Moreover, *L.*
319 *europaeum* oil contained trace of β-cryptoxanthin as well as some minor carotenoids, which
320 have not been definitively identified. Carotenoids amount in hydro-alcoholic fruit extract of *L.*
321 *europaeum* was previously estimated as 388.75 mg of β-catotene/g of dry extract (Ghali et al.,
322 20015). All-trans-zeaxanthin has been identified as the most abundant carotenoid in
323 saponified *L. barbarum* fruit extract (Inbaraj, Lu, Hung, Wu, Lin, & Chen 2008).
324 Furthermore, all-trans-zeaxanthin has been shown to be present in large quantity (83% of total
325 carotenoids) in the seeds of *L. barbarum* (Potterat 2010). Carotenoids have been demonstrated
326 to be effective in preventing chronic diseases such as skin cancer and cardiovascular disease
327 (Wang et al., 2010). There are many line of evidence supporting the protective role of
328 zeaxanthin against the development of age-related eye disease (Potterat, 2010). α−Tocopherol
329 (307.76 μg/g) was also identified in the *L. europaeum* oil extract.

330 A simple chromatographic method using a reversed-phase HPLC analysis combined with
331 ELSD detection was used for the primary evaluation of *L. europaeum* TAGs (HPLC-ELSD

chromatographic profile in Fig. S2). The identification of the main peaks was performed by the use of a standard TAGs mix and the comparison with the TAGs profiles of wheat germ (Amaral et al., 2004) and black cumin (Khoddami, Ghazali, Yassoralipour, Ramakrishnan, & Ganjloo, 2011) reference oils, obtained by SFE-CO_2 and analyzed in the same experimental conditions (Fig. S2). The main *L. europaeum* TAGs (Table 3) were LLL (39.87%) and OLL/PLL (39.43%). These data were, therefore, consistent with the fatty acid profile, which indicated over 50% linoleic acid content in the oil. Table 3 also shows some of the possible minor TAGs components.

Fats and oils play an important role in the food and pharmaceutical industries, being widely used for human nutrition and nutraceutical applications. There is an increasing interest in the lipid characteristics of nonconventional vegetable oils as they seem to be an interesting source of bioactive phytochemicals/functional nutrients (carotenoids, fat-soluble vitamins, essential unsaturated fatty acids, and phenols) with unique chemical properties and health benefits (Ramadan, Sharanabasappa, Seetharam, Seshagiri, & Moersel,; Alasalvar et al., 2006; Al Ashaal, Farghaly, Abd El Aziz, & Ali, 2010). SFE-CO_2 has been amply used for the extraction of fixed oils from vegetable matrices as an alternative to organic solvent-based extraction (Piras et al., 2009; Piras et al., 2013; Rosa et al., 2012). Supercritical CO_2 is an inert, non-toxic, environmentally safe solvent, and SFE extracts, generally recognized as safe (GRAS), are of superior quality for food products as compared to those obtained by conventional organic solvent extraction methods (Ghafoor, Park, & Choi, 2010).

The special fatty acid composition as well as the presence of valuable amounts of lipid-soluble bioactive compounds like carotenoids and α-tocopherol, make SFE *L. europaeum* fruit oil a special component for nutritional application. *L. europaeum* fruit oil appeared to be a rich source of valuable nutrients and functional components with potential health benefits.

3.2. Total phenolic and flavonoid contents

We also determined the total phenolic and flavonoid contents of *L. europaeum* fixed oil using the Folin–Ciocalteu reagent method and the aluminium chloride colorimetric assay, respectively. The total phenolic content (TPC) was expressed as milligrams of gallic acid equivalents per gram of oil extract (mg GAE/g oil) and the total flavonoid content (TFC) as milligrams of quercetin equivalents per gram of oil extract (mg QE/g oil). The results are summarized in Table 4. The oil was characterized by a relatively high amount of TPC (13.6 mg GAE/g of oil) and TFC (6.8 mg QE/g of oil). Fruits of *Lycium* species (in particular *L. barbarum* and *L. chinense*) have been reported to possess high levels of flavonoids and phenolic acids (Wang et al., 2010; Potterat 2010; Yu et al., 2005). TP values of 1.12 and 0.91 mg GAE/g of fresh fruit were found in the acetone and EtOH extracts, respectively, obtained from *L. europaeum* berries, while TFC values were detected as follows: 3.7 and 4.2 mg of rutina equivalents/g of fresh fruit in the acetone and EtOH extracts, respectively (Turker et al., 2012). The results of this study showed that *L. europaeum* berry oil is a good source of phenolic compounds, important group of dietary phytochemicals. Recently, the presence of a wide range of phenolic compounds in oils has been shown to contribute to their human health benefits, including anticancer, antiviral, antioxidant, hypoglycemic, hypo-lipidemic, and anti-inflammatory activities. (Alu'datt et al., 2017; Wang et al., 2010).

