Antioxidative Properties and Ability of Phenolic Compounds of *Myrtus communis* Leaves to Counteract *In Vitro* LDL and Phospholipid Aqueous Dispersion Oxidation

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Abstract: Antioxidant activities of *Myrtus communis* leaf phenolic compounds (McPCs) were investigated on 2,2′-9-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{+}$•) and oxygen radical absorbance capacity (ORAC) tests or on oxidation of biological models, human low-density lipoprotein (LDL) and phospholipid aqueous dispersion (L-α-phosphatidylcholine stabilized by bile salts). Two extraction techniques, microwave-assisted (MAE) and conventional (CE), were used to isolate McPCs, producing similar results of phenolic compound content. $ABTS^{+}$• assay showed clearly that myrtle extracts exhibited a stronger scavenging effect than butylated hydroxyanisole and α-tocopherol, with a slight advantage for myrtle CE extract. In ORAC assay, the both McPC extracts were similarly less effective than the pure compounds as caffeic acid and myricitrin (myricetin 3-*O*-rhamnoside) but stronger than butylated hydroxytoluene. Moreover, myrtle CE and MAE extracts, and myricitrin were able to inhibit similarly the production of conjugated dienes and to prolong the lag phase (Tlag) during Cu^{2+}-induced LDL oxidation with a dose-response effect. The cryo-electron microscopy observations on studied phospholipid dispersion stabilized by bile salts (BS) revealed the presence of bilayer vesicles and micelles. In 2,2′-azobis (2-amidinopropane) hydrochloride–induced phospholipid/BS oxidation, myrtle CE and MAE extracts gave similar effects to α-tocopherol and caffeic acid but myricitrin showed a higher protective effect than myrtle extracts. We showed also that no synergic or additive effect between α-tocopherol and myrtle extracts or caffeic acid in α-tocopherol–enriched phospholipid/BS dispersion, but myricitrin showed an additive effect and thus promoted the total antioxidant activity. These data showed that myrtle extract could be used as potential natural antioxidants, food stabilizers, or natural health products.

Keywords: $ABTS^{+}$• test, AAPH-mediated phospholipid dispersion oxidation, low-density lipoprotein Cu^{2+}-mediated oxidation, *Myrtus communis*, ORAC test

Practical Application: We show that microwave-assisted extraction could be an alternative method for plant phenolic compound recovery allowing important gain in time extraction. We report inhibition of low-density lipoprotein oxidation *in vitro* initiated by Cu^{2+} ions. We report that myrtle extract may be a source of natural antioxidants to counteract phospholipid peroxidation as well as α-tocopherol.

Introduction

Lipid oxidation is a complex phenomenon induced by oxygen in the presence of initiators such as heat, free radicals, photosensitizing pigments, and metal ions (Laguerre and others 2007) and plays an important role in many pathological processes such as atherosclerosis, cancers, Alzheimer, and Parkinson diseases. Oxidative modification of plasma low-density lipoproteins (LDL) is believed to have a crucial role in the pathogenesis of atherosclerosis (Beung 2000). In addition, oxidative stress has been recognized as a main component of post-prandial dysmetabolism (Heine and Dekker 2002; Bloomer and others 2010), suggesting that lipid oxidation could occur during digestion process. In fact, the work of Kenmogne-Domguia and others (2014) showed that during the *in vitro* digestion, emulsified lipids oxidized during the gastric step, in the presence of a low pH gastric medium, but also during the intestinal phase (presence of oxygen and oxidant substances) and that high quantities of oxidation products as malonaldehyde (MDA) and 4-hydroxy-2-hexenal (4HHE) were formed. Indeed, these oxidation products may be absorbed in the small intestine (Singh and others 2009), which may increase the oxidative stress in the plasma after their absorption. Thus, the inhibition of lipid peroxidation during digestion is an important role of radical-scavenging dietary antioxidants. It is now recognized that diets rich in antioxidants derived from fruits and vegetables are associated with lower risks of cardiovascular diseases and cancers (Ishimoto and others

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2012). Thus, interest in naturally occurring antioxidants has considerably increased for use in food, cosmetic, and pharmaceutical products (Djeridane and others 2006). Many medicinal plants contain large amounts of antioxidants (phenolic acids and flavonoids), which have gained much attention as a potential source of natural antioxidant phenolic compounds for improving the quality of healthcare.

Myrtus communis L. is one of the important aromatic and medicinal species from the Myrtaceae family (Wannes and others 2010) and is typical of the Mediterranean maquis, which grows spontaneously in many countries (Chryssavgi and others 2008**)**. Myrtle extracts have been reported to possess antiinflammatory, antimutagenic, antigenotoxic, and antihyperglycaemic effects. Recent reports showed also that myrtle leave extracts have strongest antioxidative activity and highest phenolic content compared with other myrtle plant parts (Chryssavgi and others 2008; Wannes and others 2010). The main class of *M. communis* leaf phenolic compounds was hydrolysable tannins, but the presence of phenolic acids (caffeic acid) and flavonoids (myricitrin, a myricetin 3-*O*-rhamnoside) were also shown (Romani and others 1999; Wannes and others 2010). These compounds showed strong scavenging activities in many studies and can be health-promoting compounds.

To isolate antioxidants from plants, different extraction methods can be used. Recently, a microwave-assisted extraction (MAE) method became an alternative extraction technique, which proved to be considerably more effective and economical. It provides higher recoveries, requires considerable less time and smaller solvent consumption compared to conventional extraction (CE) method.

The objectives of this study were to investigate for the first time the effects of antioxidant phenolic compounds from Algerian *M. communis* leaves extracted by both methods, MAE and CE methods. For this, different antioxidant activity tests were used to evaluate the scavenging capacity of myrtle extracts. In fact, in addition to the chemical analyses ($ABTS^{+}$• and ORAC), which are generally used to measure the capacity of a molecule to reduce a stable artificial free radical (by hydrogen or electron transfer), we were interested in the direct evaluation of the antioxidant activity of myrtle extracts in lipid system models oxidized *in vitro*: human LDL Cu^{+2}-oxidation and 2.2′-azo-bis-2-amidinopropane hydrochloride (AAPH)-induced L-α phosphatidylcholine aqueous dispersion oxidation. The last model assayed was used to simulate the duodenal (pH 6.5) and intestinal conditions (the pH was adjusted at 7.4: data not shown) of lipid peroxidation may occurring in small intestine during lipid digestion. For this, we used bile salts as physiological detergent substances to stabilize the phospholipid aqueous dispersion, and AAPH as generator of free radicals to initialize the peroxidation. The cryo electronic microscopy was applied to visualize structure and size difference of lipid formed at pH 6.5 and 7.4 (data not shown) in the system used.

Materials and Methods

Chemicals and standards

All chemicals used were analytical grade. Egg yolk phosphatidylcholine (EYPC), $CuCl_2$, butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and 2N Folin-Ciocalteu reagent were purchased from Sigma Aldrich Chemical Co. (Saint Quentin Fallavier, France). Gallic acid and 2,7-dichloro-fluorescein were obtained from Merck (Darmstadt, Germany). Methanol used for chromatography was High Performance Liquid Chromatography (HPLC)-grade supplied by Merck. Ethanol used for preparing standard solutions was from Prolabo (Paris, France). Chloroform was from Prolabo. AAPH was from Biovalley (Conches, France). Myricitrin was purchased from Roth Sochiel EURL (Lauterbourg, France).

Plant material preparation

M. communis leaves were collected in the region of Bejaia (Algeria), and dried in an oven at 40 °C until constant weight, then crushed and sieved to have a size less than 125 μm. The samples were stored in the dark at room temperature.

