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Lipid nanocarriers based on natural oils with high activity against oxygen free radicals and tumor cell proliferation

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ABSTRACT

The development of nano-dosage forms of phytochemicals represents a significant progress of the scientific approach in the biomedical research. The aim of this study was to assess the effectiveness of lipid nanocarriers based on natural oils (grape seed oil, fish oil and laurel leaf oil) in counteracting free radicals and combating certain tumor cells. No drug was encapsulated in the nanocarriers. The cytotoxic effect exerted by bioactive nanocarriers against two tumor cells, *MDA-MB 231* and *HeLa* cell lines, and two normal cells, *L929* and *B16* cell lines, was measured using the MTT assay, while oxidative damage was assessed by measuring *the* total antioxidant activity using chemiluminescence analysis. The best performance was obtained for nanocarriers based on an association of grape seed and laurel leaf oils, with a capacity to scavenge about 98% oxygen free radicals. A dose of nanocarriers of 5 $mg \cdot mL^{-1}$ has led to a drastic decrease in tumor cell proliferation even in the absence of an antitumor drug (e.g. about 50% viability for *MDA-MB 231* cell line and 60% viability for *HeLa* cell line). A comparative survival profile of normal and tumor cells, which were exposed to an effective dose of 2.5 $mg \cdot mL^{-1}$ lipid nanocarriers, has revealed a death rate of 20% for normal *B16* cells and of 40% death rate for *MDA-MB 231* and *HeLa* tumor cells. The results in this study imply that lipid nanocarriers based on grape seed oil in association with laurel leaf oil could be a candidate to reduce the delivery system toxicity and may significantly improve the therapeutic efficacy of antitumor drugs in clinical applications.

1. Introduction

Combining natural and synthetic remedies is very useful in biomedical research in order to develop a safety therapeutic strategy for a maximum treatment efficiency and low side effects [1,2]. Currently, aromatic plant species are primarily recommended and used to treat several diseases due to the therapeutic properties of their essential oils such as anti-inflammatory, antibacterial, anti-rheumatic, free radical-scavenging activities and antitumor activities [3–5]. Laurel essential oil isolated from leaves of *Laurus nobilis* L. (LLO) contains many active compounds including 1,8-cineole (eucalyptol — the major monoterpenoid from LLO), terpinyl-acetate, pinene, limonene and so on whose properties have been assessed for their efficacy and tolerability in various anticancer therapies. It was reported that nonpolar *Laurus nobilis* leaf extract had the ability to induce cytotoxicity and apoptosis towards SK-N-BE(2), SH-SY5Y and C6 brain tumor cell lines [6]. The antiproliferative activity of laurel volatile oil against human breast adenocarcinoma was proved by Jelnar et al. [7]. Other in vitro study demonstrated inhibition of cancer cell growth by laurel leaf oil in HT-29, HCT-116, Caco-2, and SW-480 human cancer cell lines, which were accompanied by variable levels of elevated apoptosis [8]. Both seed and leaf essential oils from laurel plant exhibited a scavenging effect on DPPH radical and inhibited proliferation of the K562 tumor cell line [9]. A comparative study claiming the effect of *Laurus* essential oil and its main components on α-glucosidase and reactive oxygen scavenging activity has revealed that inhibition of lipid peroxidation manifested by the essential oil was more pronounced than those of 1,8-cineole and limonene alone [10]. The synergistic effect of *Laurus nobilis* L. leaf extracts was also demonstrated by Kauroinovic et al. in an in vitro and in vivo antioxidant activity study [11].

In conjunction, the natural oils enriched in ω-3, −6, and −9 fatty acids completed the health-promoting effects of essential oils. Broad pharmacological activities of *grape seed oil* associated to its composition have already been demonstrated [12–14]; e.g. enhancing the cardiovascular health, combating the free radicals, reducing the symptoms in gastric ulcers, inhibiting the growth of certain types of cancers in cell cultures. The anticancer effects of grape antioxidants have been demonstrated in both in vitro and in vivo models [15,16]. The antioxidant compounds from grapes, especially proanthocyanidins have been shown to induce cell cycle arrest and apoptosis in cancer cells by activating DNA damage checkpoint cascade [17], and to inhibit angiogenesis in human breast cancer xenografts in vivo [18] as well as cancer progression in

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rodent models [19]. An irreparable DNA damage leading to G2/M arrest and apoptosis selectively in head and neck squamous cell carcinoma was also reported by Shrotriya et al. [20]. In addition, grape seed antioxidants were also found to selectively inhibit the human melanoma A375 and Hs294t cells, inhibiting over-expression of COX-2 and prostaglandin E2 receptors, or modifying estrogen receptor pathways, resulting in cell cycle arrest and apoptosis [21].

