Impact of Interactions between Ferulic and Chlorogenic Acids on Enzymatic and Non-Enzymatic Lipids Oxidation: An Example of Bread Enriched with Green Coffee Flour

Urszula Gawlik-Dziki 1,*, Jarosław Bryda 1, Dariusz Dziki 2, MichałŚwieca 1, Ewa Habza-Kowalska 1 and Urszula Złotek 1

1 Department of Biochemistry and Food Chemistry, University of Life Sciences, Skromna Str. 8, 20-704 Lublin, Poland; brydajarek@gmail.com (J.B.); michal.swieca@up.lublin.pl (M.Ś.); ewa.habza1@gmail.com (E.H.-K.); urszula.zlotek@up.lublin.pl (U.Z.)

2 Department of Thermal Technology and Food Process Engineering, University of Life Sciences, Doświadczalna Str. 44, 20-280 Lublin, Poland; dariusz.dziki@up.lublin.pl

* Correspondence: urszula.gawlik@up.lublin.pl; Tel.: +48-81-4623327; Fax: +48-81-4623324

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Abstract: This study investigated the activity, mode of inhibition, and interactions between lipoxygenase (LOX) inhibitors and compounds able to inhibit lipids oxidation (LPO) derived from green coffee beans (GCBs), wholemeal wheat flour (WF), and bread enriched with GCB, in comparison with pure chemical standards of the main active components—ferulic (FA) and chlorogenic acids (CGA). Both GCB and WF contain potentially bioaccessible and bioavailable LPO inhibitors acting synergistically. Both FA and CGA presented nearly additive interaction. Potentially bioaccessible LOX inhibitors from GCB and WF acted synergistically, whereas moderate antagonism was found for potentially bioavailable compounds. The activity of CGA and FA was quite similar (EC50 = 18.56 and 22.36 µg DW/mL, respectively) and synergistic action between these components was found. The LPO activity of functional breads was positively correlated with the percentage of GCB (R^2 = 0.98). Ferulic acid and CGA acted as competitive LOX inhibitors, whereas potentially bioaccessible compounds from bread enriched with GCB demonstrated an uncompetitive mode of action. Green coffee beans, WF, and the proposed functional product could be helpful in dietary therapy and prevention of so-called lifestyle disorders related with the lipid metabolism. Moreover, this paper highlights the need to study the interactions between the active ingredients of newly designed functional products.

Keywords: ferulic acid; chlorogenic acid; lipoxygenase; lipids peroxidation; interactions

1. Introduction

Lipid peroxidation is a process generated naturally in small amounts in the body, mainly by the effect of the action of several reactive oxygen species (ROS) or enzymes. The destruction of membrane lipids and the end-products of such lipid peroxidation reactions are especially dangerous for the viability of cells, and even tissues [1,2]. Oxygenation of polyunsaturated fatty acids containing a (cis, cis)-1,4-pentadiene system is catalyzed by lipoxygenases (LOXs). The main substrates for LOXs are arachidonic and docosahexaenoic acids released from membrane phospholipids by phospholipases A2 (PLA2) [3,4]. Evidence is accumulating in support of the direct involvement of 5-LOX in the progression of different types of cancer, including prostate, lung, colon, and colorectal cancers [5],
likewise, lipid peroxidation has been implicated in disease states such as atherosclerosis, asthma, Parkinson's disease, kidney damage, preeclampsia, and others [6].

Several studies have illustrated the effectiveness of using pure phenolic compounds for preventing enzymatic and non-enzymatic lipid oxidation [7]. In the last decade, agents that specifically inhibit the LOX metabolic pathway have been developed to treat inflammatory diseases, such as asthma and arthritis [5].

The most commonly occurring phenolic compounds in foods are ferulic (FA) and chlorogenic (CGA) acids. Ferulic acid is commonly found in plant-derived food, especially cereals, fruits, and vegetables. Ferulic acid is a strong membrane antioxidant known to positively affect human health, effectively scavenge free radicals, and inhibit lipid peroxidation. Due to their strong antioxidant properties, it exhibits a wide range of therapeutic effects against cancer, diabetes, and cardiovascular and neurodegenerative diseases [8]. Chlorogenic acid, one of the most abundant polyphenolic compounds in the human diet, is an important component of coffee; it exerts many biological properties, including antibacterial, antioxidant, and anticarcinogenic activities. Recently, the roles and applications of CGA, particularly in relation to glucose and lipid metabolisms, have been highlighted [9]. The estimated daily intake of the natural phenolics by an adult ranges from about 20 mg to 1 g. Such an intake and bioavailability may be important to human health [10]. One way to increase the daily intake of phenolics is traditional food fortification. Raw materials especially rich in the above compounds are green coffee beans (GCBs) and wholemeal wheat flour (WF). Thus, given the biological value of the raw materials, we propose a new functional product—bread enriched with GCB [11,12]. Importantly, when used as a functional additive for functional whole meal bread preparation (up to 3% w/w), GCB significantly improves the product’s antioxidant activity without any deterioration of sensory properties [11].