Extraction procedure

MAE method. A domestic microwave oven (NN-S674MF, LG, Japan, 32 l, 1000 W; variable in 100 W increments, 2.45 GHz) modified in our laboratory was used for extraction of myrtle phenolic compounds (McPC). A preliminary study was conducted to determine the effects of solvent type (water, ethanol/water [50/50, v/v], acetone/water [50/50, v/v], and of irradiation time [30 to 120 s]). Irradiation power at 700 W and 1/20 m/v ratio (1 g of leaves per 20 mL of solvent), respectively, were fixed. It was found that ethanol/water (50/50, v/v) or acetone/water (50/50, v/v), and 60 s of irradiation time provided the maximal recovery of McPCs (data not shown). In our study, the solvent used for extraction was chosen as ethanol/water (50/50, v/v) because it is less dangerous than acetone. The leaf suspension was irradiated by microwaves according to the following cycle: 45 s power-on (to keep temperature not rising above 80 °C), 10 s power-off (for cooling), and again 15 s power-on. The sample was filtered with a sintered glass at 0.45 μm using a vacuum pump. Two other additional extractions were carried out for recovering the totality of McPCs. Then, volume was adjusted to 50 mL. The obtained extract was stored at 4 °C until use.

CE method. The optimal parameters obtained with the microwave extraction will be applied in this case: 20 mL of ethanol/water (50/50, v/v) were added to 1 g of powder and let macerate during 60 min with magnetic agitation. After that, the process was the same as for MAE method.

Quantification of McPCs

Determination of total phenolic content. Total McPC concentration was estimated by Folin–Ciocalteu's assay, with absorbance monitored at 760 nm (Mondé and others 2011). The spectrophotometric measurement was repeated 3 times for each extract and the average data was interpolated in a gallic acid calibration curve and expressed on a dry weight basis as mg of gallic acid equivalents per g of dry weight sample (mg GAE g^{-1} DW).

Determination of flavonoid content. The total flavonoids were measured by a colorimetric method as described previously (Quettier-Deleu and others 2000). Each analysis was repeated 3 times. The flavonoid concentration was expressed as milligrams of rutin equivalents per g of dry weight sample (mg RE g^{-1} DW).

Measurement of antioxidant activities

2,2′-9-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{+}$•) radical cation decolorization assay. The spectrophotometric analysis of $ABTS^{+}$• scavenging activity was determined according to a method (Re and others 1999) based on the ability of antioxidants to quench the long-lived cation radical, in comparison to that of BHA a synthetic antioxidant, or α-tocopherol. The $ABTS^{+}$• was produced by reacting 7 mM ABTS in H_2O with 2.45 mM potassium persulfate, stored in the dark at room temperature. Before use, the $ABTS^{+}$• solution was diluted

to get an absorbance of 0.700 ± 0.020 at 734 nm with ethanol. Then, 1 mL of $ABTS^{+\bullet}$ solution was added to 1 mL of McPCs or standard ethanolic solutions at different concentrations. Inhibition was evaluated as percentage reduction of absorbance for each sample concentration expressed as mg GAE L^{-1} for McPCs or as mg L^{-1} for pure standards. The IC_{50} (concentration providing 50% inhibition) was calculated.

Oxygen radical absorbance capacity (ORAC) assay. ORAC values were measured with fluorescence spectrometer (Victor^2 Wallac–Perkin-Elmer) by inhibition of 2′,7′dichlorofluorescein (DCF; Ishimoto and others 2012) with slight modifications. Briefly, all samples and reagents were dissolved in 10 mmol phosphate L^{-1}/150 mmol NaCl L^{-1} buffer (PBS) at pH 7.4. 50 μL test samples or 50 μL Trolox solutions (0 to 20 μmol L^{-1}), 100 μL DCF solution (50 nmol L^{-1}), and 100 μL AAPH solution (20 mmol L^{-1}) were added to the wells of a 96-well plate. The fluorescence was recorded every 1 min for 90 min at 485-nm excitation and 535-nm emission wavelengths. A calibration curve of ORAC levels was obtained by plotting the period of time needed to obtain 50% fluorescence decay versus the Trolox concentrations. ORAC levels were expressed as mole of Trolox equivalent (TE) per mole of antioxidant (pure compounds) or mole of GAE (extracts). Gallic and caffeic acids were used as controls. Their respective ORAC values of 1.3 ± 0.4 and 4.1 ± 0.5 mol TE mol^{-1} (means ± SD) were found close to those previously reported (Ishimoto and others 2012).

LDL isolation and evaluation of PC extract effect on LDL oxidation mediated by Cu^{2+} ions. LDL was isolated from fresh plasma of healthy human subjects, obtained from the "French Blood Establishment," in accordance with its ethical rules, and oxidizability was monitored at 234 nm for 5 μmol L^{-1} Cu^{2+} oxidation as previously indicated (Mondé and others 2011). Briefly, isolated LDL was diluted to 1 μmol apoB L^{-1}, added with the various McPC concentrations to be tested, and then 10-fold diluted in oxygenated PBS at pH 7.4. For rendering the antioxidant abilities, we used the notion of specific antioxidant activity (SAA), which was calculated as the slope of the linear relationship obtained between relative lag time (rTlag), and concentrations of the different tested compounds (Mondé and others 2011). Relative Tlag was defined as $[Tlag^+/Tlag^-] \times 100$, with $^+$ and $^-$ denoting LDL with and without antioxidants, and Tlag was defined as the time corresponding to the end of the 1st kinetic phase during, which optical density (OD) do not or only slowly increase. An increased protection ratio was calculated using the following formula: protection ratio (%) $= [(Tlag^+ - Tlag^-)/Tlag^-] \times 100$, with $^+$ and $^-$ denoting LDL with and without tested antioxidants. Finally, the CD_{max} value was calculated as OD_{max}/ε, where OD_{max} corresponded to maximal oxidized product accumulation that was determined graphically by drawing a tangential line at the highest point corresponding to the end of the oxidation propagation phase, and ε is the specific absorption coefficient of CDs. The rate of oxidation propagation (R_p) was then expressed as mol CD_{max} mol $apoB^{-1}$ min^{-1}. An inhibitory ratio was finally calculated using the following formula: inhibitory ratio (%) $= [R_p^- - R_p^+/R_p^-] \times 100$.

Preparation of phospholipid aqueous dispersions with EYPC and bile salts and evaluation of McPC extract effects on oxidation mediated by AAPH. An appropriate quantity of EYPC was dissolved in chloroform. An aliquot of EYPC solution was dried under nitrogen, and bile salts (50%-cholic and 50 %-desoxycholic sodium salts) were added. For samples enriched with α-tocopherol, α-tocopherol was added to the EYPC solution before drying. The aqueous dispersion was prepared by adding 10 mL of PBS at pH 6.5, to make stock dispersions of 2.96 and 3.38 g L^{-1} of EYPC and bile salt concentrations, respectively. Then, sonication was carried out, under a stream of N_2 and in an ice bath, with 3 to 5 30-s irradiation cycles with a delay time of 10 s, until complete dispersion of EYPC. The aqueous dispersion was incubated at 37 °C during 30 min, and filtered to 0.2 μm. Samples were sealed under nitrogen and stored at −80 °C.

The oxidative stability of the emulsion was determined by monitoring the formation of conjugated dienes (CDs) with a UV-Visible thermostatic Spectrometer (Uvikon-XL; Bio-TeK Instruments) at 245 nm and 37 °C for 150 min with measurement intervals of 1.5 min. For this, to 1 mL of phospholipid aqueous dispersion were added 0.5 mL of AAPH (as oxidant agent) with a final concentration of 5 mmol L^{-1}, and 0.5 mL PBS containing different tested concentrations of McPCs or pure standards. Oxidation results were calculated as the ratio of AUC of tested antioxidants to that of control, and were expressed as percentages (Lorrain and others 2010). The AUC value was calculated by the integration of the area under the curve (AUC) obtained by CD concentrations *versus* time (until 150 min).

