CHICKPEA CHELATING PEPTIDES INHIBIT COPPER-MEDIATED LIPID PEROXIDATION

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ABSTRACT

BACKGROUND

Transition metals produce radical oxygen species (ROS) promoting lipid peroxidation processes that favor the development of cardiovascular and neurodegenerative diseases. In addition, the oxidation of lipids present in food may affect the quality of food products. Therefore, antioxidants counteracting these metals pro-oxidant effects may have high potential for pharmacology and food industries. In this work, we studied the capability to inhibit the copper mediated lipid peroxidation of peptides fractions purified from a chickpea protein hydrolysate in different lipids substrates: β-carotene, mixture of unsaturated fatty acids and low density lipoprotein (LDL).

RESULTS

Peptide fractions with the highest histidine content are the most antioxidant. This antioxidant effect is mainly due to the capability of histidine to bind copper and act as hydrogen donor through its imidazole ring.

CONCLUSIONS

These results suggest that chickpea proteins are a potential source of antioxidant peptides which may be included as ingredients into functional foods with beneficial health effects. In addition, these antioxidant peptides may be useful to protect food products from lipid peroxidation processes increasing their quality and shelf life.

KEY WORDS: Chickpea, Protein hydrolysate, Antioxidant peptides, Chelating peptides, LDL.

INTRODUCTION

Copper is an essential transition metal that is implicated in several metabolic process such as mitochondria respiration, iron metabolism or free radical detoxification [1].This metal acts as cofactor of many enzymes involved in redox reactions, such as cytochrome c oxidase, ascorbate oxidase, or superoxide dismutase. Following to its absorption from the diet, copper is bounded to proteins such as serum albumin and storage in the liver or delivered to cells through the bloodstream [2]. In addition, similarly to others metals such as iron, copper may produce reactive oxygen species (ROS) via Fenton reactions, causing damage in biomolecules (DNA, lipids and proteins) and promoting the development of different diseases such as atherosclerosis, cardiovascular diseases, cancer and neurological degenerative diseases[3]. On the other hand, ROS may also contribute to the oxidation of lipids present in food via lipid peroxidation processes, which involve chain reactions of free radicals with polyunsaturated fatty acids. These lipid peroxidation processes have a negative impact in flavor, texture, nutritive value, and shelf life of foods products and may produce toxins under extreme conditions[4].

In the last years, the use of natural antioxidants is becoming of interest. In particular, bioactive peptides with antioxidant activity have been extensively studied (for review see [5, 6]). Bioactive peptides are amino acids sequences, between 2 and 15 residues, which are inactive within the original protein but may exert beneficial effects once that they are released during the gastrointestinal digestion or by enzymatic hydrolysis process [7]. Thus, peptides with several beneficial activities such as antioxidant, opioid, antimicrobial, immunomodulators, antithrombotic, hypocholesterolemic, antihypertensive, or

chelating have been reported [8-10]. Food products containing bioactive peptides would increase their nutritional value. Therefore, once that bioactive peptides bioavailability is demonstrated, which depends of their resistant to intestinal absorption and gastrointestinal digestion, these bioactive peptides may be potentially used as functional food ingredients. Milk proteins, such as caseins or whey proteins, have been the most used source of bioactives peptides [6, 11, 12]. However, bioactive peptides isolated from vegetable proteins hydrolysates are becoming important since they are a more economical and accessible alternative that proteins from animal sources [13].

Several studies have described the production of chelating peptides, which also exert antioxidant activity in the presence of transition metals, from plant proteins such as *Phaseolus vulgaris* (bean) [14], *Jatropha curcas* (physic nut) [15], *Helianthus annuus* (sunflower) [16], *Zea mays* (corn) [17] *Ginkgo biloba* (maidenhair tree) [18] or *Ipomoea batatas* (sweet potato)[19]. Also, in previous studies we have shown that hydrolysis of *Cicer arietinum* L. (chickpea) proteins with the digestive enzymes pepsin and pancreatin produced copper and iron chelating peptides which were purified by affinity chromatography through immobilized copper [20, 21]. These peptides may prevent the pro-oxidant effect of transition metals by metal ion chelation. Hence, in the present work we study the capability of these chickpea chelating peptides to inhibit the copper-mediated lipid peroxidation in three different lipid systems: β-carotene, unsaturated fatty acids mixture and low density lipoprotein (LDL). These peptides may be of high interest for the pharmacology industry to treat or prevent diseases related with the oxidative damage or mineral deficiencies. In addition, they also may have potential for the food industry to be included into functional foods with

antioxidant and mineral fortification properties as well as to increase the quality and shelf life of food products.

MATERIALS AND METHODS

Materials.

Kabuli Chickpea seeds were purchased in a local market. β-carotene, low density lipoprotein (LDL), butylated-hydroxy-toluene (BHT), sodium dodecyl sulphate (SDS), fatty acids mixture and thiobarbituric acid (TBA) were from Sigma (Sigma Chemical Co., MO, USA). Tween 20 was from VWR. Reagents were of analytical grade and purchased from Sigma and Merck (Darmstadt, Germany).

Methods.

Purification of chickpea chelating peptides.

Chickpea chelating peptides were purified as previously described [20]. Briefly, chickpea protein isolate (5%, w/v) was sequentially hydrolyzed with the digestive enzymes pepsin and pancreatin (E/S 1/20, w/w) at 37°C using a hydrolysis reactor (B. Braun Biotech Internacional GMBH, Melsungen, Germany). Hydrolysis with pepsine was carried out at pH 2.5 for 180 min. Next, pancreatin was added and the pH was increased to 7.5. After 180 min, hydrolysis was inactivated at 80°C for 20 min. Next, peptides fractions were purified from this chickpea protein hydrolysate by affinity chromatography in a FPLC AKTA-purifier system (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) using copper as immobilized ligand. Peptides retained by the copper column were eluted by pH gradient (7.4 to 4.0) in 50 mM sodium acetate, 0.5 M sodium chloride buffer at 1 mL/min flow. Finally, peptides

fractions collected were further fractioned by size exclusion chromatography using a Superdex-peptide 10/300 GL column (GE Healthcare) (**Figure 1**).

β-carotene bleaching assay.