Generally, estimation of the bioactivity of a newly designed functional food is a very complicated issue and there are several reasons for this:

(i) a given activity of phenolic compounds studied in vitro (after their isolation from the food) does not have to be in accordance with the same activity demonstrated in the human organism [13]. In order to study the impact of digestion on the biological activity of food and beverages, in vitro models based on human physiology are widely used.

(ii) interactions between bioactive components naturally occur in raw materials.

(iii) interactions occur between nutraceuticals and food matrix components (starch, proteins, lipids).

(iv) processing has an impact during food production (e.g. cooking, baking, storage).

Thus, the aim of this study was to evaluate the activity, mode of inhibition and interaction between potentially bioavailable LOX inhibitors and compounds able to protect lipids against oxidation derived from green coffee beans, wholemeal WF and a functional product—bread enriched with GCB, in comparison with pure chemical standards—ferulic and chlorogenic acids.

2. Materials and Methods

2.1. Chemicals

α-amylase, pancreatin, pepsin, bile extract, linoleic acid, ammonium thiocyanate, haemoglobin, pepsin, chlorogenic, ferulic, lipoxygenase (LOX), and PBS (phosphate buffered saline, 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4 at 25 °C) were purchased from Sigma–Aldrich (Poznan, Poland). Standard ferulic and chlorogenic acids were purchased from Sigma–Aldrich Fine Chemicals (St. Louis, MO, USA). Liquid chromatography grade methanol (MeOH) was purchased from Avantor Performance Materials (Gliwice, Poland). Liquid chromatography grade water was prepared using a Millipore Direct-Q3 purification system (Bedford, MA, USA). All others chemicals were of analytical grade.
2.2. Plant Materials

Green coffee beans were obtained from Cofeina Romuald Zalewski, Marki, Poland. The functional supplement—flour from green coffee beans—was prepared according to Dziki et al. [11]. Wholemeal wheat flour type 2000 (protein content 14 mg/100 g, ash content 1.89 mg/100 g, moisture content 13.6 mg/100 g; WF) were purchased in the local supermarket in Lublin, Poland.

2.3. Bread Preparation

The flour used in the formula of control bread (C) was wholemeal wheat bread flour (600 g), type 2000 (WF). The flour was replaced with GCB flour at 1 g/100 g, 2 g/100 g, 3 g/100 g, 4 g/100 g, 5 g/100 g levels (GC1, GC2, GC3, GC4, and GC5, respectively). The control and enriched breads were prepared accordingly as described previously [11].

2.4. Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry LC-ESI-MS/MS Analysis of Phenolic Acids

Dried and powdered control bread and bread enriched with 3% GCB addition (2 g) were placed in the stainless-steel cell of Dionex ASE 100 accelerated solvent extractor (Sunnyvale, CA, USA). The extraction conditions were optimized (methanol concentration 80% (v/v), temperature: 80 °C). Three cycles (for 15 min) of extraction were performed, extracts after combination and concentration under reduced pressure were dissolved in 10 mL of 80% methanol. Five mL of this extract was diluted with 5 mL of water and applied to Sep-Pack C18 Cartridges (500 mg, Waters, Milford, MA, USA) previously activated with 10 mL of methanol and 10 mL of water. The cartridge was eluted with 10 mL of 80% methanol. The combined eluates were concentrated under reduced pressure, then dissolved in 2 mL of 50% methanol in volumetric flask. Prior to further analysis extracts were filtered again through 45-µm nylon filters.

Phenolic acids identification and quantification were determined by reversed-phase high-performance liquid chromatography and electrospray ionization mass spectrometry (LC-ESI-MS/MS) according to the method previously described [14] with slight modifications.

2.5. Extracts Preparation

Buffer extracts containing potentially masticable compounds (BE), extracts after in vitro digestion containing potentially bioaccessible compounds (DE), and extract after absorption in vitro containing potentially bioavailable compounds (AE) were performed according to Minekus et al. [15] with some modifications [12].

2.6. Inhibition of Linoleic Acid Peroxidation

Antioxidant activity was determined as the degree of inhibition of the peroxidation of linoleic acid according to Kuo et al. [16] with the following modification: instead hemoglobin, 10 mmol/L FeCl₂ in water was used. All measurements were performed in four replicates. Antioxidant activity was determined as EC₅₀—extract concentration providing 50% of activity was based on a dose-dependent mode of action.

2.7. Inhibition of Lipoxygenase Activity (LOXI)

Lipoxygenase activity was determined spectrophotometrically at 20 °C by measuring the increase of absorbance at 234 nm over a 2 min period [17] adapted for microplate reader (Epoch 2 Microplate Spectrophotometer, BioTek Instruments, Winooski, Vermont, USA). Briefly, the reaction mixture contained 240 µL 0.066 M phosphate buffer, 10 µL LOX solution (167 U/mL), 10 µL of extracts solution. After preincubation of the mixture at 30 °C for 10 min, the reaction was initiated by adding 40 µL of 2.5 mmol/L linoleic acid. One unit of LOX activity was defined as an increase in absorbance of 0.001 per minute at 234 nm. All measurements were performed in four replicates.
Antioxidant activities were determined as EC50—extract concentration providing 50% of activity based on a dose-dependent mode of action.