Extraction of lipids, determination of fatty acid (FA) composition, and α-tocopherol content

Total lipids were extracted from aqueous dispersion according to a slight modification of Folch's technique (Folch and others 1957). Briefly, a mixture containing 250 μL of dispersion and 750 μL of 9 g NaCl L^{-1} was homogenized with 4 mL of chloroform/methanol (2/1, v/v) containing 50 mg L^{-1} of BHT as an antioxidant. This homogenate was centrifuged to recover total lipids in the organic phase, which were then washed using 9 g NaCl L^{-1} and recentrifuged. The total recovered organic phase was evaporated under N_2 and the residue was taken again by 2 mL of a chloroform/methanol (2/1, v/v) mixture, and then stored at −20 °C until further analyses. Preparation of the methylated FAs and gas chromatography analyses were carried out according to our usual technique (Aoun and others 2011).

α-Tocopherol was determined after extraction as previously described (Mondé 2011) with slight modifications. Briefly, 1 mL of aqueous dispersion was extracted by ethanol/hexane (1/1, v/v) containing δ-tocopherol as an internal standard. Extracted products were measured after HPLC separation with spectrophotometric detection (at 292 nm), on a Lichrocart® 125-4 (5 μm-particle size) column (Merck, France), using a water/methanol mixture (3/97, v/v) mobile phase at 0.8 mL min^{-1} flow rate for 12 min.

Cryo-transmission electronic microscopy

Sample freezing was performed using a semiautomatic plunge freezing instrument (cryoplunge CP3; Gatan Inc.). Briefly, 3 microliters of phospholipid aqueous dispersion at 3 g/L were applied to glow discharged quantifoil R 2/2 grids (Quantifoil Micro Tools GmbH, Jena, Germany), blotted for 2 s and then flash frozen in liquid ethane. Cryo-transmission electron microscopy (cryo-TEM) observations were carried out on a JEOL 2200FS FEG operating at 200 kV under low-dose conditions (total dose of 20 electrons/$Å^2$) in the zero energy loss mode with a slit width of 20 eV. Images were recorded with defocus ranging from 1.5 to 2 μm.

Statistical analysis

Analyses were carried out in 3 times or more and results were reported as mean values ± standard deviation (SD). Data were compared on the basis of the mean values. Differences among

means of variety groups were tested using a Tukey–Kramer HSD (logiciel JMP version 7.0) with a significance level of 0.05.

Results and Discussion

Quantification of *M. communis* phenolic compounds

Total McPC content was determined by Folin–Ciocalteu's assay. The both MAE and CE methods gave almost very close levels of phenolic compounds (176.4 ± 6.8 and 181.0 ± 6.3 mg GAE g^{-1} DW, respectively), but with a slight advantage for the CE method ($P < 0.05$). Results of flavonoid content showed that MAE method was more effective than CE one (25.6 ± 0.9 and 22.7 ± 0.6 mg RE g^{-1} DW, respectively).

According to our results, MAE method showed to be an alternative to the conventional one, as previously shown for many plants (Proestos and Komaitis 2008). Microwave energy offers a rapid transfer of energy to the extraction solvent and raw plant materials. This extracting method also results in rupture of the plant cells and quickly release of intracellular products into the solvent. McPC contents obtained with the 2 extraction procedures were

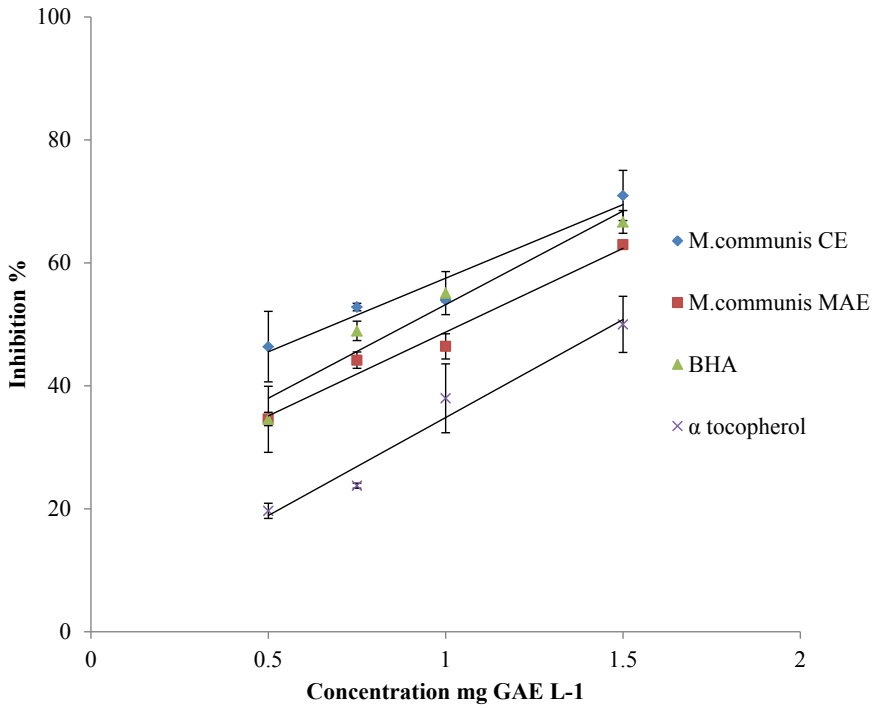

Figure 1–Scavenging activity of *M. communis* extracts on ABTS radical. Results are expressed as means of 3 experiments ± SD ($n = 3$).

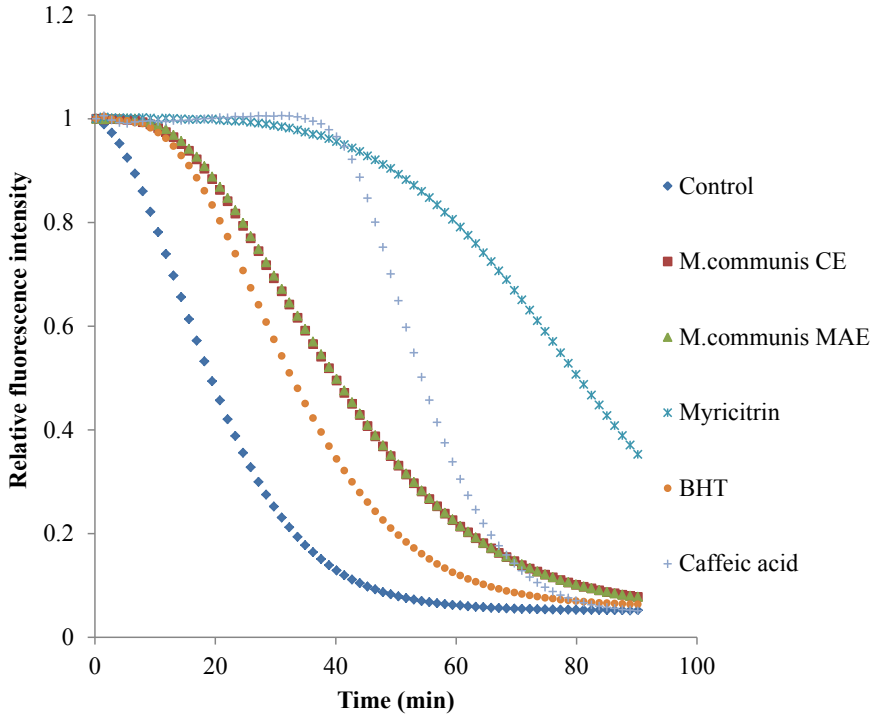

Figure 2–Fluorescence decay curve of fluorescein during the ORAC assay in the presence of various phenolic compounds (1 μmol L^{-1}). The results are the mean of 3 experiments ($n = 3$).

found to be 2 times lower than that found in methanolic extract of Greece myrtle leaves (Chryssavgi and others 2008).