3.3 Radical scavenging activity

Free radical scavenging activity of *L. europaeum* fruit oil was determined by DPPH and ABTS scavenging assays. Results showed that the fixed oil was effective in reducing the stable radical $DPPH^{\bullet}$ to the yellow-colored diphenylpicrylhydrazine, indicating that this oil was active in $DPPH^{\bullet}$ radical scavenging. The fixed oil showed a lower activity in comparison to the positive control, with EC_{50} values of 1.28 mg/mL and 0.0072 mg/mL, respectively. This is not surprising as Trolox is a single molecule, whereas oil extract is a mixtures of numerous compounds. Thus, the real concentration of active compound is lower than the EC_{50} value. The ABTS assay confirmed the antioxidant activity of the *L. europaeum* fruit oil.

383 Fruits of *L. barbarum* and *L. chinense* have been shown to be rich in antioxidant
384 compounds (tocopherols, flavonoids, phenolic acids, and carotenoids) (Wang et al., 2010;
385 Potterat 2010; Yu et al., 2005). Several studies have been focused on the noticeable
386 antioxidant and free radical-scavenging activities of extracts and polysaccharides obtained
387 from *L. barbarum* fruits (Wang et al., 2010; Potterat 2010; Yu et al., 2005). A previous study
388 found an excellent antioxidant activity of *L. barbarum* seed oils, obtained by different
389 extraction methods, in DPPH radical scavenging assay (Gouliang et al., 2011). Moreover,
390 ethanolic, hydro-methanolic, and acetone extracts obtained from *L. europaeum* fruits
391 exhibited DPPH free radical scavenging capacities and the ability to protect lipids, proteins
392 and DNA against H_2O_2-induced oxidative damage (Turker et al., 2012; Ghali t al., 2015). Our
393 finding provides evidence that *L. europaeum* fruit oil is a source of natural antioxidants like
394 phenolic compounds, α-tocopherols, and carotenoids, responsible for the protective effect
395 observed in the DPPH and ABTS scavenging assays.

396 *3.4. Cytotoxic activity of fixed oil in cancer Caco-2 cells*

397 *L. europaeum* oil was evaluated for cytotoxicity in colon cancer Caco-2 cell cultures. Fig.
398 1A shows the cell viability, expressed as % of the control, induced in undifferentiated Caco-2
399 cells after 24 h of incubation in the presence of different concentrations of fixed oil (25-500
400 μg/mL) by AlamarBlue and MTT assays. The treatment with oil induced a significant
401 reduction in cell viability, in comparison with control, from the concentration of 50 μg/mL
402 (35% reduction) by MTT test and 100 μg/mL (28% reduction) by AlamarBlue assay. EtOH,
403 used to dissolve oil extract, was not toxic in cancer cells and cell viability, measured at the
404 maximal tested dose (2.5%), was 93 and 96% by AlamarBlue and MTT assays, respectively.
405 Colon cancer is one of the most common forms of cancer in the more developed countries and
406 epidemiological studies suggest an association between quantity and quality of dietary fat and
407 colon cancer risk (Sala-Vila, Folkes, & Calder, 2010). Undifferentiated Caco-2 cells, a line of

