Food Bioscience 37 (2020) 100686

Contents lists available at ScienceDirect

Food Bioscience

journal homepage: www.elsevier.com/locate/fbio

Antithrombotic properties of Spirulina extracts against platelet-activating factor and thrombin

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ARTICLE INFO

Keywords:
Spirulina
Phycocyanobilin
Polar lipids
Platelets
Anti-inflammatory

ABSTRACT

Spirulina (Arthrospira maxima) components have shown several health-benefits, including immunomodulatory and anti-inflammatory properties. Even though a few studies have examined the effects of some *Spirulina*-derived ingredients on platelets, none studied the effects on platelet aggregation induced by inflammatory and thrombotic mediators, namely platelet-activating actor (PAF) and thrombin. In the present study, the antithrombotic properties of compounds extracted from *Spirulina*, namely phycocyanobilin (PCB), phycocyanin-protein, polysaccharides (PS) and lipid extracts, but also of bioactive HPLC-separated lipid-fractions, were assessed in washed rabbit platelets (WRP) activated by PAF and thrombin. All extracts showed strong anti-PAF and anti-thrombin activities in WRP. PCB showed the strongest inhibitory effects on PAF/thrombin-induced platelet aggregation, with a stronger anti-thrombin effect, followed by the relative strong anti-PAF inhibitory effects of the total lipid extracts. HPLC-separation of *Spirulina* lipid extracts into lipid subclasses' fractions showed that specific polar lipid fractions of glyco-lipids and PAF-like phosphatidylcholine moieties exhibited the strongest anti-PAF effects. Some of these lipid-fractions and PCB, when assessed at higher concentrations on WRP showed also an anti-PAF agonistic effect, with the PS extract exhibiting the strongest platelet-aggregatory effect. In conclusion, the results suggested that *Spirulina* seems to be a sustainable source of bioactive compounds with strong anti-PAF and anti-thrombin properties, and thus a potential candidate for developing food supplements and nutraceuticals against inflammation, thrombosis and related disorders. However, more studies are needed to explore the potential and safety of further use of *Spirulina*.

1. Introduction

Spirulina are microscopic photosynthetic and filamentous cyanobacteria (blue–green algae) belonging to the family Oscillatoriaceae that have been used since ancient times as a food source (Sotiroudis & Sotiroudis, 2013; Wu et al., 2016). Apart from its crude protein (60–70%, w/w) and vitamins (4%, w/w), *Spirulina* is also rich in essential amino acids (EEA), minerals, essential fatty acids (EFA), and antioxidants (Sotiroudis & Sotiroudis, 2013; Wu et al., 2016). It is now used as a nutraceutical food supplement since it contains prophylactic and therapeutic nutrients, but also a number of unexplored bioactive compounds (Deng & Chow, 2010; Kulshreshtha et al., 2008; Sotiroudis & Sotiroudis, 2013; Wu et al., 2016). *Spirulina* is generally recognized as safe (GRAS) for human consumption supported by its long history of use as a food source and its favorable safety profile in animal studies (Deng & Chow, 2010; Kulshreshtha et al., 2008; Sotiroudis & Sotiroudis, 2013; Wu et al., 2016). *Spirulina* has shown therapeutic functions such as antioxidant, anti-bacterial, anti-viral, anti-cancer, anti-inflammatory, hypolipidemic, anti-allergic and anti-diabetic functions (Chei et al., 2020; Deng & Chow, 2010; Ku et al., 2015; Kulshreshtha et al., 2008; Mazokopakis, Papadomanolaki, et al. 2014, Mazokopakis, Starakis, Papadomanolaki, Mavroeidi & Ganotakis, 2014; Ngo-Matip et al., 2015; Ouhtit et al., 2014; Serban et al., 2016; Sotiroudis & Sotiroudis, 2013; Wu et al., 2016).

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https://doi.org/10.1016/j.fbio.2020.100686
Received 11 January 2019; Received in revised form 25 June 2020; Accepted 27 June 2020
Available online 10 July 2020

The mechanisms of the beneficial bioactivities of *Spirulina* are not fully understood, while it may have additional unstudied compounds. Most studies have used separate bioactive molecules or different extracts derived from *Spirulina*, such as phycocyanin (the main biliprotein and *Spirulina*'s holoprotein), its linked tetrapyrrole and chromophore phycocyanobilin (PCB), polysaccharide extracts, lipid extracts or specific molecules (Deng & Chow, 2010; Gemma et al., 2002; Ku et al., 2015; Kulshreshtha et al., 2008; Liu et al., 2016; Mazokopakis, Papadomanolaki, et al. 2014, Mazokopakis, Starakis, et al. 2014; McCarty, 2007; Ngo-Matip et al., 2015; Ouhtit et al., 2014; Romay et al., 2000; Serban et al., 2016; Shih et al., 2009; Sotiroudis & Sotiroudis, 2013; Wu et al., 2016). For example, Spirulina proteins, such as C-phycocyanin, have been found to inhibit NO and prostaglandin E(2) over-production through suppressing iNOS and COX-2 induction (Romay et al., 2000; Shih et al., 2009) and attenuation of TNF-alpha formation (Gemma et al., 2002; Shih et al., 2009) and neutrophil infiltration into inflammatory sites (Shih et al., 2009), but also a moderate inhibition of PLA_2 activity (Romay et al., 2000).

Apart from the above-mentioned mechanisms, phycocyanin derived from *Spirulina* has also been studied in platelet models by examining its inhibitory mechanisms on platelet aggregation induced by agonists such as arachidonic acid or collagen (Chiu et al., 2006; Hsiao et al., 2005). Consumption of an aqueous cyanophyta extract (ACE), containing a high dose of phycocyanin (~1 g phycocyanin/day, based on the highest dose GRAS by the U.S. Food and Drug Administration) (Jensen et al., 2016) showed safety based on anticoagulant activity and platelet activation status, since no changes were observed for P-selectin expression, serum P-selectin levels, activated partial thromboplastin time, thrombin clotting time, or fibrinogen activity (Jensen et al., 2016). Nevertheless, further studies are needed to fully elucidate the whole spectrum of the effects of phycocyanin extracts derived from *Spirulina* on platelet pathophysiology.

With respect to *Spirulina* related lipid molecules, some biological activities have been attributed to a sulphoquinovosyl diacylglycerol (SQDG), which is a natural sulpho-glycolipid found in all photosynthetic plants, cyanobacteria, and algae, and has been shown to have anti-tumor, anti-viral and anti-inflammatory activities (Berge et al., 2002; Chirasuwan et al., 2009; Gustafson et al., 1989; Liu et al., 1998; Shirahashi et al., 1993; Vasange et al., 1997). This sulphonic-acid containing polar glycolipid (SQDG) has also been found to strongly inhibit inflammation induced by platelet-activating factor (PAF) through an antagonistic effect on PAF-receptor in human neutrophils, but also through inhibiting its biosynthesis (Liu et al., 1998; Vasange et al., 1997). Based on these results, a European patent has been approved for the use of this glycolipid as a new PAF-receptor antagonist for the prophylaxis or treatment of inflammatory skin diseases, especially psoriasis (Bohlin et al., 2004).

However, the inhibitory effect of such lipid molecules and several *Spirulina* derived lipid extracts, polysaccharide-extracts and protein-extracts (e.g., phycocyanin and PCB) have not been studied against platelet aggregation induced by the inflammatory and thrombotic platelet-agonists, PAF and thrombin.