Antioxidant activities of McPCs

The $ABTS^{+\bullet}$ assay allowed to determine the electron-donating capacity of antioxidant compounds and showed that myrtle extract obtained by CE method and BHA standard exhibited higher antioxidant capacity (Figure 1) with an IC_{50} of 0.68 ± 0.10 mg GAE L^{-1} and 0.89 ± 0.07 mg L^{-1}; respectively, followed by MAE method with an IC_{50} of 1.05 ± 0.02 mg GAE L^{-1}, and finally the lowest antioxidant capacity was obtained by α-tocopherol standard with an IC_{50} = 1.49 ± 0.11 mg L^{-1} ($P < 0.05$) compared to the 3 other compounds. Hence, when data are expressed in terms of dry weight (instead of GAE), the myrtle (CE and MAE) extracts with an IC_{50} value of 3.79 ± 0.13 and 5.95 ± 0.22 mg DW L^{-1} respectively, became less effective than the 2 pure compounds tested. This supposes that in myrtle extract, there are other compounds devoid of antioxidant activity that led to underestimate the antioxidant activity of the extract when the result was expressed on dry weight basis. Our results were in accordance with a previous work on Moroccan myrtle leaves showing the ability of myrtle extract to scavenge $ABTS^{+\bullet}$ radical (Amensour and others 2010). It was also found that MAE method presented a less scavenging activity than CE one. These results lead to the hypothesis that microwave-induced heating of extraction solvent and this might cause a thermal degradation of some antioxidant compounds present in the extract during the irradiation phase, as shown previously (Liazid and others 2007). These authors concluded that all the compounds studied are stable up to 100 °C, whereas at 125 °C there is significant degradation of epicatechin, resveratrol, and myricetin. It has also been found that phenolic compounds having a greater number of hydroxyl substituents are more easily degraded under the extraction conditions, which let suppose particularly a high potential degradation of myricetin derivatives possibly present in the extract. Moreover, we can also hypothesize that in both extracts, there is a difference of antioxidant substance composition due to the both used different extraction procedures.

The 2nd chemical assay used was ORAC test, which could be considered as a preferable method because of its biological relevance to the *in vivo* antioxidant efficacy (Oueslati and others 2012) and where the operating mechanism is H-atom transfer reactions from the phenols to AAPH-derived peroxyl radicals. Figure 2 showed that myrtle extracts obtained by MAE and CE methods showed similar results with an ORAC value of 1.59 ± 0.07 and 1.57 ± 0.13 mol TE mol GAE^{-1}, respectively, and were less effective ($P < 0.05$) than pure standards as myricitrin and caffeic acid, with ORAC values of 6.3 ± 0.4 and 4.1 ± 0.5 mol TE mol^{-1}. However, they were more effective than BHT as synthetic antioxidant with an ORAC value of 0.7 ± 0.1 mol TE mol^{-1}.

These values confirm that an increase in the number of hydroxyl groups, particularly in the B-ring of flavonols (myricetin) is generally correlated with an increase in antioxidant activity (Cao and others 1997). Moreover, myricitrin has a slightly increased ORAC value than myricetin, its aglycon form (4.9 ± 0.2 mol TE mol^{-1}, as previously found by Ishimoto and others 2012), possibly via its rhamnoside moiety. A recent work (Oueslati and others 2012) showed also that pure antioxidant as caffeic acid or quercetin have a stronger scavenging effect than 4 acetonic extracts of Tunisian halophytes. These results may be explained by a competition for the H-donating capacity between different antioxidants present in a natural mixture.

Myrtle extracts prevent copper-induced LDL oxidation

It is important to mention that plasma concentrations achieved by flavonoids are low, usually no more than 1 μmol L^{-1} (Halliwell and others 2005), a reason to choose the concentrations used under our LDL oxidization model.

Effects of McPCs (MAE and CE) were evaluated on 5 μmol Cu^{2+} L^{-1}-mediated LDL oxidation at different concentrations (0 to 2.95 μmol GAE L^{-1}). Oxidation process was assessed by kinetic of CD formation (Figure 3) evaluated by OD at 234 nm. Myrtle extracts were able to inhibit in a dose-dependent manner LDL oxidation leading to a significant increase in lag time (Table 1).

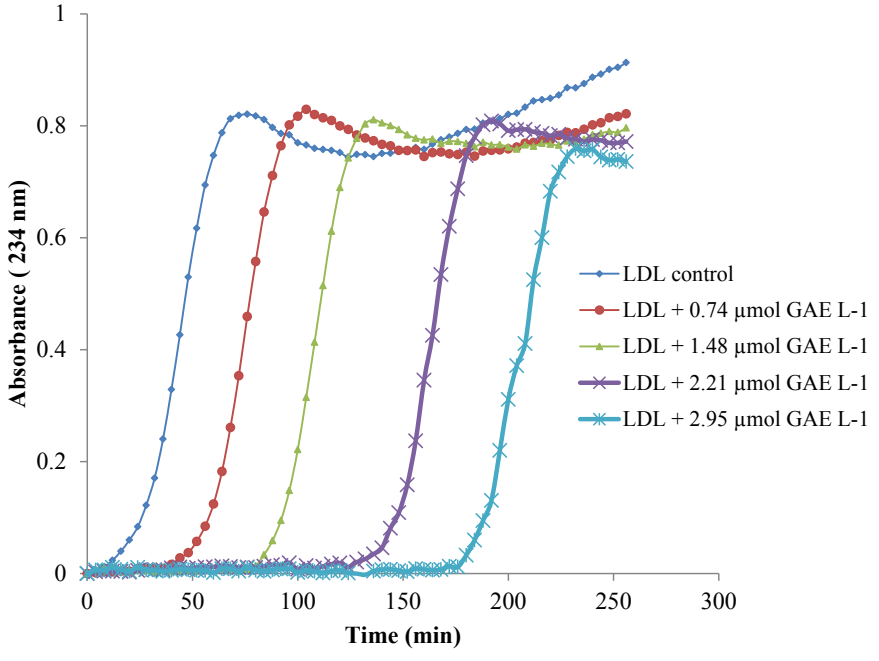

Figure 3–Kinetic of CDs production by LDL Cu^{2+}-mediated oxidation in the presence of McPCs (MAE method).
LDL (0.1 μM) is oxidized in PBS pH 7.4 at 37 °C with 5 μmol Cu^{2+} L^{-1} with McPCs added just before oxidation, or without antioxidant for LDL control. Concentrations of McPCs are evaluated as μmol GAE L^{-1} and the absorbance is continuously monitored at 234 nm. CD production is evaluated as OD/ε of CDs. Kinetic of CD production with McPCs (CE method) gives similar curves.