408 human colon adenocarcinoma, are an established cell line for oncological studies
409 investigating, for example, the anticarcinogenic effects of food constituents (Carvalho et al.,
410 2010). Several reports have been published on the growth inhibitory effect and
411 antiproliferative properties of fixed oils in carcinoma cell lines or in animal models (Al
412 Ashaal et al., 2010; Naqshbandi, Rizwan, & Khan, 2013; Rosa et al., 2012; Shih, Ho, Lia,
413 Yanga, Hou, & Cheng, 2011). Several plant-based oils rich in α-linolenic fatty acid 18:3 n-3
414 (canola and flaxseed oils) have been examined for their potential to modulate cancer cell
415 growth and death (Lin et al., 2013; Wang, Chen, & Thompson, 2005). This essential fatty acid
416 is the precursor for the formation of the long chain n-3 polyunsaturated fatty acids (n-3
417 PUFA) with anticancer activity (Gogus, & Smith, 2010). Evaluation of the cytotoxic activity
418 of *L. europaeum* oil revealed its ability to reduce viability in cancer Caco-2 cells, maybe
419 partly due to the oil content of 18:3 n-3 that accounted for 6% of total fatty acids. Aqueous
420 extracts and purified polysaccharide fractions obtained from *L. barbarum* fruits have been
421 shown to inhibit the growth of several cancer cell lines *in vitro* by apoptosis induction
422 (Potterat 2010; Wang et al., 2010; Yao et al., 2011). Moreover, the hydro-methanolic extract
423 obtained from *L. europaeum* fruits was found to inhibit cell proliferation and display
424 cytotoxic activity on human lung carcinoma cell line (A549) and rat pheochro-mocytoma
425 (PC12) (Ghali et al., 2015). The treatment of cancer cells with *Lycium* extract (MeOH:H_2O
426 80:20) induced a significant inhibition of cell viability, with a 60 and 80% viability reduction
427 in PC12 and A549 cells, respectively, at 100 μg/mL of extract.

428 *3.4. Cell viability and lipid profile of differentiated Caco-2 cells exposed to fixed oil*

429 *L. europaeum* fixed oil was tested in differentiated Caco-2 cell monolayers as an intestinal
430 epithelial cell model for the evaluation of the effect on cell lipid composition, as a preliminary
431 study of the intestinal absorption of oil components. The differentiated Caco-2 cells, retaining
432 many of the morphological and functional characteristics (such as brush border microvilli,

433 tight junctions, and dome formation) of normal small intestine polarized enterocytes, are
434 extensively used in the prediction of intestinal drug absorption and in studies of toxicity of
435 dietary lipids (Rosa et al., 2012; Travelin et al., 2002). In order to determine the non-toxic
436 dose, the oil effect on cell viability was preliminary monitored in Caco-2 cell monolayers by
437 the AlamarBlue assay, a method amply used for the evaluation of the toxic effect of natural
438 extracts and compounds in differentiated Caco-2 cells (Shappell, 2003). Fig. 1B shows the
439 viability (% control) induced in differentiated Caco-2 cells after 24 h-incubation in the
440 presence of different concentrations (25-500 μg/mL) of *L. europaeum* oil. The oil did not
441 show a toxic effect on differentiated Caco-2 cells in the tested concentration range; EtOH,
442 used to dissolve oil extract, did not affect cells viability. The present data demonstrate that *L.*
443 *europaeum* oil extract inhibited the growth of colon cancer cells without affecting, at the same
444 concentration range, viability in normal intestinal cells. It is worth noting that the hydro-
445 methanolic extract obtained from the fruits of *L. europaeum* exhibited the ability to reduce
446 viability and inhibit proliferation in cancer cells, whereas it did not show a significant
447 cytotoxic effect on normal rat cerebellum granule cells (Ghali et al., 2015).

448 *L. europaeum* fixed oil was then tested, at a non-cytotoxic concentration (100 μg/mL), in
449 differentiated Caco-2 for the evaluation of the cell lipid modulation. After 24 h of incubation
450 with the oil, cell lipid fraction was extracted and the variation of the levels of fatty acids and
451 cholesterol was analyzed with respect to control cells. Fig. 2 shows the fatty acid
452 chromatographic profiles obtained by HPLC analysis with DAD (Fig. 2A) and ELSD (Fig.
453 2B) detection and values (expressed as μg/plate) of main fatty acids and cholesterol (Fig. 2C)
454 measured in differentiated Caco-2 control cells and cell treated for 24 h with *L. europaeum*
455 fixed oil (LO, 100 μg/mL). By HPLC, the total cholesterol level was measured in control cells
456 as mean content of 43.00 ± 3.25 μg/plate. Furthermore, the content of the most abundant fatty
457 acids in control cell was detected by HPLC as follows: 81.03 ± 3.65 μg, 18.22 ± 0.71 μg,
458 15.77 ± 2.29 μg, 7.40 ± 0.62 μg, 5.28 ± 0.87 μg, 4.08 ± 0.13 μg/plate for 18:1 isomers, 18:2

