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Published in:
Foods

Document Version:
Publisher's PDF, also known as Version of record

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Download date: 23. Dec. 2020
The effects of oxidation on the antithrombotic properties of tea lipids against PAF, thrombin, collagen, and ADP

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Abstract: Tea provides health benefits, while oxidation is part of tea processing. The effect of oxidation on the antithrombotic properties of tea lipid extracts was evaluated for the first time. Total lipids (TL) extracted from fresh tea leaves and commercial tea powder, before and after 30-60 min of oxidation, were further fractionated into neutral lipids (NL) and polar lipids (PL). The antithrombotic bioactivities of tea TL, PL and NL were assessed in human platelets against the inflammatory mediator platelet-activating factor. PL were further assessed against thrombin, collagen, and adenosine diphosphate, while their fatty acid composition was evaluated by GC-MS. PL exhibited the strongest antithrombotic effects against all platelet agonists and were rich in omega-3 polyunsaturated (ω3 PUFA) and monounsaturated (MUFA) fatty acids. A decline was observed in the antithrombotic activities, against all platelet agonists tested, for PL after 60 min of oxidation, and on their MUFA content, while their overall ω3 PUFA content and ω6/ω3 ratio remained unaffected. A synergistic effect between tea phenolic compounds and PL protects them against oxidation, which seems to be the rational for retaining the antithrombotic biofunctionalities of PL at a considerable favorable cardioprotective level, even after 60 min of tea oxidation. More studies are required to elucidate the mechanisms of the favorable synergism in tea PL extracts.

Keywords: tea; cardiovascular diseases; PAF; polar lipids; platelet aggregation; PUFA; MUFA

1. Introduction

Tea, brewed from the dried leaves of the plant Camellia sinensis, is one of the most widely consumed beverages in the world with supposed health benefits, and is reported to
contain nearly 4,000 bioactive chemical compounds, including polar lipids (PL) and several of their subclasses of glycolipids, phospholipids, essential fatty acids (FA), and polyphenols such as catechins [1-8]. Tea can be mainly categorized into three types, depending on the level of tea polyphenol oxidation, i.e. green tea (non-oxidised), oolong tea (partially oxidised) and black tea (fully oxidised) [1-3]. Nevertheless, all tea types are rich in several phenolic compounds such as catechins (i.e. epigallocatechin gallate), flavonols (i.e. quercetin), phenolic acids (i.e. caffeic and gallic acids) and methylxanthines, containing approximately one-third the amount of caffeine compared with coffee [1-3]. Black tea also contains bioactive derivatives of catechins such as theaflavins and thearubigins, which are favourably formed in the expense of catechins during the stage of oxidation [1-3].

Tea health benefits have previously been associated with putative antioxidant capabilities due to its phenolic compounds that act against oxidative stress and its related unfavourable manifestations [2, 3]. However, limited evidence exist that flavonoids can actually inhibit oxidative damage in vivo, since phenolic compounds have poor bioavailability and their antioxidant activities are affected and altered by their metabolic transformation, while inhibition of atherosclerosis in animal models was not associated with markers of change in oxidative damage by these compounds [9]. The favourable effects of tea on several chronic disorders, including cardiovascular disease (CVD), seem to have little to do with the antioxidant properties of its phenolic compounds against oxidative stress, but more to do with other health related mechanisms, including the favourable effects on endothelial function, inflammation and risk of thrombosis related to platelet activation and aggregation [2, 3, 10-14].

The beneficial effects of hot water extracts of tea against platelet aggregation and CVD have been studied since the early 1990s [8]. Several tea phenolic compounds have been proposed as the active principles for inhibition of platelet aggregation, mostly by their effects against arachidonic acid (AA) and its eicosanoid related pathways, but also through their effects against other platelet agonists and inflammatory and thrombotic mediators, such as the platelet-activating factor (PAF), thrombin, collagen, and adenosine diphosphate (ADP) [2, 3, 10-14]. Tea catechins, isoprenyl gallates, and theaflavin and its galloyl esters in black tea extract were are potent inhibitors of PAF synthesis and PAF-induced platelet aggregation. These activities may be relevant to the claimed therapeutic effects of tea extracts [11, 15]. Quercetin and gallic acid are present in tea, which also inhibit platelet aggregation induced by several of the aforementioned platelet agonists [12, 13]. However, such antiplatelet effects in vitro for tea phenolic compounds are due to concentrations that cannot be attained in vivo [12-14], while consumption of such large quantities of tea polyphenols may cause unwanted side effects [14].

