Contents List available at JACS Directory

Journal of Natural Products and Resources

journal homepage: http://www.jacsdirectory.com/jnpr

Phenolic Compounds in Extracts from *Eucalyptus globulus* Leaves and *Calendula officinalis* Flowers

C.d. Santos Ferreira[1], A. Pereyra[2], A. Patriarca[1,3], M.F. Mazzobre[1,3], T. Polak[4], V. Abram[4], M.P. Buera[1,3], N. Poklar Ulrih[4,*]

[1]Departamentos de Industrias y de Química Orgánica, Universidad de Buenos Aires, Argentina.
[2]Medex, d.o.o., Linhartova 49, 1000 Ljubljana, Slovenia.
[3]National Council of Scientific and Technical Research (CONICET), Buenos Aires, Argentina.
[4]Biotechnical Faculty, University of Ljubljana, Jamnikarjeva 101, 1000 Ljubljana, Slovenia.

ARTICLE DETAILS

ABSTRACT

Selection of the optimal solvent system for extraction of the phenolics from *Eucalyptus globules* leaves and *Calendula officinalis* flowers, determination of the reducing potential and identification of the phenolics in these extracts was performed. The highest content of phenolics was obtained for methanol: water extracts from both sources. All of the *Eucalyptus* leaf extracts had higher reducing potential than those from the *Calendula* flowers. Solid-phase purification of the crude extracts removed 57% to 78% of the compounds in the crude extracts. The reducing potential of the purified extracts varied from 0.17 to 2.92 mg caffeic acid/g dry weight. The extracts from *Eucalyptus* leaves and *Calendula* flowers both contained chlorogenic acid, rutin and quercetin 3-glucuronide. Ellagic acid derivatives were identified only in the leaves of *Eucalyptus*, while beside caffeic acid and salicylic acid, quercetin 3-glucuronide, and pinobanksin 3-acetate was found in the *Calendula* flower extract for the first time.

1. Introduction

The quest for natural antioxidants from herbs, species, fruits and vegetables for dietary, cosmetic and pharmaceutical uses has become a major industrial and scientific research challenge over the last two decades. Many efforts have been devoted towards gaining more extensive knowledge regarding their antioxidant activities and potential applications [1, 2]. Extracts from such natural materials contain diverse classes of secondary metabolites, such as phenolic compounds (phenolics), terpenoids and alkaloids [3].

Eucalyptus globulus belongs to the Mirtaceae family, and it is a very common tree throughout the world. Its leaves, bark and fruit have been used as topical formulations in China as traditional remedies for treatment of inflammation and healing of wounds. In South America and Africa, tea prepared from its leaves has been used for symptomatic treatment of respiratory tract and throat diseases, and as a medication for diabetes [4]. Parts of the eucalyptus are known as a rich source of biologically active terpenoids, tannins, flavonoids and phloroglucinol derivatives [5]. Antioxidant activity has also been reported for the essential oils and wood and bark from *E. globulus* [6].

Calendula officinalis, or marigold, belongs to the Asteraceae family, and it is an herb of ancient medicinal repute. It grows both as a wild plant and as a common garden plant throughout Europe and America. In traditional and homeopathic medicine, it has been used for skin complaints, wounds, burns, conjunctivitis, poor eyesight, menstrual irregularities, varicose veins, haemorrhoids, duodenal ulcers, and others [7]. The yellow or golden-orange *Calendula* flowers are normally used as a food additive, to confer both colour and flavour to foods [8]. These flowers can be used as fresh or dried, and can be made into tea or used as a spice, tincture, ointment or cream. The pharmacological activity of *Calendula* is related to the content of several classes of secondary metabolites, such as flavonoids, sterols, carotenoids, tannins, saponins, triterpene alcohols, a bitter principle, mucilage and resin [9].

Phenolics are antioxidants, and there are numerous different antioxidant assays that have been used to demonstrate their antioxidant activity. Recently, Foti reviewed and discussed the uses and abuses of one of the most widely used assays for antioxidant activity – the 2,2-diphenylpicrylhydrazyl (DPPH) assay [10]. The final conclusion of Foti was that the DPPH assay is not appropriate to be considered as an antioxidant assay, due to the difference between the peroxyl radicals formed in cells and the DPPH radicals used in the assay, and due to large effects of polar solvents on the rate and mechanism of H-atom transfer from H-donor compounds (e.g. phenolics, which are excellent H-donors through their functional groups rich in electrons) to DPPH radicals. Here, the data from HPLC with electrochemical detection and DPPH assays are presented, to define the reducing potential of the extracts.