459 n-6, 16:0, arachidonic acid (20:4 n-6), 16:1 n-7, and docosahexaenoic acid (DHA, 22:6 n-3),
460 respectively, as reported in Fig. 2C; minor amounts were measured for eicosapentaenoic acid
461 (EPA, 20:5 n-3, 3.88 μg/plate), eicosatrienoic acid (20:3, 2.81 μg/plate), and α-linolenic acid
462 (18:3 n-3, 2.39 μg/plate). The incubation of differentiated Caco-2 cells with *L. europaeum* oil
463 induced a significant change in the fatty acid composition, with a marked increase in the cell
464 levels of the essential fatty acids 18:2 n-6 and 18:3 n-3, that reached values of 2 and 2.3 times
465 higher than that of control cells, respectively. Also the cellular amounts of 16:0 and 18:1 n-9
466 significantly increased, although to a minor extent (approximately 170 and 120% of control
467 value, respectively). Incorporation of oil into cells did not induce a change in the levels of the
468 other fatty acids and cholesterol. The oil was able to induce significant modifications in cell
469 fatty acid composition, with an increase in the levels of the essential fatty acids, indicating a
470 process of absorption of these important bioactive components in intestinal cells. These
471 polyunsaturated fatty acids are important for the maintenance of biofunctions in mammals
472 (Hornstra, 2000; Ariffin et al., 2009). A significant accumulation of 18:3 n-3 and 18:2 n-6 has
473 also been observed in differentiated Caco-2 cells after 24 h-treatment with Maltese mushroom
474 (*Cynomorium coccineum*) fixed oil, characterized by high levels of 18:1 n-9 (38% of total
475 fatty acids), 18:2 n-6 (20%), and 18:3 n-3 (11%) (Rosa et al., 2012). The incorporation of *C.*
476 *coccineum* oil into cells was also associated with some increase in the cellular level of
477 intermediates involved in the process of elongation and desaturation of 18:3 n-3 (such as 20:3,
478 20:5 n-3, and 22:5 n-3) (Rosa et al., 2012). This has not been observed in differentiated Caco-
479 2 cells treated with *L. europaeum* oil, probably due to the different amounts of 18:3 n-3 (6%)
480 and 18:2 n-6 (52%) with respect to *Maltese mushroom* oil (Goyens, Spilker, Zock, Katan, &
481 Mensink 2006).

482

483

4. Conclusions

The chemical composition, nutritional and biological properties of fixed oil extracted by SFE from the fruits of *L. europaeum* were investigated. The oil showed high levels of lipid-soluble antioxidants (all-trans-zeaxanthin, all-trans-β-carotene, and α-tocopherol), total phenols and total flavonoids. The essential fatty acid 18:2 n-6 and TAGs derived from it were the major oil lipid constituents. The oil showed radical scavenging activity and induced a significant in vitro inhibitory effect on the growth of colon adenocarcinoma cells. Moreover, 24 h-treatment with oil extract induced, at a non-cytotoxic dose, significant modifications in the fatty acid profile of differentiated Caco-2 cells, indicating the intestinal absorption and bioavailability of *Lycium* oil essential fatty acids (18:2 n-6 and 18:3 n-3).

The results of this study showed remarkable nutritional/nutraceutical value of *L. europaeum* fixed oil, and qualify it as a potential resource for human nutrition/food fortification. Its consumption as a part of a normal diet could help in the maintenance of human health and prevention of several disease conditions.

Further studies are needed to optimized the SFE extraction condition in order to improve the percentage yield of the oil extracted form *L. europeum* fruits.

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Figure captions

Fig. 1. Viability, expressed as % of the control, induced by incubation for 24 h with different concentrations (25-500 μg/mL) of *Lycium europaeum* fixed oil in cancer and differentiated Caco-2 cells by AlamarBlue assay (A) and in cancer Caco-2 cells by MTT test (B). Three independent experiments are performed and data are presented as mean ± SD; *** = $P < 0.001$; ** = $P < 0.01$; * = $P < 0.05$ versus Control.

