Liposomal Formulations from Phospholipids of Greek Almond Oil. Properties and Biological Activity[§]

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Z. Naturforsch. **59 c**, 330–334 (2004); January 14/February 12, 2004

The seeds of the almond tree [(*Prunus dulcis* (Mill.) D. A. Webb. (syn. *Prunus amygdalus*)] were collected in two different periods of maturity and were studied for their lipid content. The total lipids (TL) were extracted by the Bligh-Dyer method and the lipid classes have been isolated by chromatographic techniques and were analyzed by HPTLC coupled with a flame ionization detector (HPTLC/FID) and GC-MS. The oils were found to be rich in neutral lipids (89.9% and 96.3% of total lipids) and low in polar lipids (10.1% and 3.7% of total lipids) for the immature and mature seed oils, respectively. The neutral lipid fraction consisted mainly of triacylglycerides whereas the polar lipids mainly consisted of phospholipids. GC-MS data showed that the main fatty acid for both oils was 9-octadecenoic acid (oleic acid). The unsaturated fatty acids were found as high as 89.4% and 89.7%, while the percentage of the saturated fatty acids was found 10.6% and 10.3% for the immature and mature seed oils, respectively. Liposomes were prepared from the isolated phospholipids using the thin lipid film methodology, and their physical properties were characterized. Cytotoxicity was found absent when assayed against normal and cancerous cell lines. These new formulations may have future applications for encapsulation and delivery of drugs and cosmetically active ingredients.

Key words: Almond Oil, *Prunus dulcis*, Liposomes

Introduction

The almond tree [(*Prunus dulcis* (Mill.) D. A. Webb. (syn. *Prunus amygdalus*, family Rosaceae)] is cultivated *e.g.* in Mediterranean countries, and the oil is mainly produced from almonds grown in these countries (Evans, 1996). The almond oil is extensively studied on account of its nutritional (Spiller *et al.*, 1992), industrial and medical importance (Davis and Iwahasi, 2001). It is also used as an excellent carrier oil for other materials (Shafic, 1990; Cicinelli *et al.*, 1992; Gardner and Solomou, 1984). Furthermore, almond seeds are used both as a snack and as an ingredient in other food products (Sathe and Sze, 1997). Because of the high content of monounsaturated fatty acids almond seeds can decrease the cholesterol levels and play an important role in prevention of cardiovascular pathologic conditions (Spiller *et al.*, 1992). Almond oil is widely used in many cosmetic formulations (Hotellier and Delaveau, 1972), because the beneficial action of almond oil on skin is known for centuries. Almond oil is a component of skin hydrating creams, anti-wrinkle and anti-ageing products (Jackson, 1992).

Liposomes are hollow spheres of lipid bilayers, which are mainly formed of phospholipids and are widely used as carriers of active ingredients to human tissues and also as lipid transfer vesicles to the skin (Hatziantoniou *et al.*, 2000). The objective of this study was the isolation and the identification of the components of the total lipid fraction of the almond oil as well as the preparation of new liposomal formulations which can serve as drug delivery systems having new controlled release properties as well as active ingredients in cosmetics.

[§] This work is a part of F. Malisiova's Master of Science Thesis.

0939–5075/2004/0500–0330 $ 06.00

Experimental

Materials

Almond seeds [(*Prunus dulcis* (Mill.) D. A. Webb. (syn. *Prunus amygdalus*, family Rosaceae)] were collected (collector Dr. C. Demetzos) in two different periods, at the end of June 2000 (immature seeds: sample A) and at the end of September 2000 (mature seeds: sample W) in the village of Fourni, prefecture of Lasithi, East Crete island (Greece). The plant material was identified by Dr. D. Perdetzoglou (University of Athens). Lipids used as reference compounds were: cholesteryl ester, triacylglycerol (TG), cholesterol (CH), phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA), cardiolipin (CL), sphingomyelin (SM), ceramides (types III and IV), galactosylcerebrosides (types I and II), all from Sigma Chemical Co (St. Louis, MO). Organic solvents were analytical grade from Labscan Ltd., Ireland. Salts and buffers were from Sigma Chemical Co.

