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ORIGINAL PAPER

In vitro anti-atherogenic properties of traditional Greek cheese lipid fractions

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Abstract Given that platelet activating factor (PAF) is a crucial inflammatory phospholipid mediator that is implicated in the mechanism of atherogenesis, the presence of PAF inhibitors in food reinforces their nutritional value in terms of protection against cardiovascular diseases. The aim of the present study was to evaluate the anti-atherogenic (anti-inflammatory) properties of two different types of Greek cheese: Kefalotyri and Ladotyri. Total lipids (TL) of both types of cheese samples were extracted by the method of Bligh and Dyer and separated into total polar lipids (TPL) and total neutral lipids (TNL) by countercurrent distribution. TPL were further separated by preparative thin-layer chromatography (TLC). TL, TPL, TNL and the obtained polar lipid fractions after TLC separation were tested to determine their biological activity towards atherosclerosis based on the in vitro inhibition of PAF-induced platelet aggregation. Both types of cheese samples exhibited strong biological activity, and their lipids were potent PAF inhibitors. Comparing the two types of cheese samples, Ladotyri cheese polar lipid fractions were found to exhibit stronger inhibitory properties than those of Kefalotyri cheese. The fact that both types of cheese were found to contain PAF inhibitors highlights their nutritional value in terms of cardio-protection.

Keywords Cheese · Polar lipids · Platelet aggregation · Atherosclerosis

1 Introduction

An increasing amount of scientific evidence confirms that many chronic diseases such as cardiovascular diseases (CVDs), hypertension and cancer are connected to an

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unbalanced diet. On the other hand, Mediterranean diet has been suggested to have a protective role against chronic diseases, mainly CVDs and cancer, as well as obesity and type 2 diabetes (Panagiotakos et al. 2005).

Milk and dairy products are significant components of Mediterranean diet since they can be consumed daily (Kastorini et al. 2010). These products can be characterized as mandatory since they contain components with a hypocholesterolaemic effect such as calcium, linoleic acid, conjugated linoleic acid (CLA), antioxidants and lactic acid bacteria or probiotic bacteria. Calcium plays an important role in mediating vascular contraction and vasodilatation, muscle contraction, nerve transmission and glandular secretion, while linoleic acid is beneficial in reducing CVD risk in human subjects (Rogelj et al. 2000).

Dairy products provide more nutritional benefits than milk itself, while their beneficial effects on the cardiovascular system have been shown to be superior to those of milk. This phenomenon has been attributed to the fact that the microorganisms *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, which are involved in the fermentation of yogurt and cheese, have been found to aid the formation of lipids that inhibit platelet activating factor's (PAF) activity (Antonopoulou et al. 1996).

PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (Demopoulos et al. 1979), is a potent inflammatory phospholipid mediator that is implicated in the mechanism of atherogenesis (atheromatosis). A mechanism for the initiation of atherogenesis has been proposed by our team providing a biochemical explanation of the epidemiological data showing that the Mediterranean diet can act protectively against atherogenesis and CVDs (Demopoulos et al. 2003).

Linking dairy consumption to CVDs is feasible today given that there is a growing body of epidemiological, clinical and experimental evidence suggesting that regular cheese intake may reduce the risk of cardiovascular outcomes. Even though recent studies focus on lipid markers of CVDs, scientific data indicate that markers of inflammation are strong predictors of atherosclerotic CVDs. However, there is limited research on the effects of dairy products on inflammatory mediators, such as PAF (Huth and Park 2012). This study aimed to contribute to fill in this gap.

According to the DAFNE project database, Greeks consume on average 55–60 g of cheese daily (Naska et al. 2006). Kefalotyri cheese is one of the most popular cheeses consumed on a daily basis in Greece. It is a hard, salty, yellow cheese made from sheep's and/or goat's milk, and it is produced in several areas of Greece. Ladotyri is a Protected Designation of Origin (PDO) dairy product in the European Union, exclusively produced in Mytilene Island of Greece. It is also a hard, yellow cheese made from sheep's and goat's milk, while its characteristic is that it is packaged with a layer of olive oil, which allows it to be preserved for a longer time and contributes to its characteristic flavour. Both of these types of cheese are exported worldwide (GAIN 2010).

The aim of the current study was to determine and compare the in vitro biological activities of lipid fractions obtained from these two kinds of cheese. These activities were assessed in terms of the ability of the lipids to induce washed platelet aggregation and/or to inhibit PAF-induced platelet aggregation.