Fig. 2. Fatty acid chromatographic profile obtained by HPLC analysis with DAD (at 200 nm) (A) and ELSD detection (B), and values (expressed as μg/plate) of main fatty acids and cholesterol (Chol) (C) measured in differentiated Caco-2 control cells (Control) and cell treated for 24 h with *Lycium europaeum* fixed oil (LO, 100 μg/mL). Results were expressed as a mean ± standard deviation (SD) of three independent experiments involving duplicate analyses for each sample. ** = $P < 0.01$; * = $P < 0.05$ versus Control.

Table 1

Fatty acid composition (% of total fatty acids, g/100 g) by GC of *Lycium europaeum* fixed oil obtained by SFE (at 300 bar and 40 °C). Composition of wheat germ and black cumin seed oils obtained at the same experimental condition is reported for comparison.

Fatty acid			*L. europaeum*	Wheat germ[a]	Black cumin[b]
L	Lauric	12:0	0.03 ± 0.01	-	0.05 ± 0.01
M	Myristic	14:0	0.18 ± 0.04	0.16 ± 0.02	0.30 ± 0.03
P	Palmitic	16:0	18.41 ± 1.53	17.15 ± 0.09	13.51 ± 0.55
Po	Palmitoleic	16:1	2.02 ± 0.20	0.29 ± 0.05	0.26 ± 0.02
S	Stearic	18:0	2.43 ± 0.11	0.98 ± 0.07	2.83 ± 0.26
V	Vaccenic	18:1 *n-7*	0.84 ± 0.28	0.87 ± 0.05	0.48 ± 0.08
O	Oleic	18:1 *n-9*	13.53 ± 0.15	19.97 ± 0.09	22.53 ± 0.51
L	Linoleic	18:2 *n-6*	52.01 ± 1.29	51.55 ± 0.18	55.31 ± 0.67
Ln	α-Linolenic	18:3 *n-3*	6.49 ± 0.18	6.42 ± 0.09	0.25 ± 0.02
γLn	γ-Linolenic	18:3 *n-6*	1.63 ± 0.03	-	-
A	Arachidic	20:0	0.31 ± 0.08	0.36 ± 0.04	0.14 ± 0.01
Ec	Eicosenoic	20:1 *n-9*	0.58 ± 0.04	1.39 ± 0.01	0.24 ± 0.02
Dd	Docosadienoic	20:2	-	-	2.39 ± 0.18
	Saturated	SFA	21.36 ± 1.43	18.67 ± 0.04	16.86 ± 0.68
	Monounsaturated	MUFA	16.69 ± 0.49	22.53 ± 0.00	23.51 ± 0.49
	Polyunsaturated	PUFA	60.14 ±1.49	57.97 ± 0.27	58.15 ± 0.59

Oil analysis was performed in quadruplicate and all data are expressed as mean values ± standard deviations (sd); (n = 4). [a]Piras et al. (2009); [b]Piras et al. (2013).

Table 2

Composition of main fatty acids (expressed as mg/g oil) and liposoluble antioxidants (μg/g oil) of *Lycium europaeum* fixed oil measured by HPLC.

Compound	Amount
Fatty acids	*mg/g oil*
16:0	35.02 ± 1.65
16:1 *n-7*	2.88 ± 0.12
18:1 *n-9*	57.92 ± 2.57
18:2 *n-6*	208.54 ± 3.93
18:3 *n-3*	30.56 ± 0.11
18:3 *n-6*	6.60 ±0.07
Liposoluble antioxidants	*μg/g oil*
all-*trans*-zeaxanthin	754.62 ± 31.74
all-*trans*-β-carotene	332.15 ± 48.00
α-tocopherol	307.76 ± 80.90

Oil analysis was performed in quadruplicate and all data are expressed as mean values ± standard deviations (*sd*); (*n* = 4).

Table 3

Composition of main triacylglycerols (expressed as % oil) of *Lycium europaeum* fixed oil measured by HPLC-ELSD analysis.

Triacylglycerols	ECN	% Oil
LnLnLn	36	0.15 ± 0.01
LnLLn	38	0.24 ± 0.04
LLLn	40	3.58 ± 0.04
LLL	42	39.87 ± 0.46
OLLn/LnLP	42	1.65 ± 0.08
OLL/PLL	44	39.43 ± 0.24
OOL/POL	46	9.41 ± 0.14
POO/OOO	48	1.72 ± 0.56
Others	-	4.28 ± 0.54

Oil analysis was performed in quadruplicate and all data are expressed as mean values ± standard deviations (*sd*); (*n* = 4).