Total lipid extraction

After peeling, the almond seeds were weighed and the total lipids were extracted by the Bligh-Dyer method (Bligh and Dyer, 1959). The $CHCl_3$ was removed and evaporated by a rotary evaporator at 30 °C and the residue (a yellow oil) was weighed. The remaining oil was diluted with $CHCl_3$/MeOH (9:1, v/v) to a final mass fraction of 12% (w/v) and stored in the dark at – 18 °C. The total lipid extract of the immature seeds was classified as oil A, while the total lipid extract from mature seeds was classified as oil W.

Isolation of lipid classes

The separation of neutral and polar lipids of the extracted almond oil was performed by Vacuum Liquid Chromatography (VLC) using silica gel 60 (230–400 meshes, Merck, Darmstadt, Germany). The solvent mixtures for the isolation of the lipid classes were: $CHCl_3$ (100%) for the neutral lipids, $CHCl_3$/MeOH (90:10 v/v) for the sphingolipids and MeOH (100%) for the phospholipids (oil A), and $CHCl_3$ (100%) for the neutral lipids and MeOH (100%) for the phospholipids (oil W) (Karleskind, 1996). The isolated lipid classes were monitored by TLC and classified as neutral lipids (sample I), sphingolipids (sample II), and phospholipids (sample III).

Qualitative and quantitative analysis of the lipid classes

The analysis of the almond oil lipid classes was carried out with a High Performance Thin Layer Chromatograph coupled with a flame ionization detector (HPTLC/FID) (Iatroscan MK – 5new, Iatron Lab. Inc., Tokyo, Japan). Hydrogen flow rate was 160 ml/min, airflow rate 1900 ml/min, scan speed 30 s/scan. As stationary phase Chromarods – SII (Iatron Lab. Inc.) in set of 10 rods was used.

Chromarod development and scanning

Three samples (1 μl each) were analyzed on the Chromarods: Almond oil's neutral lipids (sample I), sphingolipids (sample II) and phospholipids (sample III). Sample I was developed by *n*-hexane/diethyl ether/acetic acid (70:30:1 v/v/v), sample II by chloroform/methanol/water (90:10:1 v/v/v) and sample III by chloroform/methanol/water (65:25:4 v/v/v) (Karleskind, 1996). After development the Chromarods were dried at 100 °C for 1 min and scanned. Each sample was analyzed on four Chromarods and the average was calculated.

Identification of fatty acids in total lipids

The free fatty acids were converted into the corresponding methyl esters by 2 N KOH in methanol and vortexed for 2 min. The methyl esters were then extracted by *n*-hexane and analyzed using GC-MS. A Hewlett-Packard (HP) 6890 gas chromatograph equipped with a 30 m HP-5 fused silica gel capillary of 0.25 mm film thickness was coupled to a Hewlett-Packard 5973 mass spectrometer. The column was temperature programmed as follows: 170 °C for 15 min, temperature increase 2 °C/min until 220 °C where it stayed for 30 min; injection port temperature 250 °C, detector port temperature 250 °C; Helium gas carrier pressure 53.1 kPa; split ratio 1:20. The injection volume was 1 μl and the fatty acids were identified using the Wiley 275k MS Database (Coudrec, 1995).

Liposome preparation

Liposomes were prepared from almond oil phospholipids using the thin lipid film hydration