2.8. Isobolographic Analysis of Interaction

Dose-normalized isobolograms were performed according to Chou [18]. All measurements were performed in four replicates. The quantification of interaction as a synergism or antagonism was done by the general Equation (1) for n-drug combination at x% inhibition [18] using combination index (CI) for interaction interpretation:

\[
CI = \frac{(D_1)}{(D_X)_1} + \frac{(D_2)}{(D_X)_2} = \frac{1}{(DRI)_1} + \frac{1}{(DRI)_2}
\]

where: CI is the sum of the dose of drugs that exerts x% inhibition when combined. In the denominator \(D_X\) is for D “alone” that inhibits a system x%. If CI value is equal, smaller or greater to 1, an additive, synergistic or antagonistic effect is indicated.

2.9. Theoretical Approach

For the estimation of interaction of wheat bread enriched with GCB flour, the interaction factor (IF) was determined [19]:

\[
IF = \frac{A_M}{A_T}
\]

where, \(A_M\) = the measured activity of a mixture of samples, and \(A_T\) = the theoretically calculated mixture activity (based on the dose response of single components at various concentrations). An \(IF < 1\) indicates synergistic interaction; \(IF > 1\) indicates antagonism; \(IF = 1\) indicates additional interactions.

2.10. Statistical Analysis

All experimental results were mean ± SD of four parallel measurements and data were evaluated by two-way analysis of variance (Tukey test) using Statistica 6.0 software (StatSoft, Inc., Tulsa, OK, USA). The statistical tests were carried out at a significance level of \(\alpha = 0.05\).

3. Results and Discussion

The chemical compositions of WF and GCB used in this study have previously been described [14]. The major phenolic in GCB used for functional bread production was 5-caffeoylquinic acid (5-CQA; 39.92 mg/g DW). Significant amounts of 5-feruloylquinic acid (5-FQA), 4-caffeoylquinic acid (4-CQA), and 3-feruloylquinic acid (3-FQA) were also found. The dominant fraction of phenolic acids in WF consists of trans- and cis-ferulic acids (257 and 165.57 mg/100 g DW, respectively) [20].

As presented in Table 1, baking increased ferulic acid content (compared to WF). These results were in accordance with literature data [21]. Moreover, the GCB flour addition significantly enriched wholemeal wheat bread with 5-CQA (Table 1).

A key factor in the activity of plant phenolic compounds is their bioavailability, and this was determined based on the model of the human gastrointestinal tract.

We confirmed that both raw materials contain potentially bioaccessible and bioavailable compounds able to protect lipids against oxidation. In cases of potentially mastication-extractable (BE) and bioavailable (AE) compounds, higher activity was found for samples obtained from green coffee. Unexpectedly, the activity of bioaccessible in vitro (DE) compounds derived from both raw materials (i.e., GCB and WF) was comparable. Importantly, the activity of bioaccessible and bioavailable in vitro compounds able to protect lipids against oxidation from both raw materials was higher than those obtained for buffer extracts (Figure 1B).
Table 1. Ferulic and chlorogenic acids content (μg/g DW) in control bread and bread enriched with green coffee flour addition.

|            | trans-FA | cis-FA | 5-CQA |
|------------|----------|--------|-------|
| C          | 341.81 ± 3.21 a | 220.21 ± 7.09 a | nd    |
| GC1        | 338.41 ± 5.88 b | 219.01 ± 6.95 b | 322.38 ± 14.85 a |
| GC2        | 335.97 ± 5.48 b c | 214.94 ± 5.82 c | 644.76 ± 15.11 b |
| GC3        | 332.82 ± 5.76 d | 213.58 ± 3.64 d | 895.5 ± 12.57 c |
| GC4        | 325.46 ± 6.01 e | 210.32 ± 3.88 e | 1325.34 ± 34.21 d |
| GC5        | 323.62 ± 4.85 f | 208.56 ± 2.45 f | 1647.72 ± 46.32 e |

C—control bread, GC1–GC5—bread with 1–5 g/100 g green coffee bean addition, respectively, trans-FA—trans ferulic acid, cis-FA—cis ferulic acid, 5-CQA—5-cafeoylquinic acid. Results were expressed as mean ± SD (n = 9). The values designated in columns by the different letters (a, b, c) are significantly different (p < 0.05).

Figure 1. Lipids-preventing (LPO) potential of green coffee beans and wholemeal wheat flour component in comparison with pure ferulic and chlorogenic acids. (A) Isobole curve for chlorogenic and ferulic acids, (B) LPO potential of buffer extractable (BE), bioaccessible (DE), and bioavailable (AE) green coffee beans, and wholemeal wheat flour compounds and pure ferulic and chlorogenic acids, (C) Isobole curve for BE, DE, and AE green coffee beans and wholemeal wheat flour compounds, (D) Estimation of strength of interactions of buffer extractable (BE), bioaccessible (DE), and bioavailable (AE) green coffee beans and wholemeal wheat flour compounds and pure ferulic and chlorogenic acids.