Table 4

Total phenol content (TPC), total flavonoid content (TFC) and antiradical effects of *Lycium europaeum* fixed oil.

	TPC (mg GAE/g oil)[a]	TFC (mg QE/g oil)[b]	ABTS ($EC_{50,}$ mg/mL)[c]	DPPH ($EC_{50,}$ mg/mL)[c]
Fixed oil	13.60 ± 0.60	6.78 ± 0.52	0.81 ± 0.01	1.28 ± 0.01
Trolox[d]	-	-	0.0013 ± 0.0004	0.0072 ± 0.0011

Each value represents the mean ± *sd* (*n* = 3).

[a]GAE: gallic acid equivalent. [b]QE: quercetin equivalent. [c]EC_{50} value is the concentration of antioxidant required to quench 50% radicals in the reaction mixture under the experimental condition. [d]Positive control

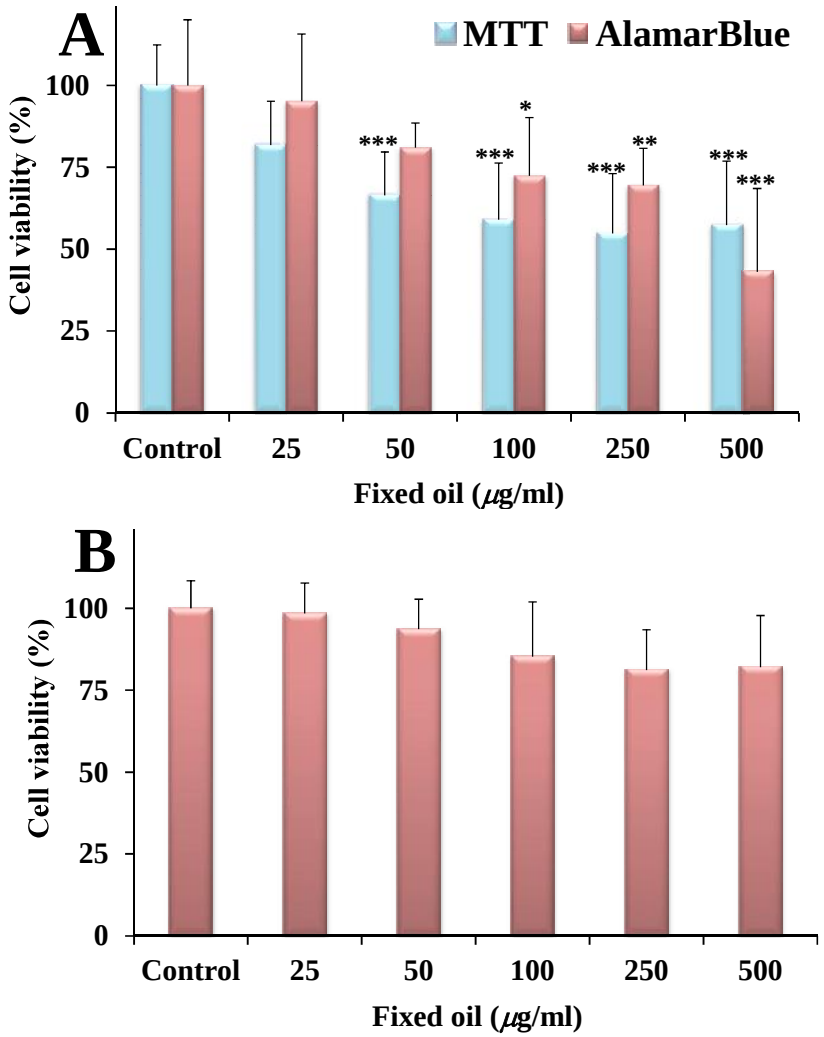
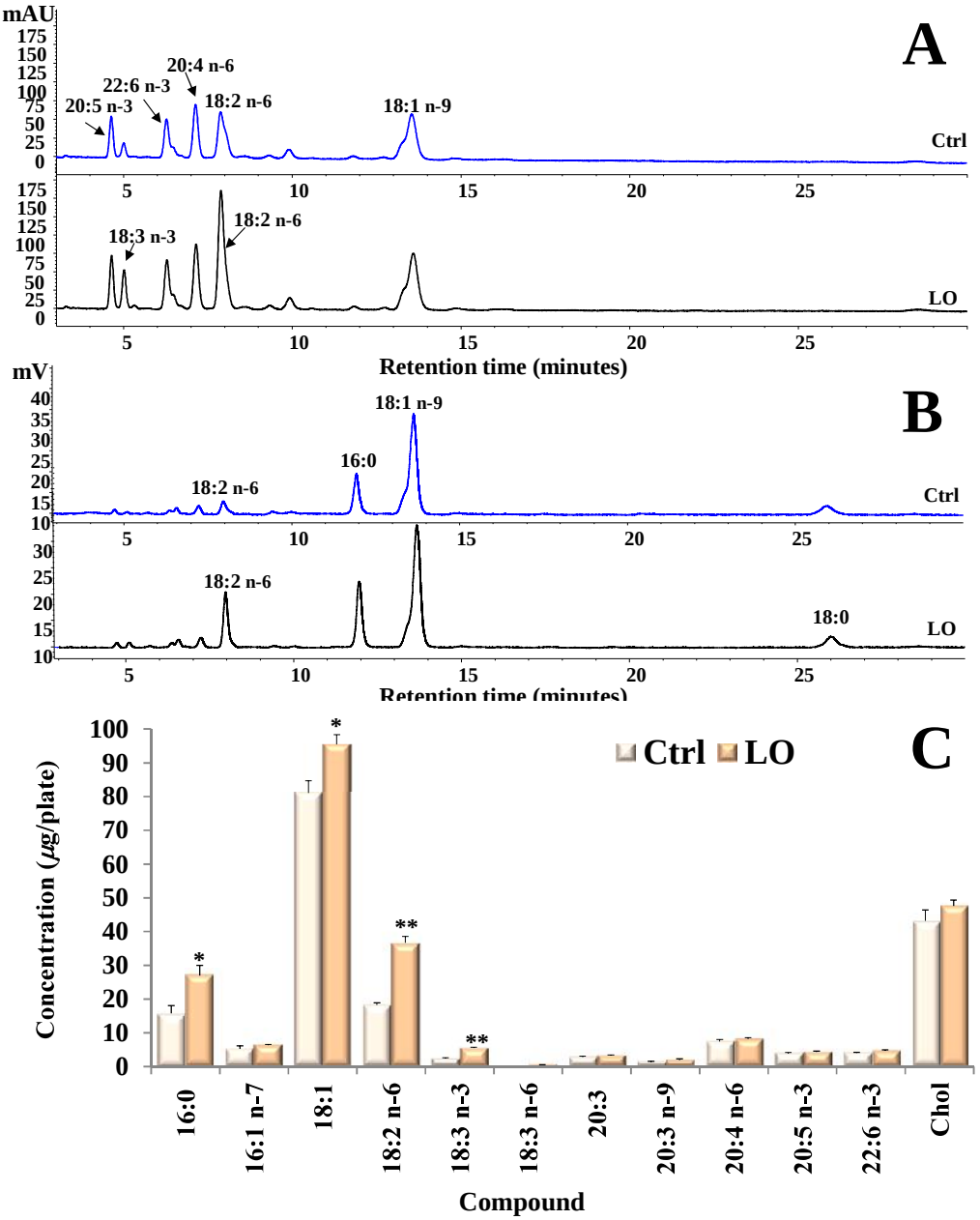

A
MTT
AlamarBlue
Cell viability (%)
100
75
50
25
0
***
***
*
***
**
***
***
Control
25
50
100
250
500
Fixed oil (μg/ml)
B
Cell viability (%)
100
75
50
25
0
Control
25
50
100
250
500
Fixed oil (μg/ml)

Fig. 1.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mAU
20:5 n-3
22:6 n-3
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18:2 n-6
18:1 n-9
Ctrl
18:3 n-3
18:2 n-6
LO
Retention time (minutes)
B
mV
18:1 n-9
16:0
18:2 n-6
Ctrl
18:2 n-6
18:0
LO
C
Ctrl
LO
Concentration (μg/plate)
16:0
16:1 n-7
18:1
18:2 n-6
18:3 n-3
18:3 n-6
20:3
20:3 n-9
20:4 n-6
20:5 n-3
22:6 n-3
Chol
Compound

Fig. 2.

Supplementary Material

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