Many studies and significant efforts have been made to develop targeted delivery systems that can provide higher effectiveness in prevention and treatment of cancer with minimal effect on normal cells [22]. Lipid based nanoformulations, with particular emphasis on nanostructured lipid carriers (NLC) are among the most attractive strategies for encapsulation and delivery of antitumor drugs [23,24]. NLC are spherical particles with mean diameters in the 50–500 nm range, made by biocompatible lipids and surfactants [25,26], with proven safety and improved delivery features [27]. In their pure form, NLC are composed of a solid lipid core consisting of solid lipids blended with a liquid lipid [28], but new and efficient vegetable lipid based nanocarriers were recently reported by the authors for co-delivery of antioxidant, UV-A and UV-B filters [29,30]. The benefit of these nanocarriers with high content of vegetable active compounds is highly advantageous as the bioactive principles of phytochemicals can complement or even enhance the biological activity of the synthetic drug [31,32].

Conventional chemotherapy usually employs high doses of toxic antitumor drugs which often induce severe hazardous effects on healthy organs [33,34]. In addition, the delivery systems can contribute to the overall toxicity of a pharmaceutical product [35]. Alternatively, oral delivery of anti-cancer drugs is usually compromised by the low penetration and limited distribution [36] of the drug in solid tumors and its degradation during the first-pass metabolism [37]. Consequently, an ideal therapy for certain tumor cells could be based on the development of new approaches in delivery systems that can successfully meet both requirements, to improve drug and carrier safety and to provide complementary biological effect by using a combination of natural bioactive compounds and synthetic drugs.

Over the past decade solid lipid nanoparticles and nanostructured lipid carriers have been extensively investigated in the delivery of antitumor drugs such as oridonin [38], doxorubicin [39], methotrexate [40], and tetracyclines (CMT) [41]. However, to the best of our knowledge the positive/negative effect of delivery systems was not a topic of great interest. Although the pharmacological and biological effects of LLO have been thoroughly investigated in the literature, its presence inside lipid nanocarriers that could be directly associated with therapeutic efficacy has not been addressed yet. As a result, in the present work we focused on the evaluation of the effectiveness of several lipid nanocarriers prepared by association of plant and animal oils, e.g. *grape seed oil* or *fish oil* with *laurel leaf oil*, to counteract the oxygen free radicals and to combat certain tumor cells without encapsulating any antitumor drug. The chemiluminescence method and cellular proliferation tests were employed in order to demonstrate the efficiency of developed bioactive lipid nanocarriers. The study intends to underline the ability of vegetable oils to confer valuable bioactive properties to conventional lipid nanocarriers. The association of classical lipid nanocarriers with vegetable oils will significantly contribute to the removal of carrier cytotoxicity simultaneously with adding supplementary biological effects.

2. Experimental

2.1. Materials

Sodium cholate, polyoxyethylene sorbitan monolaurate (Tween 20), L-α-phosphatidylcholine, dimethylsulfoxide, and hydrogen peroxide were purchased from Merck (Frankfurter, Germany). Poloxamer 407 (block copolymer of polyethylene and polypropylene glycol) was supplied by BASF Chem Trade GmbH (Burgbernheim, Germany) and Tris[Hydroxymethyl] aminomethane (Luminol) was purchased from Sigma Aldrich Chemie GmbH (Seelze, Germany). n-Hexadecyl palmitate (*CP*) was obtained from Acros Organics (USA), glycerol monostearate (*GM*) from Cognis GmbH (Monheim, Germany) and Myritol 318 (*My*) was from Merck (Germany). Grape seed oil (GSO) with a fatty acid content of 59% linoleic, oleic, palmitic and stearic acids was from Manicos SRL (Romania). The essential oil fraction of laurel (LLO) concentrated in eucalyptol, α-terpinyl acetate and α,β-pinene [42] was obtained from dried leaves (collected from Amanos Mountain) and was provided by the Department of Field Crops, Faculty of Agriculture (Turkey). The fish oil (*FO*) with the composition of 33% eicosapentaenoic acid (*EPA*), 22.3% docosahexaenoic acid (*DHA*) and 5.6% docosapentaenoic acid (*DHA*) was supplied by Henry Lamotte Oils Gmbh (Bremen, Germany).

2.1.1. Cell cultures and treatments

HeLa human cervical cancer cell line, *MDA-MB 231* human breast cancer cell line, *B16* mouse melanoma cell line and *L929* mouse fibroblast cell line were purchased from the American Type Culture Collection (ATCC) and routinely maintained in culture in RPMI-1640 medium added with 2 mM L-glutamine and 10% fetal calf serum (Sigma Aldrich, St. Louis, MO, USA) and incubated at 37 °C/5% CO_2 humidified atmosphere.

