Total Antioxidative Activity of Evening Primrose (*Oenothera paradoxa*) Cake Extract Measured in Vitro by Liposome Model and Murine L1210 Cells

Bożena Bałasińska*,† and Agnieszka Troszyńska‡

Department of Animal Physiology, Warsaw Agricultural University, Nowoursynowska 166, 02-787 Warsaw, Poland, and Division of Food Science, Institute of Animal Reproduction and Food Research, Polish Academy of Sciences, Tuwima 10, 10-718 Olsztyn, Poland

The antioxidative effects of evening primrose seeds extract were investigated in vitro. The oil-free cake from evening primrose seeds was extracted with two solvents: water and acetone−water (7:3) mixture. There were various contents of phenolic compounds in these extracts (580 and 180 mg/g of acetone−water and water extracts, respectively; expressed in D-catechin). Acetone−water extract was separated into five fractions according to their absorbance readings at 350 nm using a Sephadex LH-20 column. Collected fractions had maximum absorptions of their UV spectra in a broad range between 278 and 286 nm (except fraction 1), which indicated that flavonoids predominated in the phenolic compounds of evening primrose. Absorption at 325 nm for fraction 1 indicated the presence of phenolic acid in that extract. PC (L-α-phosphatidylcholine) liposome system and leukemic L1210 murine cells were used to evaluate the antioxidative activity of extracts and their fractions. In these systems the oxidation process was stimulated by addition of AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride]. Extract with acetone:water had the highest antioxidative activity measured by the PC oxidation to PC−OOH (hydroxyperoxidephosphatidylcholine). Also the same extract in the concentration range from 17.5 to 175 ng/mL significantly inhibited peroxidation of cell membranes expressed by TBARS formation in cell cultures.

Keywords: *Evening primrose (Oenothera paradoxa); antioxidants; lipid peroxidation; polyphenols; liposomes; leukemic cells*

INTRODUCTION

Reactive oxygen substances, ROSs ($O_2^{\bullet -}$, H_2O_2, $OH^\bullet$, $LOO^\bullet$, $NO^\bullet$), play an important role in the vital processes of an organism. They attack polyunsaturated fatty acids of cell membranes, causing their oxidation and finally cell damage. Lipid peroxides formed in these reactions may accelerate aging and also are considered to be responsible for many diseases, including atherosclerosis and cancer (Ames et al., 1993; Cutlar, 1984; Harman, 1982; Auroma et al., 1991; Kehrer 1993; Ohara et al., 1993; Prasad and Kalra, 1993). Epidemiologic studies demonstrated that, in the countries with high morbidity rate caused by heart diseases, the vitamin E content in human blood is significantly lower than in the countries where morbidity is low and the consumption of vitamin E as well as vitamin C is high. Similar studies concerning the relationship between the morbidity due to heart and cancer diseases and the consumption of fruits and vegetables indicated that polyphenols present in large amount in fruits and vegetables have significant impact on the morbidity decrease from these diseases (Cutlar, 1984; Osawa et al., 1990; Doll, 1990; Dragsted et al., 1993; Hertog et al., 1993). As a result of these studies attention has been focused in recent years on antioxidants of natural origin isolated from raw products (Katsuzaki et al., 1993; Nishima et al., 1992; Okamura et al., 1993; Osawa et al., 1992); some of them are already present on the market in forms of parapharmaceutical preparations.

Polyphenolic compounds are found mainly in fruits and vegetables (Hertog et al., 1992; Ortuno et al., 1995). Many polyphenols such as kaempherol, quercetin, luteolin, myricetin, and eridictyol i catechin express strong antioxidative (Bors et al., 1987, 1990; Hanasaki et al., 1994), antiinflammatory, antiallergic, and antineoplastic properties (Dragsted et al., 1993; Formica and Regelson, 1995). The high antioxidant activity of plant phenolic compounds attractive to the food industry, prompting their use as replacements for synthetic antioxidants (Lindberg Madsen et al., 1996). Since synthetic antioxidants are often toxic and may cause liver damage and deterioration of enzymatic processes (Martin and Gilbert, 1968; Halladay et al., 1980), they are more and more often criticized and in some countries the use of them in ordinary food products is even not permitted. At present there are many studies going on to further identify the new natural sources of antioxidants which might be alternatives to synthetic compounds and also play a role in preventing many diseases after being absorbed in the small or/and large intestine (Formica and Regelson, 1995).