The classic molecule of PAF is that of a 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (Demopoulos et al., 1979). Moreover, PAF and PAF-like molecules (molecules with similar structure and activities like PAF) belong to a family of molecules (the PAF-family), which are signaling phospholipid mediators of inflammation implicated in several inflammation-related diseases (Tsoupras, Lordan & Zabetakis, 2018), such as atherosclerosis and CVDs (Demopoulos et al., 2003; Tsoupras, Lordan & Zabetakis, 2018), renal disorders (Tsoupras et al., 2011, Tsoupras, Lordan & Zabetakis, 2018, 2007; Verouti et al., 2013), cancer (Tsoupras et al., 2009; Tsoupras, Lordan & Zabetakis, 2018), several persistent infections (e.g., HIV-infection, periodontitis, leishmaniosis and sepsis) (Chatzovoulos et al., 2011; Papakonstantinou et al., 2014; Tsoupras et al., 2006; 2008; Tsoupras, Chini, Mangafas, et al., 2011; Tsoupras, Chini, Tsogas, et al., 2011; Tsoupras, Lordan & Zabetakis, 2018), autoimmune disorders including psoriasis (Tsoupras, Lordan & Zabetakis, 2018) and neurodegenerative disorders (Tsoupras, Lordan & Zabetakis, 2018), etc.

Thrombin, on the other hand, is a serine protease participating in coagulant catalytic processes, such as the conversion of fibrinogen into fibrin and the activation of the V, VIII, XI, and XIII (Tsopanoglou & Maragoudakis, 2009) coagulation factors, as well as the activation of many cell types and platelets including platelet aggregation (Li et al., 2010). Increasing evidence points to an extensive cross-talk between inflammation and coagulation systems in severe chronic diseases such as sepsis and cancer, whereby inflammation leads to activation of coagulation, and coagulation also considerably affects inflammatory activity (Tsoupras et al. 2009, Tsoupras, Chini, Tsogas, et al., 2011). For example, in melanoma metastasis the PAF/PAF-receptor pathway interrelates with that of the thrombin and its receptor (PAR1) pathway (Melnikova et al., 2008). As a result, the study of bioactive molecules with anti-PAF and/or anti-thrombotic activities, especially when derived from non-toxic nutraceuticals of natural origin such as *Spirulina*, may be of great importance.

The aim of this study was to examine the putative *in vitro* anti-inflammatory and anti-coagulant effects of several lipid, protein and polysaccharide (PS) extracts and bioactive molecules derived from *Spirulina*, against the PAF and thrombin related pathways of platelet activation. Therefore, the protein extracts of *Spirulina* (phycocyanin extracts), PCB and the PS-extracts of *Spirulina*, as well as the lipid extracts of *Spirulina* and lipid fractions derived from further separation of these lipids extracts using chromatographic analysis (HPLC), were tested for their ability to inhibit PAF or thrombin induced platelet aggregation and/or to cause platelet aggregation. Moreover, lipids secreted in the culture medium were also isolated and examined, since microalgae and cyanobacteria secrete lipids and lipid containing vesicles, respectively, that are rich in sulpholipids and glycolipids (Biller et al., 2014; Kind et al., 2012).

2. Materials and methods

2.1. Spirulina (Arthrospira) cultures

Freeze-dried *Spirulina (Arthrospira) sp.* powder from Algae A.C. (Nigrita, Greece) was used to separate crude phycocyanin, PCB and PS extract.

Fresh biomass of *Spirulina (Arthrospira maxima)* cultures were used for the separation of lipid molecules. For the cultivation of working cultures, stock cultures of a *Spirulina (A. maxima)* strain (COMPERE 1968/3786, CCALA, Třeboň, Czech Republic), which was kindly offered by Algae A.C., was used. The microalgae cultivation was done in 1 L conical flasks using an illuminated orbital shaker incubator. The flasks were placed on the shaker at 150 rpm. The inoculum was prepared using a modified Zarrouk medium (400 mL) (Markou et al., 2015) with the following composition: 16.8 g/L $NaHCO_3$, 2.5 g/L $NaNO_3$, 0.5 g/L KH_2PO_4, 1.0 g/L K_2SO_4, 1.0 g/L NaCl, 40 mg/L $CaCl_2$, 80 mg/L Na_2EDTA, 200 mg/L $MgSO_4{\cdot}7H_2O$, 10 mg/L $FeSO_4{\cdot}7H_2O$ and 1.0 ml of trace elements stock solutions: 2.86 g/L H_3BO_3, 20 mg/L $(NH_4)6Mo_7O_{24}$, 1.8 g/L $MnCl_2{\cdot}4H_2O$, 80 mg/L $CuSO_4$ and 220 mg/L $ZnSO_4{\cdot}7H_2O$, purchased from Merck (Darmstadt, Germany). The cultures were incubated at 30 °C under constant illumination of 12 μmol photons $m^{-2}s^{-1}$ (measured in the middle of the flasks with a portable digital Lux meter (Labmatrix Manufacturing LLP, Bengaluru, India) provided by MR16 12V 50W Osram tungsten halogen lamps (Osram Light AG, München, Germany) positioned on the top of the flasks. The pH of the growth medium was initially adjusted to 9.3 by adding NaOH. *Spirulina* cells were cultured as above until the late linear growth phase, then the cells were separated from the medium with filtration. The growth of Spirulina was followed by determining the concentration of cells in diluted cell suspensions using spectrophotometric measurements at 560 nm (Cary 1E Varian UV–Vis spectrophotometer, Harbor City, CA,

USA) and the concentration was plotted on a standard curve based on dry weight (g/L).

2.2. Materials and instruments

Centrifugations were done in a 3L-R Heraeus Labofuge (Hanau Germany), a 400R Heraeus Labofug and a RC-5B Sorvall (Newtown, CT, USA) refrigerated superspeed centrifuge apart from the centrifugation at 20,000×*g*, which was done in a refrigerated Micro 22R Zentrifugen Hettich (Kirchlengern, Germany) superspeed centrifuge. PAF-induced platelet aggregation studies were done in a model 400 VS aggregometer from Chrono-Log (Havertown, PA, USA) coupled to a Chrono-Log recorder at 37 °C with constant stirring at 1200 rpm, ZHWY-211C Zhicheng Incubator Shaker (Zhicheng Instruments, Shanghai, China), for the culture of *Spirulina*. Bovine serum albumin free of fatty acids (BSA), standard synthetic PAF (1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) and standard active thrombin, dimethyl sulfoxide (DMSO), trichloroacetic acid (TCA), ammonium sulfate of biochemistry grade and analytical reagents and organic solvents were purchased from Sigma Aldrich Co. (St. Louis, MO, USA).

2.3. Extraction, separation and partial purification of phycocyanin and PCB from Spirulina

Phycocyanin was partially purified from the *Spirulina* powder. Briefly, the powder was suspended in distilled water and the protein was extracted by repeated (4 times) 6 h of freezing at −20 °C and 1 h of thawing at 25 °C in the dark. After the removal of cell debris by centrifugation using a fixed angle rotor SS-34 of the RC-5B (11,200×*g* at 4°C for 10 min), the protein in the supernatant (total protein extract) was precipitated with ammonium sulfate (70%). The solution was allowed to stand for 24 h at 4°C in the dark and the precipitate was collected by centrifugation with the same conditions. The precipitate was dialyzed against 5 L distilled water at 4°C in the dark for 24 h (three changes of dialysis buffer) in a cellulose membrane dialysis tubing (Sigma, D9652) with 12,000 Da nominal MWCO. The dialyzed protein solution was further used as partially purified phycocyanin.