2.2. Preparation of bioactive nanocarriers by association of natural oils

The nanocarriers were prepared by a combined HSH (High-Shear Homogenizer PRO250 type, 0–28.000 rpm; power of 300 W, Germany) and HPH (APV 2000 Lab Homogenizer, Germany). In brief, an aqueous phase which consisted of distilled water and 2.5% surfactant mixture (sodium cholate: Tween 20: block copolymer = 80: 12: 8, *w*/*w*) and various lipid phases (e.g. mixture of solid lipids, including GM/CP, and oils – GSO, FO, My and LLO) were separately prepared. After mixing the aqueous and lipid phases, the obtained pre-emulsions were kept under stirring at 80 °C for 5 min. The resulting emulsions were subjected to a high shear homogenization stage by applying 12,000 rpm for 1 min and then processed through high pressure homogenization, with eight homogenization cycles at 600 barr. The nanodispersions were cooled at room temperature to obtain bioactive lipid nanocarriers. In order to remove the excess of water, all dispersions were lyophilized, using an Alpha 1–2 LD Freeze Drying System, Germany.

The developed nanocarriers have been denoted as *NLC 1 ÷ 3* (prepared with 2% LLO and 15, 20 and 25% grape seed oil), *NLC 4 ÷ 6* (prepared with 2% LLO and 15, 20 and 25% fish oil) and *NLC 7 ÷ 9* (prepared with 2% LLO and 15, 20 and 25% Myritol).

2.3. Characterization of bioactive nanostructured carriers

2.3.1. Particle size analysis

Particle size measurements were performed by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments Ltd., United Kingdom). The mean particle size (Z_{ave}) and the polydispersity index (PdI) of the NLC dispersions were measured at a scattering angle of 90° and a temperature of 25 °C. Before measurements, the dispersions were diluted with deionized water to an adequate scattering intensity. The particle size data was evaluated using intensity distribution. The average diameters (based on Stokes–Einstein equation) and the polydispersity index were given as average of three individual measurements.

The size and morphology of bioactive lipid nanocarriers were examined by transmission electron microscopy (Philips 208 S, The Netherlands). The lipid nanocarriers were diluted with water (1:100, v/v) before examination. Analyses were performed without staining.

2.3.2. Zeta potential measurements

The Zeta potential (ZP) was determined by measuring the electrophoretic mobility of the lipid nanocarriers in an electric field, by using

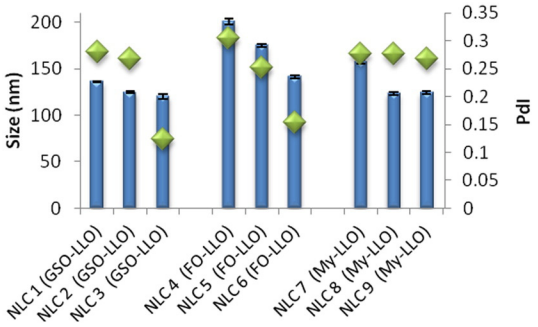

Fig. 1. Mean particle size and polydispersity index of lipid nanocarriers synthesized with mixtures of GSO/FO/My and LO, by DLS technique.

the Helmholtz–Smoluchowsky equation. Prior to the measurements, taken with the appropriate accessory of Zetasizer Nano ZS (Malvern Instruments Ltd., U.K.), the aqueous nanostructured carrier dispersion was diluted with a sodium chloride solution (0.9%, w/v), to adjust the conductivity to 50 μS/cm. All measurements were performed at 25 °C, in triplicate and the mean value was reported.

2.3.3. Differential scanning calorimetry

The changes in the crystalline states of the lyophilized bioactive nanocarriers were studied by differential scanning calorimetry (DSC). The DSC analysis was performed using a Jupiter, STA 449C differential scanning calorimeter (Netzsch, Germany). The samples (10 mg) were weighed into standard alumina pans. An empty pan was used as reference. The thermal analysis profiles were obtained as the temperature was increased from 30° to 100 °C at a rate of 5 °C/min.

2.3.4. In vitro evaluation of antioxidant activity

The in vitro antioxidant activity has been determined by the chemiluminescence method (Chemiluminometer Turner Design TD 20/20, USA). Luminol has been used as light amplifying substance and H_2O_2 in Tris–HCl buffer solution (pH 8.6) has been used as generator system for free radicals. Starting from lyophilized lipid nanocarriers with initial concentrations of 1.6% LLO and 20%, 16% and 12% GSO/FO/My, DMSO solutions were prepared with concentrations of 0.13 mg/mL. The percentage of free radical scavenging of NLCs was compared with solutions of the same concentration of vegetable oils and was calculated by using the relation:

$$\%AA = \frac{I_o - I_s}{I_o} \times 100$$

where I_o and I_s are the chemiluminescence maximum for standard, and for sample at t = 5 s.

2.3.5. In vitro cellular cytotoxicity assays

The cytotoxic effect was evaluated by MTT cell viability assay. The cell viability experiments were carried out at different nanocarrier dilutions on two tumor lines.

MTT cell viability assay was used to measure the cytotoxicity and cell viability using a standard colorimetric assay.