Apart from its phenolic content, tea also contains other polar compounds, such as polar lipids (PL) (e.g. glycolipids and phospholipids), with several of them being rich in unsaturated FA, especially oleic acid (OA) and α-Linolenic acid (ALA), the latter being an essential omega-3 polyunsaturated FA (ω3 PUFA) [4-8]. Apart from other benefits, OA, ALA and other ω3 PUFA, such as the docosahexaenoic acid (DHA) and the
Eicosapentaenoic acid (EPA), have also exhibited antiplatelet effects of their own [16-19]. However, several PL of natural origin (e.g. from plants, animals, marine sources, microorganisms of agricultural, nutraceutical and biopharmaceutical interest, foods and related products and by-products, etc.) and especially those bearing unsaturated FA, have much more potent antithrombotic properties against platelet aggregation and thus an overall protective effect against several chronic disorders including CVD [8, 15, 20-29].

Tea phenolic compounds, along with tea PL and FA (e.g. OA and ALA), have also been found to be affected by seasonal variation and several manufacturing processes [1, 4-8, 30, 31]. To our knowledge, both the antithrombotic activities of tea PL and the effect of oxidation on the biofunctionality and FA composition of tea PL compounds has not been reported so far. Therefore, the aim of the present study was to evaluate for the first time the effects of several levels of oxidation on the biological activities of tea leaf lipids, including tea PL, by assessing their antithrombotic properties in human platelets against the aforementioned platelet agonists (PAF, thrombin, ADP and collagen). In addition, the changes on the FA composition of tea PL was also evaluated, in order to elucidate the effect of each level of oxidation on the structure activity relationships of tea derived bioactive PL.

2. Materials and Methods

2.1. Materials and Instrumentation

All glass and plastic consumables, reagents, and solvents were of analytical grade and were purchased from Fisher Scientific Ltd. (Dublin, Ireland). 20G safety needles and evacuated sodium citrate S-monovettes for blood sampling were purchased from Sarstedt Ltd. (Wexford, Ireland). All platelet aggregation consumables were purchased from Labmedics LLP (Abingdon on Thames, UK). Standard PAF, thrombin, egg phospholipid extract, and BSA were purchased from Sigma Aldrich (Wicklow, Ireland), while collagen and ADP from Chronolog (Havertown, PA, USA). Centrifugations were carried out on an Eppendorf 5702R centrifuge (Eppendorf Ltd, Stevenage, UK). Spectrophotometric analysis was carried out on a Shimadzu UV-1800 spectrophotometer (Kyoto, Japan) using a quartz 1 cm cuvette.

2.2. Samples of Tea Assessed Before and After Oxidation Processing

Fresh tea leaves samples were obtained from a specific and widely available clone of the Camellia sinensis L. plant, commonly known as black tea, withered for 16-18h at room temperature, flash frozen with liquid nitrogen and grinded using a mortar and pestle. The leaves were left to defrost before they and were exposed to different levels of oxidation, before (T0) and after 30 (T30) and 60 min (T60) of oxidation in the dark. Tea leaves were oxidised in an in-house oxidation unit developed for lab-scaled R&D tea processing experiments in Queen’s university, Belfast. The oxidation unit includes a shelf to hold the samples, a thermostat heating system to control temperature and fans to ensure the flow of oxygen. When temperature stabilised at 32 °C and constant air stream (0.5 l/min), grinded tea samples (T30 and T60), were placed on the shelf within the oxidation unit. After 30 and 60 min,
the leaves were removed and immediately placed in an oven at 103 °C to inhibit the oxidation reaction by drying. Exposure to oxidation, occurred using an in-house oxidation unit prototype developed at Queens University Belfast with a constant air stream (0.5 l/min) and temperature (32°C). The freshly oxidised tea leaves were removed from the oven when they reached a dried in the oven at 103 °C until constant weight. Dried leaves were milled to a powder using a ball mill for 5 min at a speed of 500 rpm. In addition, tea powder of commercial tea (CT) from a leading tea brand in Ireland (Barry’s Tea), was also analysed as a control sample.

