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Antioxidant Properties of High-Lutein Grain-Based Functional Foods in comparison with Ferulic Acid and Lutein

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Abstract

Dietary antioxidants could alleviate oxidative damage to cellular components via their ability to scavenge reactive oxygen species and other free radicals. In this regard wholegrain foods are recognized sources of dietary antioxidants and thus they are linked with health promotion and reduced risk of chronic diseases. In the current study high-lutein wholegrain bread, cookie and muffin products were assessed in terms of antioxidant properties based on their ability to scavenge peroxyl, ABTS and DPPH radicals and compared with ferulic acid and lutein, the primary antioxidants in the products. Aqueous methanol extract and bound diethyl ether/ethyl acetate extract followed alkaline hydrolysis were employed in the antioxidant measurement. Antioxidant capacity of the products was influenced by food product, type of extract (e.g. unbound versus bound) and antioxidant assay. In the ORAC test similar antioxidant capacities were obtained for unbound phenol extracts either from fortified or unfortified products, while significant differences were observed in bound phenol extracts. Significant differences were also found between unbound and bound phenol extracts in their ability to scavenge ABTS radical cation. In the DPPH assay lutein-fortified products had scavenging capacities significantly higher than that of the unfortified ones. In general, the bound phenolic extracts contribute significantly higher to the antioxidant capacity than the unbound phenol extracts. Only the DPPH test showed the contribution of lutein to the antioxidant capacity. At a lutein level of about 1.1-1.2 mg per serving (e.g. 30g) the products would boost the daily intake of lutein and antioxidants, as well as consumption of wholegrain foods.

Keywords: Antioxidant capacity, ORAC, TEAC and DPPH assays, Lutein, Ferulic acid, high-lutein wholegrain foods.

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1. Introduction

Dietary antioxidants could prevent or mitigate oxidative damage to cellular components via their ability to scavenge reactive oxygen species and other free radicals. Studies have shown effectiveness of dietary antioxidant components such as phenolic acids, tocols, carotenoids, and anthocyanins in improving antioxidant status and reducing the oxidation of vulnerable molecules such as LDL cholesterol *in vitro* and *in vivo* [1-4]. The measurement of antioxidant capacity of foods, dietary supplements or biological materials is currently done by using several methods. A review on the determination of antioxidant capacity in foods and dietary supplements suggested three assays for standardization namely oxygen radical absorbance capacity (ORAC), Folin-Ciocalteu (FC) or total phenols method and possibly Trolox equivalent antioxidant capacity (TEAC) [5]. In a biological or food system multiple free radicals and oxidants exist in addition to the presence of a diverse array of antioxidants such as enzymes, hormones, and large and small molecules. Thus the concept of using a single antioxidant assay to evaluate antioxidant properties of a food could be inadequate. In addition, the nature and extractability of antioxidants present in foods would be a big challenge due to their diverse structures and properties. Antioxidants in food matrixes are present in unbound and/or bund form such as unbound and bund phenolic compounds. Phenolics especially phenolic acids are the main antioxidants in cereal grains and their contribution to antioxidant properties is well evident [6-7]. In the present study, the antioxidant properties were measured based on three assays, ORAC, TEAC and DPPH using unbound and bound component extracts.

Wholegrain foods are recognized sources of dietary antioxidant and fiber components, and thus they are linked with health promotion and reduced risk of chronic diseases. Recently high-lutein wholegrain bakery products have been developed to boost the daily intake of lutein and to promote consumption of wholegrain foods as a source of antioxidants and dietary fiber [8]. Lutein is a member of the carotenoid family that is essential for human health and its abundance in human body is entirely dependent on dietary intake. It is a yellow pigment found in commonly consumed foods such as spinach, kale, eggs and pasta. Lutein plays significant roles in promoting the health of eyes and skin [9] and in reducing the risk of age-related macular degeneration (AMD) [10], cataracts [11], cancer [12] and cardiovascular disease (CVD) [13].

In previous studies stability and bioavailability of lutein in the developed high-lutein wholegrain bakery products were investigated. Stability of lutein in these products showed significant reduction in lutein (28-62% loss) during baking due to oxidation and isomerization [8]. Hidalgo and others also showed carotenoids losses of 21 and 47% for bread crumb and crust, respectively [14]. Bread leavening had almost negligible effects on carotenoids losses, while baking resulted in a marked decrease in carotenoids. In pasta, the longer kneading step had significant effects on carotenoids losses, while the drying step did not induce significant changes [14]. Despite the significant losses of lutein during processing the developed fortified baked products still contain reasonable concentrations of lutein (up to 1 mg/serving) and other phenolic compounds [8]. In a more recent study bioavailability of lutein in the wholegrain bread, cookie and muffin was investigated using fasted and fed digestion model in which food products were subjected to an *in vitro* simulation of human salivary, gastric and duodenal digestion, and then followed by Caco-2 monolayer absorption [15]. The fed model resulted in much higher estimates of bioavailability of lutein and the higher fat products (cookie and muffin) resulted in higher overall bioavailability. Other work has also shown the importance of fat on bioavailability of lutein supplements consumed in combination with fat [16].

This study is a continuation of our effort in the development of high-lutein wholegrain functional foods to boost lutein and antioxidant intake as well as to increase the consumption of wholegrain foods. In this study we evaluate antioxidant properties of the products using three assays namely, ORAC, TEAC and DPPH. Bound

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and unbound component extracts of phenolic acids, the main antioxidant components, were used in the evaluation of antioxidant properties. The quality of the high-lutein wholegrain bakery products was previously evaluated using objective and subjective measurements [8].

2. Materials and Methods

2.1 Chemicals and Materials

Fluorescein, 2,2'-Azobis (2-methylpropion-amidine) dihydrochloride (AAPH), 2, 2-azino-bis (3-ethylbenzthiazoline-6sulfonic acid) (ABTS), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Trolox and ferulic acid were purchased from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON). Four authentic carotenoids that are common in cereal grains, all-*trans*-lutein (90% purity), trans-beta-apo-8'-carotenal (96% purity) and all-*trans*-β-carotene (95% purity) were purchased from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON) and all-*trans*-zeaxanthin (95% purity) and all-*trans*-β-cryptoxanthin (95% purity) were from ChromaDex (ChromaDex Inc., Santa Ana, CA, USA). All the chemicals and reagents used in the study are at the highest commercially available purity.

Einkorn wheat (*Triticum monococcum* L.) cultivar AC Knowles known for its high content of lutein [17] was obtained from the Eastern Cereals and Oilseeds Research Centre, Ottawa, ON, Canada. Other baking ingredients including corn flour, Becel margarine, baking powder, sugar, whey protein isolate and salt were purchased from the retail market in Guelph, ON, Canada. Einkorn wholegrain flour was prepared as described in our earlier study [8]. The wholegrain flour was thoroughly mixed to ensure uniformity and kept at 4 °C until processing and analyses. The bakery products were fortified with free lutein (Lyc-o-20% oil suspension) kindly provided by LycoRed (Orange, NJ). The level of lutein in the supplement was quantified and confirmed by HPLC analysis [8].

2.2 Preparation of bakery products

Three bakery products including flat bread, cookie and muffin were prepared without and with lutein fortification. Lutein fortification was performed to achieve a level of about 1 mg of free lutein per serving (30 g) in baked products. Due to the substantial reduction of lutein on baking process [8], wholegrain flours were fortified with higher levels (40-60%) than the desired target to compensate for the anticipated lutein loss during thermal processing. The baking formulas for bread, cookie and muffin and processing conditions were based on AACC approved methods or in-house standard methods as described in a previous study [8]. All the baked freshly prepared products were cooled to room temperature, then placed in a freezer (-20 °C) and freeze dried. The dried samples were passed through a sieve with 355 μm opening and kept in a freezer at -20°C until extraction and analyses.