This assay is a widely method used for the indirect determination of antioxidant activity and is based on the determination of the oxidative degradation of β-carotene [22]. A β-carotene solution at 4 mg/mL was prepared in chloroform and mixed with Tween 20 (1:1, v/v). Following to chloroform evaporation under nitrogen, absorbance at 470 nm of the β-carotene solution was adjusted to 1 through addition of 100 mM phosphate buffer (pH 7). Chickpea protein hydrolysate and peptide fractions were incubated with β-carotene solution containing 50 µM $CuSO_4$ in a 96 well microplate for 1 h at 50°C. Controls in presence and absence of 50 µM $CuSO_4$ were included as negative and positive control respectively. In addition, the synthetic antioxidant, butylated hydroxytoluene (BHT) was used as an extra positive control. The degradation of β-carotene is evaluated by measuring the decrease in absorbance at 470 nm at different times using a microplate spectrophotometer (Multiskan Spectrum, Thermo). Data was analysed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc.).

Thiobarbituric acid reactive substances (TBARS) assay.

This assay is based in the monitoring of malondialdehyde (MDA) that is produced by oxidation of polyunsaturated fatty acids. At low pH and high temperature conditions, MDA is involved in a nucleophilic addition reaction with thiobarbituric acid (TBA) generating a red fluorescent adduct with maximum absorption at 532 nm. TBARS assay was carried out in two lipid model systems,

149 fatty acids mixture and low-density lipoprotein (LDL), according to Moon &
150 Shibamoto [23] with modifications:
151 - Fatty acids: 25.1 mM unsaturated fatty acids solution (70% linolenic acid, 25%
152 linoleic acid and 5% oleic acid) was prepared in 50 mM phosphate buffer (pH 7)
153 containing 11.4 mM Tween 20. Following to sonication for 1 minute to remove
154 turbidity, chickpea protein hydrolysate and peptide fractions were incubated with
155 unsaturated fatty acids solution containing 5 μM $CuSO_4$ at 37°C for 24 h. Then,
156 50 µL samples were taken at 0, 4, 8 and 24 h and incubated with an aqueous
157 solution containing 600 µL of TBARS reagent (26 mM TBA, 918 mM
158 trichloroacetic acid and 0.25 N HCl) and 250 µL of SDS 1% (w/v) in 0.1 N NaOH
159 at 80º C for 15 min. TBA-MDA adduct was monitored by measured of
160 absorbance at 535 nm. Fatty acids solution containing 5 μM $CuSO_4$ in presence
161 and absence of BHT were included as positive and negative control
162 respectively. The assay was carried out in triplicate and the results expressed in
163 MDA concentration (µM), which was calculated from a standard MDA curve.
164 - LDL: LDL solution (1 mg/mL) prepared in 50 mM M phosphate buffer (pH 7)
165 was dialyzed at 4°C for 48 h to remove EDTA and salts. Next, LDL solution was
166 diluted to 0.5 mg LDL/mL in 0.05 M phosphate buffer (pH 7). Chickpea protein
167 hydrolysate and peptide fractions were incubated with LDL solution containing 5
168 µM $CuSO_4$ at 37°C for 24 h. TBARS assay was carried out in triplicate as
169 described above for fatty acids. Results are expressed as nmoles MDA
170 generated per mg of LDL.

171 Data was analysed using GraphPad Prism software (PRISM 5.0; GraphPAD
172 Software Inc.).

173

Statistical analysis

Statistical analyses were performed using SPSS software (IBM SPSS statistics 20). Statistical analyses for the β-carotene bleaching assay were carried out using a one-way ANOVA with Bonferroni correction post hoc test. Differences between the mean values were considered significant at $P<0.05$. In addition, statistical significant levels are subsequently depicted as follows: *indicating $P<0.05$, ** indicating $P<0.01$ or *** indicating $P<0.001$.

RESULTS

Inhibition of copper mediated β-carotene oxidation.

β-carotene belongs to the group of red, orange, and yellow pigments named carotenoids. These compounds are very susceptible to free radical mediated oxidation due to several double bonds within their structure, which are responsible of their absorbance at 470 nm and characteristic color. These double bonds are lost when β-carotene is oxidized by light (photo-oxidation) or in the presence of ROS, producing a decreased absorbance at 470 nm. Hence, antioxidants counteracting β-carotene oxidation will show a lower decrease in the absorbance. In this case, we used copper as catalyst for β-carotene oxidation.

Figure 2A shows the copper mediated oxidation, expressed as decrease in absorbance at 470 nm, in presence of BHT and increasing concentration of chickpea protein hydrolysates (CPH) (0.12 mg/mL, 0.16 mg/mL and 0.24 mg/mL). The synthetic antioxidant BHT (0.12 mg/mL) counteracted the copper mediated oxidation showing a decreased absorbance at 470 nm similar to the positive control (β-carotene in absence of copper). Increasing CHP

concentrations produced an increasedinhibition of copper-mediated β-carotene oxidation. However no significant differences are found along the inhibition activity at the different doses tested in this assay. Therefore, CPH showed only a trend to inhibit copper-mediated oxidation in a dose dependent manner.P<

In addition, purified chickpea chelating peptide fractions, F1, F2 and F3, also showed capability to inhibit copper mediated β-carotene oxidation with a higher efficacy than the original protein hydrolysate (**Figure 2B**). Thus, while at 0.12 mg/mL CPH produced a not significant inhibition of copper mediated β-carotene oxidation of 18.2% as compared to negative control, F1, F2 and F3 fractions showed a significant inhibition of 36% ($P<0.01$), 45.5% ($P<0.001$) and 40.3% ($P<0.001$), respectively (**Figure 2C**). In addition, BHT showed a significant inhibition ($p<0.001$) of β-carotene oxidation of 69.3% as compared to negative control (**Figure 2C**).

On the other hand, some peptides fractions purified by size exclusion chromatography from fractions F1, F2 and F3 also showed inhibition of copper mediated β-carotene oxidation at assay concentration (0.12 mg/mL) (**Figure 3**). Thus, within fractions purified from F1, F1A and F1F showed no decreased absorbance at 470 nm with respect to the negative control and, therefore, no antioxidant effect. In contrast, F1B, F1D and F1E showed a significant inhibition of copper mediated β-carotene oxidation with respect to the negative control by 50.7% ($P<0.01$), 63.6% ($P<0.001$) and 62.6% ($P<0.001$) respectively (**Figure 3A**). Furthermore, F2A, F2B, F2C and F2D fractions showed similar antioxidant activities decreasing significantly ($P<0.001$) the β-carotene oxidation with respect to the negative control by 50.6, 56.4, 55.4 and 43.2% respectively (**Figure 3B**). Finally, within F3 fractions, F3A and F3B were pro-oxidant

whereas F3C, F3D and F3E fractions showed a significant ($p<0.001$) antioxidant activity inhibiting the copper mediated β-carotene oxidation by 67.9, 85.2 and 78% respectively. In addition, F3D and F3E showed a higher antioxidant activity than BHT, which showed a significant ($p<0.001$) inhibition of copper mediated β-carotene oxidation of 70.4% at assay concentration (0.12 mg/mL).