Experiments conducted in a rat model showed that CGAs are not hydrolyzed in the stomach but absorbed in intact form. Based on the Caco-2 intestine epithelial cultured monolayer, a passive diffusion was indicated. Similar results were observed with caffeic acid, which is absorbed both from paracellular diffusion as well by a monocarboxylic acid transporter (MCT). Strong evidence exists that the majority of CGA is not absorbed in the proximal part of the gastrointestinal tract, unless transformed to caffeic and ferulic acids before being absorbed. At present, the influence of the type of food matrix, which influences CGA digestion and bioavailability, remains unclear and represents an interesting area for more research on factors that influence the bioaccessibility of CGAs and other important dietary polyphenols [22].
It should be emphasized that, although in all cases the activity of GCB flour extracts was higher, the differences were not stark, especially in the cases of potentially bioaccessible (DE) and bioavailable (AE) samples. This fact demonstrates the complexity of interactions with food matrix components, because the activity of AE was relatively high in both GCB and WF extracts. Additionally, the activity of pure chemicals was also comparable (Figure 1B). The potential of pure phenolic acids for lipid preservation is strongly documented in the different food systems. Ferulic acid has the ability to delay the lipid oxidation process through the decay of hydroperoxides [23]. Phenolic compounds such as caffeic acid, tannic acid, ferulic acid, catechin, gallic acid, and so on, have proved to be efficient antioxidants in different fish model systems [7]. Phenolic compounds, depending on their mode of action, can show different antioxidative activities in different seafood systems [23]. Medina and others [24] demonstrated that the overall order of antioxidant efficiency for hydroxycinnamic acids in chilled horse mackerel was caffeic acid > ferulic acid = chlorogenic acid = α-coumaric acid. Among the pure phenolic compounds, caffeic acid, tannic acid, and gallic acid have been used for retardation of hemoglobin (Hb)-mediated lipid oxidation in fish muscle. Caffeic acid inhibits Hb-mediated lipid oxidation by various mechanisms. It strongly inhibits perch Hb-mediated lipid oxidation in washed cod mince during storage. Caffeic acid might bind with perch Hb, thus preventing its autoxidation. Caffeic acid and gallic acid are able to lower lipid oxidation of washed bighead carp mince induced by myoglobin or Hb throughout storage [7].

It must be taken into account that, in natural systems, phenolic compounds do not occur singly but in mixtures, which generally modulates their activity. Thus, we decided to analyze the activity of whole extracts from WF and GCB and determine their interactions in comparison with pure chemical standards.

According to isobolographic analysis, active compounds derived from both raw materials acted synergistically. However, the strength of synergism differed significantly depending on the kind of extract. The highest level of synergism was found in the case of extracts after simulated digestion (CI = 0.54). The results were compared with those obtained for pure chemical standards. As expected, ferulic and chlorogenic acids were able to protect lipids against oxidation. Their activity was comparable (EC$_{50}$ about 67 µg DW/mL) (Figure 1B). As presented, these compounds demonstrated a nearly additive interaction (CI value about 0.94) (Figure 1A,D).

In the food science literature, chelating power is indicated as the main reason for LPO ability because iron-chelating capacity plays an important role in preventing lipid oxidation induced by Fe$^{2+}$ [10]. In the cited study, ferulic acid displayed greater antioxidant activity than caffeic acid but was less efficient in inhibiting the Fe$^{2+}$-LO. This low-antioxidant efficiency of ferulic acid could be caused by the inability of ferulic acid to chelate Fe$^{2+}$ in the Fe$^{2+}$-LO system. In our previous study, we confirmed the comparable chelating power of ferulic and chlorogenic acids [25]. Interestingly, their activity (63.06 and 54.15 µg/mL, respectively) was similar to the LPO ability presented in this paper.

Results presented in Figure 1B–D clearly indicate the role of the activity of all extract components, the food matrix and the digestion stage in the generation of LPO activity. In the case of DE and AE, synergism was found, whereas pure chemicals (principal compounds of WF and GCB) and BE components exhibited a nearly additive interaction. Thus, drawing inferences concerning biological activity based only on knowledge of the activity of chemically pure compounds can lead to erroneous conclusions.

Compounds derived from GCB and WF acted also as effective LOX inhibitors. As expected, the highest level of activity was determined for GCB extracts. Importantly, LOX inhibitors from GCB and WF were potentially bioaccessible and bioavailable in vitro (Figure 2B). Interestingly, the activity of pure chlorogenic and ferulic acids was quite similar (18.56 and 22.36 µg/mL, respectively). This fact indicates the role of the food matrix in causing biological activity. Additionally, the isobole curve for ferulic/chlorogenic acids has a concave shape which indicates their synergistic action as LOX inhibitors (Figure 2A). Combination index (CI) analysis confirms this observation, the CI from pure chemical patterns is about 0.5, which confirms synergism. Also, hydrophilic (BE) and potentially bioaccessible
(DE) compounds derived from GCB and WF demonstrate synergistic interaction (Figure 2C); however, the strongest synergism (CI = 0.40) was found for extracts after simulated digestion (Figure 2D). Most importantly, moderate antagonism was found in the case of potentially bioavailable compounds (AE)—the isobole has a convex shape, and the CI value (1.22) indicates moderate antagonism (Figure 2B,D).