PCB was prepared from partially purified phycocyanin as follows: The ammonium sulfate phycocyanin, before re-suspension and dialysis, was loaded on a vacuum filtration apparatus (Büchner funnel connected to a Büchner flask and a vacuum pump) and extensively washed successively with 2 L of anhydrous n-hexane and 2 L of anhydrous MeOH for a period of 40–60 min to remove *Spirulina* pigments. PCB was then cleaved from the dried protein powder using methanolysis as described (Beuhler et al., 1976) without converting the diacid pigment to its dimethyl ester. The concentration of phycocyanin was estimated spectrophotometrically as follows (Sode et al., 1991): Phycocyanin (mg/mL) = (A_{615}-0.474 X A_{650})/5.34; A_{615} = a_{615}-a_{750}; A_{650} = a_{650}-a_{750} where a_{615}, a_{650} and a_{750} is the absorbance of the sample at each wavelength. PCB concentration was determined by measuring the absorbance of a diluted sample in HCl–MeOH (5% w/v) at 690 nm (ε = 37900 M^{-1} cm^{-1}) (Cole et al., 1967).

The potential PAF-like activity and/or inhibitory effect against PAF, and thrombin biological activities of the phycocyanin and PCB extracts were measured as explained below (section 2.6).

2.4. Extraction, separation and purification of PS from Spirulina

The extraction, separation and partial purification of PS from *Spirulina* powder was done as previously described (Hayashi et al., 1996). Briefly, the freeze-dried powder of *Spirulina* sp. (5 g) was extracted with boiling H_2O (50 mL) for 10 min. After centrifugation with the SS-34 in the RC-5B (16,100×*g* at 4°C for 10 min), the supernatant (40 mL) was treated with 10% TCA. After 1 h at −20 °C, the supernatant (30 mL) obtained by centrifugation at the same conditions was dialyzed for 48 h against distilled H_2O. After the centrifugation (16,100×*g* at 4°C for 30 min) the supernatant was precipitated with 3 volumes of ethanol (96%). After leaving the solution in a refrigerator for 2 h the precipitate containing the PS was dried under vacuum at room temperature (20–22 °C) for 24 h to a constant weight and was dissolved in water. Its potential PAF-like activity and/or inhibitory effect against PAF and thrombin biological activities were measured as explained below (section 2.6).

2.5. Extraction, separation and purification of bioactive lipid molecules from Spirulina cells and culture medium supernatants/filtrates

Cells of *Spirulina* working cultures were separated from the growth medium (supernatant/filtrate) by filtration on a Whatman filter paper No. 3, purchased from Sigma Aldrich Co. (St. Louis, MO, USA). Total lipids of *Spirulina* cells (biomass) were extracted into $CHCl_3$ according to the method of Bligh and Dyer (1959), vaporized with a Heidolph rotary evaporator Laborota 4000 (Heidolph Instruments GmbH & Co. KG., Schwabach, Germany) with a stream of N_2, re-dissolved in a small volume of $CHCl_3$:MeOH at a ratio 1:1 v/v and stored in −20 °C, for a maximum of 8 wk. The same procedures were used to extract total lipids from the growth medium supernatants/filtrates of the *Spirulina* cultures.

Further separation of the total lipid extracts of both the cell biomass and the filtrate medium to several lipid subclasses/fractions was done using a one-step HPLC separation system of bio-functional lipid compounds from natural sources, as previously described by Tsoupras et al. (2012). Briefly, the HPLC-analysis was done at room temperature on an HP HPLC Series 1100 liquid chromatography model (Hewlett Packard, Palo Alto, CA, USA), by applying the one-step HPLC lipid-separation procedure on a semi-preparative reversed phase column Luna 5u C8 (2) (100 Å × 15 cm) (Phenomenex, Torrance, CA, USA), with a mobile phase being a gradient of HPLC-high-purity acetonitrile (ACN) and water (W) as follows. For 0–2 min after the injection of the sample a mobile phase of ACN:W at 40:60 v/v was used and for 2–26 min a gradient was used until a ratio of ACN:W 100:0 v/v, which was used until the end (80 min) of the experiment. The flow rate on all HPLC procedures was 3 mL/min. During HPLC separation, lipid fractions were manually collected according to absorbance (208 nm), evaporated under a stream of nitrogen, re-dissolved in $CHCl_3$:MeOH (1:1 v/v) and stored at −20 °C for a maximum of 4 wk.

A small volume of solutions in $CHCl_3$:MeOH (1:1 v/v) of total lipids extracted from *Spirulina* cells and cell-medium/filtrate, as well as lipid molecules from each HPLC fraction, were evaporated under a stream of nitrogen, re-dissolved in BSA (0.25% w/v in saline), and their potential PAF-like activity and/or inhibitory effect against PAF and thrombin biological activities were measured as explained below (section 2.6).

2.6. Biological assay in WRP

The antagonistic effects (inhibitory effects) against PAF biological activities on platelets and/or the agonistic effects (PAF-like activity for activating platelets), were measured for all the above mentioned *Spirulina*-derived samples/extracts using biological assays on platelet aggregation of WRP, as previously described (Tsoupras et al., 2006; 2008; Tsoupras, Chini, Tsogas, et al., 2011; 2012). The samples tested for such antagonistic/agonistic effects were the total proteins extract, the crude phycocyanin, the PCB, and the PS extracts that were extracted from *Spirulina* sp powder., but also the total lipids extracted either from *Spirulina* cells or from cell-medium/filtrate of *Spirulina* working cultures, as well as each one of the lipid fractions derived from the HPLC separation procedure of these two total lipids extracts of *Spirulina*. In addition, some of these extracts were also tested for their potential inhibitory effect towards thrombin induced aggregation of WRP as previously described (Tsoupras, Chini, Tsogas, et al., 2011).

The lipid samples, as well as the synthetic PAF, were dissolved in BSA (0.25% w/v in saline), whereas the samples of phycocyanin and PS and thrombin were dissolved in saline and the sample of phycocyanobilin in DMSO. For this reason the effects of saline and DMSO were also assayed

with WRP to evaluate their effects.

Samples were tested for their ability to inhibit PAF or thrombin induced aggregation of WRP (antagonistic effect) in the aggregometer (Havertown, PA, USA). Briefly, the platelet aggregation induced by PAF ($1–15 \times 10^{-11}$ M final concentration, in the aggregometer cuvette) or thrombin (0.10–0.01 International Units (IU)) was measured as PAF or thrombin induced aggregation in WRP before (considered as 0% inhibition) and after the addition of various concentrations of the sample. The plot of percentage inhibition (within the range of 20–80%) versus different concentrations of the sample was linear. The concentration of the sample that inhibited 50% of PAF or thrombin induced platelet aggregation was calculated. This value is defined as the concentration for 50% inhibition, namely the IC_{50} value (half maximal inhibitory concentration). The lower the IC_{50} value the stronger its inhibitory effect against PAF/thrombin-induced aggregation of platelets.

The aggregatory (agonistic) effect of samples on platelets was also studied as previously described (Chatzovoulos et al., 2011; Tsoupras et al., 2006; 2012). Briefly, various concentrations of each sample were added to the aggregometer cuvette and the aggregation of platelets induced by the sample was measured. A linear relationship was found between the concentration (within a specific range that is usually higher than the IC_{50} value) that induce platelet aggregation within the range of 20–80% of the maximum-reversible aggregation of platelets. The amount in mg of the sample needed to induce 50% of platelet aggregation is defined as the EC_{50} value (half maximal effective concentration). The lower the EC50 value the stronger its agonistic effect on platelet aggregation. In desensitization (a) and cross-desensitization (b) experiments, platelets were activated by the addition of the test sample (a) or PAF (b) to the platelet suspension at a concentration that caused reversible aggregation. Stimulation with the test sample (a or b) was done immediately after complete disaggregation as previously described (Chatzovoulos et al., 2011; Tsoupras et al., 2006; 2012).