Eucalyptus and *Calendula* are two of the most commonly used plants for medical and cosmetic purposes, although they are known to have completely different activity spectra. Their biological activities have been mainly related to their essential oils. However, phenolics present in polar solvent extracts from *Eucalyptus* and *Calendula* have been less characterised in terms of their antioxidant properties. The aim of the present study was to determine inexpensive yet effective solvent(s) for the extraction of the phenolics from *Eucalyptus* leaves and *Calendula* flowers, so as to develop mixtures of these extracts that have high antioxidant activity, but different biological effects from these two natural sources.

2. Experimental Methods

2.1 Plant Materials and Phenolic Standards

Dry *Eucalyptus* (*Eucalyptus globulus*) leaves and marigold (*Calendula officinalis*) flowers were from a local herbal drugstore in Buenos Aires, Argentina. Gallic acid (G7384), chlorogenic acid (C3878), caffeic acid (C0625), *p*-coumaric acid (C9008), ellagic acid (E2250), luteolin (L9283), pinocembrin (P5239), quercetin (Q0125), myricetin (M6760), and galangin (282200) were from Sigma-Aldrich (Steinheim, Germany), and apigenin (10798), chrysin (27214), and kaempferol (600109) were from Fluka AG (Buchs, Switzerland). For the HPLC and HPLC–tandem mass spectrometry (MS/MS) analysis, acetonitrile, methanol (both HPLC grade), ethanol, and formic acid were from Merck (Darmstadt, Germany), and ammonium formate was from Fluka. Ultrapure water was from a Milli-Q apparatus (Millipore S.A., France).

*Corresponding Author
Email Address:* natasa.poklar@bf.uni-lj.si(Nataša Poklar Ulrih)

2.2 Extraction

Dry *Eucalyptus* leaves (200 g) or dry *Calendula* flowers (250 g) were mixed with 2.5 L chloroform, ethanol, methanol or methanol: water (70:30; v/v), agitated using a shaker for 8 h at room temperature, and then filtered (N° 389; Sartorius AG, Göttingen, Germany). The remaining material was rinsed with the corresponding solvent. The filtrates were concentrated under reduced pressure at 40 °C in a rotary evaporator (Rotavapor R 210; Buechi, Germany) and lyophilised in a freeze dryer (Alpha 1-2 LD *plus*; Christ GmbH, Germany) for 48 h. The freeze-dried samples were stored over silica gel and under nitrogen in a desiccator, and in the dark (covered with aluminium foil to avoid light), due to the presence of light-sensitive compounds [11].

2.3 Solid-Phase Extraction

Solid-phase extraction (SPE) was used as a purification step, with Strata-X, 33 µm, SPE cartridges (6 mL, 200 mg sorbent) from Phenomenex (Torrance, CA, USA), and a 12-port Visiprep SPE vacuum manifold (Supelco, St. Louis, MO, USA). Syringe filters (13 mm nylon membrane, 0.22 µm) were from Restek (Bellefonte, USA).

The freeze-dried extracts (~50 mg) were dissolved in 4 mL 25% methanol in 20 mmol/L ammonium formate (pH 2.4), and then loaded onto the SPE cartridges that had previously been conditioned with 2 mL methanol and 2 mL of 20 mmol/L ammonium formate. The extract was allowed to pass through the cartridges, and the cartridges were washed with 2 mL of 15% methanol in 20 mmol/L ammonium formate and vacuum-dried for 3 min. Finally, the retained phenolics were eluted with 2 mL methanol. These purified extracts were dried and redissolved in 1% aqueous formic acid:methanol (1:1; v/v), filtered (0.22 µm nylon filters) and analysed by HPLC with electrochemical detection (HPLC-ED), and by liquid chromatography (LC)-MS/MS.

2.4 Total Phenolics

2.4.1 Spectrophotometric Analysis

UV-Vis spectra of the crude extracts from *Eucalyptus* leaves and *Calendula* flowers were obtained from 200 nm to 800 nm using a UV-Vis spectrophotometer (Cary 100 Bio; Varian, Walnut Creek, CA, USA). An aliquot of each crude extract (1 mL) was centrifuged at 1,000× g for 10 min; the supernatant was 50-, 100- and 200-fold diluted before analysis.

2.4.2 Folin–Ciocalteu Method

The total phenolics in the extracts were determined using the Folin–Ciocalteu method [12]. An aliquot of 200 µL Folin–Ciocalteu reagent was diluted in water (1:3; v/v), and added to 200 µL SPE-purified extract along with 125 µL 20% Na_2CO_3 solution. The mixture was centrifuged at 13,800× g for 10 min (5415 C; Eppendorf, Hamburg, Germany), and the absorbance of the resulting blue-coloured solution was measured at 765 nm after 40 min, against a blank containing the corresponding solvent. All of the determinations were repeated in triplicate. Gallic acid (GA) was used as the standard, with the concentrations of the total phenolics expressed as mg GA/g dry weight (dw) plant material, and the data are means ±standard deviation.