Inhibition of copper mediated fatty acids oxidation.

Antioxidant activity of chickpea chelating peptides was further analysed in a mixture of unsaturated fatty acids. Malondialdehyde (MDA) is one of the end products of the peroxidation of polyunsaturated fatty acids and, therefore, its quantification is considered a good index of lipid peroxidation process. The most widely used method to measure MDA formation is the "thiobarbituric acid reactive substances" (TBARS) assay [24]. Copper mediated MDA generation was determined after incubation of copper with fatty acids and chickpea chelating peptides for 4, 8 and 24 hours.

Figure 4A shows fatty acids peroxidation, expressed as MDA formation (μM), in presence of BHT and CPH. CPH showed a decreased MDA formation, as compared to the control, in a dose dependent manner. Thus, after 24 h, CPH displayed a decreased MDA formation as compared to control by 12.4, 69.8 and 75.4% at 1, 2.5 and 5 mg/mL respectively. However, BHT showed a decreased MDA formation as compared to control of 97.4% at 0.1 mg/mL, being 10-fold more effective than the protein hydrolysate.

On the other hand, chickpea copper chelating peptide fractions, F1, F2 and F3, showed a higher decreased MDA formation than the original protein hydrolysate (**Figure 4B**). Hence, F1 decreased copper mediated fatty acid

oxidation by 45.4% as compared to control. In addition, F2 as well as F3 showed a full inhibition of the fatty acid peroxidation after 24 h and at the maximum assayed concentration (0.2 mg/mL). Moreover, F3 was the most active peptide fraction showing this antioxidant effect also at lower concentration (0.15 mg/mL), similarly to the synthetic antioxidant BHT (**Figure 4B**).

Inhibition of MDA formation by size exclusion peptide fractions purified from F1, F2 and F3 was also studied (**Figure 5**). F1 fractions did not show antioxidant activity at assay concentration (0.03 mg/mL). Moreover, F1A fractions showed a pro-oxidant effect, which was also observed in the β-carotene assay (**Figure 5A**). Among F2 fractions, F2A did not show antioxidant activity. However, F2C and F2D showed full inhibition of fatty acids peroxidation at 0.03 mg/mL after 24 h. F2B also displayed a potent antioxidant effect decreased MDA formation of 94.1% as compared to control (**Figure 5B**) at the same assay conditions. Finally, among F3 fractions, just F3D and F3E showed antioxidant activity letting to a decrease in MDA formation as compared to control of 100 and 93.6% respectively (**Figure 5C**).

Inhibition of copper mediated low-density lipoprotein oxidation.

Low-density lipoprotein (LDL) is highly sensitive to lipid oxidation by transition metals, such as copper, due to its high content in polyunsaturated fatty acids. Oxidation of LDL plays an important role in the development of atherosclerosis. Hence, potential prevention of LDL oxidation by nutraceuticals is becoming of interest for the food industry. Protein hydrolysates from different natural sources have shown inhibitory activity of LDL oxidation in the presence of copper. Thus, pepsin hydrolysates of algae [25], egg white proteins [26] and

Ipomoea proteins [27] have been reported to show antioxidant activity. However, this is the first time to our knowledge that copper chelating peptides have been assayed for their LDL protective activity against copper mediated oxidation. Copper binds to apolipoprotein B-100 present in LDL promoting its oxidation through lipid radical generation [28].

Figure 6A shows copper mediated LDL oxidation expressed as nmoles MDA obtained per mg LDL, in presence of BHT and chickpea protein hydrolysate. CPH showed inhibition of copper mediated LDL oxidation in a dose dependent manner. Thus, after 24 h incubation, CPH decreased MDA formation as compared to control by 26.8 and 73.9% at 0.02 and 0.1 mg/mL respectively. However, this antioxidant effect was more than 10-fold lower than for BHT, which showed full MDA formation inhibition at 0.01 mg/mL.

However, copper chelating peptide fractions F1, F2 and F3 showed an antioxidant effect comparable to BHT, displaying full inhibition of copper mediated LDL oxidation at 0.02 mg/mL after 24 h incubation (**Figure 6B**).

On the other hand, F1E and F1F provided the highest antioxidant activity among size exclusion F1 fractions inhibiting MDA formation after 24 h by 53.5 and 37.4% as compared to control at assay concentration (0.03 mg/mL) (**Figure 7A**). F2 fractions showed a higher antioxidant activity being F2B the most active (**Figure 7B**). Thus, F2B showed full inhibition of the copper mediated LDL oxidation as compared to control at assay concentration (0.03 mg/mL) after 24 h incubation. F2A, F2C and F2D showed a reduced MDA formation as compared to control by 77.1, 89.5 and 82.8% respectively, at the same assay conditions (**Figure 7B**). Finally, F3C, F3D and F3E showed full inhibition of

298 copper mediated LDL after 24 h at assay concentration, showing similar efficacy
299 than BHT (**Figure 7C**).

300 **DISCUSSION**

301 In this work we show, for first time to our knowledge, the antioxidant
302 activity of peptide fractions purified from a chickpea protein hydrolysate
303 obtained by sequential hydrolysis with the digestive enzymes pepsin and
304 pancreatin. In particular, we have studied the capability of these peptide
305 fractions to inhibit the copper mediated oxidation in three different lipid systems:
306 β-carotene, mixture of unsaturated fatty acids and low density lipoproteins
307 (LDL).

308 In general, an increased antioxidant activity is observed from the parental
309 protein hydrolysate to peptides fractions purified by affinity and size exclusion
310 chromatography. We hypothesized that this is due to an increased proportion of
311 antioxidant peptides along the purification process. Accordingly, a
312 heterogeneous mixture of peptides is present in the parental hydrolysate
313 protein, including no active peptides that may produce antagonist effects
314 decreasing the antioxidant activity. In contrast, peptide fractions are more
315 homogeneous, evading these antagonist effects and increasing possible
316 agonist effects between active peptides.

317 Some of these peptides fractions have previously shown copper chelating
318 activity [20] and, therefore, they are expected to inhibit copper pro-oxidant effects.
319 Thus, a positive correlation between copper chelating activity and antioxidant
320 activity is observed reinforcing that antioxidant effects are mainly due to the
321 formation of copper chelates which counteract the pro-oxidant effect of this
322 metal. Hence, F2B, F2C, F2D, F3D and F3E, which were reported to be the

most copper chelating peptide fractions, were the most antioxidant peptide fractions. This antioxidant activity may be due to the high histidine content of these peptide fractions (15-34%) (**Table 1**). Histidine binds copper through its imidazole ring inhibiting the copper mediated reactive oxygen species (ROS) production [28]. In addition, these antioxidant effects may be also due to the capability of this imidazole ring to act as hydrogen donor reducing lipid radicals that are produced in lipid peroxidation process [29].