Biological assays were done several times ($n > 3$) to determine precision.

2.7. Statistical analysis

One way analysis of variance (ANOVA) was used to determine the significant differences ($p \leq 0.05$) between IC_{50}/EC_{50} values against PAF and thrombin. Fisher's LSD multiple comparison test was used to determine the significant statistical differences between analyses ($p \leq 0.05$). Although a $p \leq 0.05$ was generally used, the authors have also chosen to use $p \leq 0.01$ for some of the data to indicate the greater significance of the differences. The data were analyzed using the Statistical Package for the Social Sciences version 25 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Inhibitory (antagonistic) and aggregatory bio-activities of Spirulina-derived total protein, phycocyanin, PCB, PS and total lipid extracts against PAF and thrombin

Total protein, phycocyanin, PCB and PS extracts derived from *Spirulina* sp., as well as total lipid extracts derived from *Spirulina (A. maxima)* cultures, showed strong inhibitory effects against both PAF and thrombin in WRP. The inhibitory effect of each tested extract against PAF/thrombin-induced platelet-aggregation of WRP is expressed as an IC_{50} value and shown in Table 1.

The purified PCB showed the strongest inhibitory effect against both PAF and thrombin induced aggregation of WRP (Table 1) ($p \leq 0.01$ when compared with the total proteins extract and the partially purified phycocyanin). The PCB preparation showed higher inhibitory effect against the thrombin-induced aggregation of platelets when compared to its relative inhibitory effect against PAF-induced platelet aggregation ($p \leq 0.05$); the IC_{50} values of its anti-thrombin effect (~0.09 μg), were ~5 times lower than the relative IC_{50} values of its anti-PAF effect (~0.47 μg). Apart from its comparison with the protein extracts, both the anti-PAF and anti-thrombin effects of purified PCB were also found to be stronger than all the other extracts tested ($p \leq 0.05$ when compared to PS extract from *Spirulina* sp. powder and to total lipids extracts from *Spirulina* cultures) (Table 1).

Even though the partially purified phycocyanin showed a lower inhibitory effect (than that of PCB) against both PAF and thrombin induced aggregation of WRP (Table 1), however its anti-PAF and anti-thrombin effects on platelets were found to be almost one order of magnitude stronger than those of the total protein extract (Table 1). However, in contrast to PCB, the anti-PAF effect of the partially purified phycocyanin extract was similar to its anti-thrombin effect ($p > 0.05$), while the anti-PAF effect of the total protein extract was also found to be similar to its anti-thrombin effect. Unlike PCB, both partially purified phycocyanin and total protein extract showed at least one order of magnitude lower anti-PAF and anti-thrombin effects on platelets than the PS extract from *Spirulina* sp. powder and the total lipids extracts from *Spirulina* cultures ($p \leq 0.01$).

Apart from PCB, the crude extracts of PS showed also a strong inhibitory effect against the PAF-induced platelet aggregation (Table 1), with IC_{50} values higher than the relative ones of the PCB ($p \leq 0.05$), but within the same order of magnitude with the relative ones of the lipid extracts from *Spirulina* cultures ($p > 0.05$). Moreover, the crude extracts of PS also showed a strong inhibitory effect against the thrombin-induced platelet aggregation, similar to the ones of the lipid extracts

Table 1
In vitro inhibitory effect of the bioactive *Spirulina*-derived total-protein, phycocianin, PCB, PS and total lipid extracts against PAF and thrombin induced platelet aggregation of WRP, and their ability to induce platelet aggregation of WRP.

Bioactive Compound	IC_{50} values (μg) against PAF-induced platelet aggregation of WRP[a]			IC_{50} values (μg) against thrombin-induced platelet aggregation of WRP[a]			Platelet aggregation/cross desensitization against either PAF or thrombin induced platelet aggregation
	Median	Min	Max	Median	Min	Max	
TL of Spirulina (Arthrospira maxima) cells	5.19	3.12	5.97	126	112	137	ND
TL of Spirulina (Arthrospira max.) culture medium filtrate	2.16	1.41	2.34	11.4	7.65	13.4	ND
PS extract from powder of Spirulina sp.	5.25	3.96	6.79	16.4	12.2	18.1	+/+[b]
Total proteins extract from powder of Spirulina sp.	129	112	155	159	146	172	ND
Phycocyanin	41.1	27.9	47.1	49.1	43.9	54.1	ND
PCB	0.471	0.182	0.661	0.921	0.312	0.122	+/+[b]

WRP: washed rabbit platelets; TL: total lipid extract; PS: polysaccharide extract; PCB: phycocyanobilin; ND: Not Detected.

[a] IC_{50} values reflect the inhibitory strength of each extract tested against PAF/thrombin-induced platelet aggregation in WRP and is expressed as median of μg of each compound that when present in the aggregometer cuvette (with a total volume of platelet suspension being that of 250 mL) can cause 50% of inhibition on PAF/thrombin-induced aggregation of WRP. The lower the IC_{50} value the stronger the inhibitory effect against PAF/thrombin-induced aggregation of WRP.

[b] +/+ stands for detected platelet aggregation/positive cross desensitization test towards either PAF or thrombin induced platelet aggregation.

of the cell-medium filtrates ($p > 0.05$), but significantly stronger when compared to the effect of the lipid extracts of the cells ($p \leq 0.05$). Furthermore, the anti-thrombin and anti-PAF effects of PS were found to be stronger than the relative ones of both the total protein extract and the partially purified PC ($p \leq 0.01$).

Apart from PCB and the PS extract, the extract of total lipids of *Spirulina* cells also showed a strong inhibitory effect against PAF-induced aggregation of WRP (Table 1). Similar amounts of the total lipids extract of the *Spirulina* medium/filtrate also strongly inhibited PAF-induced aggregation of WRP, with IC_{50} values within the same order of magnitude with the relative ones of the lipid extracts of cells ($p > 0.05$) (Table 1). However, at least one to two orders of magnitude higher quantities of the extract of total lipids of the *Spirulina* cells were needed to inhibit by 50% the thrombin induced aggregation of WRP ($p \leq 0.01$) (Table 1). Thus, the anti-thrombin effect of the total lipids extract of *Spirulina* cells was found to be similar to that of the total protein extract ($p > 0.05$) but much lower than the relative anti-thrombin effects on platelets of all the other extracts tested ($p \leq 0.05$).

In the case of the total lipid extract of the filtrate, ~5 fold higher quantities were needed to inhibit by 50% the thrombin induced aggregation of WRP when compared to its similar anti-PAF effect ($p \leq 0.05$) (Table 1). Thus, the anti-PAF activities against platelet aggregation of both total lipid extracts were significantly stronger ($p \leq 0.05$) when compared to their relative anti-thrombin effect of platelet aggregation. Even though the anti-thrombin effect of the total lipids extract of the cell medium filtrate on platelets was found to be significantly lower than that of the PCB, it was found to be similar to that of the PS extract and stronger ($p \leq 0.05$) to that of the total lipids extracts from cells, of the total protein extracts and of the partially purified phycocyanin.

Moreover, from all these extracts, only the PS extract and the PCB preparation were found to induce platelet aggregation (Table 1). In the cross desensitization tests, platelets aggregated by these extracts were not reactivated by PAF or thrombin and inversely these lipids could not reactivate platelets that were firstly aggregated by PAF or thrombin (Table 1). The aggregatory effects of these samples on rabbit platelets are expressed as EC_{50} values and are shown in Fig. 1. The lower the EC_{50} value for a sample with aggregatory effects on platelets, the higher the potency of inducing platelet aggregation. Concerning this effect at least two to three orders of magnitude higher amounts of the PCB preparation were needed to induce 50% platelet aggregation (EC_{50} value ~170 μg) (Fig. 1), than those needed for its inhibitory effect (IC_{50} value) (Table 1). However, the amount of the PS-extracts that were needed to induce 50% platelet aggregation (EC_{50} value ~23 μg), were found to be ~4 times higher than that needed for its inhibitory effect against PAF (IC_{50} value) (Table 1). Thus, the agonistic effect on PAF-induced platelet aggregation of the PS-extract was found to be significantly stronger than all the other samples tested, including PCB (Fig. 1) ($p \leq 0.01$ in all these comparisons).

