Synthesis and Antioxidant Activity Evaluation of Novel Broom and Cotton Fibers Derivatives

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Received 17 April 2009; accepted 10 June 2009
DOI 10.1002/app.30928
Published online 28 July 2009 in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Novel antioxidant biopolymers composed by broom or cotton fibers, and *trans*-ferulic acid, α-lipoic acid, and α-tocopherol, were prepared. The acids were directly linked to fibers microfibrils hydroxylic groups using *N*,*N*-dimethylacetamide/lithium chloride (DMAc/LiCl) as solvent with dicyclohexylcarbodiimide (DCC) and *N*,*N*-dimethylaminopyridine (DMAP). Broom or cotton tocopherulate were prepared in the same conditions by using carboxylated fibers. Ester linkages were confirmed by using Fourier transformed infrared spectroscopy; biopolymers substitution degrees (DS) were determined by volumetric analysis. Antioxidant activity in inhibiting lipid peroxidation, in rat-liver microsomal membranes, induced *in vitro* by two different sources of free radicals, 2,2′-azobis (2-amidinopropane) (AAPH) and *tert*-butyl hydroperoxide (*tert*-BOOH), was evaluated. Finally, biocompatibility of these biomaterials was also tested by controlling glutathione-S-transferase (GST) basal concentrations. Our results strongly suggested that the better derivatives were the ferulate ones. However, all obtained biomaterials, showing excellent antioxidant properties and biocompatibility, could be successfully applied in biomedical and in packaging areas. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 3177–3183, 2009

Key words: fibers; biomaterials; antioxidants; infrared spectroscopy; biomedical and packaging areas

INTRODUCTION

During storage all foods, beverages and pharmaceutical preparations inexorably undergo gradual changes. In fact auto-oxidation process, which involves a free radical chain mechanism, is responsible for the formation of off-flavors, rancidity, color changes, and undesirable chemical compounds detrimental to health. In addition, mono or polyunsaturated fatty acids or both, which are very sensitive to oxidation, fastly deteriorate especially at high temperatures. Susceptibility to oxidation depends upon the degree of unsaturation.[1] Since every product including foodstuffs, pharmaceuticals, photochemicals, adhesives, and polymer precursors undergo oxygen degradation, there is a well-recognized need for methods and compositions that can counteract oxygen damaging effects.[2] Antioxidants are often used by industries to delay the oxidation process. Particularly, preservatives with antioxidant activity are commonly added to packaged foods to scavenge oxygen radicals. However, many preservatives used for food, textile, medicine, and other personal care products have been associated with adverse side effects. The options to solve these problems are as follows: (1) reduction of oxygen concentration,[3] (2) lowering storage temperature, (3) scavenging of metal ions (mainly Fe, Cu) with sequestrants, (4) coating antioxidants with substances that allow for sustained release,[4] (5) mixing of antioxidants with carriers such as synthetic polymers, (6) antioxidants immobilization on macromolecules of various nature.[5] The last alternative is one of the most important and rapidly developing trends in modern polymer chemistry and could furnish systems in which the antioxidant is yet fully functional and then able to interrupt chain reactions.[6] In fact, antioxidant activity of some common synthetic antioxidant preservatives, including butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), can depend on their quantity that is limited by the U.S. Food and Drug Administration because these compounds are suspected to be carcinogenic.[7] Consequently, there is a need for a new class of antioxidant preservatives that are less toxic to humans and animals. From this point of view, natural fibers seems available for this purpose. Such properties of fibers as nontoxicity facilitate their wide application for creation of antioxidant materials.[8] In this work, the synthesis of novel antioxidant broom and cotton fibers (Fig. 1) was described. The ability of these biopolymers to act as antioxidant agents were

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Contract grant sponsor: University Funds.