2 Materials and methods

2.1 Reagents and instrumentation

All reagents and solvents were of analytical grade and supplied by Sigma-Aldrich (St. Louis, MO, USA) and Merck (Darmstadt, Germany). Fatty acid methyl ester (FAME) standards were of GC quality and supplied by Sigma-Aldrich (St. Louis, MO, USA), as well as bovine serum albumin (BSA) and PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). The chromatographic material used for the preparation of the thin-layer chromatography (TLC) plates in the laboratory was silica gel G-60 supplied by Merck (Darmstadt, Germany), and the polar lipid standard used for TLC separation was a mix standard of hen egg yolk supplied by Sigma-Aldrich (St. Louis, MO, USA). Platelet aggregation was measured in a Chrono-Log (Havertown, PA, USA) aggregometer (model 400-VS) coupled to a Chrono-Log recorder (Havertown, PA, USA).

2.2 Cheese samples

Two different kinds of cheese, namely Kefalotyri and Ladotyri, were purchased from the local market. The origin of the Kefalotyri cheese sample was Elassona, Greece, while the Ladotyri cheese sample came from Mytilene Island, Greece. Both samples were hermetically sealed in plastic wrapping.

2.3 Isolation of lipids

Total lipids (TL) were extracted from 200 g of each cheese sample according to the Bligh-Dyer method (Bligh and Dyer 1959). One tenth of the TL was weighed and stored in sealed vials under nitrogen atmosphere at −20 °C until used, while the rest of it was further separated into total polar lipids (TPL) and total neutral lipids (TNL) by countercurrent distribution chromatography (Galanos and Kapoulas 1962). Countercurrent distribution chromatography allows excellent recovery of TPL from TNL, and the obtained TPL contain glyco- and phospholipids. TPL were weighed and further separated by preparative TLC. All lipid fractions obtained were stored under nitrogen atmosphere at −20 °C for further analysis.

2.4 Fractionation of TPL by preparative TLC

The TLC glass plates (20×20 cm) were coated with silica gel G-60 and activated by heating at 120 °C for 60 min. The thickness of the TLC plates was 1.0 mm (preparative TLC). Approximately 50 mg of TPL of each sample was applied to the TLC plates. A developing system consisting of chloroform/methanol/water 65:35:6 (*v*/*v*/*v*) was utilized for the separation of TPL. The plates were stained under iodine vapours. Nine bands appeared after the separation of TPL of the Kefalotyri cheese sample, and 12 bands appeared after the separation of TPL of the Ladotyri cheese sample. After staining of the TLC plate with iodine vapours, the bands were scraped off, and lipids were extracted from silica gel according to the Bligh-Dyer method (Bligh and Dyer

1959). The chloroform phase was evaporated to dryness under nitrogen, and lipids were weighed, redissolved in 1 mL chloroform/methanol 1:1 (*v*/*v*) and stored at −20 °C.

2.5 Biological assay on washed rabbit platelets

TL, TPL, TNL and purified polar lipid fractions of Kefalotyri and Ladotyri cheese samples obtained by the above TLC separation were tested for their biological activity according to the washed rabbit platelet assay (Demopoulos et al. 1979). Briefly, the samples being examined and PAF were dissolved in 2.5 mg BSA.mL^{-1} saline (0.90% *w*/*v* NaCl). Various amounts of the examined sample's polar lipids, ranging from 0.6 to 38.6 mg, were added into the aggregometer cuvette, and their ability to aggregate washed rabbit platelets and/or to inhibit PAF-induced platelet aggregation was determined. Washed rabbit platelet concentration was approximately 500,000 platelets.μL^{-1}. In order to determine the aggregatory efficiency of either PAF or the examined samples, the maximum reversible PAF-induced aggregation was evaluated, and the 100% aggregation point was determined. The plot of the percentage of the maximum reversible aggregation (ranging from 20 to 80%) versus different concentrations of the aggregatory agent was linear. From this curve, the concentration of the aggregatory agent, which induces 50% of the maximum reversible PAF-induced aggregation, is calculated. This value is defined as the amount of the sample that induces an equivalent to PAF EC_{50}, namely equivalent concentration for 50% aggregation. In order to determine the inhibitory properties of the samples' polar lipids, various amounts of polar lipids being examined, ranging from 0.6 to 38.6 mg, were added into the aggregometer cuvette, and their ability to inhibit PAF-induced aggregation was determined. The platelet aggregation induced by PAF (29.59×10^{-11} M, final concentration in the cuvette) was measured as PAF-induced aggregation in washed rabbit platelets before (considered as 0% inhibition) and after the addition of various amounts of the sample being examined. Consequently, the plot of percent inhibition (ranging from 20 to 80%) versus different concentrations of the sample is linear. From this curve, the concentration of the sample, which inhibited 50% the PAF-induced aggregation, is calculated. This value is defined as IC_{50} namely, inhibitory concentration for 50% inhibition.