All tests were performed in triplicate in 96 flat well plates with 100 μL medium at a density of 1 × 104 cells per well. The stock solution was diluted with culture medium to attain the desired concentrations. After 24 h, cells were exposed to various concentrations of NLC 3 and NLC 9 for 24 and 48 h. Then cell viability was determined by the MTS CellTiter96 Aqueous Non-Radioactive Cell Proliferation Assay developed by Promega (Promega Corporation, Madison, USA). The cells were incubated for 4 h at 37 °C. The yellow tetrazolium MTT (3-(4, 5-

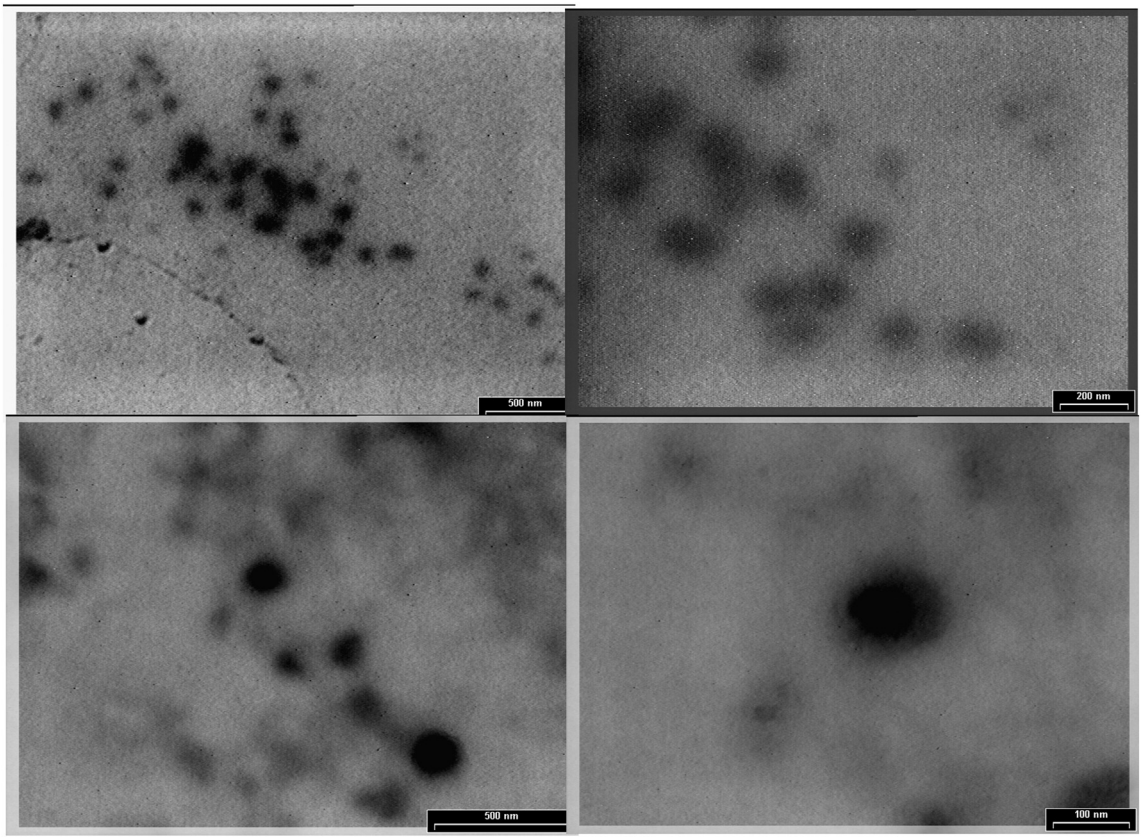

Fig. 2. TEM images of NLC 3 and NLC 9.

dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH.

The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means (λ = 570 nm, with the absorbance at 630 nm as the background correction) using Genios/Tecan spectrophotometer. The effect on cell proliferation was expressed as the percentage of cell viability. Untreated cells were taken as 100% viable.

3. Results and discussion

A comparative study regarding the influence of the lipid type and the composition on the particle size of nanocarriers prepared with several natural oils is shown in Fig. 1. The mean particle size of obtained formulations showed a lowering of the average diameters after increase of GSO/FO/My and LLO percent from 17 to 27% (Fig. 1). This size decrease could be associated to different intern assembles of the long fatty chains from GSO and FO which lead to a more confined lipid core. The size behavior is also in agreement with the obvious decrease of lipid crystallinity (e.g. more disordered lipid network) as the natural oil percentage was increased from 15 to 25% (Fig. 4).

In particular, the best results in terms of size and polydispersity index were obtained for the lipid nanocarriers preponderantly prepared with GSO, with Z_{ave} ranging from 120 nm to 165 nm. On the other hand, the NLCs prepared with fish oil were characterized by a higher mean diameter. Polydispersity values, as shown in Fig. 1a, were lower than 0.2 for nanocarriers prepared with 25% GSO or FO indicating a relatively narrow size distribution, especially for NLC 3 (PdI = 0.123). Photomicrographs of selected NLC 3 and 9 revealed that both kinds of nanocarriers prepared with grape seed oil or fish oil are spherical in shape with particle size less than 150 nm (Fig. 2).

