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Evaluation of Antioxidant Effect of Different Extracts of *Myrtus communis* L.

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Oxidative stress is involved in the pathogenesis of numerous diseases. Nevertheless, no optimal natural antioxidant has been found for therapeutics, therefore polyphenol antioxidants have been looked for in myrtle leaves, a plant that in folk medicine has been used as anti-inflammatory drug. Antioxidant-rich fractions were prepared from myrtle (*Myrtus communis* L.) leaves liquid–liquid extraction (LLE) with different solvents. All myrtle extracts were very rich in polyphenols. In particular, hydroalcoholic extracts contain galloyl-glucosides, ellagitannins, galloyl-quinic acids and flavonol glycosides; ethylacetate extract and aqueous residues after LLE are enriched in flavonol glycosides and hydrolysable tannins (galloyl-glucosides, ellagitannins, galloyl-quinic acids), respectively. Qualitative and quantitative analysis for the single unidentified compound was also performed. Human LDL exposed to copper ions was used to evaluate the antioxidant activity of the myrtle extracts. Addition of these extracts did not affect the basal oxidation of LDL but dose-dependently decreased the oxidation induced by copper ions. Moreover, the myrtle extracts reduce the formation of conjugated dienes. The antioxidant effect of three myrtle extracts decreased in the following order: hydroalcoholic extracts, ethylacetate and aqueous residues after LLE. The extracts had the following IC_{50}: 0.36, 2.27 and 2.88 μM, when the sum of total phenolic compounds was considered after the correction of molecular weight based on pure compounds. Statistical analysis showed a significant difference among hydroalcoholic extracts vs. the ethylacetate and aqueous residues after LLE. These results suggest that the myrtle extracts have a potent antioxidant activity mainly due to the presence of galloyl derivatives.

Keywords: LDL; Oxidation; *Myrtus communis* L; Hydrolysable tannins; Flavonols

Abbreviations: LDL, low density lipoproteins; LDLox, oxidized low density lipoproteins; HDL, high density lipoproteins; MDA, malonaldehyde; LLE, liquid–liquid extraction; HPLC, high performance liquid chromatography; IC_{50}, concentration that gives 50% of inhibition; DAD, diode array detector

INTRODUCTION

Oxidative stress appears to be involved in the development of several diseases, such as inflammation, diabetes mellitus and atherosclerosis.[1] In this context, there is substantial evidence that oxidized low density lipoproteins (LDLox) play a major role in atherosclerosis.[2] Dietary antioxidants are likely to be important in maintaining the cellular redox status.[3] However, the diverging epidemiological and interventional studies emphasize[4,5] the need for new antioxidant or new antioxidant mixtures.

In the last years, many researchers have focused their attention on flavonoids; these molecules are

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effective antioxidants *in vitro*[6,7] and at higher dietary intakes may reduce the risk of some chronic diseases.[8] More recent data have reported the potential role of green tea, rich in galloyl derivatives, in protection against many diseases[9] including cardiovascular ones.[10] We focused our attention on *Myrtus communis* L. for the presence of galloyl derivatives in the leaf tissues.[11] In addition, this plant has been empirically used for medicinal purposes[12–16] with many indications, including diabetes mellitus, and the myrtle hydroalcoholic extract has a hypoglycaemic effect in streptozotocin-induced diabetes.[17] Furthermore, the ethanol extract of the leaves has some anti-inflammatory activity when examined with carrageenan test in rats.[18] Finally, the aqueous extract of the leaves has antibacterial activity.[19] The presence of galloyl derivatives in myrtle leaf tissues[11] encourages better identification, characterisation and quantification of the *Myrtus communis* L. polyphenols and testing of their antioxidant activity. Thus, different extracts, containing various amounts of polyphenol classes or individual polyphenolic compounds, were prepared and evaluated for their antioxidant activity.