Evening primrose is a oleaginous plant, and its seeds are rich in unsaturated fatty acids, which are very sensitive to the process of oxidation. It is speculated that to prevent oxidation evening primrose seeds may contain some antioxidative substances. The oil of evening primrose seeds may contain some antioxidants; however, the majority of these compounds is present in the cake, and therefore such a cake is considered to be a good source of natural antioxidants.

† Warsaw Agricultural University.
‡ Polish Academy of Sciences.

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The aim of our studies was to evaluate the antioxidative activity of evening primrose cake extracts together with their fractions in vitro using the phosphatidylcholine (PC) liposome system and leukemic L1210 murine cells as experimental models. In our experiments 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) was used to initiate peroxidation. AAPH undergoes thermal decomposition in relatively low temperatures, forming with the same velocity two hyperoxide radicals (LOO•). They immediately participate in the reactions with polyunsaturated fatty acids by initiating their chain oxidation.

MATERIALS AND METHODS

Materials. L-α-Phosphatidylcholine (typ III-E; PC; Sigma Co.), hydroxyperoxidephosphatidylcholine (PC−OOH; Sigma), diethylenetriaminepentoacetic acid (DTPA; Sigma), tris(hydroxymethyl)aminomethate; Sigma, butylhydroxyanisole (BHA; Sigma), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH; Wako Pure Chemical Industries, Ltd., Osaka, Japan), mouse lymphocytic leukemia L1210 cell line (supplied by the American Type Culture Collection, Rockville, MD), RPMI-1640 medium (Sigma), fetal calf serum (FCS; Gibco BBL, Paisley, Scotland), L-glutamine, gentamicin, fungizone, and thiobarbituric acid (TBA; Sigma).

Analytical Instruments. The instruments used are listed as follows: HPLC (Shimadzu); detector, UV SPD-10A; 235 nm, column C8 Nova Pak (3.9 × 150 mm Waters Millipore); pump, LC-10AD; recorder, C-R6A (LiposoFast-Basic, Avestin, Canada); liquid chromatography Sephadex LH-20 column (1.6 × 95 cm); spectrophotometer Beckman DU 7500.

Preparation of Extracts and Their Fractionation. The oil-free cake from evening primrose (*Oenothera paradoxa*) seeds was extracted with an acetone−water mixture (7:3 (v/v)) or water alone at 50 °C for 2 h (1:5 (w/v)) in a shaking incubator. Extracts were then filtered, and acetone was evaporated under a vacuum; the residue and water extracts were refrigerated and lyophilized. Acetone−water extract (1.1 g) was fractionated by liquid chromatography using a Sephadex LH-20 column with a mobile phase of acetone−water and the UV spectrophotometer set at 350 nm. The eluates indicated by peaks were collected, concentrated by rotary evaporation to dryness, and weighed. The extracts and fractions were analyzed for the total content of phenolic compounds, UV spectra, and antioxidative activity.

Analytical Methods. The contents of phenolic compounds were determined according to Julkunen-Tiitto (1985). A 20 μL aliquot of the extract, or fractions (1 mg/mL solvent) of four replicates, or various amounts (20−70 μL) of D-catechin (0.5 mg/mL solvent) standard solution were made up to 1.0 mL with distilled water. Then, 0.5 mL of Folin−Ciocalteu reagent (1 N) was added to the tubes and vortexed. This was followed by the addition of 2.5 mL of Na_2CO_3 (20%), and the tubes were again vortexed. After incubation for 35 min at room temperature, the tubes were centrifuged as before and absorbance was read against a blank at 725 nm. The concentration of total phenols (expressed as milligrams per gram of extract) was calculated according to the standard curve. The UV spectra were obtained with a Beckman DU 7500 diode array spectrophotometer. Antioxidative activity was determined by a liposome method (Terao et al., 1985). An aliquot (50 μL) of extracts or fractions in methanol (2.5 mg/mL) was added to the solution (0.7 mL) of peroxide-free egg-yolk phosphatidylcholine (PC) dissolved in chloroform. After removing solvents in the stream of nitrogen followed by vacuum, the residue was dissolved in Tris-HCl buffer (0.7 mL, 10 nm/L, pH 7.4) with 0.5 mmol/L diethylenetriaminepentaacetic acid (DTPA), vortexed and exposed to ultrasonic waves for 30 s. The liposomes were produced by extruding the sample (0.6 mL) 21 times in a LiposoFast Basic apparatus with a polycarbon membrane of 100 nm pore size. The liposomes (0.5 mL) were suspended in 0.5 mL Tris-HCl buffer and placed in a light-protected shaker water bath at 37 °C. After 5 min, AAPH was added to the sample (final concentration was 1 mM) as an initiator of radicals. The amount of phospatidylcholine peroxides (PC−OOH), analyzed during 7 h of incubation at 72 min intervals, was determined by a HPLC method using the Shimadzu system, C_8 Nova Pak column (3.9 × 150 mm, Waters Millipore), and a methanol−water mixture (94:4 (v/v)) as a mobile phase, including a 235 nm fixed UV SPD-10 A detector, LC-10 AD pump, and C-R6A recorder. The amount of peroxides was calculated from a standard curve prepared with PC−OOH titrants.