The association of grape or fish oils with laurel leaf oil has led to strongly electronegative zeta potentials (Fig. 3a), which reveals a high surface charge of unloaded lipid nanostructures. This may be attributed to the charge components from the oils composition which could be

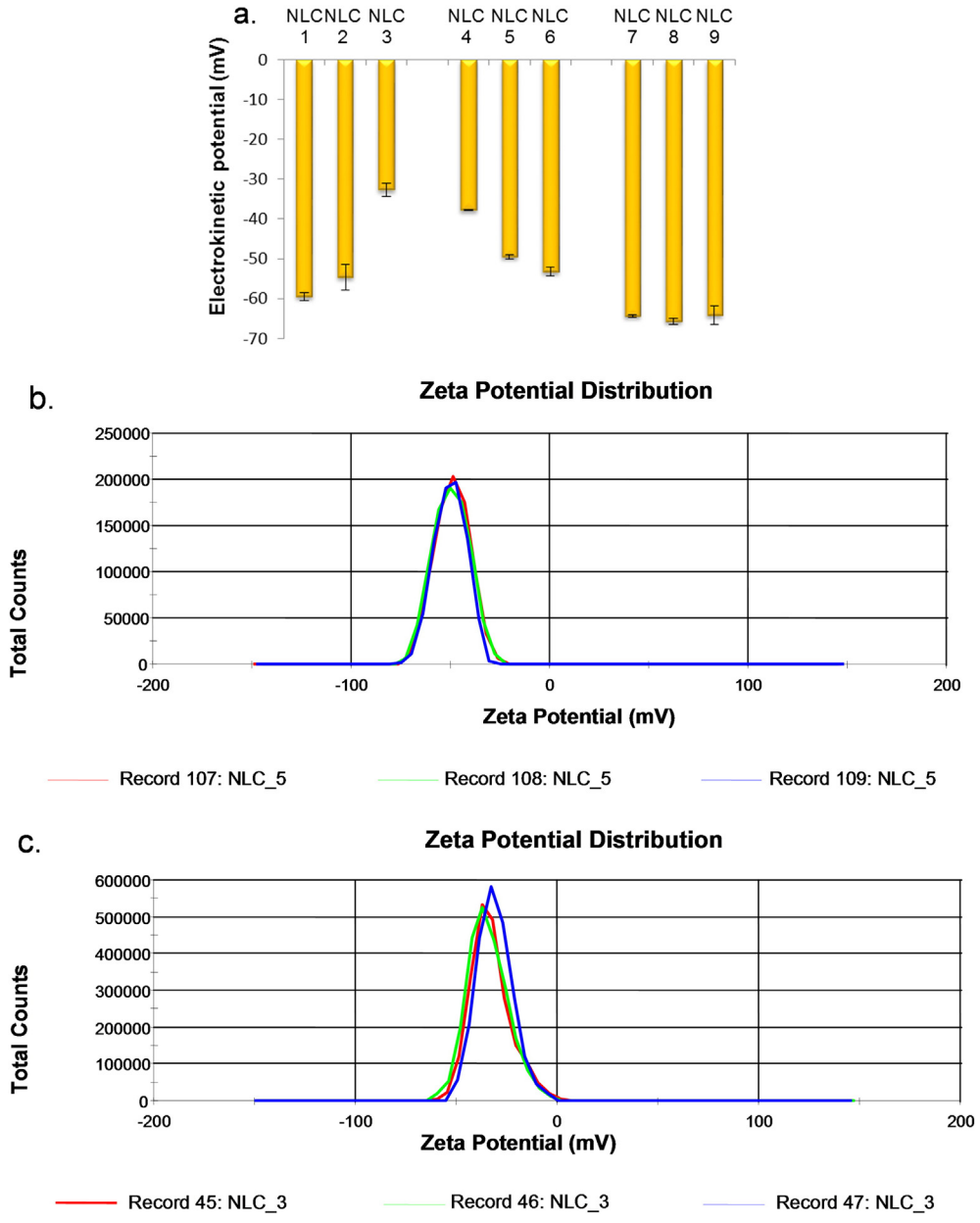

Fig. 3. Physical stability of lipid nanocarriers.

also encountered in the outside surfactant shell. In most cases, the electrokinetic potential is lower than −50 mV. The lower the zeta potentials, the higher repulsion forces between nanocarriers are encountered and therefore a low probability of particles aggregation in time and a higher stability of developed formulations are achieved. A selected example of electrokinetic profile for two lipid nanocarriers is shown in Fig. 3b, c.

Besides the promising results obtained for the size and stability of lipid based nanocarriers, the scanning calorimetry reveals a significant change of endothermic peaks in terms of a decrease in lipid crystallinity due to the partial formation of lower energy lipid modifications (Fig. 4). The profile of DSC curves and the value of enthalpy suggest a highly disordered lipid matrix which implies a large space to accommodate different types and number of active compounds. The degree of disorder increases with increasing the content of vegetable/animal oil from 17% to 27% (Fig. 4). In addition, the lipid nanocarriers prepared with 25% GSO and 2% LLO have a more disordered structure with enthalpy of 127 J/g.