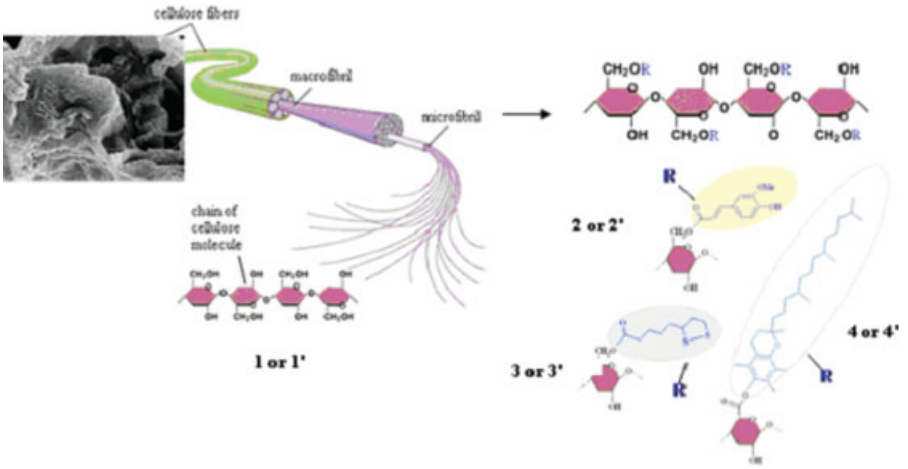

Figure 1 Schematic representation of the synthesis and chemical structures of the antioxidant fibers. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

considered investigating, in rat-liver microsomal membranes, their capacity in inhibiting the lipid peroxidation[9] induced by two different sources of free radicals, 2,2′-azobis (2-amidinopropane) (AAPH) which exogenously produces peroxyl radicals by thermal decomposition, and *tert*-butyl hydroperoxide (*tert*-BOOH), which endogenously produces alkoxyl radicals by Fenton reactions. Moreover, their biocompatibility was also studied by monitoring glutathione-S-transferase (GST) levels, a detoxifying enzyme responsible of xenobiotics conjugation with reduced glutathione to enhance their elimination.[10] Functionalized broom fibers have been assigned to the food packaging area and so, to estimate their biocompatibility, basal levels of a GST epatic isoform extracted from mitochondria of rat liver were observed.[11] On the other hand, functionalized cotton fibers have been assigned to the biomedical field and so an epithelial-like GST isoform extracted from mitochondria of rat fibroblasts has been used to confirm their biocompatibility.[12]

EXPERIMENTAL

Materials

Natural cellulose fibers from broom and cotton were supplied by Department of Chemistry-University of Calabria, Italy. *Trans*-ferulic acid (FA), α-Lipoic acid (αLA), α-Tocopherol (αT), *N*,*N*-dimethylacetamide (DMAc), lithium chloride (LiCl), dicyclohexylcarbodiimide (DCC), *N*,*N*-dimethylaminopyridine (DMAP), thionyl chloride ($SOCl_2$), potassium chloride (KCl), ethylenediaminetetraacetic acid (EDTA), sucrose, 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES), trichloroacetic acid (TCA), hydrochloric acid (HCl), nitric acid (HNO_3), butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA), *tert*-butylhydroperoxide (TBOOH), and 2,2′-Azo-bis(2-amidopropane) dihydrochloride (AAPH) were supplied by Sigma (Sigma Chemical Co, St. Louis, MO). Methanol, ethanol, diethyl ether, and chloroform were obtained from Fluka Chemika-Biochemika (Buchs, Swsitzerland) and Carlo Erba Reagents (Milan, Italy).

Modified Eagle's Medium nutrient mixture, fetal bovine serum, glutamine, trypsin, digitonin, sodium dodecyl sulfate (SDS), albumin by bovine serum, protease inhibitors (aprotinine, sodium-ortovanadate, leupeptine, phenilmetil-sulfonic fluoride) were purchased from Sigma-Aldrich (Milan-Italy).

Measurements (characterizations)

FTIR spectra were measured on a Jasco 4200 using KBr disks. Absorbances were measured in 1 cm cuvettes using a UV-Vis spectrophotometer (V-530 JASCO).