method (Mayer *et al.*, 1990). Phospholipids were isolated from the extracted almond oil by VLC using MeOH (100%) as a solvent after the removal of neutral lipids and sphingolipids. The lipid film was prepared by dissolving the lipid mixture in chloroform, which was slowly evaporated in a flash evaporator. Multilamellar vesicles (MLVs) were prepared by adding 2 ml of 150 nM ammonium sulphate buffer (pH 5.5, 535 mOsm) and vigorous shaking in a water bath at 50–60 °C to achieve a final mass fraction of 5% lipids (w/v). The samples were then maintained above the phase transition temperature for 1 h to allow the equilibration of the water across the lipid bilayers. 10 Freeze-thaw cycles reduced the size of the vesicles and the resultant large oligolamellar vesicles (LOV) were sonicated. For the preparation of small unilamellar vesicles (SUV) the resultant large oligo-lamellar (LOV) vesicles were subjected to two sonication periods in ice bath. The resultant vesicles were allowed for 30 min to anneal at room temperature. Then the liposomal suspension was centrifuged [Fixed angle rotor, SORVAL T-880, Relative Centrifugal Force (RCF) Value = 28684, 20 min, 4 °C] in order to remove MLVs and titanium particles. The liposome size distribution and ζ-potential were evaluated by the diameter and surface charge (Zetasizer 3000HS$_A$ Malvern Instr., UK). The physical and chemical stability of the liposomal formulations were tested at 4 °C. In order to observe the two samples by fluorescence microscopy, liposomes of each kind were prepared adding PE-rhodamine to the lipid mixture (0.2%mol/mol of total lipids) (Fig. 1).

Liposome images

Liposomes were observed under an epifluorescence Zeiss Axioplan2 microscope equipped with a rhodamine long pass filter (excitation LP 510 + KP 560, beamsplitter FT 580 and emission LP 560). Observations were made with a × 40 Plan Neofluoar dry lens (NA 0.75) or a × 100 Plan Neofluar oil immersion lens (NA 1.30). Images were captured with a CCD b/w camera in front of which 2 lenses were used alternatively, providing a final magnification × 1000 or × 1500. The camera was under the control of Image Pro Plus v3.1. Following capturing, images were corrected for background by subtracting an out of focus image, and then filtered using a HiGauss filter (7 × 7 pixel size, one pass, strength 3).

Biological activity

Liposomal formulations of samples A and W were tested for their toxicity against the following cancerous cell lines: H460 (colon), MCF7 (breast), SF268 (CNS), DLD1 (colon) and OVCAR3 (ovary). All cell lines were purchased from the NCI/NHI and grown as exponentially proliferating suspension cultures in RPMI-1640 medium supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine and 50 μg/ml gentamycin and incubated at 37 °C, in humidified atmosphere and 5% CO_2. Resting peripheral blood mononuclear cells (PBMCs) isolated from healthy donors were also used for testing their toxicity. The activity of the liposomal formulations on each cell line was determined by the Sulforhodamine B (SRB) assay (Koukoulitsa *et al.*, 2002). The highest final concentration tested was 100 μM.

Results and Discussion

Lipid classes of almond oil

The analysis of oil A showed the presence of five lipid classes (Table I). Neutral lipids were found in high amounts (89.9% of total lipids) and the predominant lipid class was the triacylglycerides (84.7%) while the polar lipids represented 10.1% of total lipids. The polar lipids were found to be consisted of 22% sphingolipids and 78% phospholipids. Further analysis of sphingolipids presented two distinctive groups of which type III and type IV ceramides were 60% of total sphingolipids. In the phospholipid class the predominant phospholipid was phosphatidylethanolamine (37% of phospholipids). The analysis of almond oil W showed the presence of neutral lipids in a very high proportion (96.3% of total lipids) (Table I). The polar lipids of oil W represented 3.7% of total lipids, phospholipids being the only detected class. In the phospholipid class different phospholipids were detected in similar rates and the predominant phospholipid was phytosphingosine (34% of phospholipids). A significant difference which was observed between phospholipids of oil A and those of oil W was an important decrease in phosphatidylethanolamine proportion, from 2.9% to 0.5% of total lipids, respectively. The most abundant fatty acid was 9-octadecenoic acid (oleic acid), representing 70.2% and 71.2% of fatty acids in oils A and W, respectively. The amount of unsaturated fatty acids is very high 89.4% and 89.7% while the percentage of the saturated fatty acids

Table I. Lipid analysis of almond oil and percentage composition (%) in oils A and W.