2.3 Analysis of carotenoids

Carotenoids in the developed bakery products were extracted with water saturated 1-butanol as previously described [18]. The carotenoid extracts were separated and quantified by high performance liquid chromatography (HPLC) using a 1100 Series chromatograph (Agilent, Mississauga, ON) as outlined in our previous study [18]. The separation was performed on a short C30 column YMC Carotenoid (10cm x 4.6 mm, packing 3μm) (Waters, Mississauga, ON). The column was operated at 35 °C and eluted with a gradient mobile system consisting of: (A) methanol/methyl *tert*-butyl ether/nano pure water (81:15:4, v/v/v) and (B) methyl *tert*-butyl ether/methanol (90:10, v/v) at 1 mL/min. The gradient was programmed as follows: 0-9 min, 100 to 75% A; 9-10 min, 75-0% A; 10-12 min, hold at 0% A; 12-13 min, 0 to 100% A; and 13-15 min, hold at 100% A. The separated carotenoids were detected and measured at 450 nm and the identity of carotenoids was based on the congruence of retention times and UV/Vis spectra with those of pure authentic standards. Four carotenoid standards (lutein, zeaxanthin, β-cryptoxanthin and β-carotene) were used for the identification and quantification. Standard solutions and regression equations used for quantifying lutein and other carotenoids are previously reported [18].

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2.4 Antioxidant assays

Extracts for antioxidant analyses were prepared from 0.5g sample in 80% methanol using IKA shaker VXB (IKA Works) for 30 min. The tube content was centrifuged at 10,000g for 10 min, and the extraction was repeated on residual pellet. Both extracts were pooled together, purged with nitrogen and kept in a refrigerator until further processing and analysis. The residual pellet obtained after removing unbound substances was processed immediately for the extraction of bound substances mainly phenolic acids. First the leftover pellet was washed with hexane, and then centrifuged at 10,000g for 15 min. The hexane supernatant was discarded. A 5 ml of 2M sodium hydroxide was added to the pellet and the content was purged with nitrogen and mixed on IKA shaker for 1hr at 70ºC. Then the mixture was cooled and acidified to pH 2 with 2M hydrochloric acid and centrifuged at 10,000g for 15 min. The acidic supernatant was transferred into a clean separatory funnel. The residual pellet was washed with 10 ml of nano pure water, then centrifuged at 10,000 g and the water supernatant was combined with acidic supernatant in the separatory funnel. The combined mixture was extracted three times 10 min each on IKA shaker with 10 ml of ethyl acetate and ethyl ether 1:1 ratio (v/v), then centrifuged and pooled together and eventually dried under nitrogen stream until dryness. The extracted residue was re-dissolved in 5 ml of nano pure water, filtered through 0.45µm Acrodisc syringe filter, stored in a freezer prior to antioxidant analyses.

Bound and unbound phenolic extracts (e.g. organic solvent and aqueous methanol extracts) obtained from bread, cookie and muffin were evaluated using three antioxidant assays including ORAC (oxygen radical absorbance capacity), TEAC (Trolox equivalent antioxidant capacity) using ABTS (2, 2azino-bis (3-ethylbenzthiazoline-6sulfonic acid) stable radical and DPPH (2, 2-diphenyl-1-picrylhydrazyl radical) assay. The TEAC and DPPH methods were previously described [19]. The ABTS scavenging capacity of food extracts was calculated as µmole Trolox equivalents/g sample, and in the DPPH test antioxidant capacity was calculated as percent of DPPH scavenging capacity. The ORAC method is based on the method of Ou and others [20]. The ORAC assay was also used to determine antioxidant capacity of pure feruic acid and lutein, the primary antioxidant components in the developed high lutein wholegrain bakery products. Twenty five µl sample extract, Trolox standard solution (0-140 µg/mL), ferulic solution (0-50 µg/mL), lutein solution (0-50 µg/mL) or nano pure water (blank) were mixed with 150 µl of fluorescein in each of the 96 micro-plate well. The mixture was conditioned at 37˚C for 15 min, then 25 µL of 2,2'–Azobis (2-methylpropion-amidine) dihydrochloride (AAPH) as a peroxyl radical generator was added to start the decaying of fluorescein. The degradation of fluorescein progressed for 60 min in the heated chamber of BioTech FLX800TBI with the following settings: the fluorescence excitation at 485 nm, the emission wavelength 528 nm, and reading was taken every min for 60 min. The micro-plate fluorescent reader was operated by Gen 5 software version 1.11.5 (BioTek). Data are presented as mean of relative ORAC values. Relative ORAC values were calculated as the ratio between the net areas (area under curve or AUC) between sample (s) and Trolox control (s) multiplied by molarity of Trolox over molarity of sample.

Relative ORAC value = $[(AUC_s-AUC_b)/(AUC_t-AUC_b)] \times molarity_t/molarity$

Where: AUC_s = area under curve for sample

AUC_b = area under curve for blank

AUC_t = area under curve for Trolox

2.5 Statistical analysis

All analyses were carried out in triplicate and the data are reported as means. Data were subjected to analysis of variance to determine significant differences between extracts and products using Minitab software (version 12, Minitab inc., State College, PA). Regression analysis was performed to measure kinetics of antioxidants. Differences were considered to be significant when $p < 0.05$.

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3. Results and Discussion

3.1 Content of lutein and other carotenoids

Lutein is the main carotenoid found in wheat and accounts for 77-83% of the total carotenoids in relatively high-lutein wheat species such as einkorn, durum, Kamut and Khorasan [18]. Einkorn is exceptionally high in lutein in comparison with the other wheat species, and thus in the current study it was used as the primary ingredient in the preparation of flat bread, cookie and muffin as staple foods. The products were fortified with lutein at level higher than the target dose to compensate for the reduction due to baking process [8]. Significant differences were observed in the content of lutein in fortified and unfortified bakery products (Table 1). The fortified products had lutein content ranging 38-42 µg/g or 1.1-1.3 mg/30g serving compared with 3-5 µg/g or 0.1-0.2 mg/30g serving in the unfortified bakery products. This shows the potential of the developed products to increase the daily intake of lutein and to boost the consumption of wholegrain foods since bread, cookie and muffin are staple foods. Lutein is linked with reduced risk of several diseases such as AMD [10], cataracts [11], cancer [12] and CVD [13]. Zeaxanthin is also common carotenoid in wheat and corn. No significant differences were observed between fortified and unfortified bakery products in terms of zeaxanthin content ranging from 0.8-0.9 µg/g (Table 1). β-Crytoxanthin was found in trace amounts in the bakery products. The bioavailability of lutein in cookie and muffin products was higher than that of the bread products due to their higher fat content [15]. The wholegrain bakery products are also good sources of ferulic acid at levels of 12.0-21.7 mg/kg in the unbound phenolic extracts to 261.1-394.9 mg/kg in the bound phenolic extracts.