Table 1–Effect of polyphenol extracts of *M. communis* leaves obtained by MAE or CE methods on the kinetic parameters of LDL oxidation mediated by Cu^{2+} ions.

concentration (μmol GAE L^{-1})	Tlag (min)		R_P (CD-mol apoB-mol^{-1} min^{-1})		CD_{max} (CD-mol apoB-mol^{-1})	
	MAE	CE	MAE	CE	MAE	CE
0	41.8 ± 1.7^{a}	48.9 ± 1.8^{a}	8.4 ± 0.2^{a}	7.3 ± 0.3^{a}	277.7 ± 7.5^{a}	246.22 ± 9.4^{a}
0.74	60.6 ± 1.1^{b}	80.0 ± 3.6^{b}	8.1 ± 0.2^{a}	6.7 ± 0.2^{ab}	276.8 ± 9.0^{a}	247.7 ± 19.5^{a}
1.48	92.9 ± 14.3^{c}	142.3 ± 2.8^{c}	8.1 ± 0.8^{a}	6.7 ± 0.4^{ab}	276.4 ± 9.9^{a}	240.4 ± 10.8^{a}
2.21	130.6 ± 13.9^{d}	182.2 ± 21.2^{d}	8.0 ± 0.3^{a}	5.9 ± 0.7^{ab}	270.3 ± 13.6^{a}	234.0 ± 5.1^{a}
2.95	180.4 ± 4.5^{e}	245.9 ± 12^{e}	7.0 ± 0.3^{b}	5.8 ± 0.3^{b}	255.5 ± 7.0^{a}	225.8.0 ± 2.6^{a}

Assays are performed in triplicate ($n = 3$) and data are expressed as mean ± standard deviation. Means followed by different letters in the same column are significantly different ($P < 0.05$). Kinetic parameters of LDL oxidation are: Tlag, lag time of oxidation curve; R_p, oxidation propagation rate; CD_{max}, maximal oxidized product accumulation evaluated as OD_{max}/ε of CDs.

Moreover, at higher concentration (2.95 μmol GAE L^{-1}), McPCs allowed to produce a significant ($P < 0.05$) increased protection ratio of 332.5 ± 28.1% and 402.9 ± 8.8% for MAE and CE methods, respectively. Moreover, the oxidation propagation rate (R_p) decreased ($P < 0.05$), giving an inhibitory ratio of 16.4 ± 5.5% and 20.7 ± 3.4% for MAE and CE methods, respectively, whereas the formation of oxidation products evaluated as CD_{max} remained constant. Finally, SAA values for McPC extracts were also evaluated, and the results were expressed as μmol GAE^{-1} L (Figure 4) in comparison with pure standards, for which SAA values were expressed as μmol^{-1} L. Higher SAA value indicated higher antioxidant activity. Myrtle extracts (MAE and CE) and myricitrin exhibited SAA values without significant difference (130 ± 5.5, 102.0 ± 7.3 μmol GAE^{-1} L, and 110.2 ± 17.3 μmol^{-1} L, respectively) on LDL containing natural α-tocopherol level (8.3 ± 0.6 mol α-tocopherol mol $apoB^{-1}$). However, these 3 SAA values were significantly higher ($P < 0.01$) than that of caffeic acid or α-tocopherol (54.0 ± 8.6 or 7.2 ± 0.3 μmol^{-1} L, respectively), values obtained on LDL exhibiting an exceptionally high α-tocopherol content (12.7 ± 1.1 mol α-tocopherol mol $apoB^{-1}$). The very low level of protection afforded by an external addition of α-tocopherol could be explained by its lack in the catechol structure affording maximal radical stabilization.

LDL oxidation is a complex, multistep mechanism involving both lipid and protein fractions. According to the previously proposed mechanism (Pinchuk and Lichtenberg 2002), (1) if the antioxidant prolongs the lag time without affecting the maximal rate, the underlying mechanism of inhibition probably involved either quenching of free radicals or prevention of the transfer of reactive intermediates (particularly hydroperoxides) to the LDL lipid core; and (2) if the maximal rate of Cu^{2+} peroxidation was decreased, the conclusion is that the mechanism involved either binding of copper ions or blocking of copper binding sites on the lipoprotein surface LDL. So, McPC protective effects could be afforded by a combination of these both mechanisms. Moreover, when myricitrin was used under the same oxidative condition, the R_p of oxidation remained constant. Myricetin, its aglycone form, was an efficient antioxidant for protecting LDL against Cu^{2+}-mediated oxidation and may act by chelating copper ions due to the presence of catechol group (3′ and 4′ OH) and/or donating hydrogen atom to generated peroxyl radicals (Vaya and others 2003). Caffeic acid, one of the most abundant phenolic acids in myrtle leaves (Romani and others 1999), inhibited strongly LDL modification with a dose-dependent effect (Mondé and others 2011). It was also found that McPCs of the CE method had a slightly higher SAA than that of MAE method and myricitrin but without a

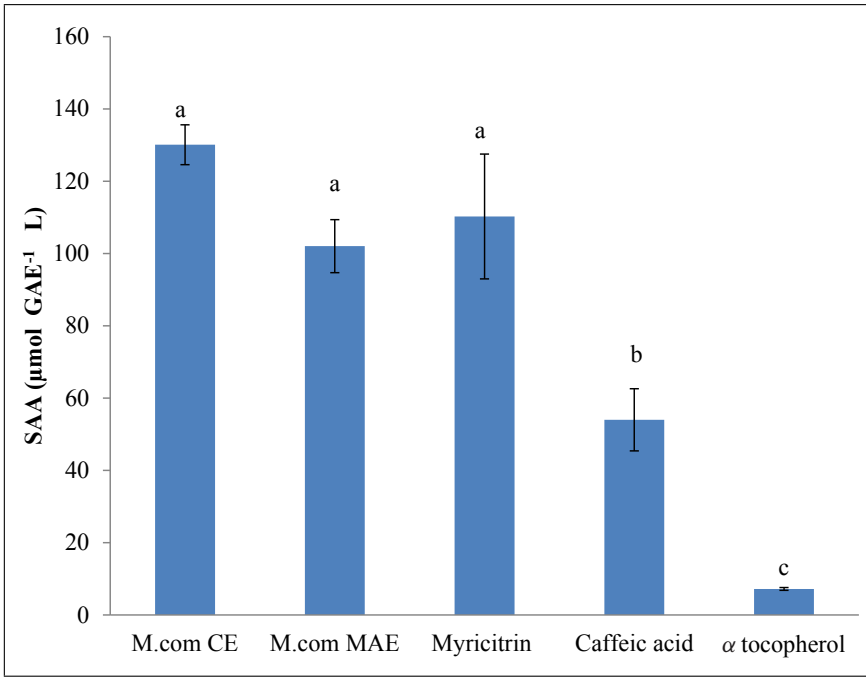

Figure 4–SAAs values of *M. communis* extracts, myricitrin, caffeic acid, and α-tocopherol for Cu^{2+}-induced LDL oxidation assay. SAA = specific antioxidant activity, defined as the slope of the relationship between relative Tlag and concentrations of tested compounds. Results are expressed as means of 3 experiments ± SD. Different letters indicate that samples are significantly different ($P < 0.05$).

significant difference (Figure 4). This result did not correlate with chemical tests, particularly the $ABTS^{+\bullet}$ assay giving statistically different values for McPCs issued from MAE and CE methods. This observation was also different with the ORAC assay, which showed that pure compound (myricitrin) had largely more inhibitory effect than the both myrtle extracts. Our results upon LDL protective effect may be interpreted by possible competition of the different compounds present in myrtle extracts. As previously shown (Meyer and others 1998), different combinations of phenolic compounds may have additive effects, except for combinations including ellagic acid and catechin, present together in *M. communis* extracts (Romani and others 1999), where ellagic acid could exert a significant antagonistic effect.

Myrtle extracts prevent AAPH-induced oxidation of phospholipid aqueous dispersion

The effect of McPCs (MAE and CE) was also evaluated on AAPH oxidation of EYPC/bile salt aqueous dispersion with the ratio of 0.8 (EYPC/BS) in PBS pH 6.5. Gas chromatography analysis of FAs showed that this aqueous dispersion contained an equivalent composition between saturated and unsaturated FAs. Major saturated FAs were palmitic (29.5 ± 4.0%) and stearic (16.9 ± 2.1%) acids. Major unsaturated FAs were in a decreasing order, oleic (25.2 ± 2.9%), linoleic (14.1 ± 1.4%), arachidonic (4.6 ± 0.5%), docosahexaenoic (3.9 ± 1.1%), and linolenic (1.7 ± 0.1%) acids.