MATERIAL AND METHODS

Materials

Gallic acid, myricetin 3-*O*-rhamnoside (myricitrin) and rutin were purchased from Extrasynthese S.A. (Lyon, Nord-Genay, France). All other reagents were of analytical grade and were purchased from Sigma (St Louis, MO, USA). All solvents were HPLC grade and were obtained from E. Merck, (Darmstadt, Germany). Myricetin-3-*O*-galactoside and 3,5 di-*O*-galloyl-quinic acid were isolated by HPLC preparative chromatography as previously reported.[20]

Preparation of *Myrtus communis* L. Extracts

The myrtle leaves, collected in southern Tuscany, were processed as previously described.[11] Briefly, the lyophilised and homogenised leaf tissue was used for extraction in 70% ethanol (pH 2); the extract was then concentrated under vacuum (Rotavapor 144 R, Büchi, Switzerland) and rinsed with ultra pure (MilliQ system, Waters Co. Milford, MA, USA) acid water adjusted to pH 2 with formic acid. It was then completely defatted with *n*-hexane and concentrated under vacuum. A part was rinsed with ethanol 70% (pH 2) for the preparation of hydroalcoholic extract. The remnant was fractionated by liquid–liquid extraction (LLE) with ethylacetate to significantly increase the extraction of flavonols and low molecular-weight phenolic compounds;[21] it was then dried under vacuum and dissolved in ethanol 70% (EtOAc from LLE). In addition, the water residue of LLE was lyophilised and dissolved in ethanol 70% (aqueous residue after LLE).

The lyophilised extracts were stored at $-20°C$ until use and remained stable for at least twelve months.

HPLC/DAD and HPLC/MS Analysis

The analysis was conducted using a HP-1100 liquid chromatograph equipped with a DAD detector and a HP 1100 MSD API-electrospray (Agilent Technologies, Palo Alto, USA) operating in positive and negative ionisation mode. Analytical conditions have previously been described.[11] Identification of individual polyphenols was carried out using their retention times, and both spectroscopic and spectrometric data. Quantitation of the single polyphenol was directly performed by HPLC-DAD using a four-point regression curve built with the available standards or isolated compounds. Curves with an $r^2 > 0.9998$ were considered. Calibration was performed at the wavelength of maximum UV-Vis absorbance applying the correction for molecular weight. In particular, galloyl-glucosides, ellagitannins and galloyl-quinic acids amounts were calculated at 280 nm using gallic acid and 3,5 di-*O*-galloyl-quinic acid as reference, respectively. Myricetin 3-(6″-*O*-galloyl-galactoside), myricetin 3-*O*-galactoside, myricetin 3-*O*-rhamnoside (myricitrin) and the other unidentified myricetin glycosides were calibrated at 350 nm using myricitrin as reference. Finally, quercetin glycosides were calibrated at 350 nm using quercetin 3-*O*-rutinoside (rutin) as reference. For example, in the case of myricetin derivatives, knowing the molecular weight of each compound (PM_M), its actual concentration was obtained applying a multiplication factor of $PM_M/464$, where 464 is the molecular weight of myricitrin.

Subjects

Twenty-eight healthy volunteers (50 ± 10 years), well matched for sex, gave informed consent to participate in the study. Individuals who reported to be non-smokers were included. People with values of total cholesterol, LDL, high density lipoproteins (HDL) and triglycerides above and below the standard values of the laboratory were excluded. None of the subjects had a family history of diabetes, hypertension, and dyslipidemia. They remained free of drugs, vitamins, amino acids, hormones, dietary supplements and

botanical remedies for fourteen days, including the day before the experiment. All the subjects consume a typical Mediterranean diet.

LDL Isolation

In the morning after 12 h of fasting, 40 ml of blood were collected by venipuncture into EDTA (1 g/l) and centrifuged immediately for 10 min at 2000*g* at 4° C. Plasma was used for LDL isolation in a TL-100 (Beckman Palo Alto, CA, USA) tabletop ultracentrifuge, according to Ref. [22] with minor modifications. To protect the LDL against oxidative alterations during ultracentrifugation, each density solution contained EDTA. Then, the samples were exhaustively dialysed in PD-10 desalting columns (Amersham Pharmacia Biotech Uppsala, Sweden) to remove the excess salt and most of the EDTA. The LDL were then immediately used.

Biochemical Determinations

Total cholesterol, LDL, HDL and triglycerides were measured as described in Franconi *et al.*[23] The protein concentration of LDL was determined according to Bradford[24] using bovine serum albumin as standard and LDL was diluted to 50 μg protein/ml. Oxidation reaction was initiated adding freshly prepared 5 μM $CuSO_4$ (final concentration). The oxidation of LDL was quantified in triplicate by thiobarbituric acid method[25] using malonaldehyde-bisdiacetale as a standard. The MDA was measured in basal condition and after 8 h of exposure to copper ions, in presence and in absence of different concentrations of myrtle extracts or pure compounds, which were added before copper ions. The conjugated diene formation was monitored at 234 nm at 37°C as described in Esterbauer *et al.*[26] The antioxidant capacity of the tested compounds was determined by continuously monitoring the kinetics of diene formation using the method originally described in Esterbauer *et al.*[26] Data derived from conjugated diene curve were expressed as time-course curve.