Oil A	
Waxes	0.5
Stearylesters	1.1
Triacylglycerides	84.7
Sterols	3.6
Total neutral lipids	*89.9*
Shingolipids	2.2
Ceramide III	1.3[a]
Ceramide IV	
Phospholipids	7.9
Phosphatidylethanolamine	2.9[b]
Total polar lipids	*10.1*
Total lipids	*100.0*

[a] 1.3% represents the percentage of ceramides in total lipids and it corresponds to 60% of the sphingolipids.
[b] 2.9% represents the percentage of phosphatidylethanolamine in total lipids and it corresponds to 37% of the phospholipids.

Oil W	
Triglycerides	95.9
Sterols	0.4
Total neutral lipids	*96.3*
Phytosphingosine	3.2[a]
Phosphatidylethanolamine	0.5[b]
Total polar lipids	*3.7*
Total lipids	*100.0*

[a,b] 3.2% and 0.5% represents the percentage of phytosphingosine and phosphatidylethanolamine in total lipids and they correspond to 33% and 14% of the phospholipids, respectively.

was found to be 10.6% and 10.3% in oils A and W, respectively.

Liposomes

The liposomal formulations were prepared from pure almond phospholipids. The liposome size distribution was determined and indicated that the sonication method resulted in LUVs exhibiting a relatively homogeneous size distribution. Table II shows the distribution mean of the two liposomal formulations prepared from phospholipids from the oils A and W. The charged surface of the liposomes may interfere with the active ingredient encapsulation capacity of the liposomes, as well as their post-administration fate. The knowledge of the lipid content plays an important role in preparation of liposomes with specific characteristics,

Table II. Physical stability of liposomal formulations at 4 °C.

	z-Average mean size [nm]			
Time [weeks]	Liposome A	PI (A)	Liposome W	PI (W)
0	202.8	0.67	218.1	0.45
1	155.3	0.62	179.3	0.32
2	157.8	0.60	172.8	0.37
4	221.3	0.66	245.3	0.31
6	181.6	0.49	253.3	0.34

	ζ-Potential [mV]			
Time [weeks]	Liposome A	SD ± (A)	Liposome W	SD ± (W)
0	– 95.0	8.5	– 70.1	2.2
1	– 96.6	9.5	– 69.9	1.7
2	– 82.6	6.5	– 74.6	4.1
4	– 80.9	2.0	– 68.3	2.1
6	– 88.6	5.4	– 94.1	6.7

because it is a key factor to the ingredient loading capacity of the liposomes and finally in the delivery of drugs and cosmetic ingredients to the targeted tissues. Concerning their physical stability, the size distribution as well as the surface charge of the two liposomal formulations remained satisfactory stable for more than a one-month period (Fig. 1). Therefore, the resultant liposomes have such physicochemical characteristics that make them suitable for further pharmaceutical and cosmetic uses.

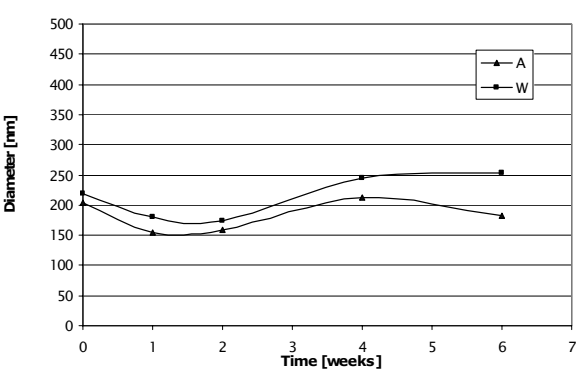

Fig. 1. Physical stability study of liposomes A and W (z-average mean).

Biological activity

The two liposomal formulations were tested for their cytotoxic activity against five cancerous cell lines, *i.e.* H460, MCF7, SF268, DLD1 and OVCAR3 as well as against resting PBMCs isolated from healthy human donors (Koukoulitsa *et al.*, 2002). Liposomal formulations were found to be nontoxic at the highest concentration tested (100 μM). Hence, it seems that the liposomes prepared from almond oil lipids may have future applications for encapsulation and delivery of anticancer drugs as well as active ingredients in cosmetics.

Acknowledgements

This study was funded by grants from the Special Account of Research (E. L. K. E.) of the National and Kapodistrian University of Athens.

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