On the other hand, F1D and F1E have also shown capability to inhibit copper mediated oxidation in β-carotene and LDL lipid systems. However, these fractions are not rich in histidine and, with the exception of F1D, did not show copper chelating activity [20]. Nevertheless, this antioxidant effect may be due to their arginine content (18-31%) (**table 1**). Arginine has shown antioxidant properties due to its guanidine group [30-32]. On the other hand, F1F (27.5% Arg) showed capability to inhibit the copper mediated oxidation only in the LDL system. This may be due to the small molecular size of this fraction, which is approximately in the free amino acids range [20], that does not favour the hydrogen donation from the guanidine group with the same efficiency than peptides of 3-4 amino acids. In addition, different studies have shown that when testing amixtures of amino acids found in antioxidant peptides, in the same ratio than in the original amino acidpeptide sequence, the antioxidant effect was not preserved [33-35]. Therefore, the amino acid sequence is essential for the activity.

CONCLUSION

In conclusion, copper chelating peptides fractions purified from an extensive chickpea protein hydrolysate have shown capability to inhibit the copper mediated lipid peroxidation in three representative systems: β-carotene,

mixture of unsaturated fatty acids and low density lipoprotein. This antioxidant effect is mainly due to the formation of copper chelates through the imidazole ring of the amino acid histidine.

These peptide fractions may have high potential as ingredients for functional food with antioxidant properties preventing the development of different diseases related with lipid peroxidation process such as cardiovascular and neurodegenerative diseases. In addition, they may also be of high interest for the food industry increasing the nutritive value and shelf life of foods products. Nevertheless, size exclusion peptide fractions were obtained in limited amounts and, therefore, purification procedure with an increased efficacy might be developed for industrial scale production. Furthermore, bioactive peptides bioavailability studies to demonstrate their resistant to intestinal absorption and gastrointestinal digestion might be also carried out before application in functional food. Hence, further investigation regarding bioavailability, cost, efficacy and safety studies might be done previously to their industrial application.

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CONFLICT OF INTEREST

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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TABLE LEGENDS

Table 1. Histidine and arginine content of chickpea protein hydrolysate and chickpea peptides fractions.

FIGURE LEGENDS

Figure 1. Chickpea copper chelating peptides purification procedure.

Figure 2. Inhibition of copper mediated β-carotene oxidation by chickpea protein hydrolysate (CPH) (0.12, 0.16 and 0.24 mg/mL) (**A**) and chickpea peptides fractions purified by copper affinity chromatography, F1, F2 and F3 (0.12 mg/mL). Controls in presence and absence of copper were included as negative (-C) and positive control (+C) respectively (**B**). Copper mediated β-carotene oxidation decrease as compared to negative control (all samples at 0.12 mg/mL) (**C**). Graph represents the mean ± standard error of the mean (SEM) of a representative experiment with each concentration point performed in duplicate. Copper mediated β-carotene oxidation was depicted as decrease in absorbance at 470 nm along 60 min incubation. ***P≤0.001, **P≤0.01, *P≤0.05 as compared to negative control.

Figure 3. Inhibition of copper mediated β-carotene oxidation by size exclusion peptide fractions purified from F1 (**A**), F2 (**B**) and F3 (**C**). Graph represents the mean ± SEM of a representative experiment with each concentration point performed in duplicate. Copper mediated β-carotene oxidation was depicted as decrease in absorbance at 470 nm along 60 min incubation. ***P≤0.001, **P≤0.01, *P≤0.05 as compared to negative control. All samples assayed only at 0.12 mg/mL due to limited amount of samples.

Figure 4. Inhibition of copper mediated lipid peroxidation in unsaturated fatty acids mixture (linolenic:linoleic:oleic 70:25:5) by chickpea protein hydrolysates (CPH) (1, 2.5 and 5 mg/mL) (**A**) and chickpea peptide fractions F1, F2 and F3 purified by copper affinity chromatography (0.15 and 0.2 mg/mL) (**B**). Copper mediated lipid peroxidation was depicted as malondialdehyde (MDA) formation after 4, 8 and 24 h incubation. Graph represents the mean ± SEM of a representative experiment with each concentration point performed in triplicate. BHT: 0.1 mg/mL.

Figure 5. Copper mediated lipid peroxidation of unsaturated fatty acids mixture (linolenic:linoleic:oleic 70:25:5) in presence of peptide fractions purified by size exclusion peptide fractions purified from F1 (**A**), F2 (**B**) and F3 (**C**) (0.03 mg/mL). Copper mediated lipid peroxidation was depicted as malondialdehyde (MDA) formation after 4, 8 and 24 h incubation. Graph represents the mean ± SEM of a representative experiment with each concentration point performed in triplicate. BHT: 0.03 mg/mL.

Figure 6. Inhibition of copper mediated lipid peroxidation in low density lipoprotein (LDL) by chickpea protein hydrolysates (CPH) at different concentrations (**A**) and chickpea peptide fractions F1, F2 and F3 purified by copper affinity chromatography (0.02 and 0.04 mg/mL) (**B**). Copper mediated lipid peroxidation was depicted as nanomoles malondialdehyde (MDA)/mg LDL after 4, 8 and 24 h incubation. Graph represents the mean ± SEM of a representative experiment with each concentration point performed in triplicate. BHT: 0.01 mg/mL.

Figure 7. Inhibition of copper mediated lipid peroxidation in low density lipoprotein (LDL) by size exclusion peptide fractions purified from F1 (**A**), F2 (**B**) and F3 (**C**) (0.03 mg/mL). Copper mediated lipid peroxidation was depicted as nanomoles malondialdehyde (MDA)/mg LDL after 4, 8 and 24 h incubation. Graph represents the mean ± SEM of a representative experiment with each concentration point performed in triplicate. BHT: 0.03 mg/mL.