2.3. Extraction and Isolation of Total, Neutral, and Polar Lipids From Tea Leaves Before (0 min) and After 30 and 60 min of Oxidation

Several tea samples of T0, T30, T60 and TC (n=3 in each case), were homogenised mechanically by a Waring blender (Fisher Scientific Ltd., Dublin, Ireland) into a mixture of solvents and their total lipids (TL) were extracted as previously described [24, 25], based on the Bligh and Dyer extraction method [32]. TL extracts of all these tea sources were further separated into their neutral lipids (NL) fraction and the PL fraction as previously described [24, 25], based on the counter-current distribution method of Galanos and Kapoulas [33].

2.4. Human Platelet Aggregation Studies Against PAF, Thrombin, Collagen and ADP of Lipid Extracts From Tea Leaves Before (0 min) and After 30 and 60 min of Oxidation

Analysis of human platelet-rich plasma (hPRP) for platelet aggregation bioassays was carried out on a Chronolog-490 two channel turbidimetric platelet aggregometer (Havertown, PA, USA), coupled to the accompanying AGGRO/LINK software package as previously described [24, 25, 34]. The Ethics Committee of the University of Limerick approved the protocol, which was performed in accordance with the Declaration of Helsinki. Healthy donors were fully aware that their blood samples were used in our study and written consent was provided.

Briefly, the blood samples were collected from each donor by a phlebotomist in sodium citrate anticoagulant and were centrifuged at 194 x g for 18 minutes at 24°C with no brake applied. The supernatant hPRP was then transferred to polypropylene tubes at room temperature for the aggregation bioassays, whereas platelet-poor plasma (PPP) was obtained by further centrifuging the specimens at 1465 x g for 20 minutes at 24°C with no brake applied. hPRP was adjusted to 500,000 platelets/µL if required by addition of the respective volume of PPP according to the absorbance of the hPRP measured in spectrophotometer.

Aliquots of standard PAF stock solution in chloroform/methanol (1:1 v/v) were evaporated under a stream of nitrogen and re-dissolved in BSA (2.5 mg BSA/mL saline) to obtain PAF solutions with final concentrations into aggregometer cuvette ranging from 0.26 nM to 0.26 µM. The examined tea TL, NL, and PL samples were also dissolved in BSA (2.5 mg BSA/mL saline). Standard stock solutions of active thrombin, collagen, and ADP dissolved in saline were further diluted in saline prior testing.
Then, 250 µl of PRP was added to an aggregometer cuvette at 37°C with stirring at 1000 rpm. The PRP was calibrated using the PPP as a blank. The maximum-reversible PAF, thrombin, collagen, and ADP induced platelet aggregation was determined as 100% aggregation, which was also used as baseline (0 % inhibition) in the absence of any lipid sample, by adding appropriate amounts of each platelet agonist in the aggregometer cuvette, in order to reach specific final concentrations; for PAF approximately 0.1-1 nM, for thrombin approximately 0.01-0.4 U/mL, for collagen approximately 1-5 µg/mL and for ADP at approximately 2-10 µM.

The PAF, thrombin, collagen, ADP induced aggregation of hPRP was calculated first at 0 % inhibition of baseline in a cuvette (100 % aggregation) in the absence of any lipid sample, whereas after the pre-incubation of hPRP with several amounts (µg) of the test lipid samples for 2 min (a different cuvette was used for each amount of the lipids tested), the same amount of PAF, thrombin, collagen, ADP was added and the reduced aggregation was calculated. Thus, a linear curve at the 20–80 % range of the percentage of inhibition against PAF, thrombin, collagen, ADP induced aggregation of hPRP to the concentrations of each lipid sample was deduced. From this curve, the concentration (µg) of the lipid sample that led to 50% of the agonist induced aggregation of hPRP was calculated as the 50 % inhibitory concentration value also known as the IC\textsubscript{50} value (half-maximal inhibitory concentration) for each sample.

The resulting IC\textsubscript{50} values were expressed as a mean value of the mass of lipid (µg) in the aggregometer cuvette ± standard deviation (SD). All experiments were performed several times (n=6) for each tea lipid sample (n=6), using a different donors blood sample for each replicate.

2.5. Gas Chromatography-Mass Spectrometry of Polar Lipids from Tea Leaves Before (0 min) and After 30 and 60 min of Oxidation

GC-MS analysis of the FA composition of tea PL was carried out as previously described [24, 25].