Table 1. Average concentration of main all *trans* carotenoids in unfortified and lutein-fortified wholegrain bakery products (µg/g)

Unfortified or control	Lutein	Zeaxanthin	β-cryptoxanthin
Bread	5.4 ±0.26	0.87 ±0.03	tr
Cookie	3.6 ±0.17	0.81 ±0.02	nd
Muffin	3.1 ±0.13	0.77 ±0.02	nd
Lutein-fortified			
Bread	42.0 ±1.21	0.91 ±0.03	tr
Cookie	38.4 ±1.53	0.89 ±0.03	tr
Muffin	40.3 ±1.29	0.86 ±0.03	tr

3.2 ORAC assay

Wholegrain foods are believed to be healthier as compared with refined foods due to their higher content of dietary fiber, antioxidants and phytochemicals. The high-lutein wholegrain bakery products were evaluated in this study for their antioxidant capacity using three assays including ORAC, TEAC and DPPH. It would be preferable to use a single test to evaluate antioxidant or radical scavenging capacity, but currently there is no single test that could characterize antioxidant properties of foods due to the presence of multiple free radicals and oxidants in foods or biological systems. Such complex system would require more than one test for antioxidant evaluation and characterization. Another issue that is considered in the evaluation process is the extractability of antioxidant components from food products. Since the vast majority of antioxidant components present in grains in bound form such as bound phenolic acids [7], two sequential extraction procedures

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using aqueous methanol to extract unbound substances (unbound phenolic extract) and diethyl ether/ethyl acetate followed alkaline hydrolysis to extract bound phenolic compounds (bound phenolic extract) were used to measure antioxidant capacity.

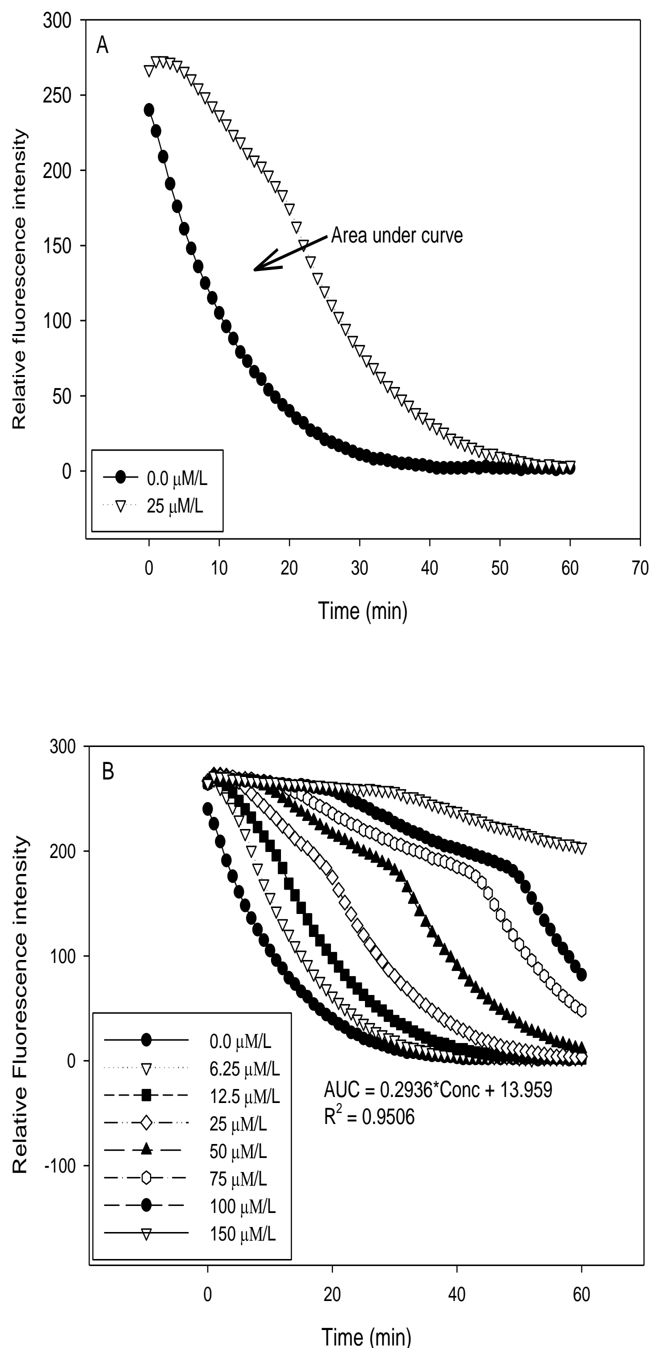

Figure 1. Trolox calibration curve (A) at the average concentration of 25 µM against blank showing the area under curve, AUC, and (B) at various concentrations (0-150 µM).

The ORAC assay measures scavenging capacity of peroxyl radical ($ROO^{\cdot}$) based on calculating the net protection area under the time recorded for the fluorescein decay curve in the presence of antioxidants, e.g. pure chemical or plant extract. The assay combines both inhibition time and inhibition percentage of free radical action by antioxidants using an area under curve (AUC) method for quantification (Figure 1A). In addition the assay uses Trolox as a control antioxidant for calibration and quantification. Figure 1B shows Trolox calibration curve with the determination coefficient (R^2=0.9506) and regression equation (AUC=0.2939*C + 13.959). ORAC method was suggested to quantify peroxyl radical scavenging capacity and the total phenols assay to quantify an antioxidant's reducing capacity [21]. In the current study antioxidant capacity of unbound and bound phenol extracts was measured with ORAC assay. The effects of different dilutions of unbound and bound component extracts on the protection of fluorescein decay are presented in Figure 2 for unbound phenol extracts of unfortified and fortified products and in Figure 3 for bound phenol extracts of unfortified and fortified products. The unbound components of unfortified (Figure 2A) and fortified (Figure 2B) bread, unfortified (Figure 2C) and fortified (Figure 2D) cookie and unfortified (Figure 2E) and fortified (Figure 2F) muffin showed that extracts diluted to 15X or 30X are appropriate for measuring ORAC value. They were found to yield similar decay curves to the control or Trolox at average concentration of 25 µM, and thus it was used to calculate relative ORAC value. It is necessary to obtain the right dilution or concentration for each extract that produces a decay curve close to the Trolox control curve in order to precisely measure the area under curve and produce accurate results. At high concentration of antioxidants such as undiluted unbound phenol extracts or those diluted to 2X, they totally inhibited the decay process (Figure 2A-F). Similarly bound phenol extracts of unfortified (Figure 3A) and fortified (Figure 3B) bread, unfortified (Figure 3C) and fortified (Figure 3D) cookie and unfortified (Figure 3E) and fortified (Figure 3F) muffin exhibited the same trend. The

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three products had similar behaviors where the crude extracts or extracts diluted 2 times showed total inhibition of fluorescein decay over the test period (60 min). This indicates the effectiveness of high-lutein functional food extracts in scavenging peroxyl radical. But at dilution of 15 or 30 times, gradual decay in fluorescein was observed. Figure 4 shows effects of ferulic acid and lutein concentrations on the fluorescein decay curve. As expected the higher concentration of ferulic acid the higher protection effect obtained (Figure 4A). For example at concentrations of 25 and 50 μg/mL ferulic acid there was total inhibition of fluorescein decay, while at concentrations of 1-5 μg/mL various levels of inhibition or protection of fluorescein against peroxyl radical were observed. A similar trend was also found with lutein, i.e. as the concentration increased the decay of fluorescein decreased (Figure 4B). Different degrees of decay inhibition were obtained at concentrations of 5.8-46.5 μg/mL of lutein. These results show that the scavenging capacity of product extracts, ferulic acid and lutein towards peroxyl radical is concentration dependent and their effectiveness as antioxidants would rely on their abundance in the diet at the physiological dose.

