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

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**Abstract**

Selection of the optimal solvent system for extraction of the phenolics from *Eucalyptus globulus* 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-glucoside. Ellagic acid derivatives were identified only in the leaves of *Eucalyptus*, while beside caffeic acid and salicylic acid, quercetin 3-glucoside, 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 Myrtaceae 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), chrys (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).

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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 [1].

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 (Belleville, 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 mM ammonium formate. The extract was allowed to pass through the cartridges, and the cartridges were washed with 2 mL of 15% methanol in 20 mM ammonium formate and vacuum-dried for 3 min. Finally, the retained phenolics were eluted with 2 mL methanol. These purified extracts were dried and dissolved 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% NaCO₃ solution. The mixture was centrifuged at 13,800 × g for 10 min (5415 C; Eppendorf, Hamburg, Germany), and the absorbance of the resulting blue-colored solution was measured at 765 nm after 5 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 LiCharrop RT-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%; 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 electrochemical 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, DPPH₂, 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. 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 and 60 min, and using GA for the preparation of the calibration curve [15]. For the crude freeze-dried samples, the 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 (AA₄₅₀nm) at time t were calculated as in Equation (1):

\[ \text{AA}_{450} = \left( \frac{\text{A}_0 - \text{A}_t}{\text{A}_0} \right) \times 100 \] 

The data are expressed as AA₄₅₀nm (%D) 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 11000) 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 150 °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 extract 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].
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 Calendula 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].

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].

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 antioxidant 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.](image)

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 and Calendula flowers determined by HPLC with electrochemical detection](table)

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 DPHH* 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 DPHH* scavenging radical in the time frame of the assay. As shown in Fig. 3, the DPHH* radicals had been scavenged immediately by the crude 2.1% dw extract of Eucalyptus leaves, while the crude 25.9% dw extract of Calendula flowers required

![Table 2 Total phenolics in the SPE purified extracts from the Eucalyptus leaves and Calendula flowers](table)
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].

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 comparison to the 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 as a predominant, and among the 39 phenolic compounds detected in purified acetone extracts from leaves of a 25-year-old E. globulus tree in northeastern 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 hydrophilic 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.

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References