2.6. Statistical Analysis

One-way analysis of variance (ANOVA) was used for all comparisons of IC\textsubscript{50} values against PAF, thrombin, collagen, and ADP platelet aggregation, while Kruskal Wallis non-parametric multiple comparison test was used for comparisons in the FA composition acquired from the CG-MS analysis. Differences were considered to be statistically significant when the \(p\) value was less than 0.05 (\(p < 0.05\)). The data were analysed using a statistical software package (IBM-SPSS statistics 25 for Windows, SPSS Inc., Chicago, IL, 17 USA).

3. Results and Discussion

It is now recognised that the favorable effects of tea against several chronic disorders, including CVD, have more to do with its favorable effects on endothelial function,
inflammation and risk of thrombosis related to platelet activation and aggregation [2, 3, 10-14]. However, several manufacturing processes affect several compounds of tea leaves, such as their phenolic and other polar compounds such as their PL and FA (e.g. OA and ALA) [1, 4-8, 30, 31].

In the present study, TL extracted from unoxidised and oxidised tea leaves (T0, T30 and T60) and from CT, were further fractionated into NL and PL fractions, and their antithrombotic activities were assessed for the first time against aggregation of human platelets, induced by the most well established platelet agonists and inflammatory and thrombotic mediators, namely, PAF, thrombin, collagen, and ADP.

The obtained amounts of TL, PL, and NL (expressed as g of lipids per 100 g of tea sample) for all tea samples (T0, T30, T60 and CT) are given in Table 1. In all these tea samples the main lipid components were found to be polar compounds, while the neutral lipids were found to be a minor component (Table 1).

Table 1. Yield of extraction of lipid content (TL, PL, and NL) of the tea samples before (0 min) and after 30 and 60 min of oxidation in comparison with commercial tea.

<table>
<thead>
<tr>
<th>Tea sample</th>
<th>TL*</th>
<th>NL*</th>
<th>PL*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>8.1 ± 1.5</td>
<td>0.8 ± 0.3</td>
<td>7.3 ± 1.2</td>
</tr>
<tr>
<td>T(0)</td>
<td>9.6 ± 4.9</td>
<td>1.6 ± 0.7</td>
<td>8.0 ± 5.6</td>
</tr>
<tr>
<td>T(30)</td>
<td>9.6 ± 0.6</td>
<td>0.3 ± 0.1</td>
<td>9.3 ± 0.3</td>
</tr>
<tr>
<td>T(60)</td>
<td>10.7 ± 4.0</td>
<td>0.9 ± 2.0</td>
<td>9.8 ± 2.0</td>
</tr>
</tbody>
</table>

*Expressed as mean values of g of lipids per 100 g of each marine source (mean ± SD, n = 6); TL: total lipids; PL: polar lipids; NL: neutral lipids; CT: commercial tea; T(0): tea leaf samples before (0 min) oxidation; T(30): tea leaf samples after 30 min of oxidation; T(60): tea-leaf samples after 60 min of oxidation; SD: standard deviation.

The yields of PL extracts in all tea samples tested (approximately 7-10 g of PL per 100 g of tea sample) were at least two times higher than previously reported ones for tea (approximately 3-5 g of PL per 100 g of tea sample) [4, 5]. The higher yield of polar compounds observed in all tea samples in the present study, seem to be related with the different extraction methods employed than those used in the previously reported studies for tea polar lipids [4, 5].

More specifically, in our study we used the well-established Bligh and Dyer extraction method for obtaining TL-extracts [32], coupled with the efficient counter-current distribution of Galanos and Kapoulas [33], as previously described [24]. When such a methodology was previously applied to other plant sources and related products that are also rich in polyphenols and bioactive polar compounds (e.g. red/white wine, musts, grapes, olive oil, sunflower oil, several kind of beers and brewery by-products), the bioactive phenolic compounds migrated to the PL fraction of the TL-extracts within these conditions [25-28, 35, 36].
Similarly, the methodology applied in the present study facilitated the recovery of the majority of the tea polar compounds within the TL extracts of tea leaves, and from them the separation of the PL fractions that are rich in bioactive polar compounds and in which tea polyphenols co-migrate. Therefore, in comparison with previous studies in tea PL [4, 5], the obtained higher amounts of PL extracts from the tea leaves of the present study, took place because of the co-migration of phenolic compounds in these tea PL-fractions in the experimental extraction conditions applied. This is related to the fact that several phenolic compounds have similar to slightly higher polarity than classic PL subclasses of glycolipids and phospholipids. A characteristic example is that of naturally occurred fatty esters of catechins in several tea varieties, namely phenolipids, with high antioxidant capacity and similar polarity and amphiphilic properties with classic PL subclasses, and thus co-migrate into PL extracts [37, 38].