2.6 Gas chromatographic analysis

Fatty acid methyl esters (FAMES) of 35 mg of TPL and 35 mg of TNL of both Kefalotyri and Ladotyri cheese samples were prepared using a solution of 0.5 N KOH in CH_3OH (KOH-CH_3OH method, reaction time 5 min) and extracted with *n*-hexane. The fatty acid analysis was carried out using the internal standard method (Nasopoulou et al. 2011). A five-point calibration curve was prepared using five solutions of heptadecanoic (17:0) acid methyl ester and heneicosanoic (21:0) acid methyl ester in ratios of 500:1,000 (*v*/*v*), 500:500 (*v*/*v*), 500:200 (*v*/*v*), 500:100 (*v*/*v*) and 500:50 (*v*/*v*), respectively. Five injections of 1 μL of each solution were analysed with a Shimadzu CLASS-VP (GC-17A) (Kyoto, Japan) gas chromatograph equipped with a split/splitless injector and flame ionization detector. The ratio of the mean area (21:0) to that of the internal standard (17:0) was used as the *y*-axis variable of the calibration curve, while the concentration (mg.kg^{-1}) of 21:0 was used as the *x*-axis variable of the calibration curve. The equation that described the calibration curve was $y=0.0012x+0.0210$ with $r=0.996$.

The ratio of the area of the analyte peak to that of the internal standard represents the y value at the above equation, and subsequently, the x value represents the analyte concentration of the fatty acid in the unknown mixture. Separation of fatty acid methyl esters was achieved on an Agilent J&W DB-23 fused silica capillary column (60 m× 0.251 mm i.d., 0.25 μm; Agilent, Santa Clara, CA, USA). The oven temperature value sequence was initially 120 °C for 5 min, raised to 180 °C at 10 °C min^{-1}, then to 220 °C at 20 °C min^{-1}, and finally isothermal at 220 °C for 30 min. The injector and detector temperatures were maintained at 220 and 225 °C, respectively. The carrier gas was high purity helium with a linear flow rate of 1 $mL.min^{-1}$ and split ratio of 1:50. Fatty acid methyl esters were identified using fatty acid methyl ester standards by matching the retention time of the relative peaks (Nasopoulou et al. 2011).

2.7 Statistical analysis

All experiment analyses were carried out in triplicate, and all results were expressed as mean value±SD. One-way analysis of variance (ANOVA) was used in order to find the significant differences. Differences were considered to be statistically significant when p was less than 0.05. The data were analysed using a statistical software package (PASW 18 for Windows, SPSS Inc., Chicago, IL, USA).

3 Results

3.1 TL, TPL and TNL contents of Kefalotyri and Ladotyri cheese samples

The amounts of TL, TPL and TNL of both cheese samples are shown in Table 1. The TL, TPL and TNL contents of both cheese samples were found to be similar ($p>0.05$) for both cheese samples.

3.2 Fatty acid profile of TPL and TNL of Kefalotyri and Ladotyri cheese samples

The fatty acid profiles of TPL and TNL of Kefalotyri and Ladotyri cheese samples are presented in Tables 2 and 3, respectively. The saturated fatty acids 14:0, 16:0, and 18:0; the monoene 18:1 *cis* (ω-9) and the ω-6 fatty acid 18:2 have been detected in the TPL fraction of both cheeses while the most abundant fatty acids were 16:0 and 18:1 *cis* (ω-9).

With regard to the TNL lipid fraction, the fatty acids that have been detected in both cheese samples were the saturated fatty acids 14:0, 16:0, and 18:0 and the monoene 18:1 *cis* (ω-9) while the most abundant fatty acids were 16:0 and 18:1 *cis* (ω-9).