Figure 2. Lipoxygenase inhibition (LOXi) potential of green coffee beans and wholemeal wheat flour components in comparison with pure ferulic and chlorogenic acids. (A) Isobole curve for chlorogenic and ferulic acids, (B) LOXi potential of buffer extractable (BE), bioaccessible (DE), and bioavailable (AE) green coffee beans and wholemeal wheat flour compounds and pure ferulic and chlorogenic acids, (C) isobole curve for BE, DE, and AE green coffee beans and wholemeal wheat flour compounds, (D) estimation of strength of interactions of buffer extractable (BE), bioaccessible (DE), and bioavailable (AE) green coffee beans and wholemeal wheat flour compounds and pure ferulic and chlorogenic acids.

Lipoxygenases are enzymes containing non-heme iron and requiring catalytic activation. This activation process involves transformation of non-active iron in ferrous state to iron in ferric state, and this is accomplished by lipid hydroperoxide oxidation [4]. Several independent studies now support the correlation between 5-LOX expression and cancer cell viability, proliferation, cell migration, invasion through extracellular matrix destruction, metastasis, and activation of anti-apoptotic signaling cascades [26]. The abilities of plant extracts and pure chemicals to inhibit LOX activity are well known and extensively studied [27,28].

While much is known about the interactions between active compounds and the food matrix [29–31], an often-overlooked problem is the interaction between the active compounds themselves. In this study, we found nearly additive interactions between CGA and FA (as LPO inhibitors) and synergism (as LOX inhibitors). Interestingly, taking into account chelating power, antagonism was found [25]. This fact may also confirm that Fe²⁺-chelating power is not the most critical factor to enable phenolics to inhibit lipids peroxidation.
An interesting issue is the question of the mechanisms of action of the studied extracts and their potential changes during digestion and absorption in vitro; thus, the next stage of our study was to determine the mechanism of action of LOX inhibitors in the light of their potential bioaccessibility and bioavailability.

Both ferulic and chlorogenic acids acted as competitive LOX inhibitors (Figure 3A). The same model of inhibition was exhibited by BE compounds from GCB (Figure 3B) and the mixture of GCB/WF (1:1, w/w) (Figure 3D), except for digested (DE) that seems to be non-competitive. Also, compounds released from GCB during digestion in vitro acted as competitive LOX inhibitors, whereas compounds potentially bioavailable demonstrated a competitive mode of LOX inhibition (Figure 3B). These results confirm those published previously [32]. Regardless of the extraction system, compounds contained in WF acted as non-competitive LOX inhibitors (Figure 3C). The same mode of action was found for potentially bioaccessible (DE) compounds from GCB/WF mixtures, whereas potentially bioavailable (AE) phytochemicals acted as competitive LOX inhibitors (Figure 3D).

![Figure 3](image-url)

**Figure 3.** Mode of LOX inhibition by pure ferulic and chlorogenic acids (A), extracts from green coffee beans (B), extracts from wholemeal wheat flour (C) and their mixture 1:1 (D).

Bishayee and Khuda-Bukhsh [5] and Hong et al. [33] proved that phenolic acids included in GCB act as LOX inhibitors and are responsible for the protective effect of coffee against inflammation, colon cancer and tumor promotion.

As published previously, chlorogenic and ferulic acids acted as competitive inhibitors of xanthine oxidase (XO). The same mode of inhibition was exhibited by their mixture (1:1). Buffer-extractable phytochemicals from GCB acted as uncompetitive inhibitors of XO. The same mode of action was found for extracts obtained after simulated absorption, whereas potentially bioaccessible compounds acted as non-competitive XO inhibitors [20].

Consumer demand for healthy diets and convenient foods creates a huge commercial market for functional foods. Food fortification is one of the major techniques used to create functional food products with predicted pro-health activities. In this context, we proposed the new functional product—wholemeal wheat bread enriched with GCB [11].

As described previously [12], GCB addition significantly increased the LPO activity of wholemeal wheat bread. Moreover, this activity was significantly influenced by the method of extraction. The highest level of activity was found in the case of extracts obtained after simulated digestion.
was observed. It is worth noting that, in the case of raw materials, moderate antagonism was found between potentially bioaccessible (DE) compounds; however, their strength was lower than that determined for samples from enriched breads. Unfortunately, active compounds were poorly bioavailable in vitro. Also, in this case enrichment of breads with GCB resulted in an increase in LPO activity. Use of isobolographic analysis is not possible in many food systems, thus, for analysis of interactions between active components of functional breads, the IF analysis was performed. Taking into account IF values, it may be concluded that LPO-active compounds, regardless of the type of extract, acted additively.