Fiber chemical modifications

Broom and cotton fibers were pretreated to obtain delignificated materials. Pretreated broom and cotton fibers were subjected to following heterogeneous reactions after drying overnight at 80°C under reduced pressure until a constant weight.[13]

Synthesis of ferulate broom (2) and cotton fibers (2′)

Lithium chloride (1.5 g) was dissolved in 25 mL of DMAc for 30 min at 80°C under N_2.[14] After cooling to 40°C, the LiCl/DMAc binary solvent mixture was added to fibers (0.155 g) [broom (**1**) or cotton fibers (**1′**)]. The heterogeneous reaction mixture so obtained was further cooled to 25°C and DCC (1.77 g, 86 mmol), DMAP (0.06 g, 5 mmol), and finally, FA (1.67 g, 86 mmol) were added. After heating to 40°C

with stirring for 72 h, the fibers were collected by filtration and finally washed initially with several aliquots of hot methanol, to remove dicyclohexylurea (DCU), and then with chloroform and diethyl ether.[15] The biopolymers were dried under vacuum to furnish **2** and **2′** that was characterized by FTIR spectroscopy (Fig. 1). Yield **2** (ferulate broom): 0.341 g; yield **2′** (ferulate cotton): 0.347 g.

Synthesis of lipoate broom (3) and cotton fibers (3′)

This derivative was prepared by procedure as described earlier for **2** and **2′** since αLA is inclined toward homopolymerization in high-temperature conditions. The same procedure was described in the previous section and same amounts of reagents and solvents were employed (1.77 g, 86 mmol of αLA).[15] Yield **3** (lipoate broom): 0.347 g; yield **3′** (lipoate cotton): 0.345 g.

Synthesis of tocoferulate broom (4) and cotton fibers (4′)

Lithium choride (0.185 g) was dissolved in 12 mL DMAc for 30 min at 80°C under N_2. After cooling down to 40°C, 0.5 g of 6-carboxycellulose, (corresponding to 0.47 mmol/g of COOH), respectively, from broom and cotton, were added. The obtained heterogeneous mixture was further cooled to 25°C and added of DCC (0.05 g, 26.4 mmol), DMAP (0.03 g, 23 mmol), and finally α-T (0.11 g, 26.4 mmol). The heterogeneous reaction mixture was stirred at room temperature for 72 h, washed with hot methanol, with several aliquots of acetone and the polymers were collected by filtration.[15] The polymers were dried under vacuum to furnish **4** and **4′** (Scheme in Fig. 1) and were characterized by FTIR. Yield **4** (tocoferulate broom): 0.598 g; yield **4′** (tocoferulate cotton): 0.725 g.

Quantitative analysis of ester groups

A sample of 50 mg ester derivative was dispersed in 5 mL of 0.25*M* ethanolic sodium hydroxide solution under reflux for 17 h. The dosing in return of the excess of soda was realized by titration with 0.1*N* HCl (first equivalent point).[15] The moles of hydrochloride acid used between the first and second equivalence points correspond to the moles of free esters. The substitution degree (DS) was determined by the following eq. (1):

$$\mathrm{DS} = \frac{\mathrm{MM}_{\text{glucose unit}}}{\left(g_{\text{sample}}/n_{\text{free ester}}\right) - \left(\mathrm{MM}_{\text{free ester}} - \mathrm{MM}_{\mathrm{H_2O}}\right)} \quad (1)$$

$n_{\text{free ester}} = (V_{2^\circ\text{ e.p.}} - V_{1^\circ\text{ e.p.}})$ * [HCl]; $\mathrm{MM}_{\text{glucose unit}}$: molecular mass of glucose unit; g_{sample} : weight of sample; $n_{\text{free ester}}$: mol of free ester; $\mathrm{MM}_{\text{free ester}}$: molecular mass of free ester; $\mathrm{MM}_{\mathrm{H2O}}$: molecular mass of water.