The lipid structures in the phospholipid aqueous dispersion studied were characterized by cryo-transmission electron microscopy (cryo-TEM), a well-adapted technique for observing structures in complex fluids such as mixed micellar dispersions, to identify the lipid structures formed by addition of the surfactant bile salts to phosphatidylcholine dispersion (Almgren and others 2000). All images revealed the presence of 2 different types of lipid structures, clearly indicating heterogeneity of the dispersion (Figure 5A and 5B). Unilamellar vesicles of about 200 nm in diameter are predominantly found in our lipid dispersion. These structures, hollowed and delineated by a bilayer are consistent with liposomal forms. It should be mentioned that some larger vesicles can also be present, as showed by a vesicle of 346 nm in diameter in Figure 5C and that membrane of some rare vesicles can locally be disrupted (white arrow). Small dense particles, ranging from 15 to 40 nm in diameter, are also observed in the dispersion, suggesting the presence of mixed micelles (black arrows, Figure 5). Our results are consistent with a previous work

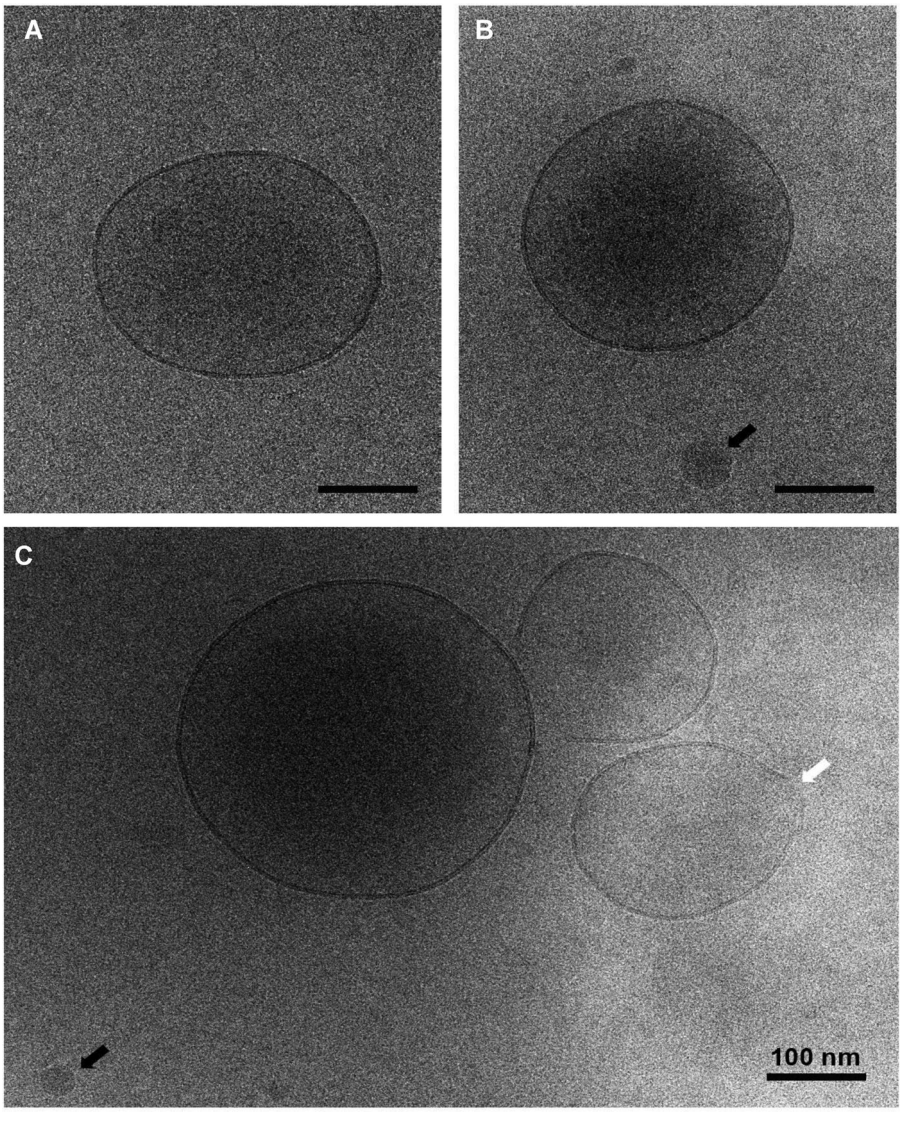

Figure 5–Cryo-electron microscopy images of phospholipid aqueous dispersion formed by lecithin (EYPC) and bile salts at pH 6.5. (A and B) Representative frozen-hydrated lipid vesicles observed in phospholipid dispersion, with diameter ranging from 200 to 250 nm. Small dense micelles can also be visualized as shown by black arrows. C, lipid vesicle having diameter larger than 300 nm can occasionally be observed as well as breach in liposome lipid bilayer (black arrow). Scale bars, 100 nm.

(Hildebrand and others 2004) that studied the solubilization of 1, 2-dipalmitoyl-*sn*-glycero-3-phospha-tidylcholine liposomes by bile salts, and demonstrated that this surfactant agent form large mixed vesicles and small mixed micelles. In our study, the presence of a large mixed vesicles (diameter > 200 nm) may be explained by the coalescence phenomena corresponding to fusion of vesicles. This is supported by the presence of punctual membrane breaking observed in our dispersion images (Figure 5C).

During aqueous dispersion AAPH oxidation at pH 6.5 (Figure 6), McPCs and myricitrin showed that they were able to minimize the formation of CDs evaluated from OD_{max}, with a dose-dependent effect. In fact, when McPC (MAE and CE) concentrations increased, AUC values decreased significantly ($P < 0.05$) with a linear relationship giving equivalent IC_{50} values of 16.1 and 15.5 μmol GAE L^{-1}, respectively). Myricitrin and caffeic acid were more than and as efficient as myrtle extracts, respectively. However, these both standard compounds gave a 2nd degree polynomial relationship with an IC_{50} value of 8.1 μmol L^{-1} for myricitrin and only a maximum inhibition value of 57.7% obtained for a concentration of 17.7 μmol L^{-1} for caffeic acid.

This observation coincided with results found in the ORAC assay showing that myricitrin was more effective than myrtle extracts. A previous study (Gulcin 2006) showed also that caffeic acid was an effective antioxidant on peroxidation of linoleic acid emulsion. It was important to mention also that linoleic acid was the main oxidizable polyunsaturated FA found in our phosphatidylcholine/bile salt dispersion. Moreover, the antioxidant activity of myricetin and myricitrin in bulk methyl linoleate system thermally oxidized at 40 °C was analyzed (Hopia and Heinonen 1999). Their results showed that the antioxidant activity difference between myricetin and its rhamnoside derivative was small but statistically significant, and they were more active than equivalent concentrations of α-tocopherol. Under our conditions, no significant difference was obtained between myricitrin and α-tocopherol protection. Comparable results were also obtained for quercetin and rutin, its glycolysated form compared to α-tocopherol (Lorrain and others 2010) on sunflower oil-in-water emulsions, stabilized by egg yolk phospholipids and oxidized by methmyoglobin. This suggested that these hydrophilic antioxidants were as efficient as the lipophilic α-tocopherol to protect sunflower oil-in-water emulsions or mixed phospholipid/bile salt dispersions against oxidation mediated by lipophilic metmyoglobin-derived or by hydrophilic AAPH-derived peroxyl radicals, respectively.