Cell Culture. Mouse lymphocytic leukemia L1210 cell cultures were maintained in RPMI-1640 medium supplemented with 10% (v/v) FCS, 0.2% (w/v) L-glutamine, gentamicin (50 μg/mL), and fungizone (2.5 μg/mL) in an atmosphere of 5%:95% CO_2−humidified air at 37 °C and routinely subcultured every 2 or 3 days. AAPH was prepared as a stock solution (10 mM) in RPMI-1640 immediately prior to experiment. Peroxidation was initiated by the addition of 1 mM AAPH, and the incubation was continued for the next 24 h. Extract from evening primrose dissolved in ethanol (at the final concentrations 17.5, 175, and 350 ng/mL, expressed as D-catechin) was added 1 h before the start of the reactions. After 24 h, aliquots were removed for the determination of both cell viability (trypan blue method) and lipid peroxidation (TBA test).

Assessment of Cell Viability. The cells were seeded in triplicate at an initial density of approximately 3×10^5cells/mL. Control flasks contained 10% FCS/RPMI (growth promoting medium). Appropriate cultures were treated with control medium supplemented with 1 mM AAPH. Before and 24 h after, AAPH administration cells were counted on a hemocytometer and their viability was determined by the trypan blue dye exclusion method.

Thiobarbituric Acid Method (Rice-Evans et al., 1991). A 1 mL aliquot of 15% TCA in 0.25 M HCl and 1 mL of 0.37% thiobarbituric acid (TBA) in 0.25 M HCl were added to 1 mL of cell suspension (3×10^6). The mixture was vortexed and heated for 40 min in 100 °C. After cooling 4 mL of buthanol was added. Absorbance of the organic layer was measured at 532 nm against a blank. The malondialdehyde (MDA) concentration was calculated according to the standard curve using 1,1,3,3-tetramethoxypropane as a standard. Lipid peroxidation was expressed as nanomoles of MDA per nanomole of phospholipids (kit Bio Merioux).

RESULTS

The content of phenolic compounds amounted to 580.23 and 170.87 mg of polyphenols in acetone−water and water extracts, respectively; results were expressed as D-catechin per gram of extract.

Figure 1 represents alterations in PC oxidation due to different concentrations of acetone−water extract. A rise in antioxidant content inhibits PC oxidation, which means that smaller amounts of PC−OOH are formed and the inhibition time of oxidative reaction is longer. Already 72.5 μg/mL of extract expressed as D-catechin significantly prevents PC oxidation for 7 h.

Comparison of the activity and the time of oxidative action using various extracts with the results obtained after BHA treatment is presented in Figure 2. The amounts of extract and BHA were identical and amounted to 72.5 μg/mL. Progress in the reaction has been monitored during 7 h. The strongest antioxidative effect was observed using acetone−water extract. The amount of newly formed PC−OOH was also similar when water extract and BHA were used as an antioxidant.

Separated fractions of acetone−water extract using the Sephadex LH-20 column are presented in Figure 3. Acetone extract (1.1 g) was separated into five fractions

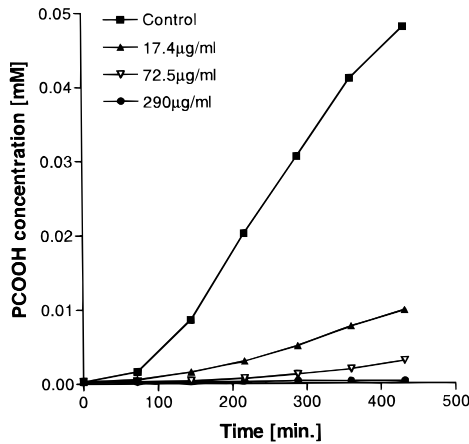

Figure 1. Time-related changes in peroxides content (PC−OOH) during incubation of phosphatidylcholine with different amounts of evening primrose cake acetone−water extract. Concentrations of extract were 17.4, 72.5, and 290 μg/mL expressed as D-catechin. AAPH (1 mM) was used as an oxidant. Results are the mean from three repetitions.