Statistical Analysis

The IC_{50} were calculated by Graph Pad Prism 3.02. Statistical analysis was performed with the same program and statistical significance was accepted after ANOVA followed by Turkey's multiple range test. Difference were considered statistically significant at $P < 0.05$.

RESULTS

Identification and Characterisation Polyphenols in the *Myrtus communis* L. Extracts

Hydroalcoholic extract, EtOAc from LLE and aqueous residue after LLE were analysed by HPLC/DAD and HPLC/MS. The chromatographic profile of the hydroalcoholic extract is illustrated in Fig. 1 and the following compounds were identified: gallic acid, 5-*O*-galloyl-quinic acid, 3,5-di-*O*-galloyl-quinic acid and the flavonols myricitrin, myricetin 3-(6″-*O*-galloylgalactoside), myricetin 3-*O*-galactoside, quercetin galloylgalactoside and quercitrin. The EtOAc and aqueous residues from LLE chromatographic profiles

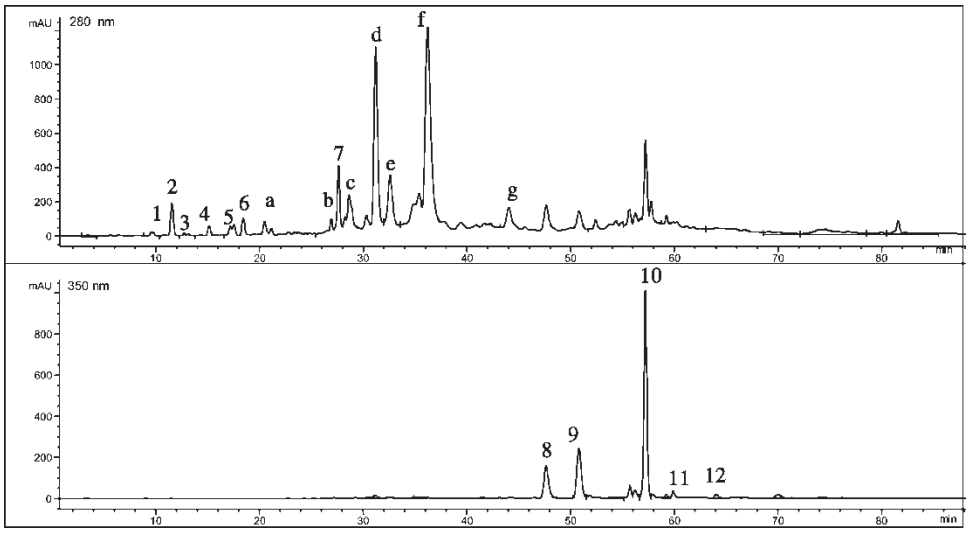

FIGURE 1 Chromatographic profile, acquired at 280 and 350 nm, of the hydro-alcoholic myrtle leaf extract: (1) Galloyl glucoside; (2) Gallic acid; (3) Digalloyl glucoside; (4) 5-*O*-galloylquinic acid (theogallin); (5) Galloylquinic acid; (6) Digalloyl glucoside; (7) 3,5-*O*-digalloylquinic acid; (8) Myricetin 3-(6″-*O*-galloylgalactoside); (9) Myricetin 3-*O*-galactoside; (10) Myricitrin; (11) Quercetin galloylgalactoside; (12) Quercitrin; a–g. Galloyl glucosides (ellagitannins).