3.2. *Evaluation of the inhibitory (antagonistic) and aggregatory bio-activities of HPLC-derived lipid fractions of total lipids extracts from Spirulina cultures against PAF in WRP*

The separation of the total lipid extracts of both the cell-biomass and the culture-medium filtrate in several lipid subclasses was done as previously described in a one-step HPLC-based separation of lipids in polar lipids (PL) fractions (glyco-lipids, phenolo-lipids and phospholipids) and in neutral lipids (Tsoupras et al., 2012). A characteristic chromatogram of this separation is shown in Fig. 1. Lipid fractions were manually collected according to absorption values from the detector (208 nm). In addition the elution times of well established phospholipid standards (PC, L-PC and SM) are also shown in Fig. 2.

Each one of these lipid fractions, which were derived from the HPLC-based separation of the total lipid extracts of either *Spirulina* cell-biomass or culture medium-filtrates, were further tested for their putative inhibitory effect against PAF-induced platelet aggregation of WRP. The IC_{50} values of the lipid molecules contained in each fraction against PAF induced aggregation of WRP, along with their retention times are shown in Table 2. Smaller quantities of lipids fractions of the total lipid extract of culture medium-filtrates from *Spirulina maxima* cultures in the stationary phase were needed to inhibit 50% of PAF-induced platelet aggregation than the relative ones of the lipids fractions derived from

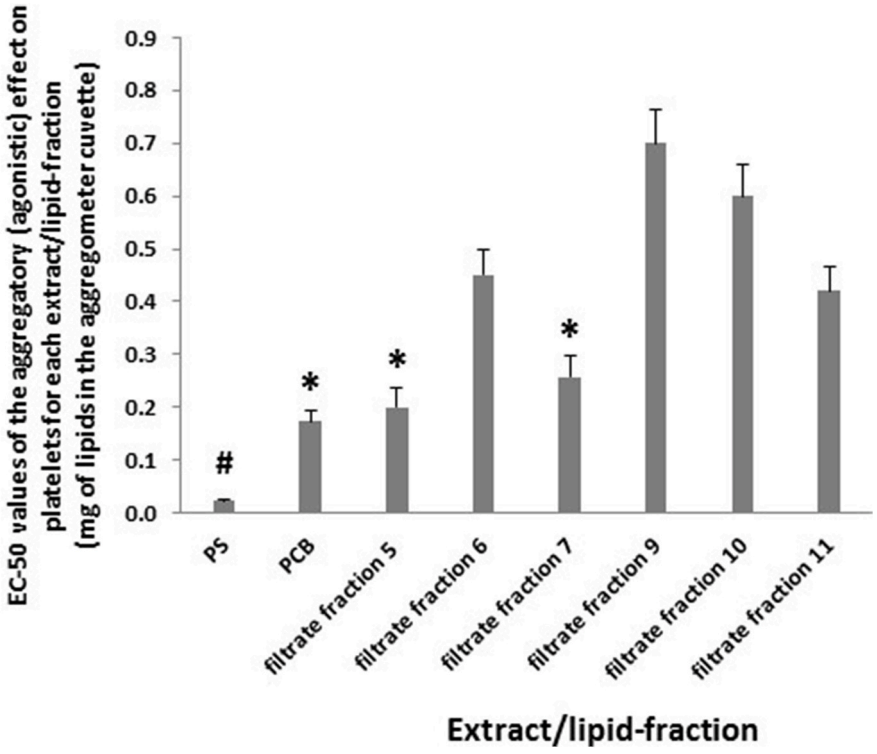

Fig. 1. The aggregatory (agonistic) biological activities of PS, PCB and lipid fractions 5–7 and 9–11 from the HPLC analysis of the total lipids extracts of *Spirulina* cultures medium-filtrates on platelets. #indicates significant difference ($p \leq 0.05$) for the EC_{50} value of PS when compared with all the other samples tested. *indicates significant difference ($p \leq 0.05$) for the EC_{50} values of PCB and lipid fractions 5 and 7 when compared with all the other samples tested. EC_{50} values: half-maximal effective concentration inducing 50% of platelet aggregation; PS: polysaccharides extract; PCB: phycocyanobilin; WRP: washed rabbit platelets.

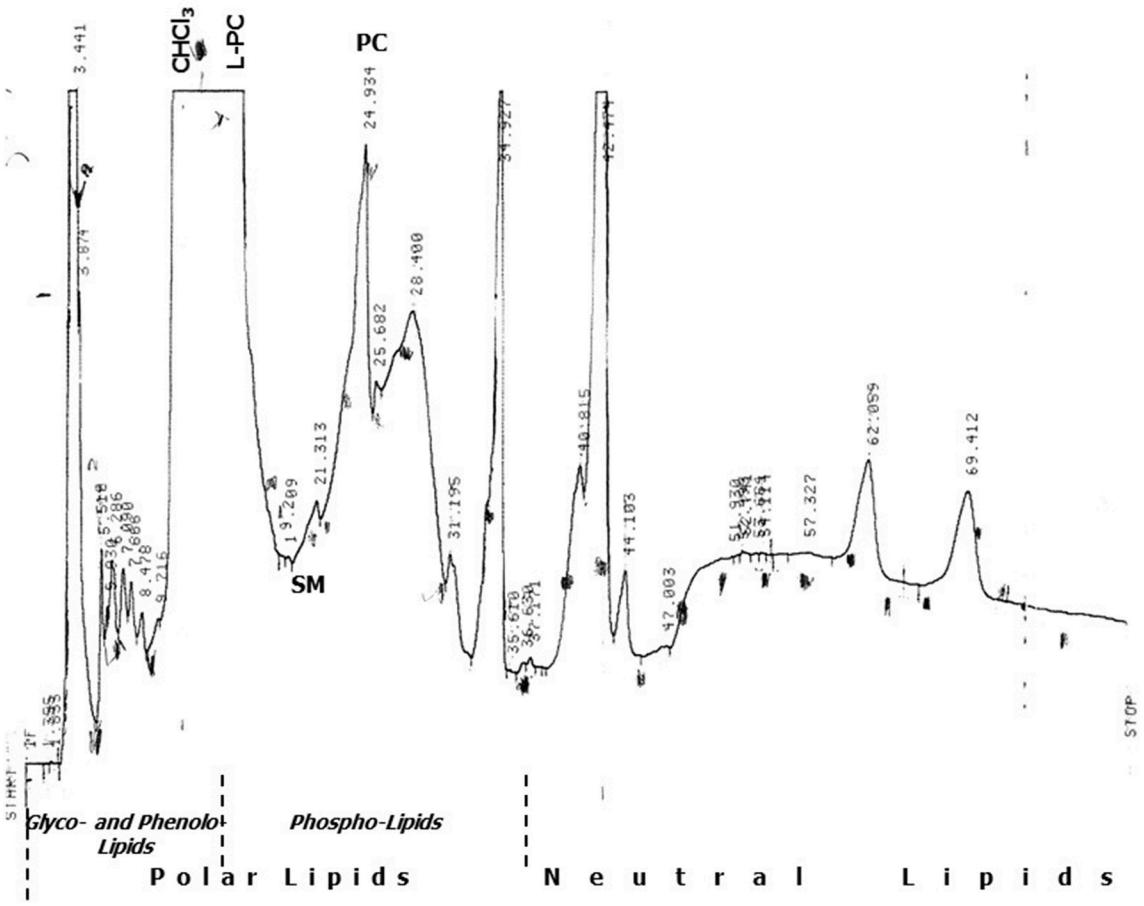

Fig. 2. ***Representative chromatograph of the HPLC-separation of total lipids of Spirulina maxima cells-biomass and culture medium filtrates in the stationary phase.*** $CHCl_3$: chloroform; PC: phosphatidylcholine, L-PC: lyso-phosphatidylcholine, SM: sphingomyelin.

the total lipid extract of *Spirulina* cells in this phase (Table 2).