Table 1

	His (%)	Arg (%)
CPH	3,0 ± 0,0	0,5 ± 0,1
F1	3,2 ± 0,0	24,9 ± 0,3
F2	17,4 ± 0,1	5,8 ± 0,2
F3	22,9 ± 0,0	5,9 ± 0,1
F1A	3,0 ± 0,3	4,7 ± 0,9
F1B	6,2 ± 1,0	7,1 ± 1,1
F1C	8,1 ± 0,1	10,5 ± 1,5
F1D	8,1 ± 0,2	18,2 ± 3,4
F1E	6,3 ± 2,2	30,9 ± 0,2
F1F	3.6 ± 3.5	27.5 ± 8.1
F2A	8,8 ± 0,1	6,4 ± 0,8
F2B	15,3 ± 0,4	3,9 ± 0,1
F2C	23,7 ± 0,7	5,2 ± 0,9
F2D	33,4 ± 2,3	7,1 ± 0,9
F3A	6,4 ± 3,2	6.3 ± 0.1
F3B	18,7 ± 4,7	5,7 ± 0,1
F3C	23,2 ± 3,2	5,1 ± 0,1
F3D	25,1 ± 11,3	5,2 ± 0,0
F3E	59,8 ± 5,3	9,7 ± 0,1

Modified from Torres-Fuentes, Alaiz and Vioque (2011).

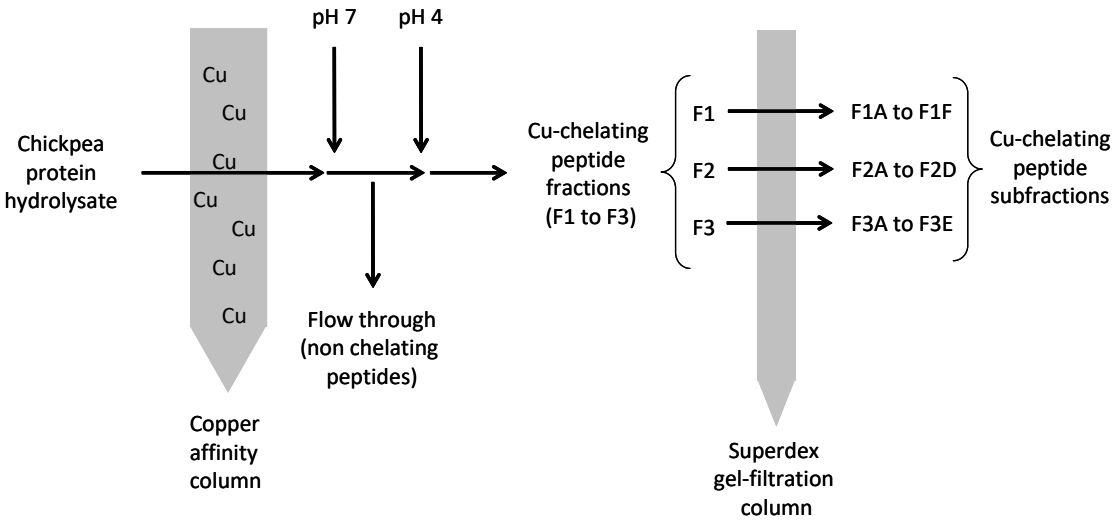
pH 7
pH 4
Cu
Cu
Cu
Cu
Cu
Cu
Cu
Chickpea protein hydrolysate
Flow through (non chelating peptides)
Copper affinity column
Cu-chelating peptide fractions (F1 to F3)
F1
F2
F3
F1A to F1F
F2A to F2D
F3A to F3E
Cu-chelating peptide subfractions
Superdex gel-filtration column

FIGURE 1

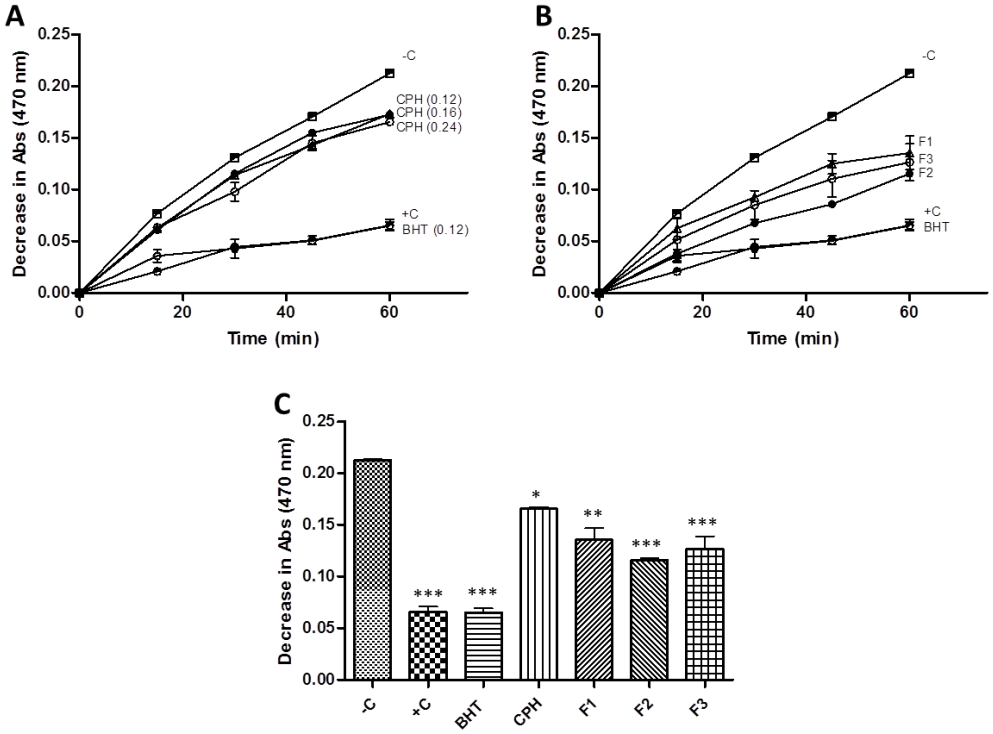
A
Decrease in Abs (470 nm)
0.25
0.20
0.15
0.10
0.05
0.00
0
20
40
60
Time (min)
-C
CPH (0.12)
CPH (0.16)
CPH (0.24)
+C
BHT (0.12)
B
Decrease in Abs (470 nm)
Time (min)
-C
F1
F3
F2
+C
BHT
C
Decrease in Abs (470 nm)
***
***
*
**
***
***
-C
+C
BHT
CPH
F1
F2
F3