**Table 2.** Comparison of lipids oxidation (LPO) and lipoxygenase (LOX) inhibitory activity (expressed as EC_{50} values) of samples obtained from enriched bread and estimation interaction between active components of wholemeal wheat and green coffee bean flour.

| Sample | LPO | | LOX | |
|--------|-----|--------|-----|----|
|        | AM [mg DW/ml] | AT [mg DW/ml] | IF | AM [mg DW/ml] | AT [mg DW/ml] | IF |
| BE     | | | | | | |
| C      | 134.69 ± 6.43 | - | - | 248.00 ± 5.55 | - | - |
| GC1    | 130.35 ± 5.98 | 134.33 | 131.33 ± 4.32 | 262.25 | |
| GC2    | 122.86 ± 6.12 | 133.76 | 115.04 ± 5.12 | 259.60 | |
| GC3    | 104.80 ± 5.13 | 133.29 | 0.98 ± 0.01 | 256.95 | 0.38 ± 0.09 |
| GC4    | 103.91 ± 4.88 | 132.83 | 83.83 ± 3.32 | 254.30 | |
| GC5    | 93.42 ± 4.35 | 132.36 | 71.71 ± 4.15 | 251.66 | |
| DE     | | | | | | |
| C      | 11.40 ± 0.54 | - | - | 44.20 ± 1.17 | - | - |
| GC1    | 10.25 ± 0.49 | 11.39 | 37.48 ± 0.99 | 46.81 | |
| GC2    | 7.53 ± 0.38 | 11.39 | 36.78 ± 1.38 | 46.33 | |
| GC3    | 6.72 ± 0.25 | 11.38 | 0.97 ± 0.01 | 45.86 | 0.77 ± 0.02 |
| GC4    | 6.34 ± 0.31 | 11.60 | 34.51 ± 1.56 | 45.39 | |
| GC5    | 4.97 ± 0.19 | 11.81 | 33.78 ± 1.33 | 44.92 | |
| AE     | | | | | | |
| C      | 84.31 ± 4.11 | - | - | 20.87 ± 1.19 | - | - |
| GC1    | 75.73 ± 3.33 | 83.93 | 33.78 ± 1.05 | 31.20 | |
| GC2    | 44.02 ± 2.01 | 83.54 | 31.86 ± 1.11 | 30.89 | |
| GC3    | 39.54 ± 1.93 | 83.16 | 33.26 ± 1.12 | 30.57 | 1.04 ± 0.02 |
| GC4    | 25.46 ± 1.17 | 82.78 | 31.05 ± 0.94 | 30.26 | |
| GC5    | 26.68 ± 1.11 | 82.40 | 29.64 ± 0.98 | 29.94 | |

LPO—ability to inhibition of lipids peroxidation, LOX—ability to inhibition of lipoxygenase, BE—buffer extract, DE—extract after digestion in vitro, AE—extract after absorption in vitro, C—control bread, GC1–GC5—bread with 1–5 g/100 g green coffee bean addition, respectively, AM—measured activity, AT—theoretically calculated activity, IF—interaction factor. Results were expressed as mean ± SD (n = 9). The values designated in columns by the different letters (e.g., a, b, c) are significantly different (p < 0.05).

All breads (control and enriched with GCB) contained buffer-extractable (BE) LOX inhibitors. Addition of GCB to bread significantly improved the LOX-inhibitory activity of buffer extracts. Most importantly, BE LOX inhibitors derived from both raw materials used for bread-making acted synergistically (Table 2). The same interaction was confirmed for raw materials before baking (Figure 2D). On this basis, it can be assumed that thermal treatment does not reduce the activity and/or compounds formed during baking (e.g., Maillard reaction products (MMPs) do not interfere with the activity or even participate in it).

Digestion in vitro released LOX inhibitors from all samples; however, the influence of GCB supplementation was less visible than in the case of buffer extracts. Also, synergistic interactions were found between potentially bioaccessible (DE) compounds; however, their strength was lower than that determined for BE compounds. LOX inhibitors derived from wholemeal wheat bread were bioavailable in vitro, while in the case of enriched breads extracts lower activity was found (EC_{50} for AE extracts ranged for about 33 to 29 mg DW/mL). Moreover, in this case an additive interaction was observed. It is worth noting that, in the case of raw materials, moderate antagonism was found.
Addition of green coffee flour into wheat bread significantly improved its LOX-inhibitory activity \(\text{green coffee flour})\) alone and the proposed functional product (i.e., wholemeal wheat bread enriched with GCB) constitutes a valuable supplement for the development of bread with enhanced functional properties. This relationship was clearly visible in the cases of BE and DE extracts [25]. Results presented in this study indicate that GCB addition significantly increased LPO and LOX activity in these extracts. Therefore, it can be assumed that there is a relationship between chelating ability and the ability to inhibit lipid peroxidation. However, a number of other factors and mechanisms that affect this activity have to be taken into account—in the case of potentially bioavailable (AE) compounds LPO increases, which may suggest that the ability to chelate in part translates into the ability to inhibit Fe-mediated lipid peroxidation. In the case of LOX inhibition, this relationship is not obvious; it is probable that Fe contained in the enzyme structure is partially protected from chelators.

Buffer extract from control bread acted as a non-competitive LOX inhibitor. It can be assumed that thermal treatment did not affect the mode of action of the active compounds.