Although there are many antioxidants commonly added in various human health products to prevent free radical damage, the reported side effects of synthetic antioxidants [43–45] make attractive the search for antioxidant and scavenger natural compounds. For almost all nanocarrier formulations developed in the present study, the antioxidant activity was maintained in the area of a high ability to scavenge the free radicals (Fig. 5). The change in antioxidant activity follows a continuous increase trend for all types of NLCs by varying the natural oil content from 17 to 27%. Compared with the nanocarriers made with triglycerides of the fatty acids C8 und C10 (My), those prepared with grape seed oil and fish oil in association with laurel leaf oil manifested a superior ability to capture the free radicals generated in situ in the chemiluminescence system, with antioxidant activity values ranging between 87.2 and 98%. This can be mainly attributed to the omega-6 and omega-9 fatty acid content in GSO (e.g. linoleic and oleic acids) and omega-3 fatty acids from FO (e.g. eicosapentaenoic and docosahexaenoic acids), but the synergism occurred in association of GSO/FO with LLO can be also responsible for the amplification of antioxidant property. These results support the utility and effectiveness of natural oils for the preparation of lipid nanocarriers endowed with biological action without the presence of any synthetic drug.

Our previous studies have shown that the antioxidant activity of lipid nanoparticles (SLN) and nanocarriers (NLC) is significantly dependent on the type of lipid matrix. For instance, moderate antioxidant activity has been identified for SLN loaded with umbelliferon (75% AA) [46] and NLC prepared with fish oil (78%), pumpkin oil (60%), squalene (52%), and amaranth oil (82%) [29,30], while the effective ability to capture oxygen free radicals (with AA > 90%) has been encountered only after entrapment of an additional active (e.g. carotene, sitosterol, lutein) together with the natural oil [47,48].

Referring to the proliferation tests of lipid-based nanocarriers against certain tumor lines, e.g. *HeLa* and *MDA-MB231* cells, or normal lines, e.g. *B16* and *L929* cells, valuable results were observed for both kinds of tested nanocarriers based on GSO/My and laurel leaf oil (Fig. 6a, b). The effect of both formulations on tumor cell viability was dependent on nanocarrier concentration, since the cell survival drastically declined by increasing the treatment concentration in the cells incubated for 48 h with 2.5 and 5 $mg \cdot mL^{-1}$ NLC 3 and 9. On the contrary, reducing the NLC concentration below 2 mg an increase in viability was observed, by means a cell proliferation which remains approximately on a platter.

Thus, it could be appreciated that contents higher than 2.5 $mg \cdot mL^{-1}$ nanocarriers based on GSO/My and LLO manifest an increased cytotoxicity against tumor cells (e.g. about 50% viability in case of *MDA-MB 231* tumor cells and about 60% viability for *HeLa* tumor cells).

The cell viability evaluated on two normal cell lines, *B16* and *L929*, has revealed the absence of lipid nanocarrier's toxicity for concentration below 0.6 $mg \cdot mL^{-1}$. The survival profile is presented in Fig. 6c and d. The lipid nanocarriers prepared by association of GSO with LLO (NLC-3) and My with LLO (NLC-9) have a similar cytotoxicity profile, and, as expected, a decrease of cell viability is observed as their concentration is increased. However, the median effective dose of 2.5 $mg \cdot mL^{-1}$ lipid nanocarriers showed an optimum balance between normal cell viability and tumor cell inhibition (e.g. 80% of *B16* normal cells remain viable as compared with 60% for *MDA-MB 231* and *HeLa* tumor cells). The survival profile of normal and tumor cells exposed to 5 $mg \cdot mL^{-1}$ lipid nanocarriers has manifested a death rate of 27% for normal *B16* cells and of 50% death rate for MDA-MB 231 tumor cells.

It is worth to note that whatever the type of lipid nanocarriers (e.g. NLC-3 prepared by association of LLO with GSO that manifested a strong antioxidant activity of 98% or NLC-9 prepared by association of LLO with My with antioxidant value of 82%), the tumor cell inhibition was similar for both kinds of nanocarriers. These results with comparable tumor cell inhibition suggested that the antioxidant activity does not interfere with the tumor inhibition mechanism. A possible explanation for this behavior is that some components from vegetable oil act together