Table 1 Content of total lipids (TL), expressed in grams per 100 g cheese (mean±SD, $n=3$), total polar lipids (TPL) and total neutral lipids (TNL), expressed as percentages of TL in Kefalotyri and Ladotyri cheese samples

Cheese sample	TL (g/100 g cheese)	TPL (% TL)	TNL (% TL)
Kefalotyri	25.16±1.22	7.11	80.79
Ladotyri	26.01±1.28	8.08	82.78

Table 2 Fatty acid profile of total polar lipids (TPL) of Kefalotyri and Ladotyri expressed in milligrams per kilogram of cheese sample (mean±SD, n=3)

Fatty acids	Kefalotyri	Ladotyri
14:0	0.027±0.001	0.025±0.001
16:0	0.125±0.006[a]	0.054±0.003[b]
16:1 (ω-7)	ND	0.001±0.0001
18:0	0.073±0.004[a]	0.021±0.001[b]
18:1 *cis* (ω-9)	0.211±0.010[a]	0.060±0.003[b]
18:2 (ω-6)	0.040±0.002	0.015±0.0008
18:3 (ω-3)	ND	0.001±0.0001

ND non-detectable

[a, b] Significantly different values within the same row between Kefalotyri and Ladotyri cheese samples (p<0.05), according to ANOVA analysis

Statistically significant elevated levels (p<0.05) were found for the fatty acids 16:0, 18:0 and 18:1 *cis* (ω-9) in the TPL fraction of the Kefalotyri cheese sample when compared to the respective fatty acid levels of the Ladotyri cheese sample (Table 2). On the other hand, the levels of fatty acids 14:0, 16:0, 18:0 and 18:1 *cis* (ω-9) in the TNL fraction of the Ladotyri cheese sample have been found to be statistically higher (p<0.05) compared to the respective fatty acid levels of the Kefalotyri cheese sample (Table 3).

3.3 Biological activity of TL, TPL, TNL and TLC polar lipid fractions of Kefalotyri and Ladotyri cheese samples

The extracted TL, TPL and TNL fractions of both cheese samples were tested for their ability to induce washed rabbit platelet aggregation or inhibit the PAF-induced platelet aggregation. The biological activities of TL, TPL and TNL fractions from both types of

Table 3 Fatty acid profile of the total neutral lipids (TNL) of Kefalotyri and Ladotyri expressed in milligrams per kilogram of cheese sample (mean±SD, n=3)

Fatty acids	Kefalotyri	Ladotyri
14:0	0.131±0.007[a]	0.350±0.018[b]
16:0	0.348±0.017[a]	0.808±0.040[b]
16:1 (ω-7)	ND	0.002±0.0001
18:0	0.082±0.004[a]	0.255±0.013[b]
18:1 *cis* (ω-9)	0.335±0.017[a]	0.599±0.030[b]
18:2 (ω-6)	ND	0.084±0.004
18:3 (ω-3)	ND	ND

ND non-detectable

[a, b] Significantly different values within the same row between Kefalotyri and Ladotyri cheese samples (p<0.05), according to ANOVA analysis

cheese samples, Kefalotyri and Ladotyri cheese samples, expressed in micrograms, are shown in Fig. 1.

According to Fig. 1, all lipid fractions (TL, TPL and TNL) of both cheese samples exhibited inhibitory activity towards PAF-induced platelet aggregation; thus, it may be suggested that these lipids act as PAF inhibitors. The IC_{50} value of the TL of the Ladotyri cheese sample (22.2±1.11 μg) (Fig. 1) was found to be significantly lower ($p<0.05$) compared to the IC_{50} value of the TL of the Kefalotyri cheese sample (44.7± 2.23 μg) (Fig. 1), indicating that the TL of the Ladotyri cheese sample contained more potent PAF inhibitors than the ones of the Kefalotyri cheese sample TL. At this point, it should be underlined that the lower the IC_{50} value, the higher the anti-atherogenic potency of the fraction. A low IC_{50} value—corresponding to a smaller lipid amount—induces the same biological effect: 50% inhibition of the PAF-induced platelet aggregation.

Moreover, the IC_{50} values of the TPL (3.92±1.38 μg) and TNL (6.90±1.15 μg) of the Ladotyri cheese sample (Fig. 1) were found to be similar (no statistical difference, $p>0.05$) compared to the IC_{50} values of the TPL (1.69±0.90 μg) and TNL (5.60± 0.92 μg) of the Kefalotyri cheese sample. However, the synergistic activity of the TPL and TNL of the Ladotyri cheese sample resulted in a more potent PAF inhibitory activity of the TL of the Ladotyri cheese sample compared to the one of the TPL and TNL of the Kefalotyri cheese sample (Fig. 1).