After simulated digestion, the uncompetitive mechanism of action was observed. This suggests that compounds formed during digestion in vitro (MMP or other high-molecular compounds) are involved in the biological activity of bread. Importantly, these compounds were not able to permeate through the dialysis membrane (AE extracts act as non-competitive LOX inhibitors), which suggests their low-molecular character (Figure 4A).

[Figure 4. Mode of LOX inhibition by extracts from wholemeal wheat bread (A) and wholemeal wheat bread enriched with 3% GCB addition (B).]

In the case of bread enriched with 3% GCB addition, an uncompetitive mode of action was observed for BE and potentially bioaccessible (DE) compounds, whereas compounds able to pass through the dialysis membrane (AE) acted as competitive LOX inhibitors (Figure 4B). However, studies concerning the anti-LOX activity of whole food are rare. Our previous studies proved that the addition of onion skin and broccoli sprouts powder significantly elevated the LPO and LOX-inhibitory activity of wheat breads [34,35].

4. Conclusions

In this study, we found nearly additive interactions between CGA and FA (as LPO inhibitors) and synergism (as LOX inhibitors), whereas interactions between extracts from raw materials (GCB and WF) differed in many cases. Thus, the inference of biological activity based only on knowledge of the activity of chemically pure compounds can lead to erroneous conclusions. Green coffee bean constitutes a valuable supplement for the development of bread with enhanced functional properties. The addition of green coffee flour into wheat bread significantly improved its LOX-inhibitory activity and its ability to protect lipids against oxidation. Thus, both the raw materials (i.e., wholemeal WF and green coffee flour) alone and the proposed functional product (i.e., wholemeal wheat bread enriched with GCB) could be helpful in dietary therapy and prevention of so-called lifestyle disorders related...
with the lipids metabolism. Moreover, this study highlights the need to study the interactions between the active ingredients of newly designed functional products.

**Author Contributions:** Conceptualization, U.G.-D.; Data curation, D.D.; Formal analysis, E.H.-K.; Investigation, J.B., D.D., M.´S., and U.Z.; Methodology, U.G.-D.; Writing—original draft, U.G.-D.

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**References**

1. Ayala, A.; Muñoz, M.F.; Argüelles, S. Lipid Peroxidation: Production, Metabolism, and Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxid. Med. Cell. Longev.* 2014, 2014, 1–31. [CrossRef] [PubMed]
2. Rikans, L.E.; Hornbrook, K.R. Lipid peroxidation, antioxidant protection and aging. *Biochim. Biophys. Acta. Acta Mol. Basis Dis.* 1997, 1362, 116–127. [CrossRef]
3. Juntachote, T.; Berghofer, E. Antioxidative properties and stability of ethanolic extracts of Holy basil and Galangal. *Food Chem.* 2005, 92, 193–202. [CrossRef]
4. Czapski, G.A.; Czubowicz, K.; Strosznajder, J.B.; Strosznajder, R.P. The lipoxygenases: Their regulation and implication in Alzheimer’s disease. *Neurochem. Res.* 2016, 41, 243–257. [CrossRef] [PubMed]
5. Bishayee, K.; Khuda-Bukhsh, A.R. 5-Lipoxygenase Antagonist therapy: A new approach towards targeted cancer chemotherapy. *Acta Biochim. Biophys. Sin. (Shanghai)* 2013, 45, 709–719. [CrossRef]
6. Suhasini, G.E.; Nirmala, M.; Varalakshmi, V.; Solomon sunder Raj, B. Antioxidant activity of newly synthesised imidazole (2-(2-benzylidenehydrazinyl)-5, 5-diphenyl-1,5-dihydro-4H-imidazol 4-one). *Int. J. Pharm.* 2012, 2, 386–391.
7. Maqsood, S.; Benjakul, S.; Abushelaibi, A.; Alam, A. Phenolic compounds and plant phenolic extracts as natural antioxidants in prevention of lipid oxidation in seafood: A detailed review. *Compr. Rev. Food Sci. Food Saf.* 2014, 13, 1125–1140. [CrossRef]
8. Srinivasan, M.; Sudheer, A.R.; Menon, V.P. Ferulic acid: Therapeutic potential through its antioxidant property. *J. Clin. Biochem. Nutr.* 2007, 40, 92–100. [CrossRef]
9. Meng, S.; Cao, J.; Peng, Q.; Peng, J.; Hu, Y. Roles of chlorogenic acid on regulating glucose and lipids metabolism: A review. *Evid. Based Complement. Altern. Med.* 2013, 2013, 801457. [CrossRef]
10. Chen, X.; Ahn, D.U. Antioxidant activities of six natural phenolics against lipid oxidation induced by Fe2+ or ultraviolet light. *JAOCS J. Am. Oil Chem. Soc.* 1998, 75, 1717–1721. [CrossRef]
11. Dziki, D.; Gawlik-Dziki, U.; Pecio, L.; Różyło, R.; Świeca, M.; Krzykowski, A.; Rudy, S. Ground green coffee beans as a functional food supplement—Preliminary study. *LWT Food Sci. Technol.* 2015, 63, 691–699. [CrossRef] [PubMed]
12. Świeca, M.; Gawlik-dziki, U.; Dziki, D.; Baraniak, B. Wheat bread enriched with green coffee—In vitro bioaccessibility and bioavailability of phenolics and antioxidant activity. *Food Chem.* 2017, 221, 1451–1457. [CrossRef] [PubMed]
13. Serrano, J.; Goñi, I.; Saura-Calixto, F. Food antioxidant capacity determined by chemical methods may underestimate the physiological antioxidant capacity. *Food Res. Int.* 2007, 40, 15–21. [CrossRef]
14. Nowacka, N.; Nowak, R.; Drozd, M.; Olech, M.; Los, R.; Malm, A. Analysis of phenolic constituents, antiradical and antimicrobial activity of edible mushrooms growing wild in Poland. *LWT Food Sci. Technol.* 2014, 59, 689–694. [CrossRef]
15. Minekus, M.; Alminger, M.; Alvito, P.; Ballance, S.; Bohn, T.; Bourlieu, C.; Carrière, F.; Boutrou, R.; Corredig, M.; Dupont, D.; et al. A standardised static in vitro digestion method suitable for food—An international consensus. *Food Funct.* 2014, 5, 1113–1124. [CrossRef] [PubMed]
16. Kuo, J.M.; Yeh, D.B.; Pan, B.S. Rapid photometric assay evaluating antioxidative activity in edible plant material. *J. Agric. Food Chem.* 1999, 47, 3206–3209. [CrossRef] [PubMed]
17. Axelrod, B.; Cheesbrough, T.M.; Laakso, S. Lipoxygenase from Soybeans: EC 1.13.11.12 Linoleate: Oxygen oxidoreductase. *Methods Enzymol.* 1981, 71, 441–451.
18. Chou, T. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. *Pharmacol. Rev.* 2006, 58, 621–681. [CrossRef]