Relative ORAC values of unbound and bound phenolic extracts (Figure 5A, B) were significantly higher than that of pure ferulic acid (0.63) and lutein (0.65). In addition the relative ORAC values of bound extracts were significantly higher than that of unbound extracts. Similar results were previously found for barley insoluble phenolics compared with soluble phenolics indicating that the contribution of bound fraction to antioxidant capacity is much higher than that of unbound phenolic compounds [7]. The relative ORAC values of vitamin C (0.95) and glutathione (0.62) are comparable to that of lutein and ferulic acid, while caffeic acid (4.37), catechin (6.76) and rutin (6.01) are close to that of the free product extracts [20]. Bound extracts of cookie had similar relative ORAC values to that of free extracts of the three bakery products, while muffin and bread products showed higher relative ORAC values than pure compounds and cookie products. Once again this indicates the strong contribution of bound antioxidant fraction and perhaps synergistic effects among antioxidant components. Other phytochemicals such as anthocyanidins exhibited relatively lower relative ORAC value (1.54-1.81) compared with flavones (2.67-4.32) [22]. Still bound extracts of the bakery products had higher relative ORAC values than the pure compounds. The relative ORAC values of the unbound phenol extracts were not significantly different between lutein-fortified and control bread, cookie or muffin products (Fig. 5A). In other words, only slight differences were observed among the three bakery products despite their distinct baking ingredients. The insignificant differences between lutein-fortified and unfortified products could be due to the low extractability of lutein in aqueous methanol and the low solubility of lutein in the buffer solution used in the ORAC assay. On the other hand, significant differences were found among the three bakery products in their relative ORAC values for bound extracts. This indicates that bound phenolics were influenced by the type of baking recipe and conditions. Bound and unbound antioxidants may behave and absorb in a different way in humans, and thus their antioxidant activity will be affected. It is believed that unbound antioxidants may become bioavailable in the GI tract, while bound phenolics or antioxidants escape the stomach or intestinal digestion/absorption to reach the colon. Ferulic acid was found to act as a potent antioxidant in isolated membranes or intact cells via the inhibition of lipid peroxidation and reactive oxygen species production [23]. In membranes its antioxidant efficiency was more pronounced than that of α-tocopherol, vitamin C and β-carotene.

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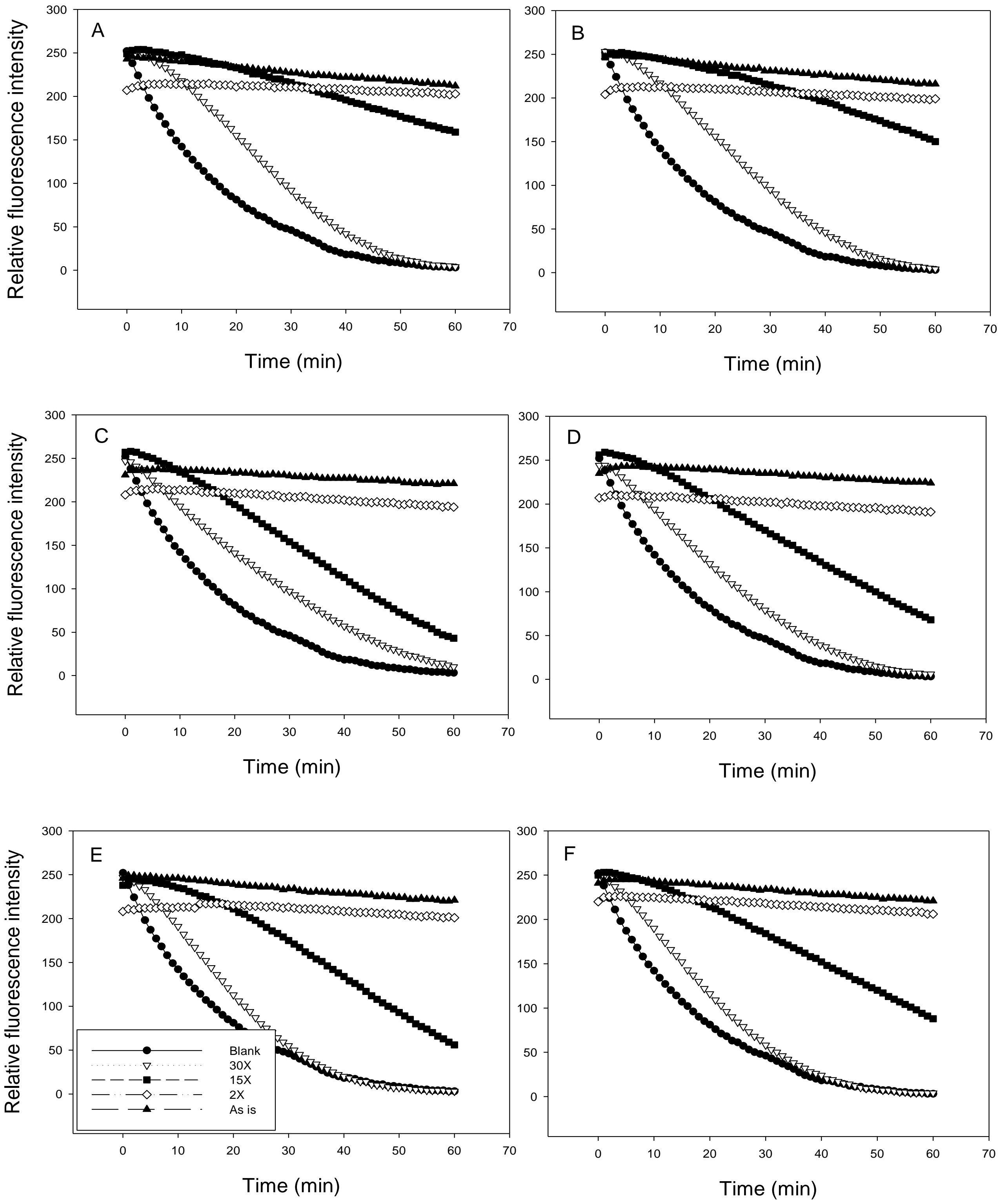

Figure 2. Fluorescein decay curve in the presence of free extracts at various dilutions (A) control bread, (B) fortified bread, (C) control cookie, (D) fortified cookie, (E) control muffin and (F) fortified muffin.

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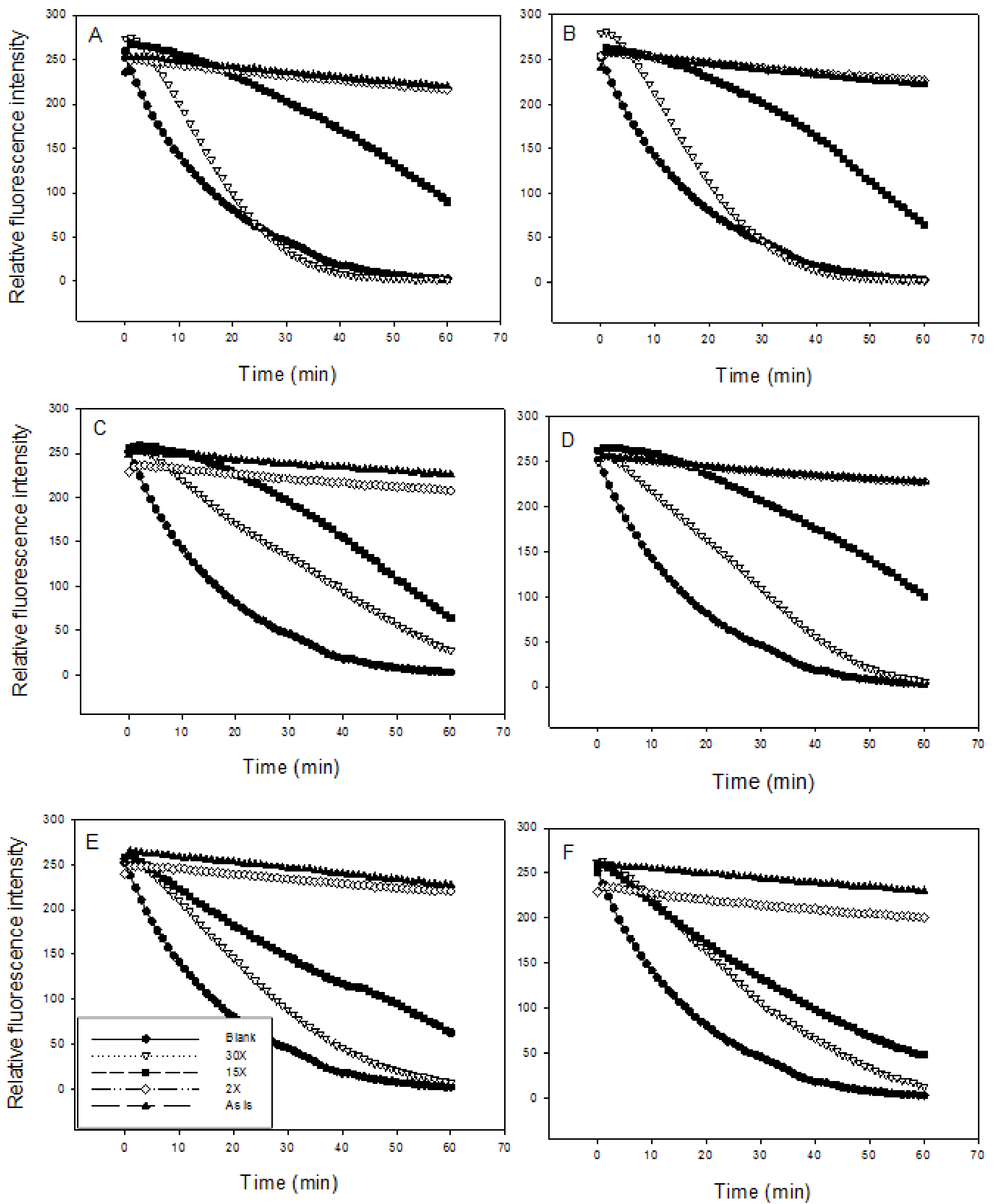