The typical profile of TPL separation on preparative TLC is shown in Fig. 2 while the biological activity of each TLC polar lipid fraction from both cheese samples, Kefalotyri and Ladotyri, expressed in micrograms, is shown in Figs. 3 and 4, respectively.

TLC polar lipid fractions 1 and 2 of the Kefalotyri cheese sample (Fig. 3) were found to exhibit a bimodal biological activity, inhibiting PAF-induced platelet aggregation at low amounts and inducing platelet aggregation at higher ones.

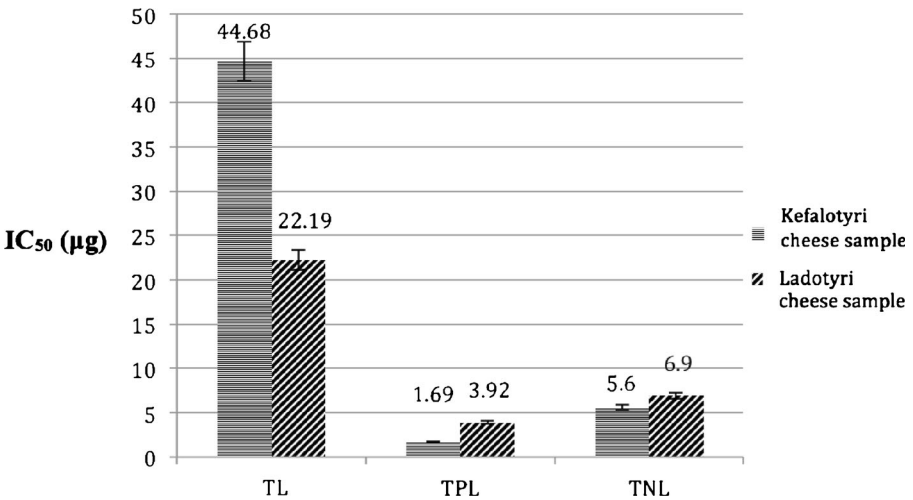

Fig. 1 In vitro biological activity of total lipids (TL), total polar lipids (TPL) and total neutral lipids (TNL) of Kefalotyri and Ladotyri cheese samples towards washed rabbit platelet aggregation, expressed in micrograms. Washed rabbit platelet concentration was approximately 500,000 platelets.μL^{-1}. The final concentration of PAF in the cuvette was 29.59×10^{-11} M

At this point it should be mentioned that some food lipid constituents could exhibit either a PAF agonistic or PAF inhibitory effect. Natural PAF agonists are considered to be the best PAF inhibitors. These molecules act through PAF receptors, inhibiting PAF biological actions at low concentrations while they may induce platelet aggregation at significantly higher concentrations. However, PAF agonists are almost five orders of magnitude less active than PAF—regarding inducing PAF-like platelet aggregation—suggesting that these compounds could minimize atherogenesis, by acting actually as PAF inhibitors at the PAF receptor level in several cells and/or tissues (Nasopoulou et al. 2007; Penna et al. 2011).

All the rest of the TLC polar lipid fractions of the Kefalotyri cheese sample were found to exhibit inhibitory activity towards PAF activity, apart from lipid fraction 7 that was found to have aggregatory properties (Fig. 3). The TLC polar lipid fractions of the Kefalotyri cheese sample, which exerted the most potent PAF inhibitory activity, were found to be lipid fractions 1, 2 and 6 (IC_{50} values 0.35, 0.34 and 0.02 μg, respectively) (Fig. 3), which had similar R_f values (0.13, 0.26, 0.65) to those of lyso-phosphatidylcholine (L-PC), sphingomyelin (SM) and phosphatidylethanolamine (PE), respectively (Fig. 2).

On the other hand, all TLC polar lipid fractions of the Ladotyri cheese sample were found to have only PAF inhibitory properties, and almost all of them were found to exhibit potent biological activity (Fig. 4). More specifically, TLC polar lipid fractions 1, 2, 3, 4, 5, 7, 8 and 10 were found to exert strong PAF inhibitory activity (with IC_{50} values of 0.16, 0.19, 0.01, 0.03, 0.15, 0.16, 0.39 and 0.24 μg, respectively; Fig. 4), while lipid fractions 2, 3, 6 and 8 were found to have similar R_f values (0.16, 0.26, 0.41, 0.65) to those of L-PC, SM, phosphatidylcholine (PC) and PE, respectively (Fig. 2).