In the case of lipid fractions derived from the total lipid extracts of the culture medium-filtrates, the polar lipid molecules contained in lipid fractions 5, 9 and 10 and the neutral lipid molecules contained in lipid fractions 28, 29 showed the stronger inhibitory effect against PAF-induced platelet aggregation of WRP (Table 2). In the case of lipid fractions derived from the total lipid extracts of the cell-biomass, only the polar lipid molecules contained in lipid fraction 4 showed strong inhibitory effect against PAF-induced platelet aggregation of WRP and within the same order of magnitude with the relative lipid fractions of the total lipid extracts derived from the culture medium filtrates. Interestingly, the polar lipid molecules contained in fraction 5 derived from the total lipid extracts of the culture medium-filtrates, were eluted with a similar retention time as the relative polar lipid molecules contained in fraction 4 derived from the total lipid extracts of the cell-biomass.

Concerning the ability of these samples to induce platelet aggregation, of all the lipid fractions tested, only the lipid fractions 5, 6, 7, 9, 10, 11 derived from the total lipids extracts of culture medium-filtrates showed an aggregatory effect on platelets (Table 2), but in much higher quantities (EC_{50} values) of at least two to three orders of magnitude than those of their IC_{50} values needed for their inhibitory effect against PAF-induced platelet aggregation of WRP (Fig. 1).

According to cross desensitization tests towards PAF, platelets aggregated by these quantities of each one of the lipid fractions of the filtrate, were not reactivated by addition of standard PAF in the aggregometer cuvette and inversely these samples could not reactivate platelets that were aggregated by standard PAF, implying that these samples induce platelet aggregation through the PAF-pathway (Table 2). For this reason the PAF-pathway related aggregatory effects of these samples on rabbit platelets are also defined as agonistic anti-PAF effects on platelet aggregation, with their relative EC_{50} values of these effects being shown in Fig. 1.

The aggregatory effects on platelets (EC_{50} values) of these lipid fractions were found to be significantly less strong than that of the PS extract ($p \leq 0.05$), while only the lipid-fractions 5 and 7 showed similar effect with the relative aggregatory effect of the PCB (Fig. 1). Lipid fractions 6, 9, 10 and 11 had the highest EC50 values and thus showed the weakest aggregatory effect on platelet aggregation (Fig. 1). Moreover, none of the lipid-fractions derived from the HPLC-analysis of total lipid extract from cells showed any aggregatory activity on platelets.

4. Discussion

Spirulina is proposed as a good source for supplements and pharmaceutical compounds (Deng & Chow, 2010; Kulshreshtha et al., 2008; Mazokopakis, Papadomanolaki, et al. 2014, Mazokopakis, Starakis, et al. 2014; Serban et al., 2016; Sotiroudis & Sotiroudis, 2013; Wu et al., 2016). For example, several health benefits have been attributed to C-phycocyanin (Chiu et al., 2006; Gemma et al., 2002; Hsiao et al., 2005; Jensen et al., 2016; Romay et al., 2000; Shih et al., 2009; Sotiroudis & Sotiroudis, 2013), while a highly bioactive sulpho-glycolipid (SQDG) has shown anti-tumor, anti-viral and anti-inflammatory activities (Berge et al., 2002; Chirasuwan et al., 2009; Gustafson et al., 1989; Liu et al., 1998; Shirahashi et al., 1993; Vasange et al., 1997). Moreover, SQDG also inhibited PAF-induced inflammation by an antagonistic effect for PAF-receptor in human neutrophils, but also by inhibiting PAF-synthesis and thus reducing PAF-levels in these cells (Liu et al., 1998; Vasange

Table 2
Inhibitory effect against PAF induced aggregation of WRP of lipid fractions derived from HPLC analysis of total lipid extracts of *Spirulina* (*A. maxima*) samples from the stationary phase of cells (a1) and filtrate (a2), respectively.

Fractions of TL extracts from cells (elution time in min)[a1]	IC_{50} values against PAF-induced aggregation of WRP (mg)[b] ± SD	Fractions of TL extracts from filtrates (elution time in min)[a2]	IC_{50} values against PAF-induced aggregation of WRP (mg)[b] ± SD	Platelet aggregation/cross desensitization against PAF-induced aggregation of WRP
1 (0–3)	2.8 ± 0.2	1 (0–3.5)	0.34 ± 0.08	ND
2 (3–5)	2.2 ± 0.2	2 (3.5–5)	0.42 ± 0.07	ND
3 (5–6.5)	1.5 ± 0.1	3 (5–7)	1.5 ± 0.1	ND
4 (6.5–11.5)	0.091 ± 0.031	4 (7–8.2)	0.061 ± 0.031	ND
5 (11.5–13.5)	0.83 ± 0.11	5 (8.2–12)	0.012 ± 0.012	+/+[c]
6 (13.5–16.5)	1.7 ± 0.1	6 (12–14)	0.11 ± 0.03	+/+
7 (16.5–19)	5.3 ± 0.3	7 (14–18)	0.054 ± 0.021	+/+
8 (19–21)	1.8 ± 0.2	8 (18–19)	0.062 ± 0.022	ND
9 (21–23)	0.52 ± 0.1	9 (19–21)	0.042 ± 0.011	+/+
10 (23–25)	0.89 ± 0.12	10 (21–22)	0.023 ± 0.013	+/+
11 (25–26)	0.59 ± 0.11	11 (22–23)	0.12 ± 0.04	+/+
12 (26–28)	1.0 ± 0.1	12 (23–25)	0.27 ± 0.06	ND
13 (28–30)	0.46 ± 0.08	13 (25–26)	ND	ND
14 (30–31.2)	0.59 ± 0.07	14 (26–28)	0.062 ± 0.024	ND
15 (31.2–33.5)	4.2 ± 0.5	15 (28–31)	0.051 ± 0.022	ND
16 (33.5–35.5)	1.2 ± 0.1	16 (31–34)	0.052 ± 0.021	ND
17 (35.5–37)	0.59 ± 0.09	17 (34–37)	0.061 ± 0.022	ND
18 (37–40)	2.3 ± 0.2	18 (37–40)	0.062 ± 0.022	ND
19 (40–43.5)	3.6 ± 0.4	19 (40–43)	0.083 ± 0.031	ND
20 (43.5–47)	ND	20 (43–46)	0.081 ± 0.032	ND
21 (47–50)	1.7 ± 0.2	21 (46–49)	0.38 ± 0.09	ND
22 (50–53)	0.59 ± 0.11	22 (49–52)	0.31 ± 0.08	ND
23 (53–56)	0.59 ± 0.09	23 (52–55)	0.071 ± 0.021	ND
24 (56–59)	0.73 ± 0.08	24 (55–58)	0.27 ± 0.07	ND
25 (59–61.5)	0.52 ± 0.06	25 (58–61)	0.48 ± 0.13	ND
26 (61.5–65)	0.59 ± 0.07	26 (61–64)	0.38 ± 0.09	ND
27 (65–67.3)	1.1 ± 0.1	27 (64–67)	0.061 ± 0.022	ND
28 (67.3–70)	1.0 ± 0.1	28 (67–70)	0.022 ± 0.012	ND
29 (70–73)	0.51 ± 0.07	29 (70–73)	0.041 ± 0.021	ND
30 (73–76)	0.69 ± 0.09	30 (73–77)	0.19 ± 0.08	ND
31 (76–79)	0.59 ± 0.08	31 (77–81)	0.063 ± 0.024	ND