The in vitro antithrombotic properties of the TL, NL, and PL extracts from all tea samples, against PAF-induced aggregation of human platelets, were expressed as IC\textsubscript{50} values (Figure 1), while the lower the IC\textsubscript{50} value against PAF for a lipid extract the stronger its antithrombotic properties.

In the present study it was found for the first time that from all the lipid extracts tested that were derived from tea leaf samples at several stages of oxidation (T0, T30 and T60) and from CT, the PL extracts were the ones that exhibited the strongest antithrombotic activities against PAF-induced aggregation of human platelets (Figure 1b).

It was also found that the TL extracts from all tea samples exhibited a potent anti-PAF effect too (Figure 1a), which was slightly lower with that of their PL fractions, whereas in contrast all NL fractions had much lower anti-PAF effects (Figure 1c). This is also in accordance with previously reported similar outcomes for TL extracts from wine and beer that were also rich in higher amounts of PL, including bioactive phenolic and classic PL compounds, with higher bioactivities from their relative less bioactive and in low amounts NL [26, 27, 36].

The FA composition of the PL extracts from all tea samples are shown in table 2. In all tea PL samples the PUFA and especially the essential ω3 PUFA ALA (18:3ω3), were the most abundant class of FA, followed by lower amounts of saturated fatty acids (SFA) such as the palmitic (16:0) and stearic (18:0) acids, and significantly less but considerable amounts of MUFA, such as OA (18:1c9). These results come in accordance with previously reported ones for this tea variety [4, 6-8, 30, 31, 38], but also for other tea varieties [39, 40].

In addition, in the majority of the tea PL, other MUFA were also present in less but considerable amounts, such as the palmitoleic (16:1c9), cis-vaccenic (18:1c11) and gadoleic (20:1c9) acids, while sizeable amounts of ω6 PUFA were also present, with the most abundant being linoleic acid (LA; 18:2ω6), followed by much less but considerable amounts of eicosatetraenoic acid (20:4ω6).
Figure 1: The antithrombotic effects of TL (a), PL (b) and NL (c) extracts from tea leaf samples, before (0 min) and after 30 and 60 min of oxidation, against PAF-induced aggregation of human platelets, in comparison to those of CT. Results are expressed as IC50 (half-maximal inhibitory concentration) values that reflect the inhibitory strength of each TL, PL and NL extract against PAF-induced platelet aggregation and is expressed as mean values of µg of lipids in the aggregometer cuvette that causes 50% of inhibition on PAF-induced aggregation of platelets in hPRP ± SD. *indicates statistical significant differences (p < 0.05). TL: total lipids; PL: polar lipids; NL: neutral lipids; CT: commercial tea samples; PAF: platelet-activating factor; hPRP: human platelet-rich plasma; SD: standard deviation.

Interestingly, much less but considerable amounts of other long chain (LC) ω3 PUFA, such as the EPA (20:5ω3) and the DHA (22:6ω3), were also detected in these tea samples for the first time. It has been previously proposed that plant sources do not contain such LC-ω3 PUFA due to lack of appropriate enzyme machinery for producing them from ALA and LA, yet Guil et al have reported the presence of low amounts of both EPA and DHA in several natural plants [40], which comes in accordance with the obtained results of the present study.
Table 2. The fatty acid profile of the PL extracts from tea leaf samples, before (0 min) and after 30 and 60 min of oxidation, in comparison to that of CT, expressed as a percentage of the total fatty acids of each sample (mean ± SD, n = 3).