TABLE I Quantitative analysis (expressed as mg/ml) of single identified compounds present in *Myrtus communis* L. leaf extracts

Extracts	Polyphenolic classes	Compounds	mg/ml
Hy	Galloyl derivatives	Gallic acid	0.259
		Mono, di-galloyl glucosides and ellagitannins	10.06
		5-*O*-galloyl quinic ac.	Traces
		3,5-*O*-di-galloyl quinic ac.	0.64
	Flavonols	Myricitrin	0.91
		Myricetin 3-*O*-galactoside	0.47
		Myricetin 3-(6″-*O*-galloylgalactoside)	0.33
		Myricetin glycosides	0.06
		Quercitrin	0.02
EtOAc	Galloyl derivatives	Gallic acid	0.73
		Mono, di-galloyl glucosides and ellagitannins	5.92
		5-*O*-galloyl quinic ac.	Traces
		3,5-*O*-di-galloyl quinic ac.	1.49
	Flavonols	Myricitrin	2.83
		Myricetin 3-*O*-galactoside	1.54
		Myricetin 3-(6″-*O*-galloylgalactoside)	1.07
		Myricetin glycosides	0.23
		Quercitrin	0.07
AR	Galloyl derivatives	Mono, di-galloyl glucosides and ellagitannins	0.30
	Flavonols	Myricitrin	0.001

Values are mean of three samples each one performed in triplicate. Standard error was omitted and it ranged among 1–3%. Hydroalcoholic extract (Hy), Ethylacetate from LLE (EtOAc), Aqueous Residue after LLE (AR). Galloyl derivatives including galloyl-glucosides, ellagitannins and galloyl-quinic acids.

showed a significant increase in flavonols and hydrolysable tannins, respectively. Further investigations are necessary to completely elucidate the galloyl-glucosides and ellagitannin structures, which were tentatively established by their UV and MS spectra. In particular for ellagitannins, the registered quasi-molecular ions $[M-H]^-$ in the mass spectra were 935, 766, 786 and 1568 m/z, probably corresponding to monomers and a dimer macrocyclic compounds, as previously reported in *Myrtaceae*.[27]

Tables I and II shows the quali-quantitative analyses of different myrtle samples. Hydroalcoholic extract was very rich in hydrolysable tannins and relatively rich in myricetin derivatives (Table I). In the EtOAc from LLE, the most abundant compounds were myricetin derivatives (Table I), while the aqueous residue after LLE mainly consisted of galloyl-glucosides and ellagitannins (99.64%), and trace amounts of myricitrin (0.36%). Finally, 3,5-di-*O*-galloyl-quinic acid was the most abundant compound among galloyl-quinic derivatives in the hydroalcoholic and the EtOAc from LLE extracts. The total polyphenol content of the analysed extracts ranged from 0.92 to 36.81 mM, being the hydroalcoholic one the richest (Table II).

LDL Test

As previously shown,[28] the exposure of LDL to copper ions produced a dramatic increase in MDA from 6.1 ± 0.7 to 108.6 ± 3.7 nmoles/mg protein ($P < 0.001$) ($n = 28$). Pilot experiments performed with extracts of myrtle leaves, pure standards and isolated compounds showed that they did not influence the MDA content in basal LDL (data not shown). Table III reports the IC_{50} of pure compounds. Although the IC_{50} for all the tested pure compounds were within a narrow concentration range, 3,5-di-*O*-galloyl-quinic acid was 1.7, 2 and 9 times more effective than rutin, myricitrin and gallic acid, respectively, in inhibiting the LDL oxidation. In addition, rutin was more active than myricitrin and

TABLE II Main polyphenolic classes in *Myrtus communis* L. leaf extracts

Polyphenolic classes	Hy (mM)	EtOAc (mM)	AR (mM)
Galloyl-glucosides and ellagitannins	30.300	1.890	0.917
Galloyl-quinic acids	2.860	4.160	nf
Myricetin glycosides	3.610	7.240	0.003
Quercetin glycosides	0.040	0.070	nf
Total	36.810	13.360	0.920

Data reported are the mean of three determination each one performed in triplicate. SE was in the range 1–3%. nf, not found; Hy, Hydroalcoholic extract; EtOAc, Ethylacetate from LLE; AR, Aqueous residue after LLE.

TABLE III IC_{50} of pure compounds measured on MDA production induced by copper ions in human isolated LDL

Pure compounds	IC_{50} (μM)
Gallic acid	20.0 ± 1.8
3,5-di-*O*-galloylquinic ac.	2.2 ± 0.1
Myricitrin	7.8 ± 0.9
Rutin	3.7 ± 0.2

IC_{50} values are means ± SE of at least seven independent experiments each one performed in duplicate.

gallic acid. Using the quantitative data shown in Table I the IC_{50} of extracts was calculated. When, the IC_{50} was calculated, considering the total polyphenol composition, total hydroalcoholic extract is the most active in inhibiting LDL oxidation (0.36 μM, $n = 7$), while, the aqueous residues after LLE and ethylacetate from LLE have 2.88 ($n = 7$) and 2.27 ($n = 8$) μM, respectively. The IC_{50} of total hydroalcoholic extract was significantly lower ($P < 0.001$) than those found for the other two extracts.