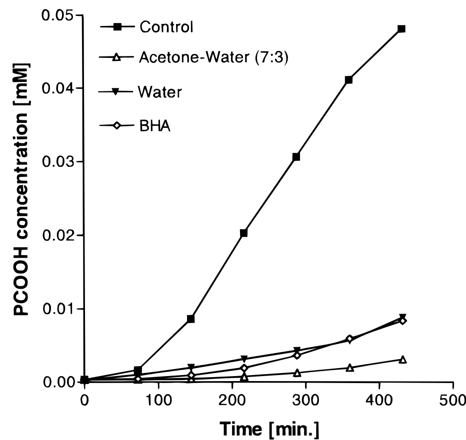

Figure 2. Comparison of antioxidant activities of water and acetone−water extracts made from oil-cake evening primrose with the antioxidant activity of BHA. Concentration of added antioxidants was 72.5 μg/mL expressed as D-catechin. AAPH (1 mM) was used as an oxidant. Results are the mean from three repetitions.

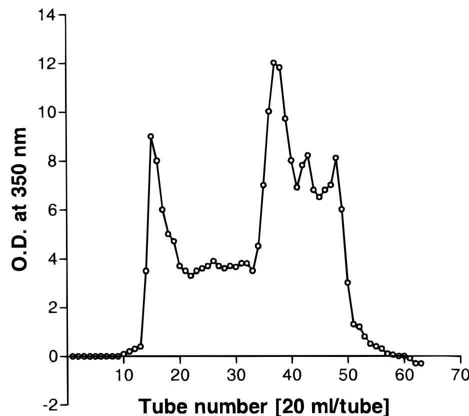

Figure 3. Sephadex LH-20 column eluation profile for extract from evening primrose cake.

according to their absorbance readings at 350 nm in which the phenol content was from 180.4 to 50.9 mg of polyphenols/g of extract (values expressed in D-catechin equivalents). Table 1 presents fractions analyzed from acetone−water extract. UV spectra of separated fractions (Figure 4) indicated absorption bands at 280 nm

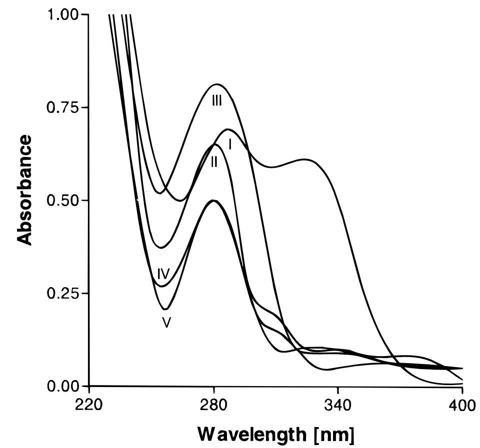

Figure 4. UV spectra in fractions from evening primrose cake acetone−water extract.

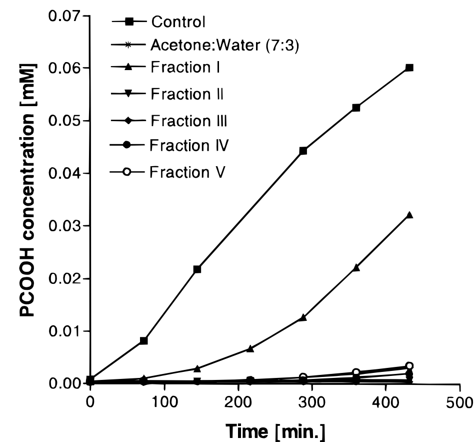

Figure 5. Time-related changes in peroxides content (PC−OOH) during incubation of phosphatidylcholine with evening primrose cake acetone−water extract and its fractions. Concentrations of added antioxidants were 72.5 μL/mg for total extract and 74.9, 71.3, 55.2, 79.8, and 72 μg/mL for I−V fractions, respectively. AAPH (1 mM) was used as an oxidant. Results are the mean from three repetitions.