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>0M</th>
<th>30M</th>
<th>60M</th>
<th>CT</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:0</td>
<td>0.700 ± 0.138&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.135 ± 0.102&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.122 ± 0.003&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.227 ± 0.019&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>14:1</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.064 ± 0.013</td>
</tr>
<tr>
<td>15:0</td>
<td>0.120 ± 0.012&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.047 ± 0.002&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>0.091 ± 0.003&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:0</td>
<td>24.60 ± 0.355&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>19.41 ± 1.117&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.38 ± 0.947&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>28.11 ± 2.014&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>16:1 c9</td>
<td>1.927 ± 0.033&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.461 ± 0.034&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.752 ± 0.073&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.217 ± 0.080&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>17:0</td>
<td>0.352 ± 0.010&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.274 ± 0.017&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.020 ± 0.025&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.332 ± 0.022&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:0</td>
<td>6.408 ± 0.241&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>8.496 ± 0.173&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.930 ± 0.124&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.013 ± 0.099&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1 c9</td>
<td>9.965 ± 0.014&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>11.95 ± 0.274&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.039 ± 0.114&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>7.840 ± 0.200&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:1 c11</td>
<td>1.500 ± 0.115&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.116 ± 0.048&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.787 ± 0.095&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.184 ± 0.043&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:2 c9, c12</td>
<td>19.48 ± 0.222&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.34 ± 0.262&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.37 ± 0.247&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.38 ± 0.839&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:3 c9, c12, c15</td>
<td>29.78 ± 0.011&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>30.84 ± 0.529&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>34.22 ± 0.282&lt;sup&gt;b&lt;/sup&gt;</td>
<td>27.44 ± 0.942&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:0</td>
<td>0.343 ± 0.064&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.442 ± 0.038&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ND</td>
<td>0.209 ± 0.032&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:1 c9</td>
<td>1.105 ± 0.044&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.698 ± 0.030&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>ND</td>
<td>0.379 ± 0.051&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:4 c5, c8, c11, c14</td>
<td>0.344 ± 0.0109&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.397 ± 0.018&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>ND</td>
<td>0.244 ± 0.033&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>20:5 c5, c8, c11, c14, c17</td>
<td>0.732 ± 0.016&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.655 ± 0.029&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.574 ± 0.050&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.339 ± 0.073&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:0</td>
<td>1.052 ± 0.015&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td>ND</td>
<td>0.319 ± 0.044&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:5 c7, c10, c13, c16, c19</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.375 ± 0.050&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>22:6 c4, c7, c10, c13, c16, c19</td>
<td>1.390 ± 0.113&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.370 ± 0.155&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.518 ± 0.142&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.122 ± 0.016&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>ω3</td>
<td>31.90 ± 0.215&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>32.86 ± 0.433&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>35.56 ± 0.176&lt;sup&gt;b&lt;/sup&gt;</td>
<td>29.28 ± 1.060&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ω6</td>
<td>19.83 ± 0.228&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.74 ± 0.271&lt;sup&gt;b&lt;/sup&gt;</td>
<td>22.37 ± 0.247&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.62 ± 0.860&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>ω6/ω3</td>
<td>0.621 ± 0.011</td>
<td>0.692 ± 0.017</td>
<td>0.629 ± 0.010</td>
<td>0.704 ± 0.055</td>
</tr>
<tr>
<td>SFA</td>
<td>32.40 ± 0.532&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>28.80 ± 0.936&lt;sup&gt;a&lt;/sup&gt;</td>
<td>31.64 ± 0.829&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>35.28 ± 1.905&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MUFA</td>
<td>15.55 ± 0.141&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.28 ± 0.299&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>10.58 ± 0.051&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.90 ± 0.287&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>PUFA</td>
<td>51.73 ± 0.413&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>55.60 ± 0.681&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>57.68 ± 0.805&lt;sup&gt;b&lt;/sup&gt;</td>
<td>49.90 ± 1.873&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Mean values (n = 3), ± standard deviation, with different letters in the same row indicating statistical significant differences between the lipid compositions when mean are compared using Kruskal Wallis non-parametric multiple comparison test (p ≤ 0.05). ω6/ω3 ratio uncertainty calculated using the following equation: ∆x/x=([∆ω]6/[ω6] + [∆ω]3/[ω3]) x ω6/ω3. Abbreviation: c = cis; CT = commercial tea; M = minutes; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids; SFA = saturated fatty acids; ND: non-detectable.
PL from several natural sources and foods that are rich in such unsaturated FA, have exhibited potent bioavailability, biofunctionality and antithrombotic properties, not only against PAF [20-29], but also against other platelet agonists such as thrombin [23-26, 28], collagen, and ADP [25]. Therefore, the much stronger anti-inflammatory and antithrombotic effects of such PL of natural origin, including plant-derived PL, against all these mediators and especially on PAF pathway and metabolism, have been translated to an overall favourable protective effect towards several inflammation-related disorders, including CVD, in which PAF and these mediators are implicated [15, 20, 22]. The strong anti-PAF effects in human platelets of the tea PL extracts that were rich in such unsaturated FA, were of similar potency and efficacy with similar anti-PAF effects in human platelets of PL extracts from other natural sources, which were also rich in ω3 PUFA and OA at their sn-2 position [23-26]. Consequently, the never before reported results in the present study concerning the strong antithrombotic bioactivities against PAF of all tea PL, which were also found to be rich in such unsaturated FA, further support the overall favorable health benefits of tea consumption.