Synthesis of carboxylic derivatives of broom and cotton fibers

1 g of fibers [broom (**1**) or cotton fibers (**1′**)] was suspended in 28.6 mL of H_3PO_4 85% under magnetic stirring; after 2 h, 0.71 g of sodium nitrite ($NaNO_2$) were added maintaining a vigorous magnetic stirring for 15 min. After 5 h, a stable foam was formed, and it was destroyed by stirring and by adding another aliquot of $NaNO_2$. This addition was repeated after 3 h. After a total reaction time of 10 h, 10 mL of formic acid 85% were added to destroy the $NaNO_2$ excess. The so-obtained biopolymer was then washed with 160 mL of ice-cold acetone and with 400 mL of ice-cold diethyl ether (esotermic reaction). Successively, carboxylated fibers were washed with distilled water until neutrality, then with an ethanolic aqueous solution 50%, and finally with absolute ethanol. These biopolymers were dried under vacuum and at high temperatures in an oil bath.[16,17] Yield (carboxylated broom fibers); 1.05 g; yield (carboxylated cotton fibers) : 1.06 g.

Determination of the carboxyl group content of cellulose samples by methylene blue sorption

A weighted-fiber sample is suspended in 25 mL of aqueous methylene blue chloride solution (300 mg/L) and 25 mL of borate buffer of pH = 8.5 for 1 h at 20°C in an 100 mL flask and then filtered. 10 mL of the filtrate are transferred to a 100 mL calibrated flask. Then, 10 mL of 0.1*N* HCl and subsequently water, up to 100 mL, are added. Then, the methylene blue content of the liquid is determined photometrically, employing a calibration plot, and from the result the total amount of free, nonsorbed, methylene blue is calculated. The carboxyl group content of the sample is obtained according to eq. (2) where *A* is the total amount of free methylene blue in mg and *E* the weight of oven-dry sample (g).[18–20]

$$\text{mmol COOH/g oven dry sample} = \frac{(7.5 - A) \times 0.00313}{E} \quad (2)$$

Microsomal suspensions preparation

Liver microsomes were prepared from Wistar rats by tissue homogenization with 5 volumes of ice-cold 0.25*M* sucrose containing 5 m*M* Hepes, 0.5 m*M* EDTA, pH 7.5 in a Potter- Elvehjem homogenizer.[21] Microsomal membranes were isolated by removal of

the nuclear fraction at 8000 *g* for 10 min and removal of the mitochondrial fraction at 18,000 × *g* for 10 min. The microsomal fraction was sedimented at 105,000 *g* for 60 min, and the fraction was washed once in 0.15*M* KCl and collected again at 105,000 *g* for 30 min.[22] The membranes, suspended in 0.1*M* potassium phosphate buffer, pH 7.5, were stored at −80°C. Microsomal proteins were determined by the Bio-Rad method.[23]

Addition of derivatized fibers to microsomes

Aliquots of derivatized broom or cotton fibers in the range of 0.5–6 mg/mL were added to the microsomes. Control microsomes received amounts of H_2O equal to those present in derivatized fibers-treated microsomes. The microsomes were gently suspended by a Dounce homogenizer, and then, the suspensions were incubated at 37°C in a shaking bath under air in the dark in the absence and in the presence of $0.25 \times 10^{-3}M$ *tert*-BOOH or $25 \times 10^{-3}M$ AAPH.

Malondialdehyde formation

Malondialdehyde (MDA) was extracted and analyzed as indicated.[24] Briefly, aliquots of 1 mL of microsomal suspension (0.5 mg proteins) were mixed with 3 mL 0.5% TCA and 0.5 mL of TBA solution (two parts 0.4% TBA in 0.2*M* HCl and one part distilled water) and 0.07 mL of 0.2% BHT in 95% ethanol. Samples were then incubated in a 90°C bath for 45 min. After incubation, the TBA-MDA complex was extracted with 3 mL of isobutyl alcohol. The absorbances of the extracts were measured by the use of UV spectrophotometry at 535 nm, and the results were expressed as nmol per mg of protein, using an extinction coefficient of 1.56×10^5 L m^{-1} cm^{-1}.