[a]Elution times of each lipid fraction derived from the HPLC-separation of the total lipids extracts of either the cell-biomass (a1) or the culture medium-filtrates (a2); [b]Biological activities of each lipid fraction against PAF-induced platelet aggregation of WRP, expressed as mean IC_{50} values (n = 3) that reflect the inhibitory strength of each lipid sample (mg of lipids in the aggregometer cuvette that causes 50% of inhibition on PAF-induced aggregation of WRP). The lower the IC_{50} value the stronger the inhibitory effect against PAF-induced aggregation of WRP; The concentration of PAF present in the aggregometer cuvette was 1–15 × 10^{-11}M; [c]+/+ detected platelet aggregation/positive cross desensitization test against PAF-induced platelet aggregation of WRP; WRP: washed rabbit platelets; SD: standard deviation; ND: not detected platelet aggregation.

et al., 1997).

Activation and aggregation of platelets are also important markers of inflammatory manifestations implicated in the onset, development and progression of several chronic disorders including atherosclerosis and CVD (Demopoulos et al., 2003; Horstman et al., 2010; Sheremata et al., 2008; Stokes & Granger, 2012; Tsoupras, Lordan & Zabetakis, 2018, 2019; Wachowicz et al., 2016). Thus, the effects of *Spirulina* derived compounds against platelet aggregation is of importance for evaluating its antithrombotic and cardio-protective properties. Inhibitory effects of *Spirulina*-derived phycocyanin protein on platelet aggregation have been reported, but only against the arachidonic acid and collagen pathways (Chiu et al., 2006; Hsiao et al., 2005).

Within the present study, apart from *Spirulina* spp. derived phycocyanin protein extracts, several other compounds of *Spirulina* were assessed against aggregation of platelets induced by PAF and thrombin, such as PCB, the PS extracts, the lipid extracts and their HPLC-derived lipid-fractions. The inflammatory and thrombotic mediators PAF and thrombin were chosen due to their high potency in inducing platelet aggregation and their central role in the development of several disorders (Tsoupras et al., 2007, 2008, 2009, Tsoupras, Chini, Mangafas, et al., 2011, Tsoupras, Chini, Tsogas, et al., 2011, Tsoupras, Lordan & Zabetakis, 2018, 2006; Chatzovoulos et al., 2011; Demopoulos et al., 2003; Li et al., 2010; Melnikova & Bar-Eli, 2007; Melnikova et al., 2008; Papakonstantinou et al., 2014; Stokes & Granger, 2012; Tsopanoglou & Maragoudakis, 2009; Verouti et al., 2013).

PCB and both protein extracts (the total protein extract and the partially purified PC extract) of *Spirulina* sp. powder strongly inhibited WRP aggregation induced by PAF or thrombin (Table 1), with PCB showing the strongest anti-PAF and anti-thrombin effects. The inhibitory effects of the phycocyanin protein and of the total protein extract of *Spirulina* against platelet aggregation induced by PAF and thrombin can be attributed to its PCB content, since PCB was derived from the partially purified phycocyanin protein and both were found to be much more active against both PAF and thrombin than the total protein extract. In addition, the anti-thrombin effect of PCB was stronger than its anti-PAF effect, suggesting that the tetrapyrrole chromophore of *Spirulina* preferentially affects the thrombin pathway rather than the PAF pathway. Moreover, the desensitization/cross-desensitization assays showed that from all these protein-samples, only PCB was found to induce platelet aggregation, through either the PAF-pathway or the thrombin pathway, but in much higher amounts (EC_{50} values) than those needed for inhibiting platelet aggregation (IC_{50} values), suggesting also an agonistic anti-PAF and anti-thrombin effect for PCB.

These strong antithrombotic properties of *Spirulina* protein extracts against both PAF and thrombin pathways further support their previously reported health benefits (Chiu et al., 2006; Gemma et al., 2002; Hsiao et al., 2005; Jensen et al., 2016; Romay et al., 2000; Shih et al., 2009; Sotiroudis & Sotiroudis, 2013).

The crude extracts of PS was also found to show a strong inhibitory effect against WRP aggregation induced by PAF or thrombin (Table 1). The anti-PAF and anti-thrombin effects of the PS extract were less strong than those of the PCB, but of similar potency to those of the total lipid extracts of *Spirulina* cell-biomass and culture medium-filtrates (Table 1). These strong anti-PAF and anti-thrombin properties of PS are a

beneficial effect for *Spirulina* derived polysaccharides that can be added to their reported antioxidant and anti-aging activities (Wang et al., 2018; Wu et al., 2016; Zhang et al., 2015).

However, higher amount of PS extract induce platelet aggregation, while the desensitization/cross-desensitization assays showed that the agonistic effect of PS on WRP seems to take place through either the PAF-pathway or the thrombin pathway. The amounts of the PS-extracts that were needed to induce platelet aggregation (EC_{50} values) were found to be higher than those of all the other samples tested and of the same order of magnitude with those needed for PS inhibitory effect (IC_{50} values against thrombin), suggesting that PS has strong aggregatory effects on platelets. This was in accordance with similar results reported for other polysaccharide extracts of marine sources such as seaweed (Manne et al., 2013), and should be of consideration when using PS from *Spirulina* in several health-related supplements and nutraceuticals.

It should also be noted that *Spirulina* sp. powder was preferably selected for protein extractions, instead of fresh cultures, to ensure high yield for bioactive PC, PCB and PS that can be more effectively extracted from powder than fresh cultures, which was the case for the lipid components of *Spirulina*. Fresh cultures of *Spirulina* were preferably selected (instead of powder) for separating its lipid extracts and molecules, since their structures and structure activity relationships can be affected by preparatory procedures (e.g., oxidation of lipids during processing for producing powder).

Beneficial effects have been previously reported for several *Spirulina* lipids, including antioxidant, anti-bacterial and anti-viral ones (Ozdemir et al., 2004; Ramadan et al., 2008). For example, pharmaceutical value has been attributed to the polyunsaturated fatty acid (PUFA) γ-linolenic acid (GLA) (Sajilata et al., 2008) and to certain sulpholipids (Ramadan et al., 2008; Sotiroudis & Sotiroudis, 2013; Tropis et al., 1996), such as the aforementioned SQDG (Berge et al., 2002; Chirasuwan et al., 2009; Gustafson et al., 1989; Liu et al., 1998; Shirahashi et al., 1993; Vasange et al., 1997). Such molecules have been found to inhibit PAF-activities, due to antagonistic effects for its receptor, and PAF-synthesis in human neutrophils (Liu et al., 1998; Vasange et al., 1997), with subsequent pharmaceutical and clinical use in psoriasis (Bohlin et al., 2004). Nevertheless, *Spirulina*-derived lipids/lipid-extracts have not been tested against the PAF and thrombin pathways of platelet aggregation.