2.5 Reducing Potential

2.5.1 HPLC with Electrochemical Detection

The freeze-dried SPE-purified extracts (as described in section 2.3) were analysed using HPLC-ED. The system consisted of an HPLC system (Waters Alliance 2690; Waters, Corp., Milford, MA, USA) with an electrochemical detector (Coulochem III, ESA Inc., Chelmsford, MA, USA), in which the electrical current from the oxidation or reduction reactions of the electrochemically active substances that were eluted from the HPLC column were measured.

The HPLC conditions were as follows: column, Purospher Star LiChrocart RP-18 (150 mm × 4.6 mm, 5 µm; Merck, Darmstadt, Germany); column temperature, 25 °C; flow rate, 0.2 mL/min; and injection volume, 5 µL. The mobile phases were 1% formic acid in water (solvent A) and acetonitrile (solvent B), with the gradient as follows (%A in B): 0-5 min, 70%-60%; 5-15 min, 60%; 15-20 min, 60%-35%; 20-25 min, 35%; 25-30 min, 35%-70%; 30-35 min, 70%. The electrochemical detector consisted of two cells in series, with E1 set at 200 mV, E2 at 900 mV, and the guard potential at 950 mV. The electrodes were connected to an electronic circuit with an amplifier that improved the signal for the data acquisition. The reducing potential of the extracts was measured in triplicate and is expressed as mg caffeic acid (CA)/g dw plant material, according to the calibration curve with CA (correlation coefficient, 0.9951).

2.5.2 Kinetics of DPPH Assay with Crude Extracts

The DPPH assay is based on electron or hydrogen atom transfer from an antioxidant to the radical oxidant 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), which is characterised as a stable free radical. When the violet-coloured solution of DPPH• is mixed with an antioxidant, the reduced compound, $DPPH_2$, is formed. The scavenging of DPPH• is accompanied by the loss of the violet colour and the decrease in absorbance can be monitored during the reaction [13, 14].

A stock solution of 71 µM DPPH in ethanol was diluted to an absorbance at 515 nm of around 0.90. The lyophilised crude extracts (10 mg) were resuspended in 100 mL water, centrifuged at 13,000× *g* (Rotanta 460R; Hettich, Germany) for 30 min, and filtered (0.45 µm; Sartorius AG, Germany). Several dilutions of the crude extracts (from 50-fold to 200-fold) were prepared in methanol: water (70:30; v/v). Then 5 µL of each diluted extract and 195 µL DPPH reagent were mixed in a 96-well microplate well. The scavenging of the DPPH• radical was followed in a microplate reader (Tecan-Safire; MTX Micro systems Inc., USA), at 515 nm for 96 min, at 2-min intervals.

2.5.3 DPPH Assay of Purified Extracts

The reducing potential of the purified freeze-dried extracts was determined using the DPPH assay in a UV-Vis spectrophotometer (Cary 100 Bio; Varian, USA) at 515 nm after 30 min and 60 min, and using GA for the preparation of the calibration curve [15]. For the DPPH assay, 1 mg freeze-dried extract was dissolved in 1 mL methanol: water (70:30; v/v). When necessary, the samples were diluted with the solvent. Then 25 µL of each sample in solution was mixed with 975 µL DPPH reagent, and the absorbance at 515 nm was measured after 30 min and 60 min, using ethanol as the blank. The antioxidant activities of the samples (AA_{DPPH}) at time t were calculated as in Equation (1):

$$AA_{DPPH} = ((A_0 - A_t) / A_0)*100 \quad (1)$$

The data are expressed as AA_{DPPH} (%) of DPPH inhibition per 1 g dry plant material. All of the analyses were performed three times, and the means ±standard deviations were calculated.

2.6 Identification of Phenolics

The LC-MS/MS system included a binary pump (model G1312A, Agilent 1100) and an autosampler (model G1330B; Agilent Technologies, Inc., USA). Reversed-phase HPLC separation was carried out using a C18 column (150 mm × 2.0 mm ID; 3 µm; Gemini; Phenomenex, Torrance, USA) protected by a C18 security guard cartridge, (4.0 mm × 2.0 mm ID; Gemini). The mass spectrometer (Micromass Quattro Micro; Waters, USA) equipped with an electrospray ionisation source was operated in negative ion mode. The mobile phase components were 1% formic acid in water (solvent A) and acetonitrile (solvent B). A mobile-phase gradient was used as follows (%A in B): 0-5 min: 90%; 5-50 min: 90%-40%; 50-52 min: 40%-20%; 52-60 min: 20%; 60-70 min: 20%-90%; 70-80 min: 90%. The column was maintained at 25 °C, with injection volume of 20 µL and flow rate of 0.2 mL/min. The mass spectra were recorded using the following operating parameters: capillary voltage, 3.0 kV, cone voltage, 25 V; and extractor voltage, 5 V. The source temperature was 100 °C and the desolvation temperature was 350 °C. The cone gas flow was set at 50 L/h, and the desolvation gas flow at 400 L/h.