Preparation of mitochondria from rat liver

Rat liver has been reduced in many small portions in a sucrose 250 m*M*, *tris*(hydroxymethyl)aminomethane chloridrate (TRIS/HCl) 10 m*M*, ethylenediaminetetraacetic acid (EDTA) 1 m*M* isotonic solution (pH = 7.4). Sucrose concentration is fundamental for not to alter hepatocytes osmotic pressure and for preventing mitochondria lysis during preparation. Moreover, TRIS/HCl can maintain proteins physiological pH and EDTA has the function of catching bivalent ions as Mg^{2+} and Ca^{2+} that could inhibit mitochondria enzymes activity. Anyway, temperature must be maintained around 4°C to avoid proteins denaturation. Hepatic fragments have to be washed many times to remove residual blood that could alter experimental results.

Small fragments were omogenized in a potter and submitted to differential centrifugations in a Sorvall RC6 centrifuge. Proteic suspension so obtained has been stored at −70°C without no loose of activity. Proteic concentration was determined using Lowry method.[25]

Mitochondria extraction from rat fibroblast (RAT-1)

Imortalized rat fibroblast RAT-1 (American Type Culture Collection, Rockville, MD) were put on Petri dishes with a MEM colture medium mixed to bovine fetal serum 10%, glutamine 1% without no antibiotics. These cells grew in an incubator at 37°C with 5% CO_2; at the confluence phase, colture medium was washed off and cells were washed with phosphate buffered saline (PBS) and trypsinized. After that cells were submitted to lysis using a lysis buffer and differential centrifugations; finally, mitochondria were suspended in a sucrose/TRIS/EDTA solution and proteic concentration was determined using Lowry method.[25]

Determination of GST activity

GST activity was assayed by the spectrophotometric method using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate.[26] Results were calculated by using the absorption coefficient (9.6 mM^{-1} cm^{-1}) of the product formed by the conjugation of GSH and CDNB. The enzyme activity was expressed as nmol/min/mg protein. Protein concentrations were measured by the method described.[25] A solution made by reduced glutathione 200 m*M*, PBS, and CDNB was employed. GST catalizes conjugation of reduced glutathione to CDNB allowing the formation of a complex absorbing at 340 nm. Absorbances values obtained were used to mesure GST activity. For these tests, control lacks of broom and cotton fibers and contains only the biological substrate and the enzyme.

RESULTS AND DISCUSSIONS

Ferulate, lipoate, and α-tocopherulate broom and cotton fibers were obtained in high yields; these results show the effectiveness of synthetic strategies used to functionalize these natural fibers. FTIR spectroscopy shows that functionalized biopolymers possess typical ester-linkage stretchings (Table I):

The most important information obtained from the FTIR (KBr) spectrum, in case of α-lipoate derivative, was that the cyclic ring of the α-lipoate moiety remains intact during the reaction. This can be concluded by the absence of a band at about 2560–2570 cm^{-1} for a S—H stretching and the presence of a

TABLE I
FTIR Data and Substitution Degree Values (DS) for Functionalized Fibers

Functionalized fibers/DS (substitution degree values)	OH stretching	CH stretching	CO stretching	COC stretching
α-Tocoferulate broom/0.45	3446 cm^{-1}	2917 cm^{-1}	1735 cm^{-1}	1262 cm^{-1}
Ferulate broom/0.56	3458 cm^{-1}	2925 cm^{-1}	1734 cm^{-1}	not evident
Lipoate broom/0.58	3438 cm^{-1}	2905 cm^{-1}	1703 cm^{-1}	not evident
α-Tocoferulate cotton/0.53	3450 cm^{-1}	2926 cm^{-1}	1732 cm^{-1}	1262 cm^{-1}
Ferulate cotton/0.47	3350 cm^{-1}	2923 cm^{-1}	1729 cm^{-1}	not evident
Lipoate cotton/0.58	3412 cm^{-1}	2900 cm^{-1}	1729 cm^{-1}	1235 cm^{-1}

band at 521 cm^{-1} for a S—S stretching. The carboxyl group of the ester function was determined at 1731 cm^{-1}.

Anyway, their substitution degree was determined to evaluate ester-linkage formation and results show an acceptable DS for these biopolymers (Table I).