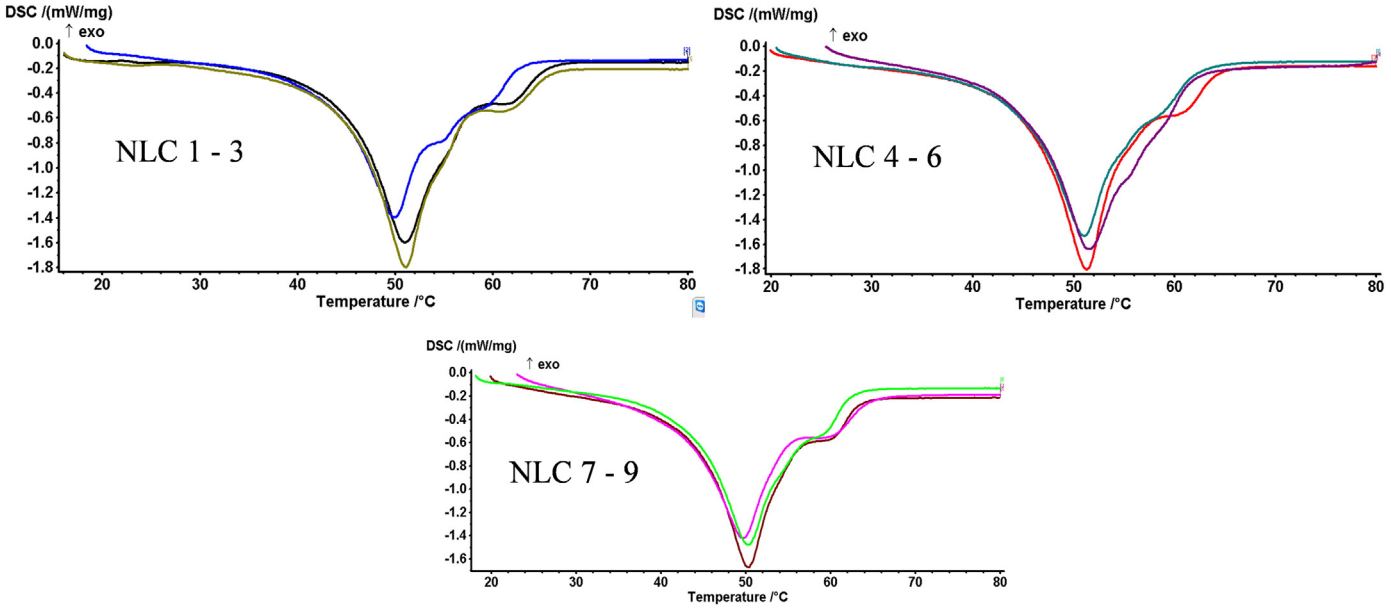

Fig. 4. Influence of type and concentration of oil in the formation of lipid nanocarriers, evidenced by differential scanning calorimetry.

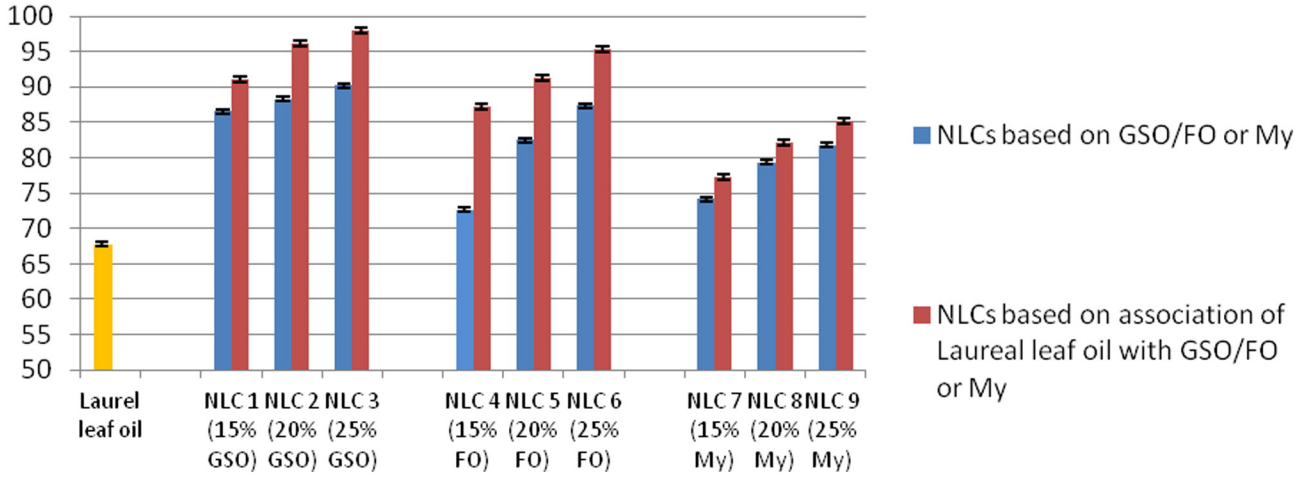

Fig. 5. Comparative evaluation of in vitro antioxidant activity.

synergistically or cumulatively, and cell growth inhibition was chemically suppressed or inactivated, mainly due to the pivotal role of LLO.

Overall, considering the diversity of bioactive compounds from grape seed and laurel oils, it is likely that the antitumor activity exerted by lipid nanocarriers to be the result of multiple cellular events associated with several mechanisms already reported in literature (e.g. antioxidant activity; induction of cell-cycle arrest and apoptosis; modulation of COX-2, Glutathione, Glutathione S-transferase enzyme activities; stimulation of the immune functions; regulation of hormone metabolism [49–53]).