Figure 3. Fluorescein decay curve in the presence of bound extracts at various dilutions (A) control bread, (B) fortified bread, (C) control cookie, (D) fortified cookie, (E) control muffin and (F) fortified muffin.

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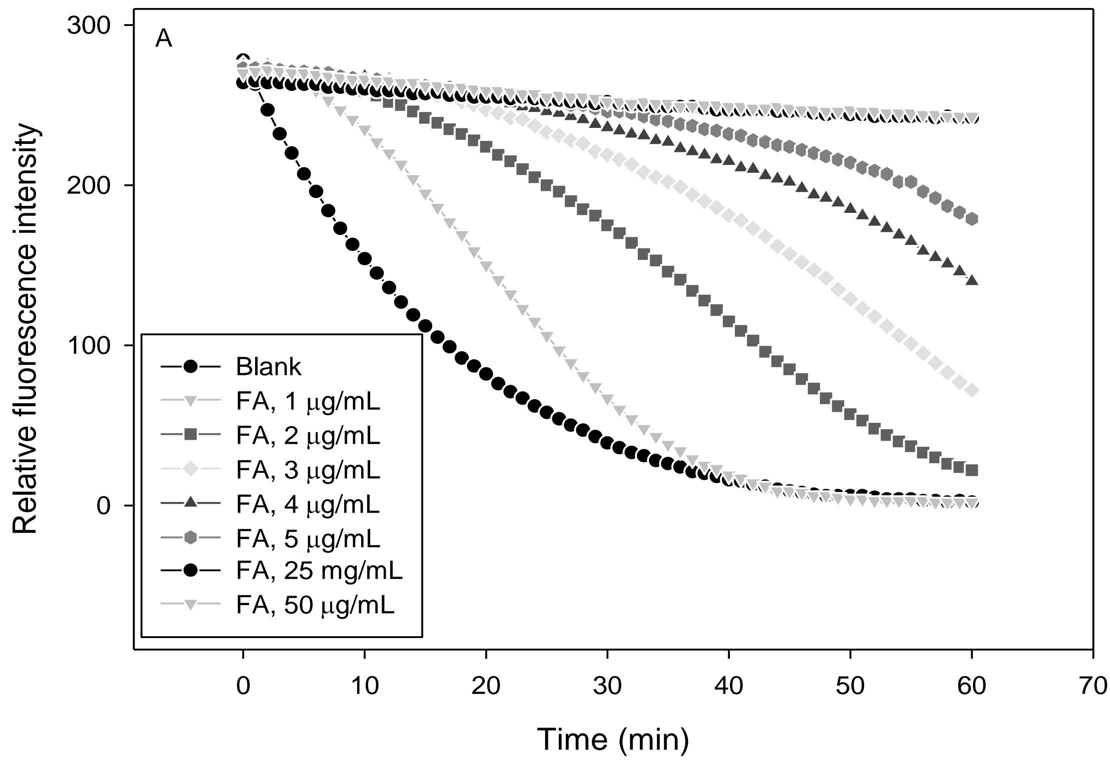

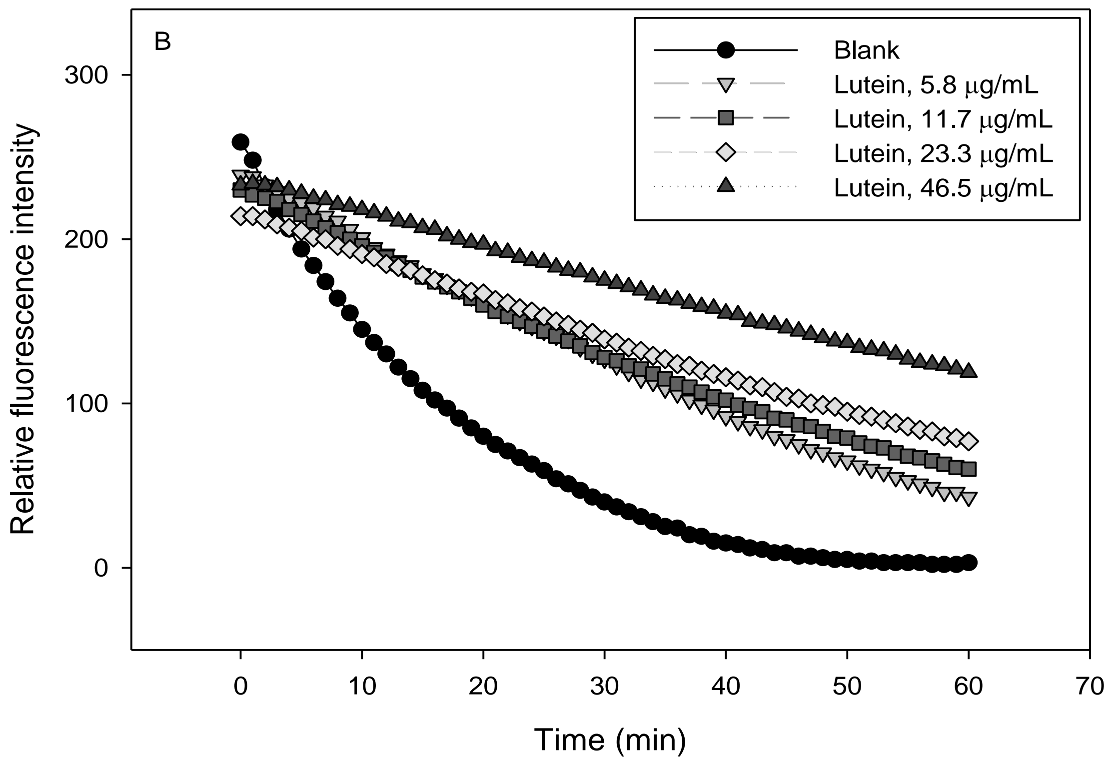

Figure 4. Fluorescein decay curve in the presence of pure ferulic acid (A) and lutein (B) at various concentrations.