Moreover, the ability of fiber derivatives to protect against lipid peroxidation, induced by two different sources of free radicals, including AAPH and *tert*-BOOH, was examined in rat-liver microsomal membranes during 120 min of incubation. To evaluate antioxidant properties of nonderivatized polymeric structures, the same experiment was performed on

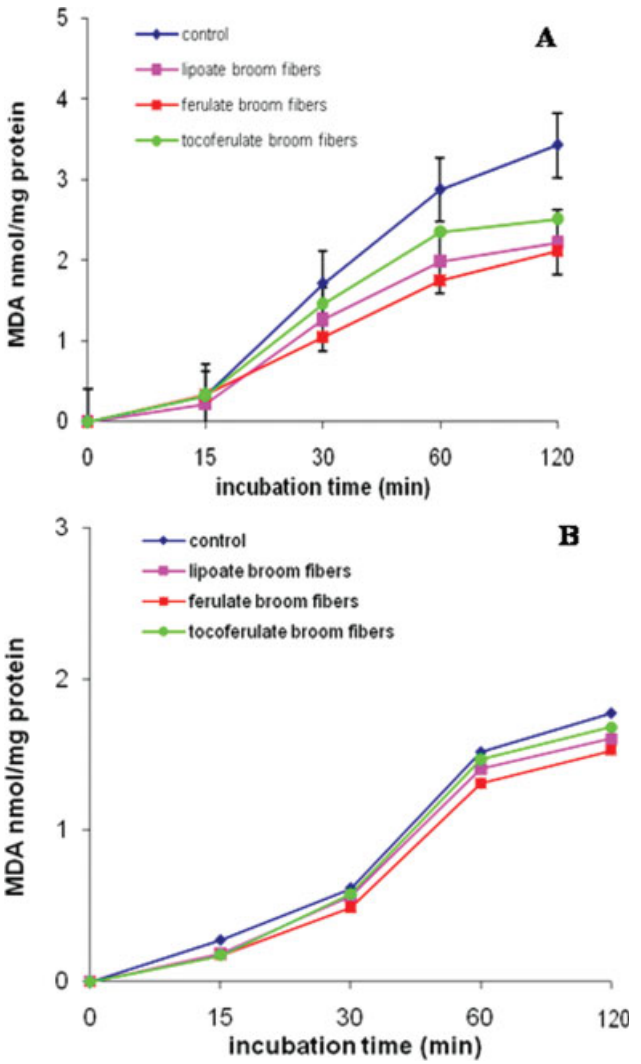

Figure 2 Effects of derivatized broom fibers, on malondialdehyde (MDA) production induced by *tert*-BOOH (A) and AAPH (B) in rat-liver microsomal membranes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