3. Results and Discussion

3.1 Extraction and Purification

Four solvents with different dielectric constants were used for the extraction of the phenolics from the dried *Eucalyptus* leaves and the dried *Calendula* flowers: chloroform, ethanol, methanol, and methanol: water (70:30; v/v). The content of extractable compounds is expressed as percentages calculated per 100 g dw of each plant material (i.e., % dw). The data for the crude extracts and the extracts purified by SPE are presented in Table 1. Methanol and methanol: water (70:30; v/v) have the highest dielectric constants, and these two solvents generally provided the highest amounts of dry extracts from both the dried *Eucalyptus* leaves and the dried *Calendula* flowers. Chloroform has the lowest dielectric constant among these solvents, and the crude extracts from the dried *Eucalyptus* leaves contained around 10%, and from *Calendula* flowers around 4%, of extractable compounds. For all of the samples, the SPE purification removed from 57% to 78% of the crude extracted material (Table 1). The loss was mainly due to the removal of polar non-phenolics, low-molecular-weight compounds such as sugars, and organic acids [16].

Table 1 Dielectric constants of solvents tested, and content of dried extractable compounds in the crude and purified extracts from *Eucalyptus* leaves and *Calendula* flowers

Solvent	Dielectric constant	Extractable compounds (% dw)			
		Eucalyptus leaves		*Calendula* flowers	
		Crude	Purified	Crude	Purified
Chloroform	4.81*	9.7	8.1	4.4	0.5
Ethanol	24.30**	8.8	2.5	7.7	1.3
Methanol	32.63**	25.3	3.6	17.0	4.4
Methanol: water (70:30; v/v)	47.11***	21.7	9.3	25.9	5.6

*Data are means: *, at 20 °C [17];**, at 25 °C [17]; ***, at 25 °C [18]*

3.2 Spectrophotometric Analysis

Fig. 1 shows the UV-Vis spectra from 200 nm to 450 nm for the crude methanol: water (70:30) extracts prepared from the dried *Eucalyptus* leaves (21.7% dw extract) and *Calendula* flowers (25.9% dw extract). It can be seen that the *Eucalyptus* leaf crude extract showed higher absorbance at 270 nm than the *Calendula* flowers. This maximum is typical for the presence of aromatic rings, and although not providing a specific measure, these direct absorbance measurements can be considered as preliminary crude evaluations of the relative amounts of the phenolic compounds [19].
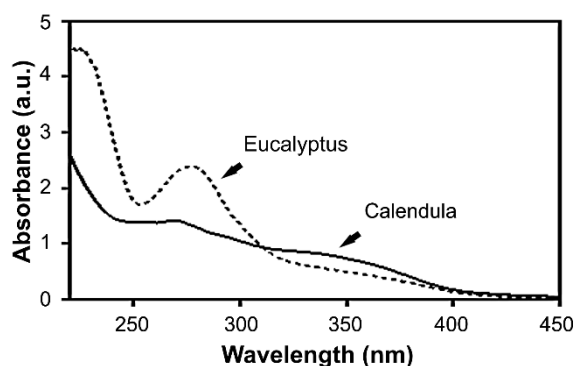
Fig. 1 UV-Vis spectra (200-450 nm) of methanol: water (70:30; v/v) extracts from the dried *Eucalyptus* leaves (21.7% dw extract) and the dried *Calendula* flowers (25.9% dw extract).

3.3 Total Phenolics

The total phenolics were determined in the SPE purified extracts from the *Eucalyptus* leaves and the *Calendula* flowers. For quantification of the total phenolics, GA was used as the standard. A linear correlation between GA concentration and absorbance was obtained with a correlation coefficient $R^2 = 0.9990$. The total phenolics are therefore expressed as mg GA/g dw of the plant material (Table 2).

For a given solvent, there were higher levels of total phenolics in the *Eucalyptus* leaves than in the *Calendula* flowers, as already suggested by the UV-vis spectra in the preliminary spectroscopic determination shown in Fig. 1. Table 2 also shows that the most efficient extraction system for the phenolics from both of these plant materials was methanol: water (70:30; v/v). The amounts of extracted phenolics with this solvent were 13 mg GA/g dw and 5.5 mg GA/g dw material from the *Eucalyptus* leaves and the *Calendula* flowers, respectively. The presence of 30% water in the methanol thus increased the total phenolics content by around 50%, which reflects the polar nature of the compounds extracted.