Myrtle extracts does not act synergistically with α-tocopherol on phospholipid aqueous dispersion oxidation

In this study, we also investigated the effect of added α-tocopherol on the stability of lipid dispersions, and possible synergic effects with other antioxidants, at pH 6.5. First, as shown in Figure 7A, at an overloading concentration of 10 μmol L^{-1}, α-tocopherol, caffeic acid, and myricitrin decreased the AUC value to 50.4 ± 7.5%, 60.3 ± 3.1%, and 44.4 ± 7.7%, respectively, as compared to the control without any added antioxidant. McPCs, at an equivalent concentration of 10 μmol GAE L^{-1}, decreased the AUC value to 67.8 ± 2.2% and 69.0 ± 1.9% for both MAE and CE methods, respectively. These results showed that α-tocopherol and myricitrin had an antioxidant effect significantly ($P < 0.05$) higher than that of both McPCs. Moreover, when McPCs (MAE or CE, 10 μGAE L^{-1}) were added in addition to α-tocopherol (10 μmol L^{-1}), protection of the dispersion was more effective (ca. a factor 1.5 or 1.6) than that provided solely by McPCs. However, this gain in efficiency is lower than that could be anticipated by an additive effect. Second, it was also investigated possible synergic effects of myrtle extracts, caffeic acid and myricitrin (Figure 7B) on α-tocopherol–enriched phospholipid aqueous dispersions. The dispersion control contains 3.7 ± 0.9 μmol α-tocopherol L^{-1}, which is naturally present in the EYPC, whereas α-tocopherol–enriched dispersion contained 10.6 ± 0.2 μmol L^{-1}. The results showed that there was no different protective effect of α-tocopherol between its external loading (decreased AUC to 50.4 ± 7.5% for a total [initial + external loading] 14.3 μmol α-tocopherol L^{-1}, as shown in Figure 7A) and its incorporation in the lipid phase prior to the aqueous dispersion formation (decreased AUC to 67.3 ± 10.9% for only 10.5 μmol α-tocopherol L^{-1}, as shown in Figure 7B). Moreover, no more additive effects were obtained by external addition of McPCs

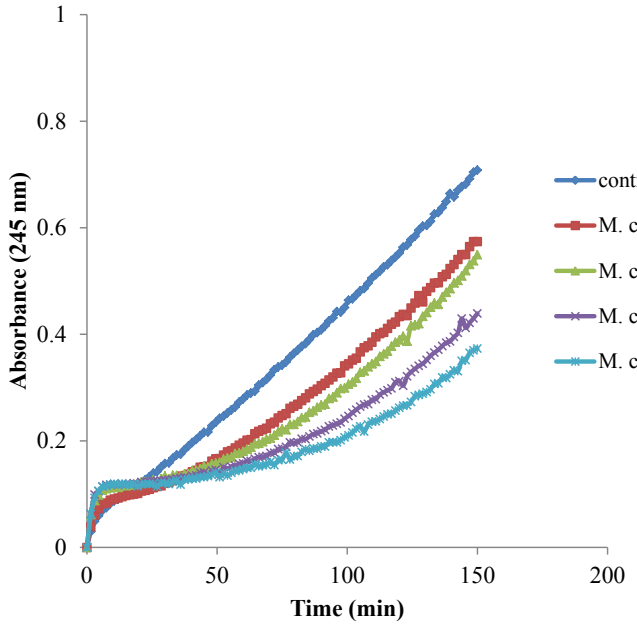

Figure 6–Kinetic oxidation of phospholipid aqueous dispersion formed by egg phosphatidylcholine and bile salts at pH 6.5, induced by AAPH-derived radical in the presence or absence (phospholipid dispersion control) of *M. communis* (M.com) MAE-PCs. Phospholipid aqueous dispersion was oxidized by 5 mmol AAPH L^{-1} with or without *M. communis* PCs (phospholipid dispersion control). Phenolic compounds were added just before oxidation. Concentrations of McPCs were evaluated as μmol GAE L^{-1} and the absorbance was continuously monitored at 245 nm. CD production is evaluated as OD/ε of CDs. Kinetic of CD production with *M. communis* CE-PCs gives similar curves.

(MAE and CE; 10 μGAE L^{-1}), or caffeic acid (10 μmol L^{-1}) on the α-tocopherol-enriched dispersion, by comparison with the control. Only a slight significant additional protective effect (ca. a factor 1.4) was obtained for external addition myricitrin (10 μmol L^{-1}) on α-tocopherol–enriched dispersion.

This finding can be supported by a previous work (Yin and others 2012) who investigated the antioxidant interaction between green tea polyphenols (GTE) and α-tocopherol against lipid peroxidation. A merely additive effect of α-tocopherol and GTE was observed in their phosphatidylcholine-based liposome system, when a synergistic effect was only observed in a sunflower oil-in-water emulsion, suggesting that this GTE extract is more efficient as antioxidant in lipid systems containing triglycerides afforded by sunflower oil. One another study (Peyrat-Maillard and others 2003) observed an antagonistic effect between α-tocopherol and some phenolic acids (particularly rosmarinic and caffeic acids) during AAPH-oxidation of an aqueous dispersion of linoleic acid. These authors explained this antagonism by the fact that a fraction of highly active acids (rosmarinic and caffeic acids) would regenerate the less active α-tocopherol, besides giving hydrogen to lipid (alkyl or peroxyl) radicals. The phenol group of α-tocopherol is located at the interface of the aqueous phase (Altunkaya and others 2009). Thus, this liposoluble antioxidant can trap free radicals generated either by lipids at the lipid–water interface where peroxidation was predominant, or by AAPH decomposition occurring into the aqueous phase. Myrtle extracts, as a mixture of different antioxidants (phenolic acids and flavonoids), probably with difference in solubility and mechanism of action, could neutralize both AAPH-initiated aqueous radicals and lipid radicals at the interface. This may explain a similar effectiveness of myrtle