FIGURE 2

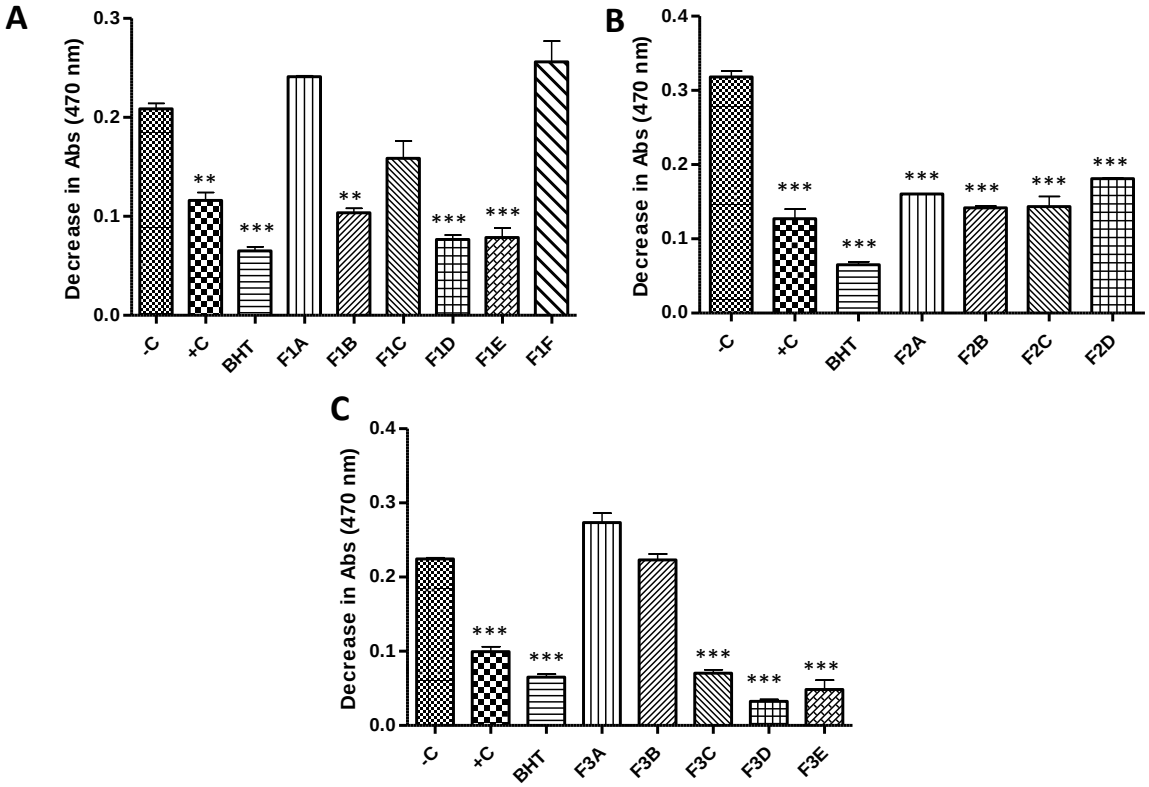
A
Decrease in Abs (470 nm)
0.0
0.1
0.2
0.3
**
***
**
***
***
-C
+C
BHT
F1A
F1B
F1C
F1D
F1E
F1F
B
Decrease in Abs (470 nm)
0.0
0.1
0.2
0.3
0.4
***
***
***
***
***
-C
+C
BHT
F2A
F2B
F2C
F2D
C
Decrease in Abs (470 nm)
0.0
0.1
0.2
0.3
0.4
***
***
***
***
***
-C
+C
BHT
F3A
F3B
F3C
F3D
F3E

FIGURE 3

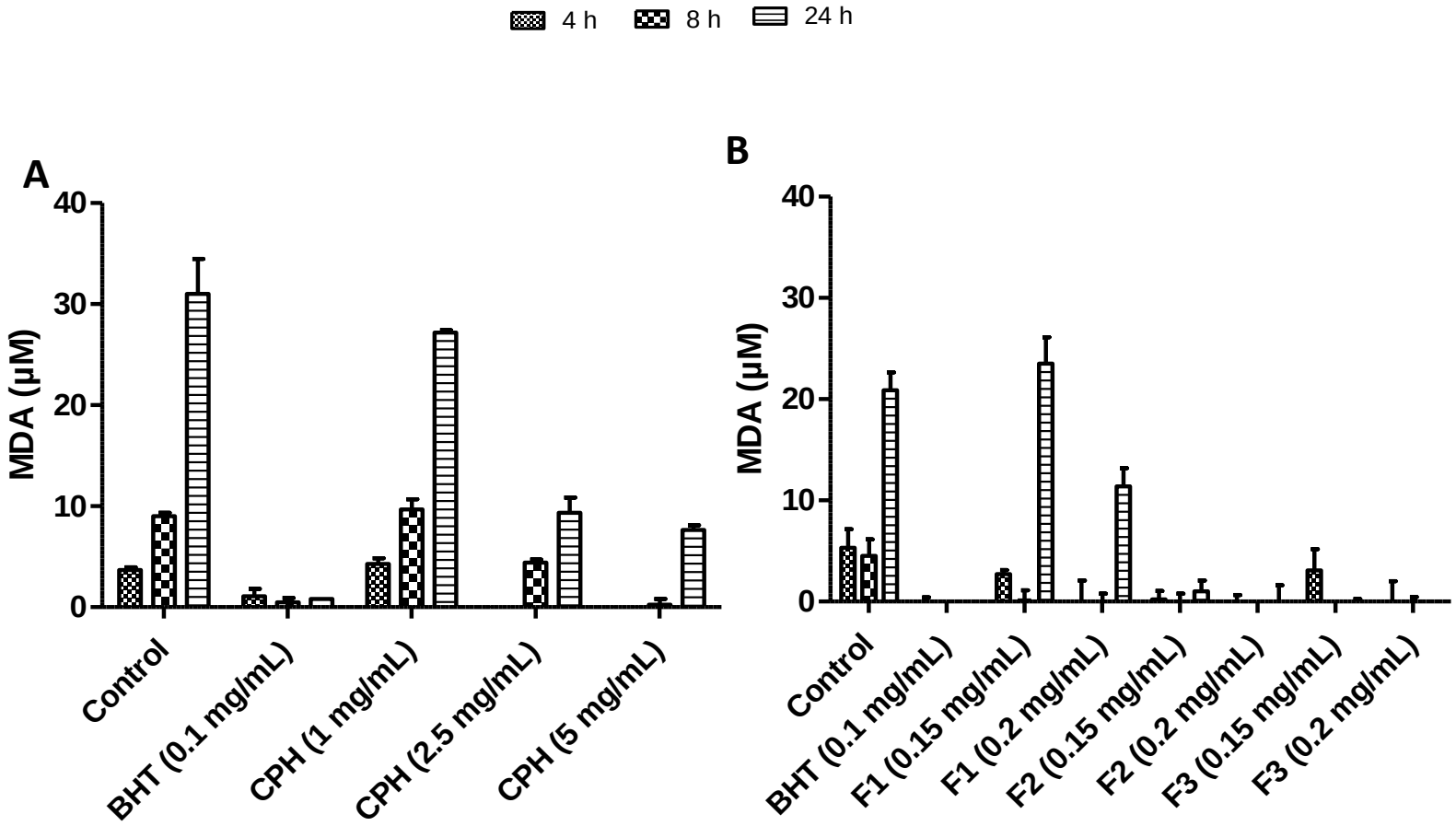
4 h
8 h
24 h
A
MDA (µM)
40
30
20
10
0
Control
BHT (0.1 mg/mL)
CPH (1 mg/mL)
CPH (2.5 mg/mL)
CPH (5 mg/mL)
B
MDA (µM)
40
30
20
10
0
Control
BHT (0.1 mg/mL)
F1 (0.15 mg/mL)
F1 (0.2 mg/mL)
F2 (0.15 mg/mL)
F2 (0.2 mg/mL)
F3 (0.15 mg/mL)
F3 (0.2 mg/mL)