The Folin–Ciocalteu method is a relatively convenient, easy and rapid method that is sensitive and reproducible, although it has a number of weak points. First, Folin–Ciocalteu is not a specific reagent, and indeed, it was developed for protein determination. A variety of compounds can react with Folin–Ciocalteu reagent, including the amino acid tryptophan. Thus, the total phenolics determined using Folin–Ciocalteu reagent reflect the reducing activity of the samples, and will at best provide only an estimation of the phenolics content of the samples [20].

Table 2 Total phenolics in the SPE purified extracts from the *Eucalyptus* leaves and *Calendula* flowers

Solvent	Total phenolics (mg GA/g dw)	
	Eucalyptus leaves	*Calendula* flowers
Chloroform	0.19 ±0.01	0.03 ±0.00
Ethanol	1.93 ±0.03	0.52 ±0.02
Methanol	8.09 ±0.03	2.30 ±0.04
Methanol:H₂O (70:30; v/v)	12.98 ±0.01	5.51 ±0.05

Data are means ±SD (n = 3)

3.4 Antioxidant Activity

3.4.1 HPLC with Electrochemical Detection

Phenolics can act as antioxidants through different mechanisms, and various methods have been developed and described for the assessment of their antioxidant activities [21]. *In-vitro* and *in-vivo* antioxidative assays can be divided into single electron transfer tests and hydrogen atom transfer tests [20]. In the present study, we determined the total antioxidant activity using HPLC-ED for two single electron transfer tests: total phenolics (an oxidation–reduction method), and DPPH free radical scavenging assay, which can in general work as a single electron transfer test [20] in polar solvents, and as a hydrogen atom transfer test in nonpolar solvents [22]. Fig. 2 shows an example of the profile obtained from the eluted crude (Fig. 2A) and purified *Calendula* extracts (Fig. 2B). The SPE purification of extracts shown in Fig. 2B as a disappearance of the eluted peaks between 5 min and 8 min promoted an enrichment of polar electrochemically active compounds, related to their reducing power.
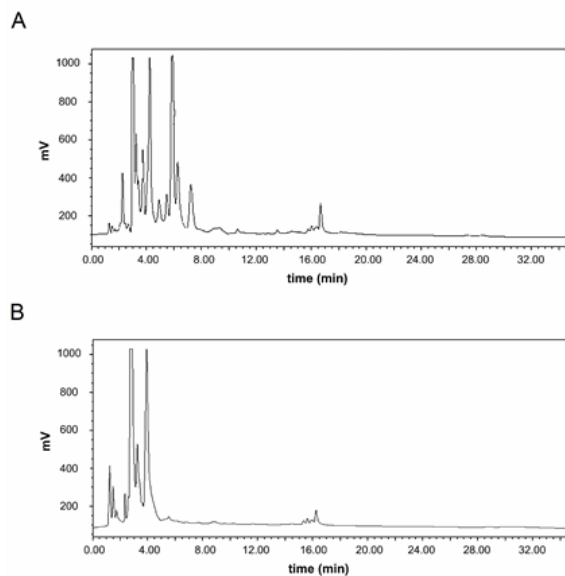
Fig. 2 HPLC-ED chromatograms for the total antioxidant activities of the crude (A) and SPE-purified (B) *Calendula* flower extracts, as obtained with the methanol: water (70:30; v/v) extraction.

The concentration–response calibration for CA as the standard demonstrated linear correlation and $R^2 = 0.9951$. The data for total antioxidant activities of these crude and SPE-purified plant extracts were expressed as mg CA/g dw, and are shown in Table 3.

Table 3 Total antioxidant activity of crude and purified extracts of *Eucalyptus* leaves and *Calendula* flowers determined by HPLC with electrochemical detection

Solvent	Total antioxidant activity of extracts(mg CA/g dw)			
	Eucalyptus leaves		*Calendula* flowers	
	Crude	Purified	Crude	Purified
Chloroform	0.10	nd	0.01	nd
Ethanol	1.62	0.32	0.34	0.17
Methanol	5.26	1.56	1.43	0.51
Methanol: water (70:30; v/v)	2.51	2.92	2.45	0.99

Data are means, with the confidence intervals of estimated as 5% of the experimental values; nd, not determined

The antioxidant activity of the purified extracts determined by HPLC-ED was linearly correlated to the total phenolics content, with a coefficient of variance of 0.989. This good correlation confirms that the total phenolics determined by the Folin–Ciocalteu method reflected their reducing power well.

3.5 DPPH Assay

The DPPH free radical scavenging activity assay is a widely used assay, although it has some shortcomings. In this system, the antioxidant activity of a compound or an extract is greater when fewer radicals of DPPH• remain in the reaction mixture, and this leads to lower final absorbance values.