Notably, the PL extracts from T0 exhibited significantly stronger anti-PAF effects in human platelets, than those of the PL extracts from T60 and CT (p<0.05 in both comparisons), while the PL extracts from T30 had an intermediate anti-PAF effect (Figure 1b). These results suggest that the more the time of the applied oxidative process in the tea leaves the lower the bioactivities of their PL against PAF-induced human platelet aggregation.

Since tea PL were the ones with the most potent anti-PAF effects, in order to fully elucidate the overall effects of oxidation we further evaluated for the first time the antithrombotic properties of PL extracts from tea leaves at several oxidation levels (T0, T30 and T60) and from CT, against human platelet aggregation induced by thrombin, ADP and collagen. The antithrombotic effects of all these tea PL extracts against thrombin, collagen and ADP were also expressed as IC$_{50}$ values and are shown in figure 2 (2a, 2b and 2c, for each platelet agonist, respectively).

In the present study, it was also found for the first time that PL extracts from all tea samples exhibited potent antithrombotic effects against aggregation of human platelets induced by thrombin, collagen and ADP. Notably, it was also observed that the antithrombotic effects of all PL extracts tested against both thrombin and collagen were comparable and in the same order of magnitude to their relative ones against PAF, while their anti-ADP effects was less potent but also in considerable levels.

Furthermore, in a similar pattern to the results obtained against PAF, it was also found for the first time that the PL extracts from T0 had more potent anti-thrombin effects than the PL extracts from T60 and CT (p<0.05 in both comparisons), while the PL extracts from T30 exhibited an intermediate anti-thrombin effect (Figure 2a). In the cases of collagen and ADP, the PL extracts from T60 had significantly less potent anti-collagen and anti-ADP effects than the PL extracts from all the other tea samples (Figure 2b and 2c) (p < 0.05 in all comparisons for collagen and borderline significance 0.05 < p < 0.10 in all comparisons for
ADP). Again, these results for thrombin, collagen and ADP, suggest that the more the time of the applied oxidative process in the tea leaves the lower the bioactivities of their PL against human platelet aggregation induced by these platelet agonists.

Figure 2: Antithrombotic effects of PL extracts from tea leaf samples, before (0 min) and after 30 and 60 min of oxidation, against thrombin (a), collagen (b) and ADP (c) induced aggregation of human platelets, in comparison to those of CT. Results are expressed as IC\textsubscript{50} (half-maximal inhibitory concentration) values that reflect the inhibitory strength of each PL extract against thrombin/collagen/ADP-induced platelet aggregation and is expressed as mean values of µg of PL in the aggregometer cuvette that causes 50% of inhibition on thrombin, collagen, and ADP-induced aggregation of platelets in hPRP ± SD. *indicates statistical significant differences (p < 0.05); #indicates borderline statistical differences (0.05 < p < 0.10); PL: polar lipids; CT: commercial tea samples; ADP: adenosine-5’-diphosphate; hPRP: human platelet-rich plasma; SD: standard deviation.

Still, the antithrombotic bioactivities of the PL extracts from all these tea samples against all platelet agonists (PAF, thrombin, collagen and ADP) remained in the same order of magnitude for each agonist, and thus similar to the ones obtained in human platelets for PL from other natural sources against PAF [23-27], thrombin [23-26, 28], collagen and ADP [25]. Consequently, although a decline was observed on the antithrombotic activities of the tea PL after 60 min of oxidation of the tea leaves, yet this reduction was not intense and as a
result the antithrombotic properties of all tea PL in human platelets against all these platelet agonists were retained at substantial levels, comparable to those for PL from other natural sources [23-27].

In addition, the PL extracts from T0 exhibited similar anti-PAF bioactivities to their anti-thrombin and anti-collagen activity, while all these antithrombotic effects were stronger than their anti-ADP activity. Instead, the PL extracts from T30 and T60 exhibited significantly stronger anti-PAF effects than their anti-thrombin and anti-collagen effects, while all these antithrombotic activities were again stronger than their anti-ADP effects for these PL samples too. These results suggest that the aforementioned preservation of the antithrombotic activities of tea PL at substantial levels, even after 60 min of oxidation of the tea leaves, was more profound in the case of their anti-PAF effects, which further implies that the PL compounds with potent anti-PAF bioactivities that are present into the tea PL extracts are more sustainable against oxidation.