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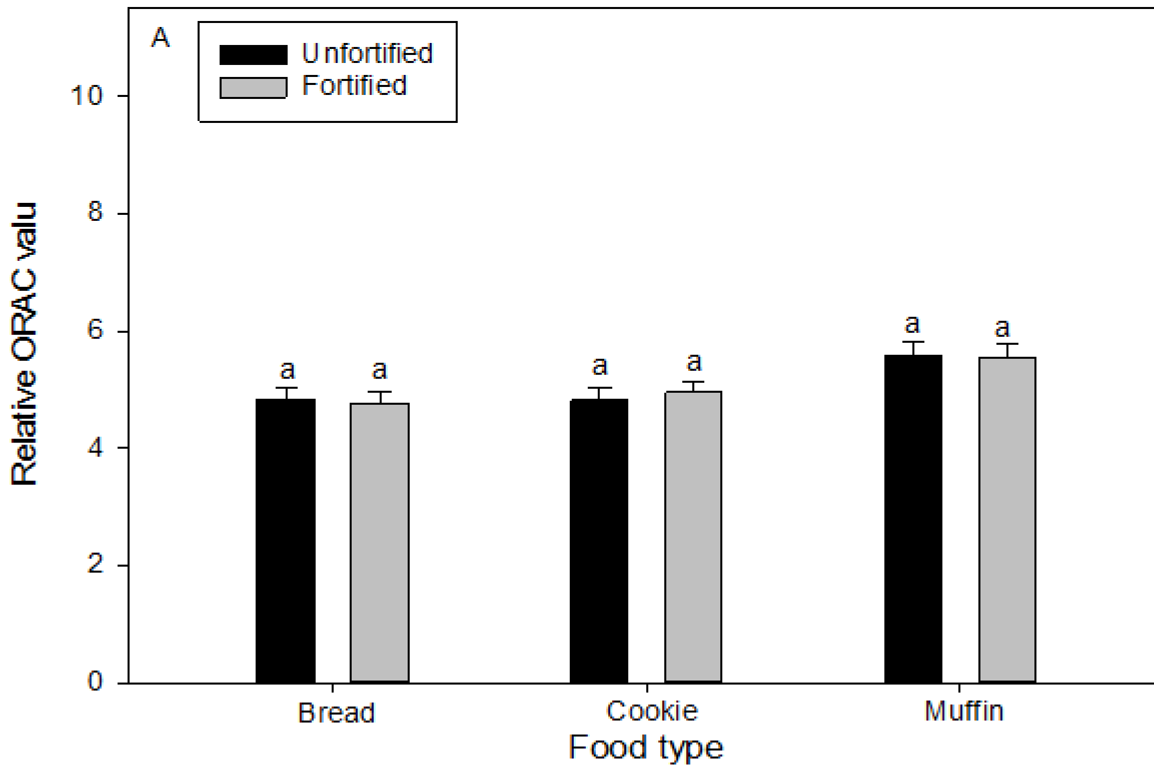

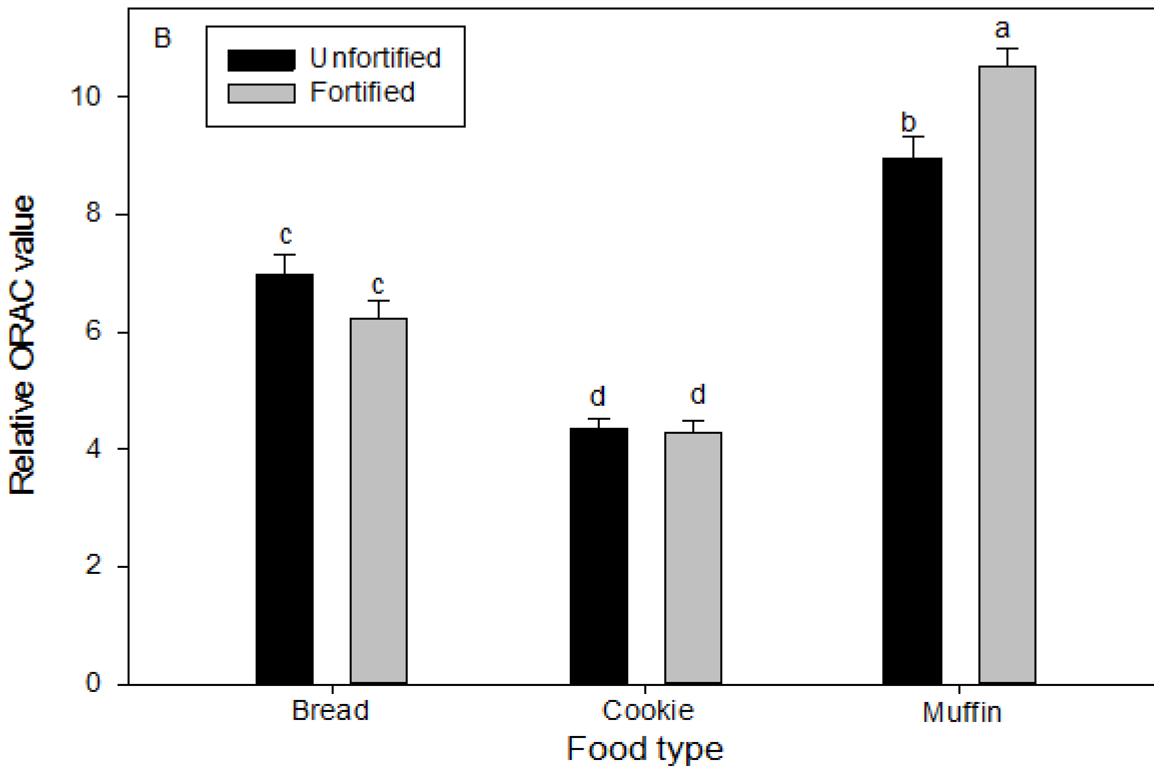

Figure 5. Relative ORAC value of control and high lutein bakery products: (A) free extracts and (B) bound extracts. Error bars represent standard deviation values. Different letters indicate significant differences between products at $p < 0.05$.

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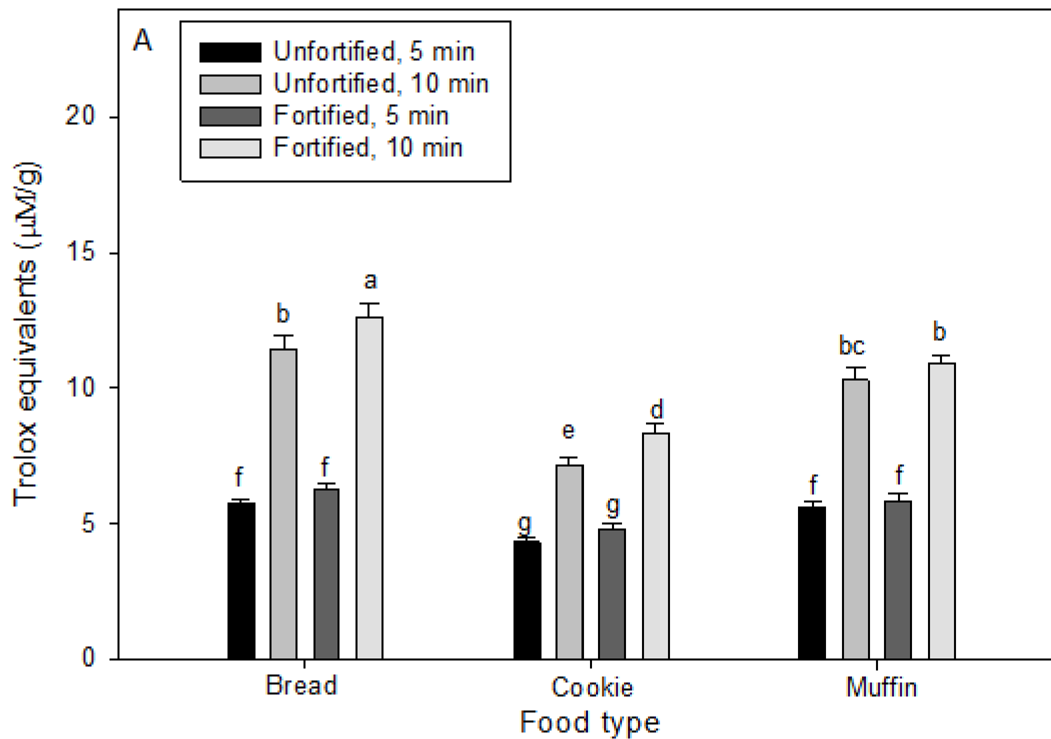