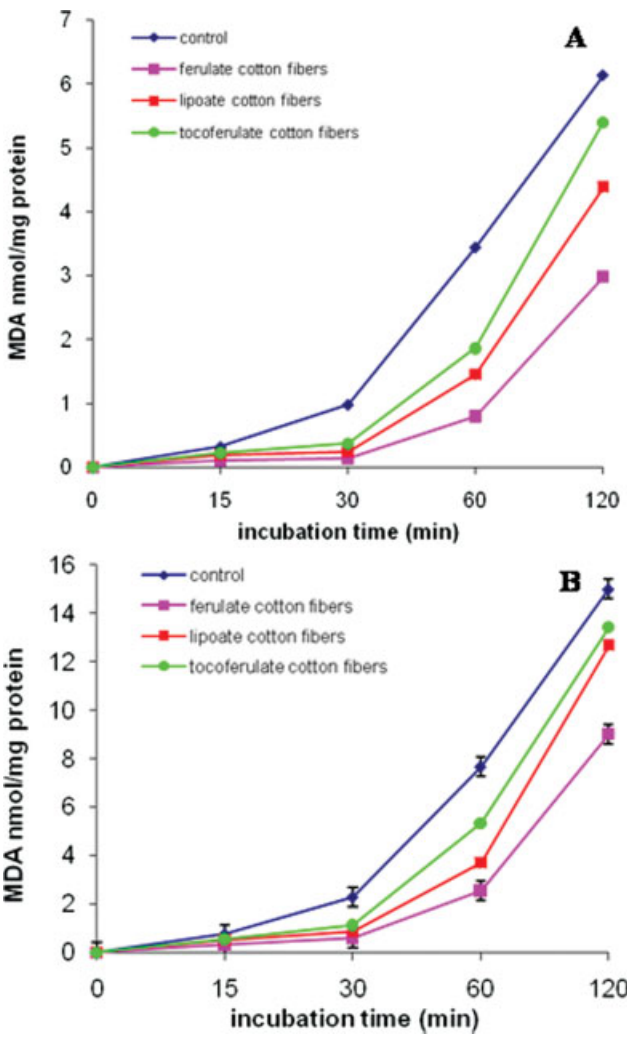

Figure 3 Effects of derivatized cotton fibers, on malondialdehyde (MDA) production induced by *tert*-BOOH (A) and AAPH (B) in rat-liver microsomal membranes. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

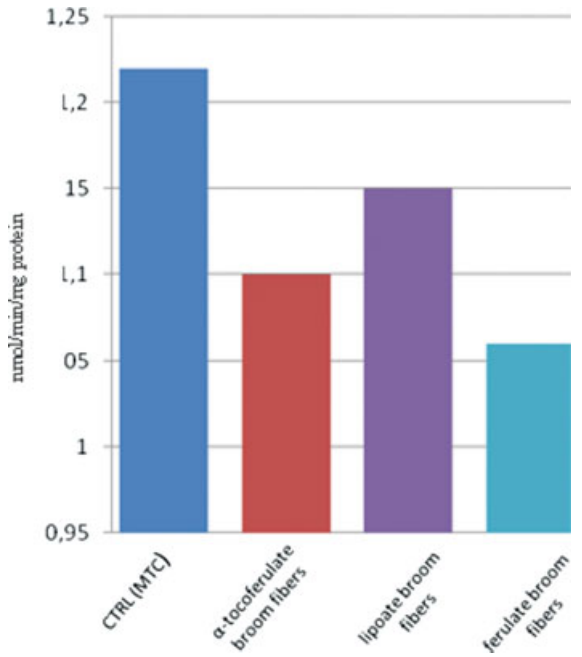

Figure 4 GST_m levels at $t = 0$ min (mmol/min/mg protein). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

not functionalized broom and cotton fibers. The data revealed that the last two have no antioxidant activity (data not shown). The effects of fiber derivatives on the lipid peroxidation were time-dependent and effected as the MDA production (in nmol mg^{-1} protein). Ferulate broom and cotton fibers were the stronger antioxidants, in protecting the membranes

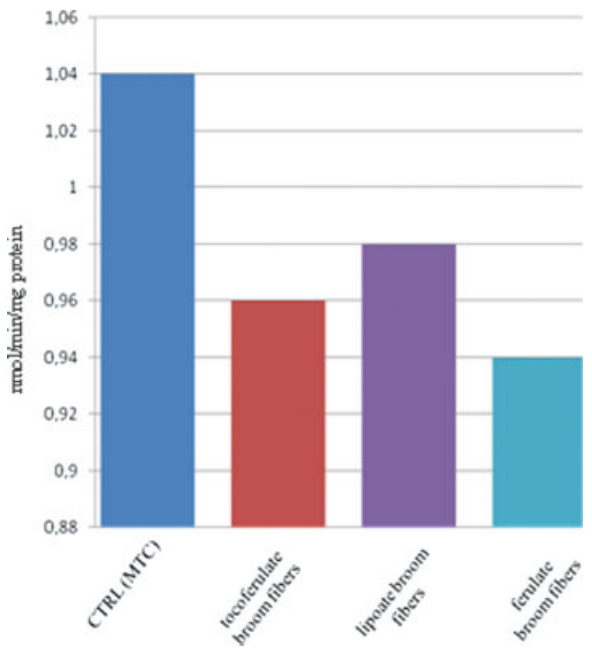

Figure 5 GST_m levels at $t = 15$ min (mmol/min/mg protein). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