The DPPH analysis was performed with different extract concentrations, to evaluate the kinetics of the DPPH• scavenging radical in the time frame of the assay. As shown in Fig. 3, the DPPH• radicals had been scavenged immediately by the crude 21.7% dw extract of *Eucalyptus* leaves, while the crude 25.9% dw extract of *Calendula* flowers required

more time to degrade the same amount of the DPPH• radical. This is most probably a consequence of the scavenging kinetics and the phenolics in the extracts. Similar observations were reported for quercetin in methanol: water (80:20; v/v), where the reaction with DPPH radicals was over after only 100 ms to 300 ms [10].
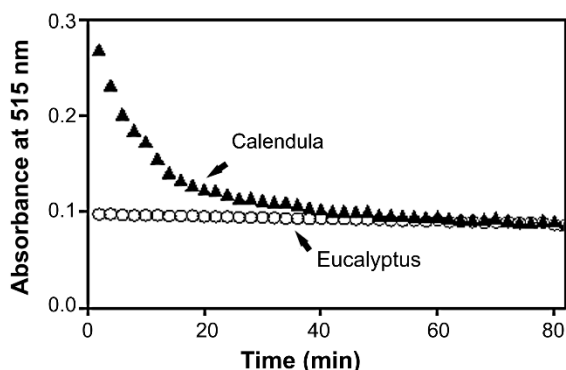
Fig. 3 Kinetics of DPPH• radical scavenging in the presence of the undiluted *Eucalyptus* leaf and *Calendula* flower crude extracts obtained with methanol: water (70:30; v/v) extraction. The extracts contained 21.7% dw *Eucalyptus* leaf crude extract *versus* 25.9% dw *Calendula* flower crude extract (see Table 1). ○ *Eucalyptus* extracts; ▲ *Calendula* extracts.

A direct relationship between the amounts of total phenolics and the antioxidant activities determined either as the total antioxidant activities or by the DPPH assay was seen for both of the purified extracts (Fig. 4). This relationship between the total phenolics and DPPH is commonly found in plant extracts, as generally the phenolic compounds are the main contributors to the antioxidative activity. However, the radical scavenging activity determined by the DPPH assay and the reducing power determined by HPLC-ED, contribute to the total antioxidant activity through different mechanisms [22]: the first acts by scavenging free radicals, and the second by promoting a reducing environment, and thus inhibiting oxidative reactions. Thus, a direct relationship between these two parameters cannot be expected always.
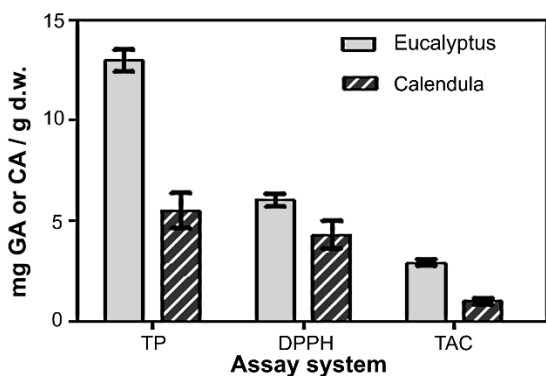
Fig. 4 Total phenolics (Folin–Ciocalteu method; mg GA/g dw), DPPH antioxidant (radical scavenging) activity (mg GA/g dw), and total antioxidant capacity (mg CA/g dw) in the methanol: water extracts from *Eucalyptus* leaves and *Calendula* flowers. Error bars represent 95% confidence intervals.

Table 4 LC-MS/MS analyses of purified methanol: water (70:30: v/v) extracts from *Eucalyptus* leaves and *Calendula* flowers

Source	Retention time (min)	m/z, (M-H)+	Major ESI fragments	Phenolic identification
Eucalyptus leaves	5.61	353.43	191.37	Chlorogenic acid [a]
	17.26	609.6	301.09	Rutin (quercetin 3-rutinoside) [a]
	17.57	463.4	301.09	Ellagic acid hexoside [b]
	20.12	477.52	301.07	Quercetin 3-glucuronide [b]
	20.38	463.66	-	Ellagic acid derivative
Calendula flowers	5.61	353.43	191.37	Chlorogenic acid [c]
	6.81	179.466	135	Caffeic acid [c]
	17.77	609.73	563	Rutin (quercetin 3-rutinoside) [d]
	19.87	137.53	93	Salicylic acid [c]
	20.77	477.69	313.86	Quercetin 3-glucuronide [e]
	39.84	313.6	201.42	Pinobanksin 3-O-acetate [e]

[a] from [6]; [b] from [5]; [c] references from [25]; [d] from [23-25]; [e] newly identified compound in dry flowers of Calendula officinalis

3.6 LC-MS/MS Analyses of the Phenolics

The results of the HPLC-MS/MS analysis of the SPE-purified methanol: water (70:30; v/v) extracts from the *Eucalyptus* leaves and *Calendula* flowers are given in Table 4. Identification of the peaks was performed according to their retention times, with comparisons to the pure standards, the MS spectra with the *m/z* of the parent ions (M-H)+, and their molecular fragments, or they were taken from literature sources. One of the major peaks is ellagic acid hexoside, with a retention time of 17.57 min. Chlorogenic acid and rutin were identified in the *Eucalyptus* leaves already [23]. Ellagic acid derivatives, with ellagic acid hexoside, were largely predominant also among the 39 phenolic compounds detected in purified acetone extracts from leaves of a 25-year-old *E. globulus* tree in northeast Algeria [5]. Quercetin 3-glucuronide was previously identified with the same group as one of the five flavonols detected [5].