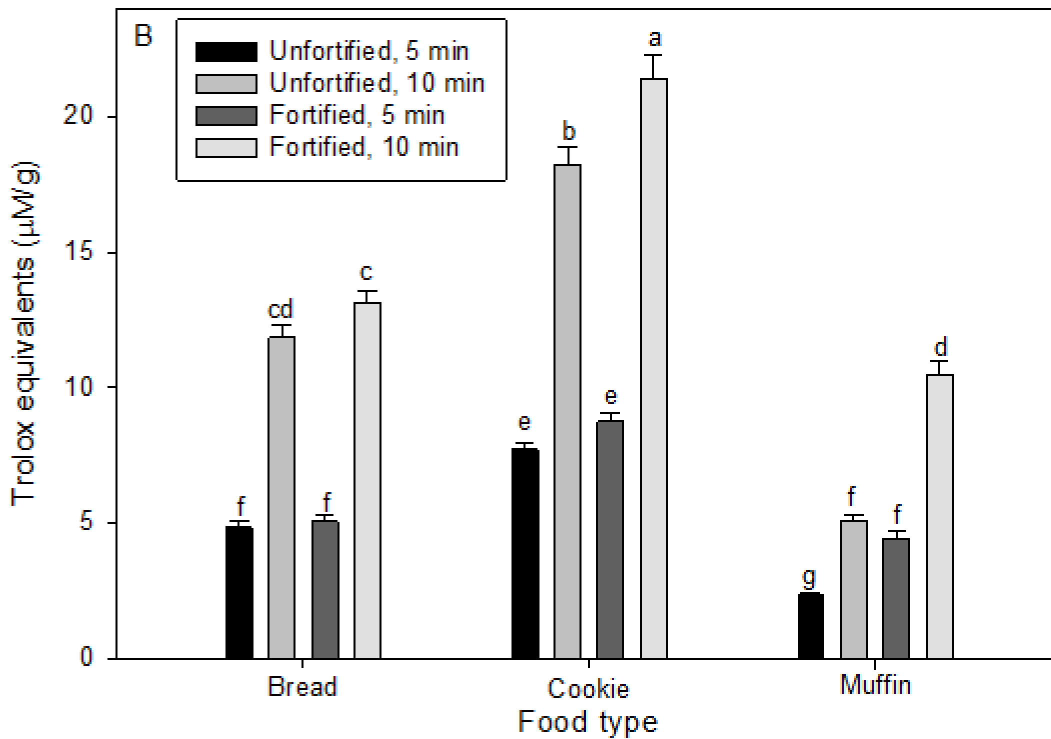

Figure 6. Trolox equivalent antioxidant capacity (TEAC) of control and high lutein bakery products: (A) free extracts and (B) bound extracts. Error bars represent standard deviation values. Different letters indicate significant differences between products at $p < 0.05$.

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3.3 TEAC assay

The TEAC assay has been used quite often in determining antioxidant capacity of foods, biological samples and pure compounds due to its simplicity and the stable nature of the ABTS radical. The TEAC values of many pure compounds have been reported, e.g. ferulic acid (1.90), *p*-coumaric acid (2.00), caffeic acid (1.00), vitamin C (1.05), α-tocopherol (0.97) and glutathione (1.28) [21]. In the current study the TEAC values for unbound phenol extracts ranged from 4.4-6.3 and 7.2-12.6 for 5 and 10 min, respectively (Figure 6A). This indicates the synergistic effects between the extractable antioxidants compared with the pure compounds. Significant differences were observed between products and the reaction time indicating that the ability of the products to scavenge ABTS radical is time dependent and subject to composition of food matrix and other ingredient in the diet. Bread unfound antioxidants had higher TEAC values as compared with cookie and muffin products. Aqueous methanol extracts of rye bread made from flours with extraction rate from 100% to 90% had TEAC values 0.51-0.88 mM/100g compared with 0.23 mM/100g for wheat rolls [24]. The study recommended the traditional rye bread for human nutrition.

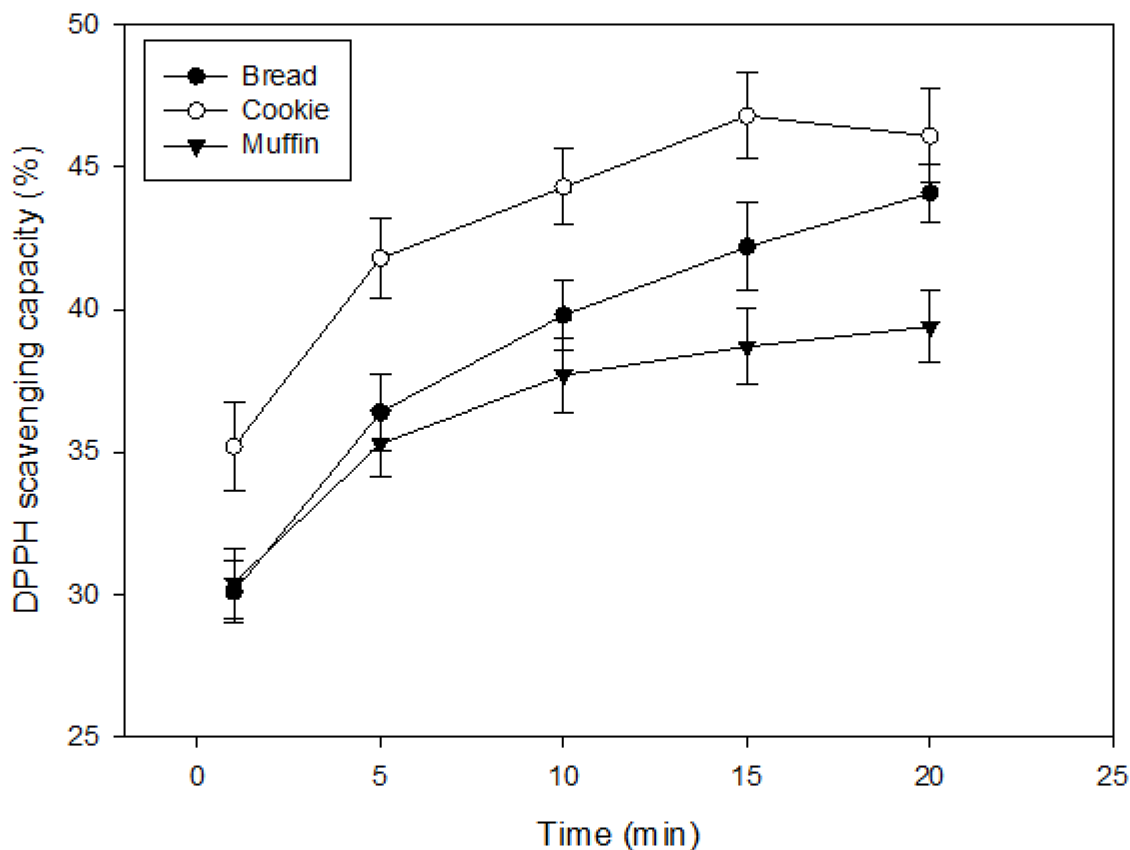

Figure 7. Kinetics of DPPH radical in the presence of free extracts of high lutein bakery products.