In the present study, *Spirulina*-derived extracts of total lipids of both cells and of culture medium-filtrates from fresh cultures of *Arthrospira maxima* were found to have strong inhibitory effects against WRP aggregation induced by either PAF or Thrombin (Table 1), with their anti-PAF effects being stronger than their anti-thrombin ones, suggesting higher specificity against the PAF-pathway. In addition, these anti-PAF effects of both lipid extracts are comparable with the relevant ones for lipid extracts derived from other cyanobacteria (Antonopoulou, Karantonis, et al., 2005; Antonopoulou, Nomikos, et al., 2005) and marine algae (Rho et al., 1996), but also from other microorganisms of biotechnological interest (Tsoupras et al., 2012), marine sources (Lordan et al., 2017; Lordan, Nasopoulou, Tsoupras & Zabetakis, 2018; Nasopoulou et al., 2011; Tsoupras, Lordan, Demuru, et al., 2018; Tsoupras, Lordan & Zabetakis, 2018; Tsoupras, Lordan, et al., 2019; Tsoupras, O'Keeffe, et al., 2019) and other foods (Lordan, Nasopoulou, et al., 2018, Lordan, Tsoupras, Mitra, & Zabetakis, 2018; Tsoupras, Lordan & Zabetakis, 2018).

The anti-thrombin effects of both these lipid extracts on WRP were found to be stronger when compared with similar anti-thrombin effect of other marine sources, which were tested in human platelets (Tsoupras, Lordan, Demuru, et al., 2018; Tsoupras, Lordan, et al., 2019; Tsoupras, O'Keeffe, et al., 2019). Such a difference may also be related to the different source and type of the platelet suspensions in the bioassays of these different studies; WRP versus human platelets in human plasma rich in platelets.

The anti-PAF and anti-thrombin effects were found in lipid extracts of both cell-biomass and medium filtrates of *Spirulina* cultures, suggesting that *Spirulina* cells secrete bioactive lipids within their cell-medium, consistent with similar results from other microalgae and cyanobacteria (Biller et al., 2014; Kind et al., 2012) and other microorganisms of biotechnological interest (Tsoupras et al., 2012). The results that the anti-thrombin effect of the total lipid extract of the culture medium-filtrates was stronger than that of the relative one of the total lipid extract of the cell-biomass, further suggested that *Spirulina* cells secrete into the cell medium lipid molecules with higher specificity against the thrombin pathway.

Based on the stronger anti-PAF effects of *Spirulina* total lipid extracts, compared to their anti-thrombin ones, both lipid extracts were further separated by using HPLC analysis into lipid subclasses and fractions, which were also assessed with WRP for their ability to inhibit PAF-induced platelet aggregation. Several of these HPLC-derived lipid fractions were found to strongly inhibit PAF-induced platelet aggregation (Table 2). Notably, some of these lipid-fractions showed the strongest anti-PAF effects of all samples tested, suggesting that the strong inhibitory effect of the total lipid extracts from both cell-biomass and the medium filtrates against PAF is derived by a synergism between these bioactive lipid subclasses.

The most bioactive lipid-fractions of the total lipid extracts of both cell-biomass and of the medium filtrates of *Spirulina* cultures were eluted with retention times where the more PL are usually eluted during such an HPLC analysis of a lipid mixture (Fig. 2). For example, the HPLC lipid fractions showing the strongest anti-PAF activity, namely fraction 4 for the *Spirulina* cell biomass lipid extract and fraction 5 for the culture medium-filtrate lipid extract, were found to be eluted in ~7–12 min and 9–12 min respectively, where glycolipids and phenolic-lipids are usually eluted (Fig. 2). These results were in accordance with previously reported strong anti-PAF effects in neutrophils of microalgae-derived sulpho-glycolipids (Liu et al., 1998; Vasange et al., 1997), but also of other glycolipids found in cyanobacteria with inhibitory effects against PAF-induced platelet aggregation (Antonopoulou, Karantonis, et al., 2005; Antonopoulou, Nomikos, et al., 2005).

Furthermore, the lipid fraction of the *Spirulina* culture medium-filtrate lipid extracts with the second strongest anti-PAF activity, namely fraction 10, was found to be eluted in ~23–25 min, where usually phosphatidylcholine (PC) and PAF-like molecules are also eluted (Fig. 2). Taking also into account that higher amounts of this lipid fraction can also induce platelet aggregation (Fig. 1) through the PAF-pathway (according to the cross-desensitization tests in platelets, Table 2), further supports that this lipid fraction contains PAF-like molecules belonging to the PC subclass of bioactive PLs with both strong inhibitory and agonistic effects against the PAF-pathways. Such PAF-like molecules have similar but slightly different structures with that of PAF, but with strong anti-PAF antagonistic activities in low doses and agonistic effects against PAF-induced platelet aggregation in higher doses, with the most representative molecules being several alkyl-acyl-phospholipids (Demopoulos & Antonopoulou, 1996; Tsoupras, Lordan, Demuru, et al., 2018; Tsoupras, Lordan & Zabetakis, 2018; Tsoupras, Lordan, et al., 2019; Tsoupras, O'Keeffe, et al., 2019).

On the other hand, none of the lipid-fractions derived from the HPLC-analysis of total lipid extract from cells showed any aggregatory activity on platelets. In addition, much smaller quantities of the most bioactive lipids fractions of the total lipid extract of culture medium-filtrates from *Spirulina* cultures in the stationary phase were needed against PAF-induced platelet aggregation than the relative ones of the bioactive lipids in fractions derived from the total lipid extract of *Spirulina* cells in this phase. These results also suggested that the most bioactive lipids of *Spirulina* have been secreted within the cell medium. This notion is also supported by the fact that the most bioactive lipid fractions from the culture medium filtrates and cell biomass, namely fraction 5 and fraction 4, respectively, were eluted with the same retention times (~9–12 min), while only bioactive fractions from the culture medium showed strong inhibitory and agonistic effects against the PAF-pathways.

5. Conclusions

The results of the present study showed that *Spirulina* derived lipid, polysaccharide and protein extracts, and especially its PCB extract, had strong antithrombotic properties against platelet aggregation induced by the inflammatory and thrombotic mediators, PAF and thrombin. *Spirulina* seems to be a good source of bioactive lipids, especially PL such as glyco-/sulpho-PL and PL molecules of the PC-family, which showed the strongest inhibitory effects against the PAF-pathway of platelet aggregation.

These results further support the nutritional value and health benefits of Spirulina and the potential use of bioactive *Spirulina*-derived protein and/or lipid compounds for developing supplements and nutraceuticals against the PAF/thrombin pathways of platelet activation/aggregation and inflammation related disorders. Nevertheless, further *in vivo* studies are needed to support these *in vitro* results on the antithrombotic properties of *Spirulina*, and to study the efficacy and safety of *Spirulina* derived components against inflammation and platelet activation/aggregation related disorders.

Author contributions

Conceptualization, A.T., C.A.D. and T.G.S.; Methodology, A.T., P.K. and G.S.; software, A.T., P.K. and G.S.; validation, A.T.; formal analysis, A.T., P.K. and G.S.; investigation, A.T. and P.K.; resources, C.A.D. and T. G.S.; writing—original draft preparation, A.T.; writing—review and editing, A.T., P.K., G.S., C.A.D. and T.G.S.; visualization, A.T., C.A.D. and T.G.S.; supervision, A.T., C.A.D. and T.G.S.; project administration, A.T., C.A.D. and T.G.S

All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors have no competing interests to declare.

Acknowledgments

The authors are grateful to Dr. Katherine M. Pappas, Associate Professor of the Department of Biotechnology and Genetics at the Faculty of Biology of the National and Kapodistrian University of Athens, 15771 Athens, Greece (kmpappas@biol.uoa.gr), for the kind donation of the HPLC C8-column.

This work was supported by the National Hellenic Research Foundation and the National and Kapodistrian University of Athens within the context of P.K.' MSc Thesis.

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