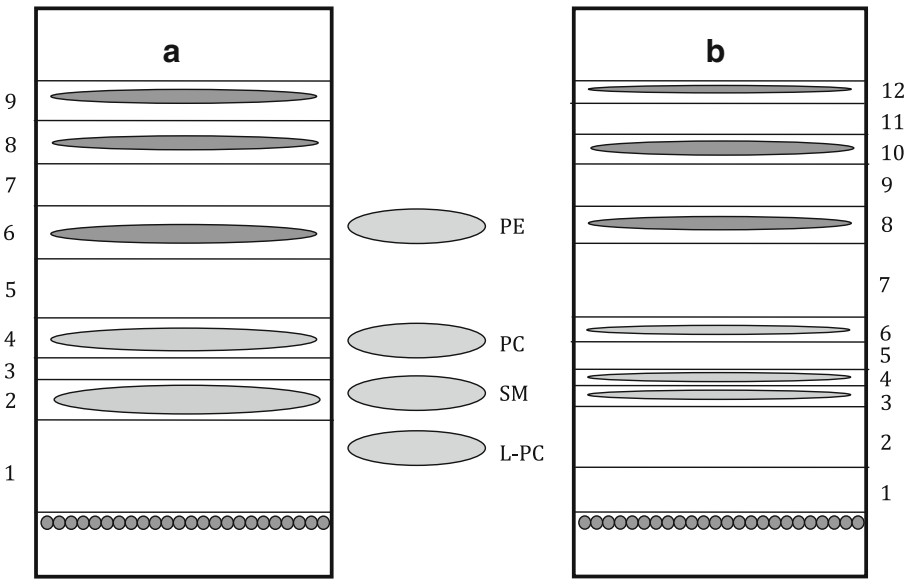

Fig. 2 Typical profile of total polar lipid (TPL) separation on preparative thin-layer chromatography (TLC). **a** Kefalotyri cheese sample, **b** Ladotyri cheese sample. *L-PC* lyso-phosphatidylcholine, *SM* sphingomyelin, *PC* phosphatidylcholine, *PE* phosphatidylethanolamine. The developing system used for the separation of TPL was chloroform/methanol/water 65:35/6 (*v*/*v*/*v*). The preparative TLC plates were stained with iodine vapours

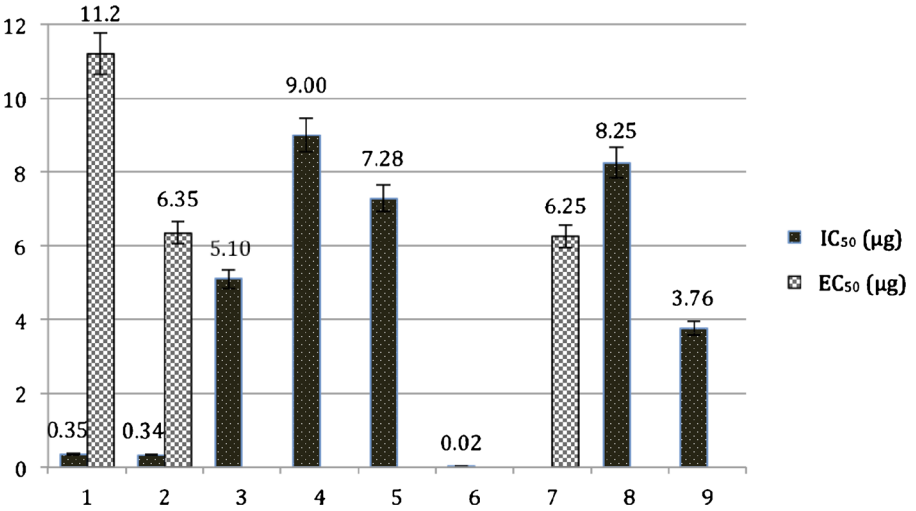

Fig. 3 In vitro biological activity of preparative thin-layer chromatography (TLC) polar lipid fractions of the Kefalotyri cheese sample towards washed rabbit platelet aggregation, expressed in micrograms. The washed rabbit platelet concentration was approximately 500,000 platelets.μL^{-1}. The final concentration of PAF in the cuvette was 29.59×10^{-11} M

By comparing the biological activities of the TLC polar lipid fractions of the two cheese samples, with the same R_f value, it could be suggested that lipid fractions 2 and 4 of the Kefalotyri cheese sample, corresponding to SM and PC, respectively, exhibited statistically significantly higher IC_{50} values ($p<0.05$), thus statistically significantly less