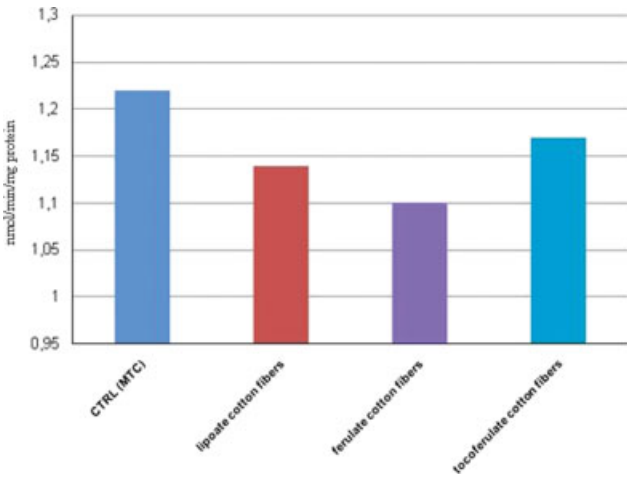

Figure 6 GST_m levels by Rat-1 at $t = 0$ (mmol/min/mg protein). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

from induced lipid peroxidation, than lipoate and α-tocopherulate ones. Particularly, all derivatives were strong antioxidants in protecting the membranes from *tert*-BOOH- than from AAPH-induced lipid peroxidation, showing in either case, higher efficiency at 30 min of incubation and the preservation of antioxidant activity up to 2 h (Figs. 2 and 3).

To evaluate the capability of functionalized broom fibers to be used in packaging area, a test using GST extracted from hepatic rat mitochondria was performed (Figs. 4 and 5).

Analogous to the test performed on rat-liver microsomal membranes, ferulate broom fibers gives the best results and generally none biopolymers can alter GST_m control levels, and so, we can deduce

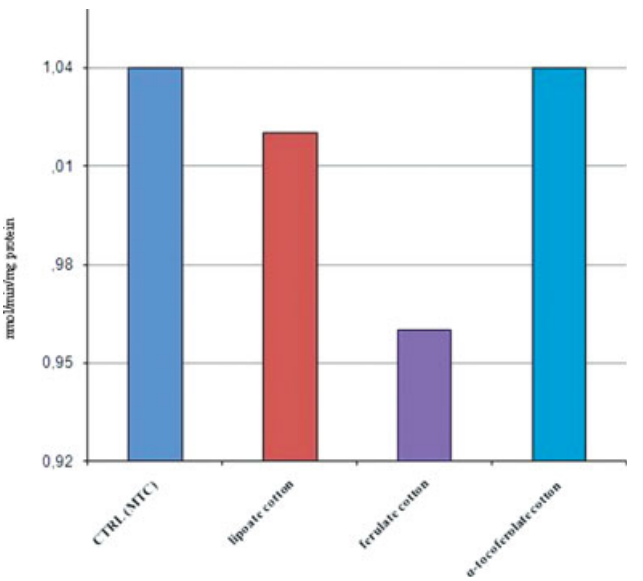

Figure 7 GST_m levels by Rat-1 at $t = 15$ min (mmol/min/mg protein). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that these biomaterials are useful for the packaging area. GST_m on rat fibroblasts test was performed on antioxidant cotton fibers (Figs. 6 and 7); this test shows that enzyme control levels are not altered by these biomaterials and that these results are in agreement with their substitution degree.

CONCLUSIONS

Broom and cotton fibers derivatives, with a variety of DS values, were successfully prepared introducing antioxidant moieties. Then, their properties were investigated to establish their applicative potential for biomedical and packaging areas. In fact, these biopolymers and the ferulate derivatives particularly, were characterized by a high-antioxidant efficacy. Moreover, after evaluation of glutathione-S-transferase (GST) levels, they showed a good biocompatibility.

On the basis of these results, one can conclude that these biomaterials can be successfully used in many fields particularly in packaging and biomedical fields.

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