FIGURE 4

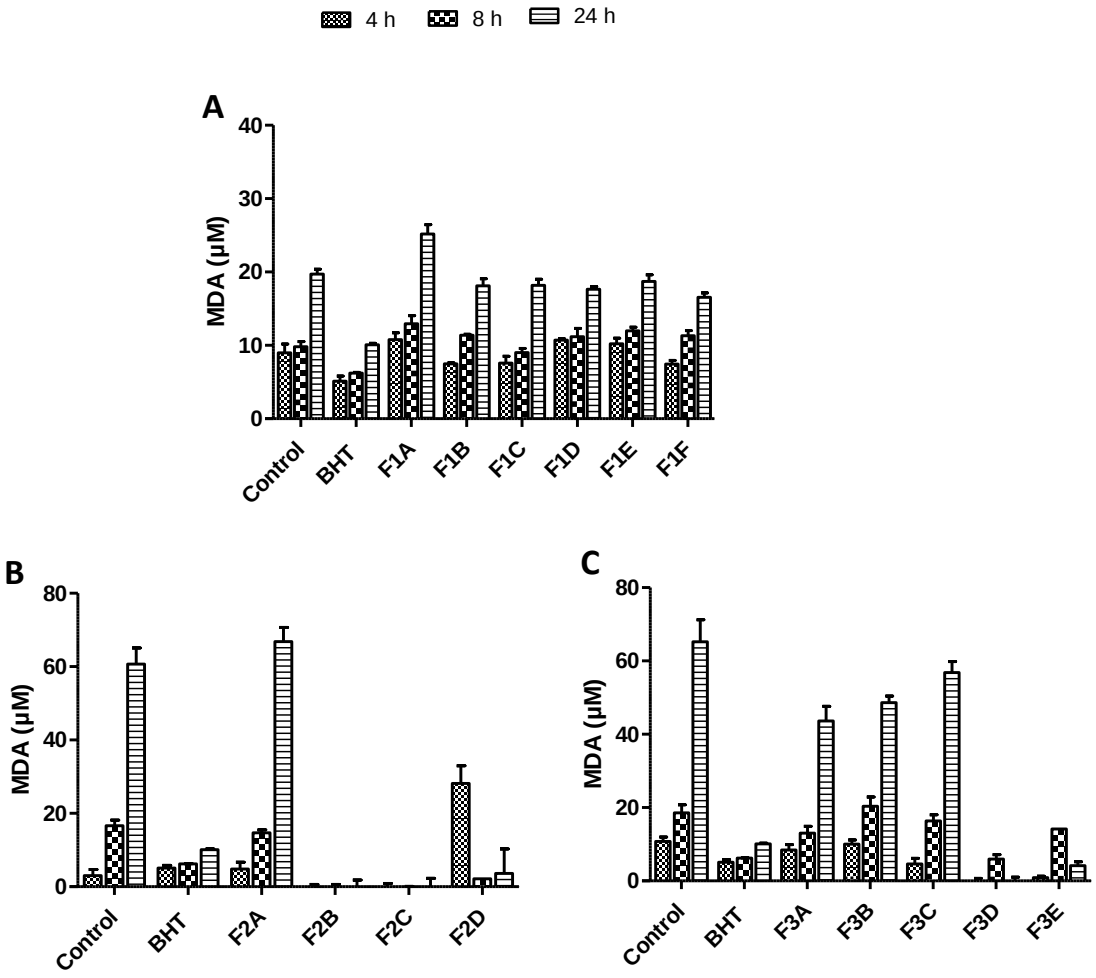
4 h
8 h
24 h
A
MDA (µM)
0
10
20
30
40
Control
BHT
F1A
F1B
F1C
F1D
F1E
F1F
B
MDA (µM)
0
20
40
60
80
Control
BHT
F2A
F2B
F2C
F2D
C
MDA (µM)
0
20
40
60
80
Control
BHT
F3A
F3B
F3C
F3D
F3E