Furthermore, in order to evaluate the reciprocal influence of single classes present in different extracts, the activities of the single polyphenol subclasses were also compared. In the total hydroalcoholic extract the hydrolysable tannins (galloyl-glucosides and ellagitannins) and galloyl-quinic acids, myricetin and quercetin glycosides had IC_{50} values of 0.41, 0.04, 0.05 and 0.001 μM, respectively. In the ethylacetate extract from LLE, hydrolysable tannins, galloyl-quinic acids, myricetin and quercetin glycosides had IC_{50} values of 0.32, 0.71, 1.23 and 0.01 μM, respectively. Finally, in the aqueous residues after LLE, the hydrolysable tannins and myricitrin had IC_{50} values of 2.79 and 0.01 μM, respectively.

The measurable LDL conjugated-diene formation did not occur in the absence of cupric ions (data not shown). The time profile of LDL oxidized with cupric ions showed a lag phase which absorption does not increase slightly followed by a propagation phase during which the absorbance rapidly increase. When LDL was oxidized with cupric ions a short lag time (45.2 ± 6.2 min; $n = 5$) was observed. Relative to LDL oxidized in the presence of myrtle extracts they had a lag time curves that were shifted to the right (Fig. 2). The lag time was 102, 165, and 224 min with ethylacetate extract from LLE, aqueous residues after LLE and hydroalcoholic extrats, respectively. The hydroalcolic extract was the most effective followed by ethylacetate extract from LLE.

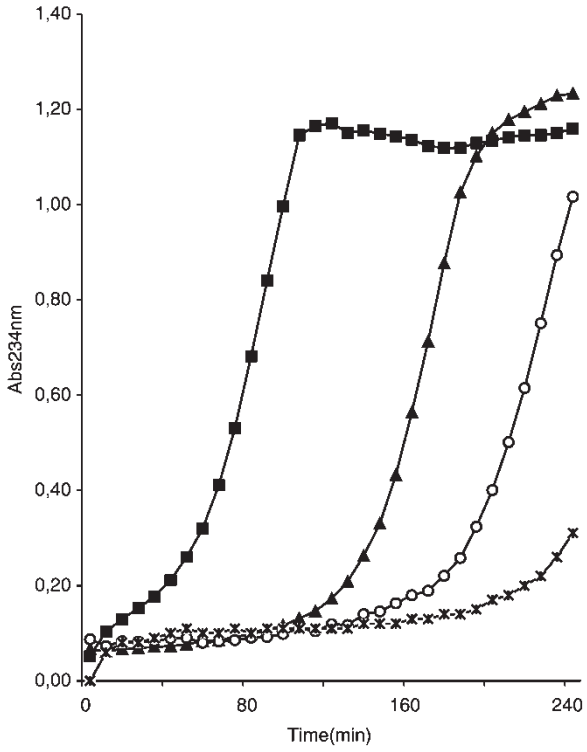

FIGURE 2 Representative curves of diene formation induced by cupric ions (■) and of the antioxidant effect of hydroalcolic extract (*), EtOAc (▲), aqueous residues after LLE (○) on lag time until the onset of LDL oxidation measured at 234 nm in isolated human LDL.

DISCUSSION

Since modern chemical and pharmacological methods were first used to study traditional molecules there has been a rapid increase in the number of known natural compounds. Among them, polyphenols including hydrolysable tannins and flavonoids have received increased attention because of some new findings regarding their biological activities. In particular, the biological activities of tannins include anti-cancer, antiviral, inhibition of lipid peroxidation,[29] whereas flavonoids exhibit anti-inflammatory activities, as well as protective effect vs. cardiovascular diseases. The *Myrtus communis* L. leaves are characterized by the presence of flavonols (myricetin and quercetin glycosides) and galloyl derivatives, which include galloyl-glucosides, ellagitannins and galloyl-quinic acids. Thus, myrtle leaves contain different polyphenolic classes as previously described by Romani *et al.*[11] In particular, the present paper offers qualitative and quantitative details of polyphenol composition. Interestingly, some of the main myrtle polyphenols are also found in green tea.[30] Recently, the potential role of green tea in protection against many diseases has been reported.[9] However, a caution should be applied to the use of high concentrations of tea for disease prevention because in rats a goitrogenic effect of green tea extract has been found[31] and recently it has been evidenced that caffeine can decrease insulin sensitivity in humans.[32]