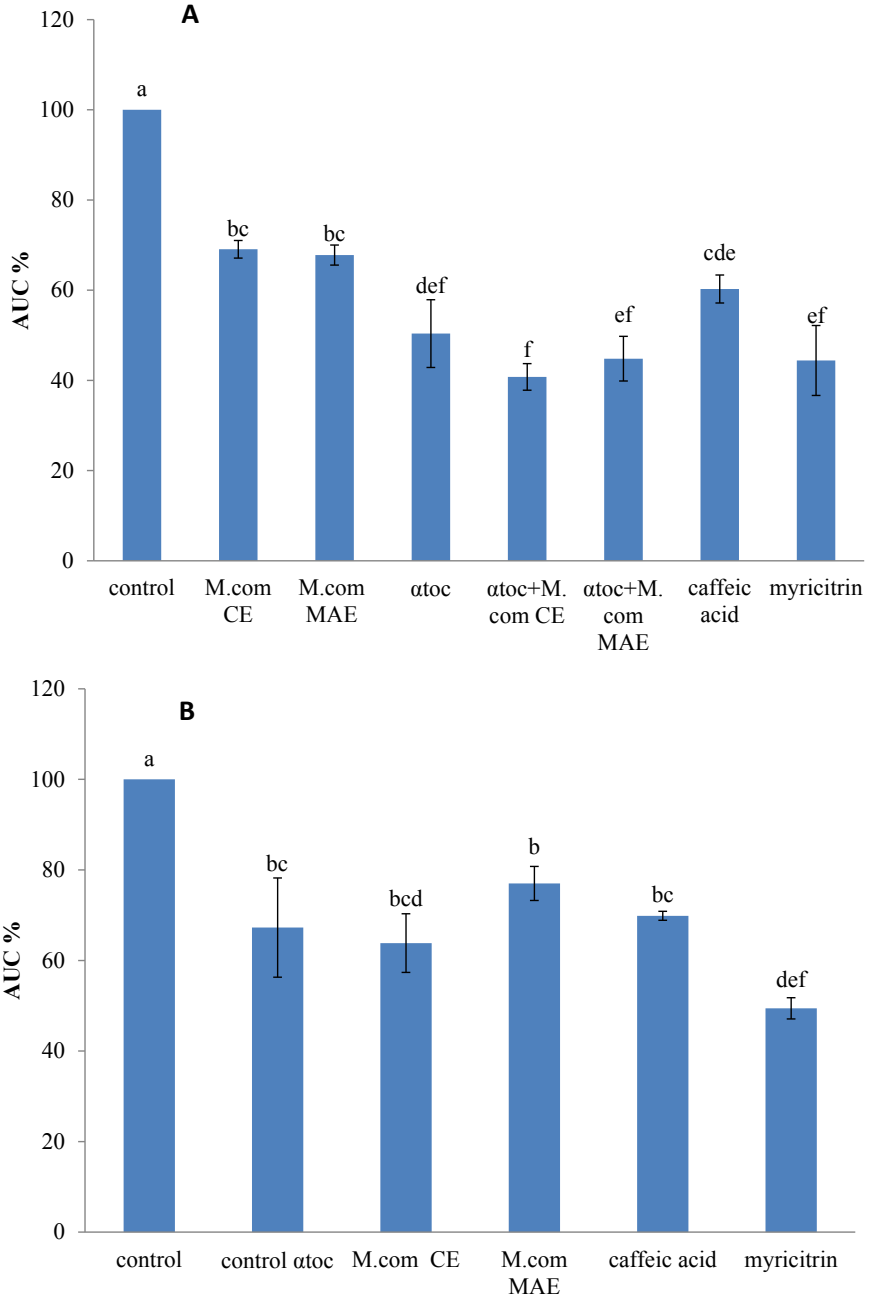

Figure 7–Evaluation of antioxidant activities and synergic or antagonist effects of myrtle extracts and pure compounds (α-tocopherol, caffeic acid, and myricitrin) on the stability of phospholipid aqueous dispersion unenriched (control) (A) and enriched (B) with α-tocopherol (control αtoc), at equivalent concentration (10 μGAE L^{-1} for myrtlePCs, and 10 μmol L^{-1} for pure compounds). AUC% (percentage of AUCs) was the ratio between (AUC^+/AUC^-) x 100, where AUC^+ and AUC^- are the AUC with and without antioxidant, respectively. Results are expressed as means of 3 experiments ± SD. Different letters indicate that samples are significantly different ($P < 0.05$). α-Tocopherol in unenriched (A) and enriched (B) phospholipid dispersion were 3.7 ± 0.9 and 10.6 ± 0.2 μmol L^{-1}, respectively.

extract and α-tocopherol, in our phospholipid aqueous dispersion model. Myricitrin was the best active antioxidant, which may be explained by a better reactivity with AAPH-derived radicals like demonstrated above in the ORAC assay, but also its possible localization near the interface where it could interact with lipid radicals.

These different observations could also explain the difference concerning McPC protective effects observed between our 2 biological models, LDL containing a large variety of lipids (cholesterol, triglycerides and phospholipids + natural α-tocopherol) with an apoproteinB-containing interface and mixed lipid dispersion containing only egg yolk phospholipids/bile salts + natural α-tocopherol. In a previous study, we showed that in oxidized LDL model, caffeic acid or phenolic compounds from extracted red wine or oil palm ripe fruits (Mondé and others 2011) were able to delay the *in vitro* LDL-α-tocopherol oxidation by metal-dependent (Cu^{2+}) or -independent (AAPH) initiation, yielding confirmation of a reducing effect on the oxidized α-tocopherol. This previous observation and this study underline that in the LDL model, McPCs are more efficient than α-tocopherol and a part of them is used to regenerate α-tocopherol.

The chemical assays showed only the ability of myrtle extracts to scavenge free radicals by electron or H atom transfer, and lack a biological significance because the peroxidation occur in a complex system where different ways of antioxidant implication may be explored. This may explained the difference found sometimes between chemical assay results (ORAC test) and lipid model oxidation results. The lipid systems used in simulated physiological conditions revealed different mechanism by which antioxidants may act against lipid peroxidation. The Cu^{+2}-induced LDL peroxidation showed a structure–activity relationship, which may explain the effectiveness of myrtle extracts and myricitrin more than α-tocopherol, which is devoid of a catechol structure. Moreover, McPCs may act synergically with α-tocopherol by regenerating tocopheroxyl radical, during LDL oxidation. In dispersed lipid system, the localization of antioxidants is more important to determine the antioxidant activity. For this, α-tocopherol was a potent antioxidant as well as myrtle extracts. Thus, McPCs possess a potent chelating and free radicals scavenging activities. Moreover, the method of α-tocopherol addition to the lipid dispersion did not affect the antioxidative properties of this lipid soluble antioxidant. Gal and others (2003) who studied the palmitoyllinoleoylphosphatidylcholine (PLPC) liposome oxidation mediated by copper (Cu^{2+}) reported that pro-oxidative effects could be observed when α-tocopherol was added externally to PLPC liposome whereas, when α-tocopherol was co-sonicated with the phospholipids, it acted as an antioxidant. Thus, the types of oxidant used determine the outcome of the antioxidant activity assay. Different interactions between phenol-containing myrtle extract, α-tocopherol, and lipid bilayers may occur (Gutierrez and others 2003). These interactions led to an antagonist effect when these antioxidants were tested together in our conditions. For the antagonism observed with the combination of McPCs and α-tocopherol in liposome model, the potential mechanism remains unclear. According to the work of Yin and others (2012), it may be suppose that this phenomena could be attributed to: (a) the physical barriers between antioxidants and the lipid derived radical; and (b) a process in which antioxidant radicals formed *via* the autoxidation of the less effective antioxidant oxidize the more effective antioxidant, thus preventing the latter to inhibit lipid oxidation. Indeed, the work of Becker and others (2007) showed that the combination of α-tocopherol and quercetin had a synergistic effect only when quercetin was at high concentration and α-tocopherol at low concentration in liposomal phospholipid oxidation. This is another aspect to consider also. The interactions responsible for this effect require further investigations with respect to their biological significance.

Conclusion

This study showed that antioxidant efficacy is dependent of the lipid substrate, and the oxidation conditions used, which may explain the difference found sometimes in antioxidant power classification of the same molecule evaluated in different tests, for example the difference found for myrtle extract scavenging effect in LDL and phospholipid assay. MAE extraction method showed to be an alternative technique to the conventional one giving the same antioxidant activities. Phenolic compounds of *M. communis* leaves possess an effective antioxidant activity toward free radical ($ABTS^{+}$• and ORAC tests), and oxidation induced by Cu^{2+} and AAPH of biological models, LDL and phospholipid/BS aqueous dispersions, respectively. Moreover, it was established in the phospholipid oxidation assay, that none of the compounds, at the tested levels, exerted any antioxidant synergism with α-tocopherol. According to all these data, McPCs could to be a good source of natural antioxidants and health promotion products. Thus, lipid oxidative stress has been recognized as a main component of postprandial digestion process, so McPCs could exert direct *in vivo* beneficial antioxidant effects by protecting dietary polyunsaturated lipids in the intestinal micellar system.

Acknowledgments

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Conflicts of Interest

No conflict of interest in our present study.

Authors' Contributions

K. Madani, M. A. Carbonneau, and J. P. Cristol supervised all the study, analyzed and interpreted the data, and they corrected the manuscript. M. Aoun analyzed FAs composition of phospholipid aqueous dispersion studied and corrected the manuscript. J. Lai Kee Him and P. Bron analyzed by cryo-electron microscopy the lipid structure in the phospholipid dispersion and corrected the manuscript. C. Lauret contributed to vitamin E analyses.

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