Table 1. Fractions (Percentage) in Extract from Evening Primrose Cake and Content of Phenolic Compounds for Each Fraction Together with Its UV Maximal Absorbance

fraction	fraction, % of the extract	content of total phenolics, mg/g of extract (as D-catechin)	λ_{max}, nm
I	25.7	180.4	286, 325
II	20.0	133.5	281
III	27.9	144.5	280
IV	6.9	50.9	279
V	19.5	131.5	280

(II−V). The UV spectrum of fraction I showed two maxima at 286 and 325 nm.

Collected fractions (I−V) were then used as antioxidants for the oxidation of PC to PC−OOH in the liposomic system. Results of their antioxidative properties are presented in Figure 5. Fractions II−V reveal similar and strong antioxidative properties. The concentration of PC−OOH and the inhibition time of the oxidation were similar in fractions II−V. In contrast to that, fraction I had low antioxidative activity, and 1 h exposure did not prevent PC oxidation. The time and inhibition rate for PC oxidation to PC−OOH were similar for both the fractions II−V and the whole acetone−water extract prepared from cake seeds of

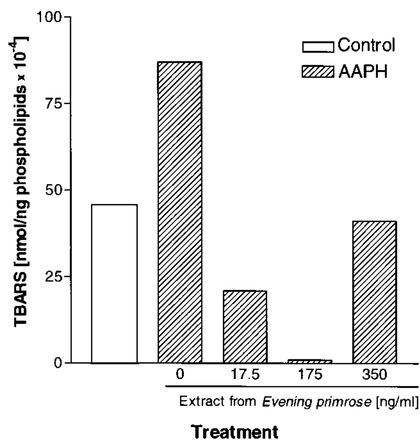

Figure 6. Effect of AAPH (1 mM) and extract from evening primrose on TBARS concentration in mouse lymphocytic leukemic L1210 cells after 24 h of incubation. Results are the mean from three repetitions.

evening primrose. The final concentration of the extract and its fractions amounted to 72.5 μg/mL for the total extract and 74.9, 71.3, 55.2, 79.8, and 72 μg/mL for I–V fractions, respectively, expressed as D-catechin.

Figure 6 shows mouse lymphocytic leukemia L1210 cell peroxidation and the effects of extract from evening primrose. The addition of AAPH to a cell culture increased the formation of TBARS. Cell viability, which was 85% in the control cell culture, decreased less than 60% over the 24 h incubation. Extract from evening primrose in the concentrations of 17.5 and 175 ng/mL significantly inhibited peroxidation in a dose-dependent manner. The addition 350 ng/mL of extract did not prevent cell oxidation. The concentration of TBARS was the same as in the control.

DISCUSSION

As it is known, the use of various solvents is a common method for the extraction of several biologically active substances, among them polyphenols. Their efficiency and individual properties depend on the solubility of each phenolic compound in a seed's cells and also on their diffusion into the extraction medium (Oszmiański et al., 1986). Alonso et al. (1991) investigated the influence of various ethanol–water mixtures on the extraction of catechins and proanthocyanidins from grape seeds. They reported more efficient extraction when the ethanol content of the extractant and the extraction time increased. On the other hand, Kallithraka et al. (1995) reported that methanol was the best solvent for the quantitative extraction of (+)-catechin, (–)-epicatechin, and epigallocatechin from grape seeds. The exact composition of evening primrose seeds extract is not known yet. Compounds present in that extract may have various structure—from small phenolic acids to flavonoids and polymerized tannins. Therefore in our studies we decided to use two solvents—water and an acetone–water mixture (7:3)—since this extraction mixture allows one to obtain the highest yield of total polyphenolic compounds (Kallithraka et al., 1995). The acetone–water mixture gained 580 mg of polyphenols/g of extract, whereas extraction yield with water alone at the same conditions was about 180 mg of polyphenolic/g of extract expressed as a D-catechin. Our data clearly demonstrate that the application of the acetone–water mixture for the extraction of evening primrose cake generates 2 times more extract than application of water alone.