FIGURE 5

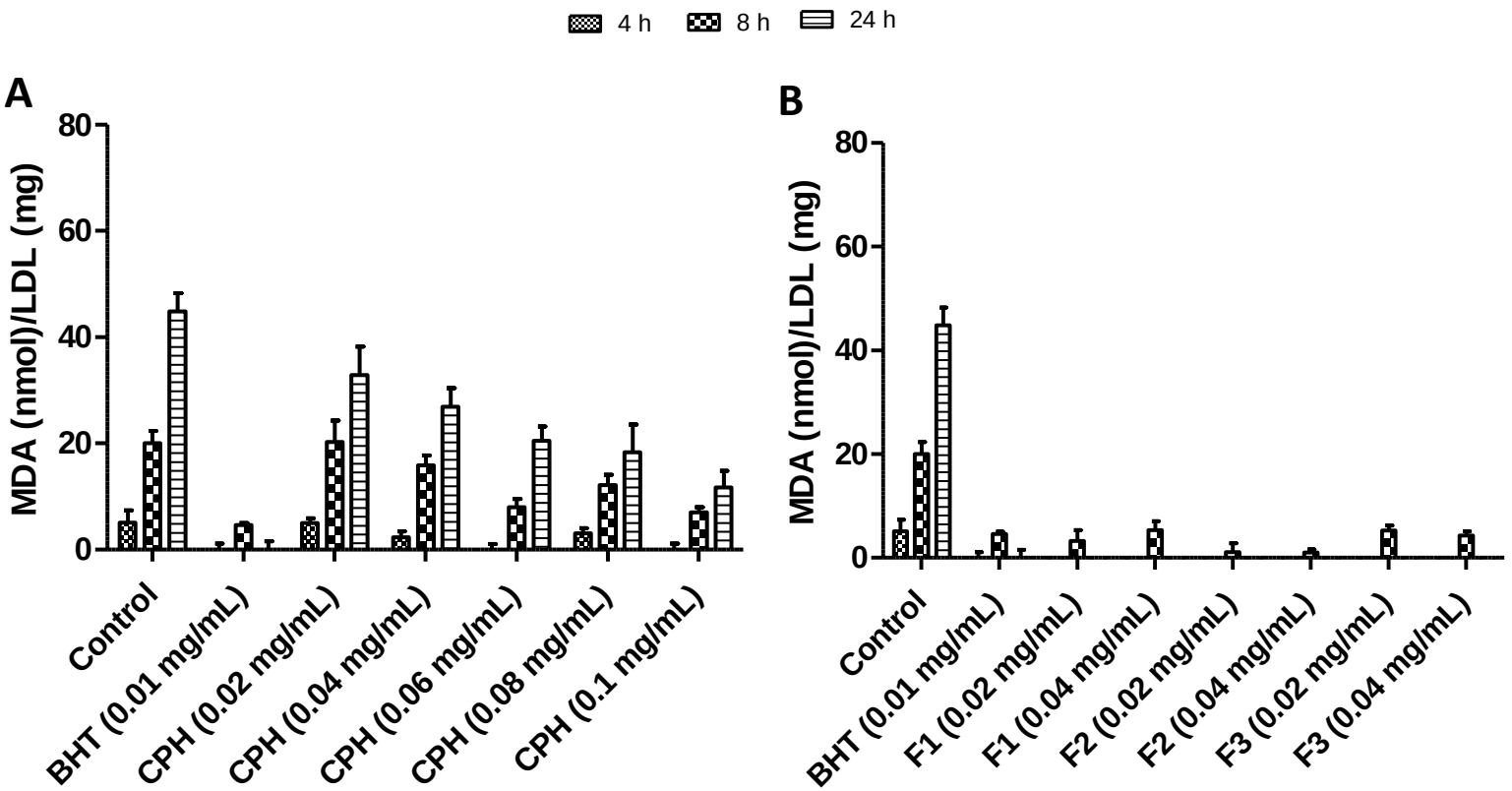
4 h
8 h
24 h
A
MDA (nmol)/LDL (mg)
80
60
40
20
0
Control
BHT (0.01 mg/mL)
CPH (0.02 mg/mL)
CPH (0.04 mg/mL)
CPH (0.06 mg/mL)
CPH (0.08 mg/mL)
CPH (0.1 mg/mL)
B
MDA (nmol)/LDL (mg)
80
60
40
20
0
Control
BHT (0.01 mg/mL)
F1 (0.02 mg/mL)
F1 (0.04 mg/mL)
F2 (0.02 mg/mL)
F2 (0.04 mg/mL)
F3 (0.02 mg/mL)
F3 (0.04 mg/mL)

FIGURE 6

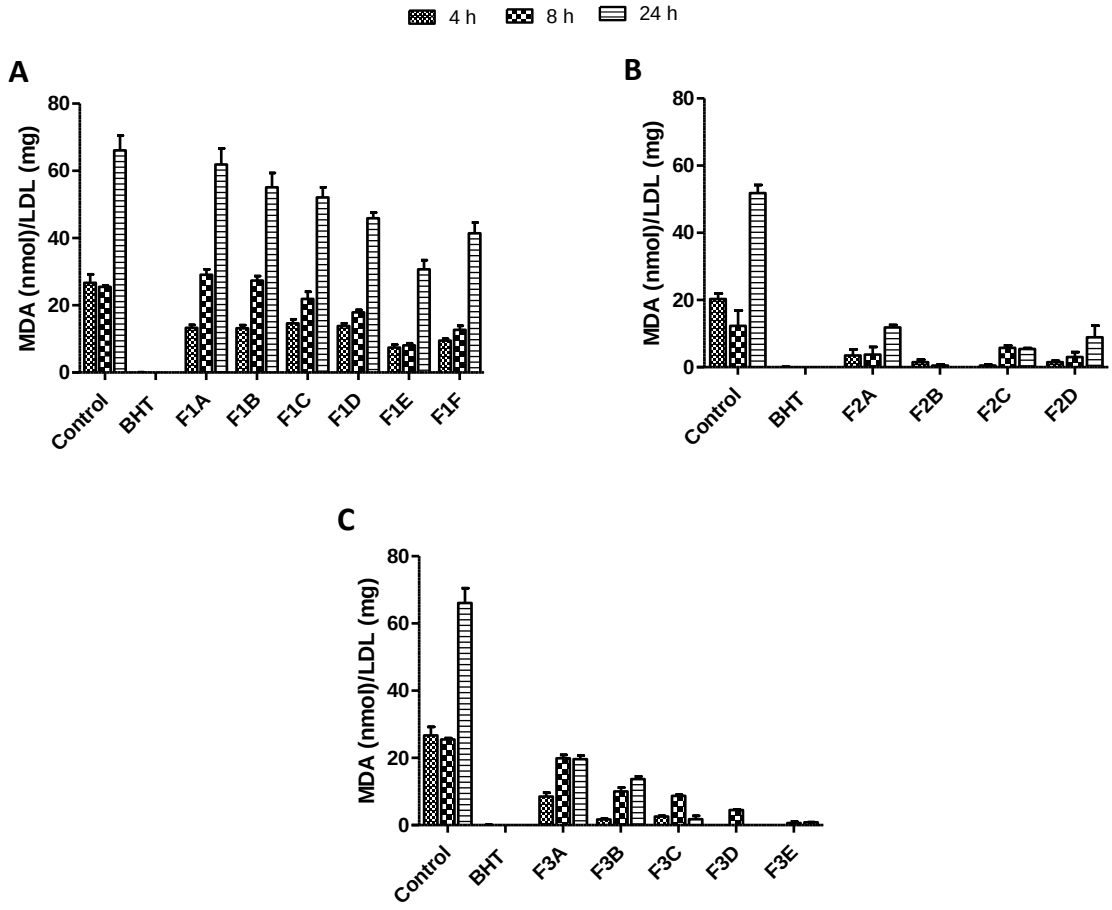
4 h
8 h
24 h
A
MDA (nmol)/LDL (mg)
80
60
40
20
0
Control
BHT
F1A
F1B
F1C
F1D
F1E
F1F
B
MDA (nmol)/LDL (mg)
80
60
40
20
0
Control
BHT
F2A
F2B
F2C
F2D
C
MDA (nmol)/LDL (mg)
80
60
40
20
0
Control
BHT
F3A
F3B
F3C
F3D
F3E

FIGURE 7