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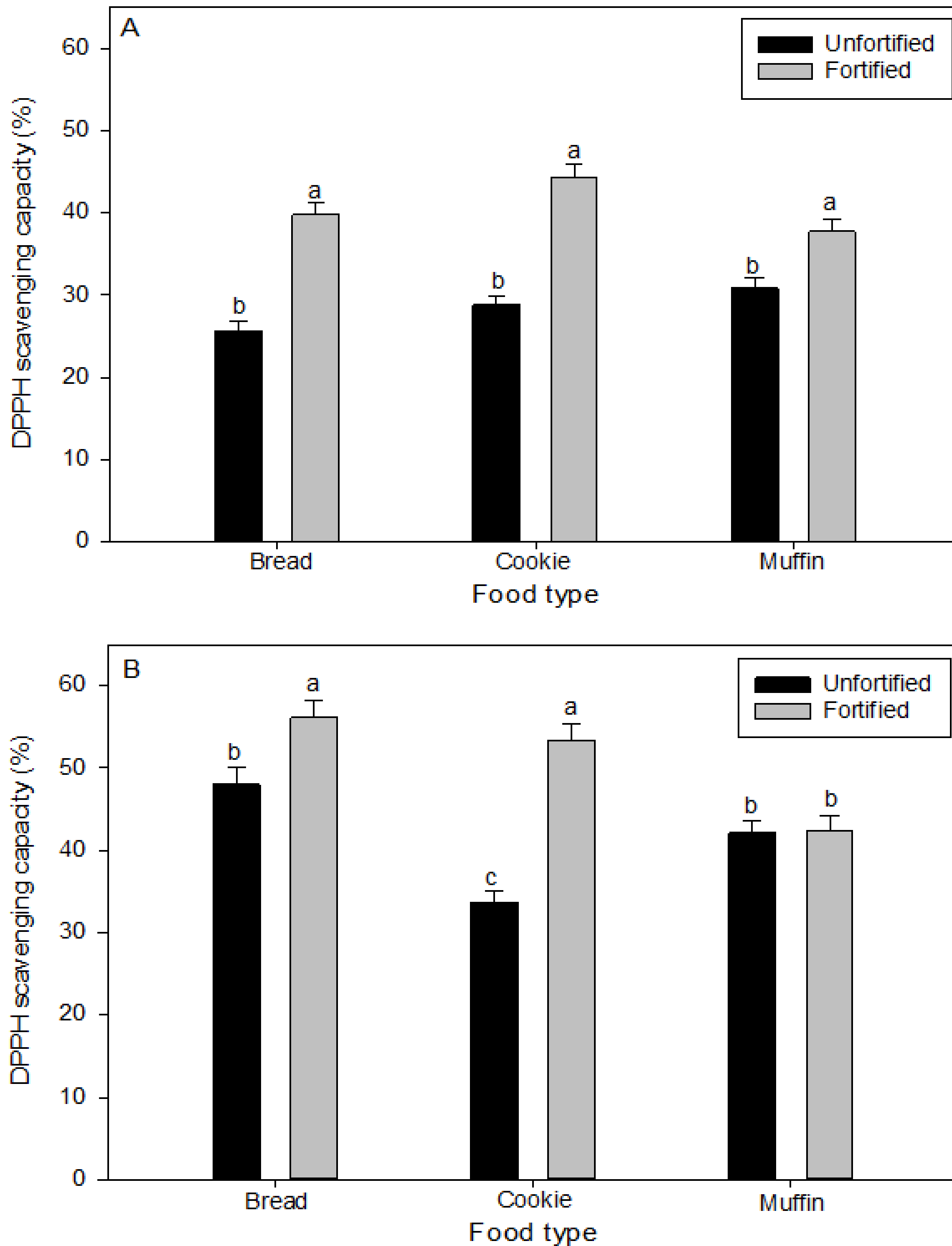

Figure 8. DPPH scavenging capacity of control and high lutein bakery products: (A) free extracts and (B) bound extracts. Error bars represent standard deviation values. Different letters indicate significant differences between products at $p < 0.05$.

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Bound phenolic extracts from high-lutein bread, cookie and muffin products also exhibited significant differences to quench ABTS radical having TEAC values ranging 2.4-8.7 and 5.1-21.4 for 5 and 10 min, respectively (Figure 6B). Once again this indicates a stronger higher contribution to antioxidant properties by bound phenolics in comparison with the unbound phenolics. Among the products high-lutein cookie had the highest TEAC values compared with bread and muffin products. Phenolic compounds were found to be active toward scavenging ABTS radical to a different extent depending upon structure of phenolic compound and reaction environment [25]. For example the TEAC values of ferulic acid were 1.32 and 1.97 in ethanol and buffer solution (pH 7.4), respectively, compared with 0.82 and 1.18 for isoferulic acid. Modern and ancient wheat products varied in their capacity to scavenge ABTS radical subject to species and cultivar [26] and type of wheat flour [19].

3.4 DPPH assay

In the DPPH assay the ability of extracts or antioxidants to scavenge the DPPH free radical is measured via the reduction in the color intensity of the radical. The color intensity of DPPH radical with no antioxidants or food extracts was stable over the test time with a constant absorbance reading (1.739) [19]. The kinetics of DPPH reaction in the presence of unbound phenol extracts is shown in Figure 7. The reaction is not linear and rapidly increased during the first 5 min, after that the reaction rate steadily increased up to 20 min. The DPPH test showed significant differences between the fortified and control bread cookie and muffin products using unbound or bound phenol extract except for muffin bound phenolic extracts (Figure 8A, B). This indicates that lutein may contribute to the antioxidant capacity in the DPPH test and the potential of antioxidant components in the extracts to contribute to the mitigation of antioxidant damage could be subject to the type of free radical species. The scavenging ability of carotenoids towards DPPH radical was previously reported [27]. The effectiveness of carotenoids as DPPH radical scavengers was increased by the length of the effective conjugated double-bond system and by the addition of hydroxyl groups on the terminal rings. For example the amount of carotenoids able to reduce the initial DPPH concentration to 50% (EC_{50}) was 3.29 moles carotenoid antioxidant per moles DPPH radical for lutein (dihydroxy carotenoid) to 0.16 for lycopene (non-oxygenated carotenoid). No significant differences were observed between the products based on the ability of unbound phenol extract to scavenge DPPH radical (Figure 8A), while significant variations were found among the bound antioxidants with fortified bread and cookie products having the highest DPPH scavenging capacity (Figure 8B). A study on antioxidant capacity of polyphenols showed that ferulic, caffeic and sinapic acids had moderate DPPH scavenging capacity compared with ellagic and quercetin being the highest and *p*-hydroxy benzoic and coumaric acids having the lowest DPPH scavenging ability [28].

In summary, the developed high-lutein staple foods would increase the daily intake of lutein and consumption of wholegrain foods as a source of antioxidants. Dietary antioxidants and lutein are essential components that play significant roles in promoting human health and reducing the risk of age-related chronic disease. The current study has demonstrated the ability of high-lutein wholegrain bread, cookie and muffin to scavenge peroxyl, ABTS and DPPH radicals. Both unbound and bound phenolic extracts were found to contribute to the antioxidant properties of the products with a higher share given by the bound phenolic compounds. In this respect, the bioavailability of lutein and antioxidant components would determine their effectiveness in the mitigation of oxidation damage.

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