High heterogenity of antioxidative substances present in plant tissues creates difficulties in the analysis of their individual properties. Therefore in biological samples the most common method is the determination of total antioxidative activity (Wang et al., 1996). In recent years there have also been some attempts undertaken to separate extracts, to collect fractions of the highest activity. It was also of interest to us to determine antioxidative properties of fractions collected after separation of acetone–water extract with the Sephadex LH-20 column and to compare these data with the results of total extract prepared from evening primrose seeds and BHA—a popular antioxidant used for food protection. Also the same substances extracted by the acetone–water mixture demonstrate higher antioxidative activity than extracted by water alone. However, an extractive mixture of acetone–water is a better diluent than water; also water extract had some interesting properties in our studies, and its antioxidative activity was similar to BHA.

Fractional separation of acetone–water extract indicates that this extract consists of various substances. The UV spectra of the fractions had the maximum absorption in a quite broad range from 278 to 286 nm and 325 nm (Table 1 and Figure 4), indicating differences in the composition of phenolic compounds. These spectra suggest that flavonoids predominate in the phenolic compounds of evening primrose (Waterman and Mole, 1994). Additional absorption at 325 nm for fraction I suggests the presence of phenolic acid in the extract (Waterman and Mole, 1994). Yoshida et al. (1995) identified in roots of *Oenothera laciniata* compounds which belong to hydrosoluble tannins.

One of the main targets of free radicals in human and animal organisms are lipids of cell membranes. ROSs are constantly generated in vivo, and organisms need them for normal functioning. These compounds however may damage biological cell structures. And although all organisms are equipped with defensive mechanisms which are an integral part of an antioxidative system (i.e., enzymes, macromolecules), nevertheless an increasing concentration of ROSs may generate, for example, atherosclerosis and cancer. A significant relationship has been found between the amount of dietary flavonoids and the mortality caused by heart diseases (Hertog et al., 1993). Since antioxidants of dietary origin may play an important role in preventing tissue damage stimulated by free radical reactions (Ames et al., 1993; Doll, 1990; Dragsted et al., 1993; Willet, 1994), a growing interest concerning these natural sources of antioxidants is observed.

Liposomes are frequently used as a model for studying lipid peroxidation in vitro. In our studies this model has been successfully used for the evaluation of antioxidants present in the extracts and for separation of cake fractions prepared from evening primrose. The HPLC method for PC–OOH measurement has been used for the evaluation of antioxidative properties of evening primrose seeds extract in the liposomic model. Time-related measurement of PC–OOH content allows estimatation of the inhibition rate of free radical oxidation and the time when this reaction is restrained. Five fractions have been collected, and each fraction shows its antioxidative ability. Fraction I has the lowest reducing potential. Other fractions, namely, II–V,

possess strong antioxidative properties. The inhibition rate of the oxidation reaction as well as the inhibition time for that reaction caused by these fractions is similar to those obtained after using the total acetone–water extract prepared from the cake of evening primrose seeds. Phenolic compounds in evening primrose seeds may have potential synergistic effects. Results of our studies with the liposomic model suggest that the use of cake extract from evening primrose may protect lipids against oxidation induced by active forms of free radicals.

Cells represent an excellent biological model for studying in vitro lipid peroxidation, since cells maintain their functional integrity. Also their defense systems remain active against oxidative challenge, what concerns also malignant cells such as, for example, murine leukemic cells line L1206 (Eskenazi et al., 1993). In our experiment AAPH was used to initiate peroxidation. TBARS production was measured as an indicator of lipid peroxidation. Extract from evening primrose prevents peroxidation but only in the concentrations of 17.5 and 175 ng/mL. The mechanism by which polyphenols inhibit lipid peroxidation seems to be rather complex, although it has not yet been clarified. Antioxidant mechanisms for flavonoids in biological systems and food products may involve free radical scavenging, metal chelation, and oxygen-free radical scavenging (Hudson et al., 1983; Thompson and Williams, 1976; Das, 1994). Polyphenols may interact with propagation reactions and with reactive species capable of initiating lipid peroxidation. When the conditions of reaction are altered by an increase of one of the reacting substances, the kinetic of reaction can also be affected. This may explain why higher concentrations of added extract could have some prooxidative potential. In our studies (Figure 6) 350 ng/mL of evening primrose extract had the same amount of TBARS as the control cells which were in a process of autoxidation.

A protection system against oxidation in living organisms consists of various compounds, which means that the administration of only one of them in experimental conditions may not be effective enough, but the data from in vitro studies with murine leukemic cells confirm that the extract from evening primrose is a good and high yielding source of biologically active substances having antioxidative properties. Further studies are needed for the approval of more frequent use of such extracts from evening primrose seeds as dietary antioxidants and conservants in the food industry.

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