The HPLC-MS/MS analysis revealed the presence of chlorogenic acid, rutin and quercetin 3-glucuronide in the purified methanol: water (70:30; v/v) extracts from both *Eucalyptus* leaves and *Calendula* flowers. Chlorogenic acid is a typical low-molecular-weight phenolic that is widely distributed in higher plants [24]. Rutin is a rutinose derivative of quercetin, and along with quercetin 3-glucuronide, it has been identified in *Eucalyptus* leaves and *Calendula* flowers previously [6, 23, 25]. Ellagic acid derivatives were found in the dried *Eucalyptus* leaves. Caffeic acid and salicylic acid were detected only in the *Calendula* flower extracts. Caffeic acid is a low-molecular-weight phenolic identified previously [23]. Salicylic acid is a phenolic phytohormone that can influence plant growth and development; it was detected earlier too [25]. Quercetin 3-glucuronide and pinobanksin 3-acetate were found in the *Calendula* flower extract for the first time. Pinobanksin is a dihydroflavonol that is also found in propolis and other honey-related products, which show many pharmacological activities [24]. All of these compounds listed in Table 4 have antioxidant properties [26]. The phenolic compositions of both of these extracts can explain their similar antioxidant behaviours and different effectiveness and kinetics of their DPPH radical-scavenging activities and electrochemical responses, as discussed in section 3.5. Indeed, further investigations can focus on the preparation of several mixtures of both of these extracts of *Eucalyptus* leaves and *Calendula* flowers, to test their antioxidant activities and for their relevant biological activities.

4. Conclusion

The aim of the present study was to determine inexpensive yet effective solvent(s) for the extraction of the phenolics from *Eucalyptus* leaves and *Calendula* flowers. Methanol and methanol:water (70:30; v/v) are highly polar and were the most effective solvents for extracting the phenolics from these sources. For the same solids content, more phenolics were extracted from the *Eucalyptus* leaves than the *Calendula* flowers. The highest antioxidant activities were obtained with the methanol:water extracts, and these strongly correlated to the phenolics content. Although not selective, the HPLC-ED method correlated well to the reducing power of the extracts, further to the total phenolics and the DPPH radical-scavenging activity of these two plant extracts.

In the methanol:water extracts from both the *Eucalyptus* leaves and the *Calendula* flowers, HPLC-MS/MS analysis revealed the presence of the following three compounds: chlorogenic acid, rutin and quercetin 3-glucuronide. Ellagic acid derivatives were found in the dried *Eucalyptus* leaves, while caffeic acid, salicylic acid, and pinobanksin were detected in the dried *Calendula* flowers only. Quercetin 3-glucuronide and pinobanksin were found in the *Calendula* flower extract for the first time.

As well as the frequently recognized valuable hydrophobic essential oils and terpenoid constituents present in medicinal plants like *Eucalyptus* and *Calendula*, and their roles that are beneficial to health, the polar phenolics and their antioxidant properties are also relevant for the complete evaluation of such plant sources of the phenolics.

References

[1] V. Abram, M. Donko, Tentative identification of polyphenols in *Sempervivum tectorum* and assesment of the antimicrobial activity of *Sempervivum* L., J. Agric. Food Chem. 47 (1999) 485-489.

[2]   S.S. Ali, N. Kasoju, A. Luthra, A. Singh, H. Sharanabasava, A. Sahu, U. Bora, Indian medicinal herbs as sources of antioxidants, Food Res. Int. 41 (2008) 1-15.

[3]   Y.S. Velioglu, G. Mazza, L. Gao, B.D. Oomah, Antioxidant activity in fruits and total phenolic in selected fruits, vegetables, and grain products, J. Sci. Food Agr. 46 (1998) 4113-4117.

[4]   K. Sugimoto, K. Nakagawa, S. Hayashi, Y. Amakura, M. Yoshimura, T. Yoshida, R. Yamaji, Y. Nakano, H. Inui, Hydrolyzable tannins as antioxidants in the leaf extract of *Eucalyptus globulus* possessing tyrosinase and hyaluronidase inhibitory activities, Food Sci. Technol. Res. 15 (2009) 331-336.