19. Gawlik-Dziki, U. Changes in the antioxidant activities of vegetables as a consequence of interactions between active compounds. *J. Funct. Foods* 2012, 4, 872–882. [CrossRef]

20. Gawlik-Dziki, U.; Dziki, D.; Świeca, M.; Nowak, R. Mechanism of action and interactions between xanthine oxidase inhibitors derived from natural sources of chlorogenic and ferulic acids. *Food Chem.* 2017, 225, 1451–1457. [CrossRef]

21. Yu, L.; Nanguet, A.-L.; Beta, T. Comparison of Antioxidant Properties of Refined and Whole Wheat Flour and Bread. *Antioxidants* 2013, 2, 370–383. [CrossRef] [PubMed]

22. Liang, N.; Kitts, D.D. Role of chlorogenic acids in controlling oxidative and inflammatory stress conditions. *Nutrients* 2016, 8, 16. [CrossRef] [PubMed]

23. Medina, I.; Gallardo, J.M.; González, M.J.; Lois, S.; Hedges, N. Effect of molecular structure of phenolic families as hydroxycinnamic acids and catechins on their antioxidant effectiveness in minced fish muscle. *J. Agric. Food Chem.* 2007, 55, 3889–3895. [CrossRef] [PubMed]

24. Chen, C.-H.; Chan, H.-C.; Chu, Y.-T.; Ho, H.-Y.; Chen, P.-Y.; Lee, T.-H.; Lee, C.-K. Antioxidant activity of some plant extracts towards xanthine oxidase, lipoxygenase and tyrosinase. *Molecules* 2009, 14, 2947–2958. [CrossRef]

25. Schneider, I.; Bucar, F. Lipoxygenase inhibitors from natural plant sources. Part 1: Medicinal plants with inhibitory activity on arachidonate 5-lipoxygenase and 5-lipoxygenase[cyclooxygenase. * Phyther. Res.* 2005, 19, 81–102. [CrossRef]

26. Języka, L.; Swieca, M.; Gawlik-Dziki, U. Nutritional and health-promoting properties of bean paste fortified with onion skin in the light of protein-phenolics interactions. *Acta Sci. Pol. Technol. Aliment.* 2015, 14, 29–36. [CrossRef] [PubMed]

27. Seczyk, Ł.; Swieca, M.; Gawlik-Dziki, U. Lipoxygenase inhibitors and antioxidants from green coffee—Mechanism of action in the light of potential bioaccessibility. *Food Res. Int.* 2014, 61, 48–55. [CrossRef]

28. Hong, J.; Smith, T.J.; Ho, C.-T.; August, D.A.; Yang, C.S. Effects of purified green and black tea polyphenols on cyclooxygenase- and lipoxygenase-dependent metabolism of arachidonic acid in human colon mucosa and colon tumor tissues. *Biochem. Pharmacol.* 2001, 62, 1175–1183. [CrossRef]

29. Gawlik-Dziki, U.; Jeżyna, M.; Ścieczkowska, I.; Pecio, Ł.; Durak, A.; Czyż, J. Effect of bioaccessibility of phenolic compounds on in vitro anticancer activity of broccoli sprouts. *Food Res. Int.* 2012, 49, 469–476. [CrossRef]