4. Conclusions

The synthesis of empty lipid nanocarriers based on an association of plant and animal oils – *grape seed oil* or *fish oil* with *laurel leaf oil* – with broad antioxidant and antitumor spectrum effectiveness has been reported. A superior ability to scavenge the free oxygen radicals was obtained for nanocarriers containing 25% grape seed oil and 2% laurel leaf oil, with an antioxidant activity value of 98%.

The cell viability of *B16* and *L929* normal cell lines has revealed the absence of lipid nanocarrier's toxicity for a concentration below 0.6 $mg \cdot mL^{-1}$. High doses of nanocarriers based on grape seed oil and laurel leaf oil exert a moderate cytotoxic effect on normal cells and a significant cytotoxic effect against the tested tumor cells. The antiproliferative effect of nanocarriers based on laurel leaf oil has been more pronounced in the case of *MDA-MB 231* tumor cell line. The tumor cell survival profile drastically declined for a treatment with 5 $mg \cdot mL^{-1}$ of bioactive nanocarrier's solutions, e.g. about 50% viability for *MDA-MB 231* tumor cell line and 60% viability for *HeLa* tumor cell line. The survival profile of normal and tumor cells that were exposed to 2.5 $mg \cdot mL^{-1}$ lipid nanocarriers has revealed a death rate of 20% for normal *B16* cells and of 40% death rate for *HeLa* and *MDA-MB 231* tumor cells. This is a promising result, since the nanocarrier formulations tested at high doses lead to a significant decrease in tumor cell proliferation even in the absence of an antitumor drug.

These preliminary in vitro antioxidant and cell viability results support the usefulness and effectiveness of bioactive natural oils to develop lipid nanocarriers with biological action as such, without the presence of

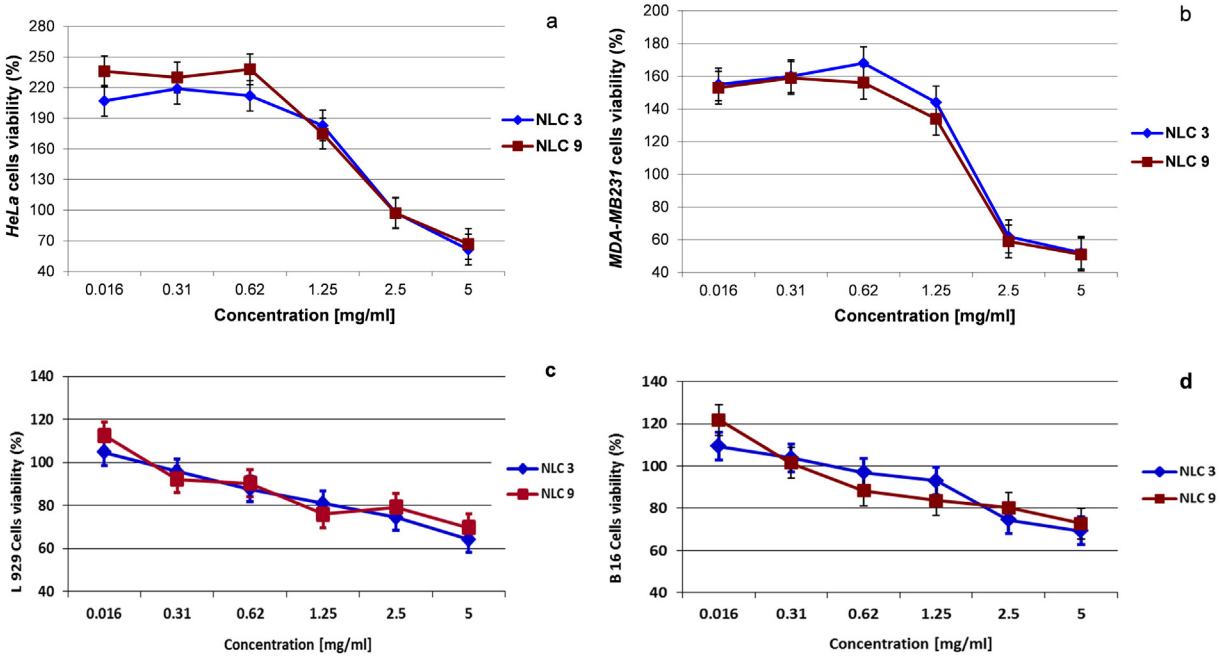

Fig. 6. The cell proliferation results of lipid nanocarriers against *HeLa* tumor cells (a), *MDA-MB231* tumor cells (b), normal *L929* cell lines (c) and normal *B16* cell lines (d).

any synthetic drug. These results could be further explored to reduce the delivery system toxicity and may significantly improve the therapeutic efficacy of antitumor drugs in clinical applications.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.msec.2015.06.019.

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