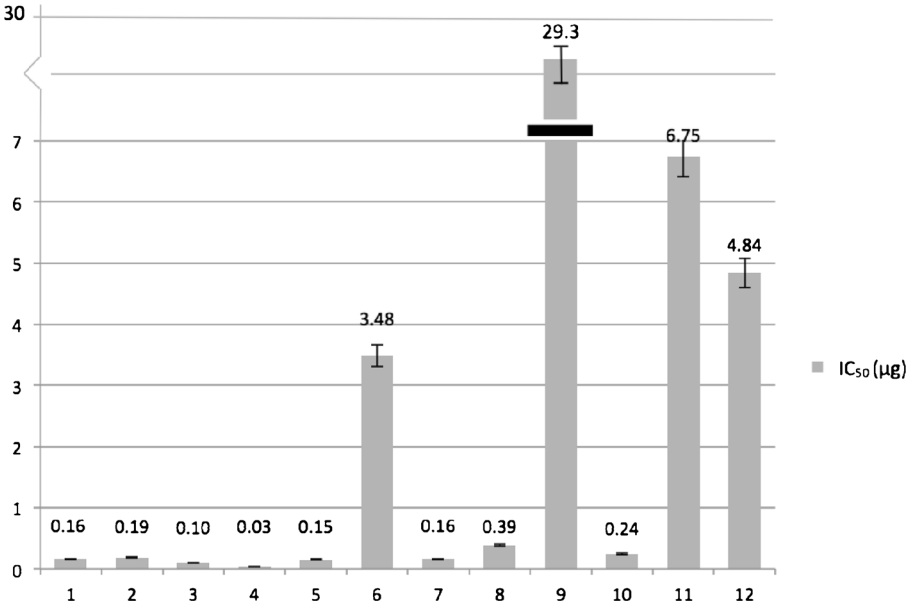

Fig. 4 In vitro biological activity of preparative thin-layer chromatography (TLC) polar lipid fractions of the Ladotyri cheese sample towards washed rabbit platelet aggregation, expressed in micrograms. The washed rabbit platelet concentration was approximately 500,000 platelets.μL^{-1}. The final concentration of PAF in the cuvette was 29.59×10^{-11} M

potent anti-atherogenic activity when compared to the PAF inhibitory activity of lipid fractions of the Ladotyri cheese sample corresponding to SM and PC (lipid fractions 3 and 6) (Figs. 2, 3 and 4). Therefore, polar lipid fractions of the Ladotyri cheese sample corresponding to SM and PC contained statistically significant more potent PAF inhibitors compared to the ones of the Kefalotyri cheese sample. Additionally, lipid fraction 6 of the Kefalotyri cheese sample, corresponding to PE, exerted a statistically significantly decreased IC_{50} value ($p<0.05$), thus more potent PAF inhibitors, compared to the PAF inhibitory activity of the lipid fraction of the Ladotyri cheese sample corresponding to PE (lipid fraction 8) (Figs. 2, 3 and 4).

4 Discussion

It is well known that dairy products contain saturated fats and that their consumption often leads to elevated cholesterol levels in plasma. This, along with the belief that cheese is 'fattening', appears to have led to the widespread conviction that dairy foods are a factor of heart disease and that their consumption should be limited (Elwood et al. 2010).

On the other hand, the anti-atherogenic effects of cheeses revealed in the present study are of utmost importance from a nutritional point of view. Our results support that cheeses may not be as 'harmful' as once believed, since they contain lipid micro-constituents that exert strong anti-atherogenic properties. More specifically, both types of traditional Mediterranean cheeses, i.e. Kefalotyri and Ladotyri, were found to contain PAF inhibitors, with the Ladotyri cheese sample exhibiting a stronger inhibitory potential. This difference could be attributed to the fact that Ladotyri is packaged in olive oil. The presence of olive oil during Ladotyri production seems to have a positive effect, increasing the nutritional value of the product, given olive oil's anti-thrombotic (Tsantila et al. 2007), anti-atherogenic (Karantonis et al. 2006; Tsantila et al. 2007) and general cardio-protective properties (Covas 2007; Urpi-Sarda et al. 2012). Additionally, the Kefalotyri cheese sample was found to contain polar lipid constituents that acted as PAF agonists, which may result in more effective anti-atherogenic activity than PAF inhibitors, since PAF agonists have been found to have better in vivo anti-atherogenic activity than PAF inhibitors (Tsantila et al. 2007; Nasopoulou et al. 2010). Therefore, the detection of such lipid micro-constituents that exhibit PAF agonistic and/or PAF inhibitory properties is a strong indication that these lipids are biologically active compounds towards PAF action and consequently towards atherogenesis.