Furthermore, the GC-MS analysis of the PL extracts from unoxidised (T0) and oxidised (T30 and T60) tea leaves revealed that the levels of ALA, their major ω3 PUFA, remained unaffected by this oxidation processing (Table 2). Therefore, even though a decline was observed in the levels of the lesser ω3 PUFA (reduction of EPA levels for both T30 and T60 and of DHA for T60), yet the overall ω3 PUFA content remained unaffected after oxidation of the tea leaves. Subsequently the favorable low levels of the ω6/ω3 ratio observed in T0, which were lower than the value of 1/1 for this ratio, were retained in the same levels during oxidation (Table 2). These results further support the favourable ω3 PUFA content of the tea PL and thus their cardioprotective properties, even after 60 min of oxidation, since it has been proposed that low levels for the ω6/ω3 ratio has favorable effects in CVD and other chronic disorders [42].

At this point it is important to stress that although the antioxidant tea polyphenols have little to do with a proposed in vivo protection against oxidative stress [9], yet they play crucial role as primary antioxidants against oxidation of lipids, including PL compounds, in several natural sources, foods, beverages, cosmetics and relevant products and lipid extracts [37, 43, 44]. In several leaves and cell membranes, a very substantial improvement in oxidative stability, bioavailability and preservation of the bioactivities of both PL and polar phenolic compounds can be achieved by a co-presence and synergism of both these polar compounds. [37, 43-47]. Subsequently, the presence of tea phenolic compounds in tea PL extracts, seem to facilitate the preservation of the bioactivities of the protected PL compounds. It is also possible that the previously reported oxidative stability of conjugative linolenic acids [48], may also contribute to the observed in this study preservation of ALA in tea PL after 60 min of oxidation.

On the other hand, in contrast to the ω3 PUFA, the overall levels of MUFA and those of individual OA (18:1c9), palmitoleic (16:1c9), cis-vaccenic (18:1c11) and gadoleic (20:1c9) acids were considerably reduced in the PL extracts of tea leaves that were oxidised for 60 min, a result that comes in accordance with previous studies in tea [30, 48, 49]. Especially the observed reduction in their major MUFA, OA, seem to be associated with the observed
decline in the antithrombotic activities of these tea PL extracts against all platelet agonists tested, since OA has favourable antiplatelet effects [18, 19]. It is also possible that the reduction of the antithrombotic activities of tea PL after 60 min of oxidation is related to an oxidation of some of the bioactive tea-phenolic compounds with reported strong antiplatelet effects, since they are labile to such a non-enzymatic oxidation processing [37]. Nevertheless, more studies of structure activity relationships are needed in order to elucidate such a notion.

5. Conclusions

Tea PL extracts were found for the first time to possess strong antithrombotic activities against the potent inflammatory mediator PAF, but also against other well-established platelet agonists, namely thrombin, collagen and ADP, while they were also found to be rich in ω3 PUFA and MUFA, with a favorable ω6/ω3 ratio. In addition, a reduction in these antithrombotic activities of the tea PL extracts after 60 min of oxidation was also observed, which was accompanied by a decline in their MUFA content. However these reductions were not so intense, while the ALA and the overall ω3 PUFA content and the ω6/ω3 ratio of tea PL remained unaffected, and thus the antithrombotic properties of all tea PL extracts even though declined, still they remained in the same order of magnitude and comparable to those of PL from other natural sources. It is possible that the presence of phenolic compounds in these PL extracts may retain their antithrombotic effects in considerable potency and protects them synergistically from the unfavourable effects of oxidation. However, more in vitro and in vivo studies are needed in order to elucidate the preservation of the favourable effects of tea PL extracts, because of the synergism of the co-existing tea phenolic compounds and other bioactive PL-subclasses.


Funding: This research received no external funding

Acknowledgments: The authors are grateful to the volunteers who took part in the study and to Elaine Ahern for her phlebotomy support. The authors acknowledge the support of the private companies for their contribution in supplying fresh tea leaves. We would also like to thank the Department of Biological Sciences at the University of Limerick, Ireland, for their continued support.

Conflicts of Interest: The authors declare no conflict of interest.

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