All extracts inhibit the lipid peroxidation and diene-formation induced by copper ions in human LDL. The effect on MDA is fairly dose-dependent within a very low concentration range. Using different methodology of extraction, we prepared different mixtures, which differ either for the whole polyphenolic content or individual polyphenols. Among the various extracts, the hydroalcoholic one, which is enriched in hydrolysable tannins, is the most active. This finding is consistent with data of Dufour and co-workers.[33] The EtOAc from LLE is enriched in flavonol glycosides, while the aqueous

TABLE IV IC_{50} (μM) of three different myrtle extracts measured on MDA production induced by copper ions in human isolated LDL

	Hy	EtOAc	AR
Galloyl-glucosides and ellagitannins	0.41	0.32	2.79
Galloyl-quinic derivatives	0.04	0.71	
Myricetin glycosides	0.05	1.23	0.01
Quercetin glycosides	0.001	0.01	

IC_{50} values are means ± SE of at least seven independent experiments each one performed in duplicate. SE was omitted and ranged among 9–15%. IC_{50} was calculated considering the total polyphenol concentration and the concentration of the single compound present in each extract (see Table I).

extract after LLE contains almost exclusively galloyl-glucosides and ellagitannins (99.64%), thus it may be considered as a single subclass (Table IV).

When we compare the IC_{50} found in the extracts with those of standards or isolated compounds, such as 3,5-di-*O*-galloylquinic acid, we find that the co-presence of different polyphenols increases the antioxidant activity. In fact, the IC_{50} of aqueous extract after LLE, which may be considered as a pure compound, is similar to that of 3,5 di-*O*-galloyl-quinic acid, which has been isolated and used as representative standard.[20]

When the ratio between galloyl derivatives and flavonols is about 1:1, such as in EtOAc extract from LLE, the IC_{50} is very similar to that of the hydrolysable tannin 3,5 di-*O*-galloyl-quinic acid. Apparently, these data indicate that in the presence of hydrolysable tannins, flavonols do not play a crucial role in inhibiting LDL oxidation. Consequently, when the galloyl derivatives and flavonols ratio is about 9:1 (see total hydroalcoholic extract) the IC_{50} is significantly lower than in the two previous extracts. As illustrated in Table III, the IC_{50} is not only influenced by the ratio between the sum of galloyl-glucosides and ellagitannins vs. flavonols, but also by the ratio between these galloyl derivatives vs. galloyl-quinic acids. The IC_{50} of galloyl-quinic derivatives is coherent with those found in a recent papers[34] which also showed that the antioxidant activity depends on the presence of galloyl substituents as well. These findings strongly suggest that the antioxidant activity of leaves is not only influenced by the polyphenolic subclasses but also by their ratio.

However, the difference in activities could be also due to pro-oxidant effects of polyphenols. At this regard pyrogallols are considerably more active than catechols as pro-oxidant. In fact, rutin which contains a catecholic ring-B is more active than myricitrin which involves a pyrogallolic group, and quercetin glycosides are much more active than the myricetin counterparts to prevent LDL oxidation.

A number of diseases are directly or indirectly linked to reactive oxygen species,[1–3] such as stroke, ischemia/reperfusion, cancerogenesis, atherosclerosis, inflammation. In this regard, it is interesting to note that consumption of food rich in flavonoids has been associated with a reduction in the risk of cancer and cardiovascular disease,[8] probably through their antioxidant effect. Interestingly, the catechol group present in flavanols and hydrolysable tannins confer a hydrophilic character to these molecules and support an antioxidant effect in aqueous domains as well.[35] These compounds can also prevent lipid peroxidation caused by initiators that act in the hydrophobic region,[35] being active both in hydro and lipid domains, and thus could be useful in disease prevention and treatment.

In conclusion, myrtle leaves are particularly rich in galloyl-glucosides, ellagitannins and galloyl-quinic acids, and to our knowledge, they have not been extensively investigated until now. Moreover, the leaf extracts have a potent antioxidant activity, which depends on chemical composition and ratio among the single phenolic subclasses. Finally, these data suggest that extracts of myrtle leaves or pure compounds present in this plant may potentially have the same effect both in inflammation and diseases associated with oxidative stress.

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