Phospholipids, such as PE and PC, have been identified in various dairy products (Contarini and Povolo 2013; Kaffarnik et al. 2013). The TLC polar lipid fractions of the cheese samples of the present study, which exhibited potent biological activity, were found to have similar R_f values to those of SM, PC and PE. It is also known that these phospholipids do not exert any biological activity towards PAF, which leads to the fact that the biologically active lipid micro-constituents present in the cheese samples of the present study may not have the typical structure of phospholipids, but they could have the structure of PC derivatives, as our team has recently reported (Nasopoulou et al. 2014).

The statistically significant increased content of 18:1 *cis* (ω-9) fatty acid content in the TNL of the Kefalotyri cheese sample, compared to that of the Ladotyri cheese sample, underlines the contribution of olive oil participation during Ladotyri production, given its high concentration in 18:1 *cis* (ω-9) fatty acid (Visioli and Galli 1998).

The concentrations of the rest of the fatty acids in the two cheese samples (Hauff and Vetter 2009), apart from 16:0, were found to be moderate, indicating a low rate of lipolysis as a consequence of the technology of these products, which does not favour lipase activity (Barbieri et al. 1994).

Dairy products are complex foods that contain fat, calcium, magnesium, potassium, vitamin D, certain amino acids and other lipid micro-constituents which may contribute to the prevention of hypertension, atherosclerosis, type 2 diabetes mellitus (T2DM) and CVDs (Chrysant and Chrysant 2013). Data derived from other studies are in good agreement with our work suggesting that fat derived from cheese could reduce CVD risk markers in rats (Abd El-Salam and Mohamed 2009). Additionally, studies have been found to underline the antihypertensive (Ramchandran et al. 2011) and cholesterol-lowering effect (Ataie-Jafari et al. 2009; Hjerpsted et al. 2011) of dairy products in hypercholesterolaemic subjects. These effects were mainly attributed to the probiotic bacteria content of dairy products. *S. thermophilus* and *L. bulgaricus* bacteria present in yogurt have been demonstrated to biosynthesize important quantities of PAF inhibitors, whereas random contamination of cow milk leads to the production of small amounts of PAF inhibitors (Antonopoulou et al. 1996). These data suggest that the cardio-protective properties of dairy products could be attributed to their probiotic content which exerts PAF inhibitory activity, as well.

The PAF inhibitory effects of olive oil polar lipids have been shown to correlate with clinical parameters, such as atheromatous plaque (Karantonis et al. 2006). Moreover, following a diet rich in PAF inhibitors has been shown to reduce platelet aggregation and increase antioxidant capacity in subjects with type 2 diabetes (Antonopoulou et al. 2006). Although the in vitro data cannot be directly translated to in vivo effects, inhibiting PAF aggregation is indicative of clinical benefits and vice versa.

The novelty of the present study is focusing towards the analysis of two traditional dairy products that have attached socio-economic implications. However, certain limitations are present. As far as the fatty acids profile of the two cheeses is concerned, short chain fatty acids have not been measured. Moreover, it has not been established yet whether the in vitro biological activity of the cheeses studied translates to actual beneficial results for humans. For this reason, animal experiments should follow to ascertain which of these compounds actually inhibits the formation of atheromatic plaque in blood arteries. However, our previous work on sea bream has shown that in vitro anti-inflammatory activities of fish (Nasopoulou et al. 2007) can be linked to beneficial in vivo anti-atherogenic clinical observations (Nasopoulou et al. 2010).

5 Conclusion

To the best of our knowledge, the present study is the first of its kind, focusing on the cardio-protective properties of Kefalotyri and Ladotyri cheese, two of the most popular hard cheese types in Greece. The conclusions derived from this study could be considered at two levels. Firstly, cheese (and in fact animal-origin food) has been shown to contain lipid micro-constituents that are active against the development of CVDs, while similar anti-atherogenic results have been obtained for the polar lipids of hen egg yolk (Nasopoulou et al. 2013).

Secondly, the obtained results could be taken into account during the further development of the cheese-making process with the scope to develop novel functional and nutritious cheese with specific cardio-protective properties.

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