[5]   L. Boulekbache-Makhlouf, E. Meudec, J.P. Mazauric, K. Madani, V. Chenier, Qualitative and semi-quantitative analysis of phenolics in eucalyptus globulus leaves by high-performance liquid chromatography coupled with diode array detection and electrospray ionisation mass spectrometry, Phytochem. Anal. 24 (2013) 162-170.

[6]   I.F. Almeida, E. Fernandes, J.L.F.C. Lima, P. Valentâo, P.B. Andrade, R.M. Seabra, P.C. Costa, M.F. Bahia, Oxygen and nitrogen reactive species are effectively scavenged by *Eucalyptus globulus* leaf water extract, J. Med. Food 12 (2009) 175-183.

[7]   M. Wichtl, Herbal drugs and phytopharmaceuticals, Medpharm Scientific Publisher, Stuttgart, 1994.

[8]   M. Hamburger, S. Adler, D. Baumann, A. Förg, B. Weinreich, Preparative purification of the major anti-inflammatory triterpenoid esters from calendula (*Calendula officinalis*), Fitoterapia 74 (2003) 328-338.

[9]   E. Vidal-Ollivier, R, Elias, F. Faure, A. Babadjamian, F. Crespin, G. Balansard, G. Boudon, Flavonal glycosides from *Calendula officinalis* flowers, Planta Med. 55 (1989) 73-74.

[10] M. Foti, Use and abuse of the DPPH·radical, J. Agric. Food Chem. 63 (2015) 8765-8776.

[11] A. Munin, F. Edwards-Lévy, Encapsulation of natural polyphenolic compounds; a review, Pharmaceutics 3 (2011) 793-829.

[12] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomolybdic-phosphotungistic acid reagent, Am. J. Enol. Viticult. 16 (1965) 144-158.

[13] W. Brand-Williams, M.E. Cuvelier, C. Berset, Use of a free radical method to evaluate antioxidant activity, Lebensm. Wiss. Technol. 29 (1995) 25-30.

[14] L. Bertalanič, T. Kosmerl, N. Poklar Ulrih, B. Cigic, Influence of solvent composition on antioxidant potential of model polyphenols and red wines determined with 2,2-diphenyl-1-picrylhydrazyl, J. Agric. Food Chem. 60 (2012) 12282-12288.

[15] D.O. Kim, K.W. Lee, H. Lee, Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals, J. Agric. Food Chem. 50 (2010) 3713-3717.

[16] J. Dai, R.J. Mumper, Plant phenolics: extraction, analysis and their antioxidant and anticancer properties, Molecules 15 (2010) 7313-7352.

[17] CRC, Handbook of Chemistry and Physics, *65*th Ed., Ed. R.C. Weast, CRC Press, Boca Raton, 1984.

[18] P.S. Albright, L.J. Gosting, Dielectric constants of the methanol water system from 5 to 55 ºC, J. Am. Chem. Soc. 68 (1946) 1061-1063.

[19] J.S. Harbertson, S. Spayd, Measuring phenolics in the winery, Am. J. Enol. Vitic. 57 (2006) 280-288.

[20] J.B.L. Tan, Y.Y. Lim, Critical analysis of current methods for assessing the in vitro antioxidant and antibacterial activity of plant extracts, Food Chem. 172 (2015) 814-822.

[21] C. Rice-Evans, Screening of phenolics and flavonoids for antioxidant activity, In: Antioxidant Food supplements in human health, Eds: L. Packer, M. Hiramatsu, T. Yoshikawa, Academic Press, San Diego, 1999, pp.239-253.

[22] C. Sanchez-Moreno, Methods used to evaluate the free radical scavenging activity in foods and biological system, Food Sci. Tech. Int. 8 (2002) 121-13.

[23] D.N. Olennikov, N.I. Kashchenko, New isorhamnetin glycosides and other phenolic compounds from *Calendula officinalis*, Chem. Nat. Prod. 49 (2013) 833-840.

[24] I. Martos, F. Ferreres, F.A. Tomás-Barberán, Identification of flavonoid markers for the botanical origin of eucalyptus honey, J. Agric. Food Chem. 48 (2000) 1498-1502.

[25] G. Rigane, S. Ben Younes, H. Ghazghazi, R. Ben Salem, Investigation into biological activities and chemical composition of *Calendula officinalis* L. growing in Tunisia, Int. Food Res. J. 20 (2013) 3001-3007.

[26]   T. Yoshida, K. Mori, T. Hatano, T. Okumura, I. Uehara, K. Komagoe, et al, Studies on inhibition mechanism of autoxidation by tannins and flavonoids, V. Radical scavenging effects of tannins and related polyphenols on DPPH radical, Chem. Pharm. Bull. 37 (1989) 1919-1921.