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Analytical Methods

Phytochemical constituents and *in vitro* radical scavenging activity of different *Aloe* species

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ABSTRACT

The phytochemical profile of *Aloe barbadensis* Mill. and *Aloe arborescens* Mill. was investigated using colorimetric assays, triple quadrupole and time-of-flight mass spectrometry, focusing on phenolic secondary metabolites in the different leaf portions. Hydroxycinnamic acids, several characteristic anthrones and chromones, the phenolic dimer feralolide and flavonoids such as flavones and isoflavones were identified. The stable radical DPPH· test and the ORAC assay were then used to determine the *in vitro* radical scavenging. The outer green rind was the most active, while the inner parenchyma was much less effective. The 5-methylchromones aloesin, aloeresin A and aloesone were the most active among the pure secondary metabolites tested. The results suggest that several compounds are likely to contribute to the overall radical scavenging activity, and indicate that leaf portion must be taken into account when the plant is used for its antioxidant properties.

1. Introduction

Aloe plants have long been used in traditional medicine for their curative and therapeutic properties and, albeit different plant species are known worldwide, *Aloe barbadensis* Miller L. (trivially labeled as *A. vera*) and *Aloe arborescens* L. are the most extensively cultivated in the world (Liao, Sheng, & Hu, 2008). Various pharmacological activities and therapeutic effects have been ascribed to *Aloe* species, such as anti-inflammatory, antimicrobial, wound healing, anti-tumour and antioxidant properties (Jones, 2008; Lucini, Pellizzoni, Molinari, & Franchi, 2012; Pellizzoni, Ruzickova, Kalhotka, & Lucini, 2012). Interest in antioxidant properties of *Aloe* species is mainly related to their therapeutic and curative uses, but there is also growing interest in the use of health-promoting food additives and natural antioxidants for extending the shelf life without the need for synthetic antioxidants.

The leaf of *Aloe* plants is heterogeneous; different leaf portions have a different composition and are likely to have different biological properties. The outer green rind is rich in anthrones, chromones and their glycosides, while the inner gel contains most of the characteristic polysaccharides (Hamman, 2008). Many authors believe that the various biological activities related to the different *Aloe* species should be ascribed to a synergistic action of several compounds rather than a single chemical substance (Dagne, Bisrat, Viljoen, & Van Wyk, 2000; Hamman, 2008).

A. barbadensis Mill. has been shown to protected against pro-oxidant membrane and cellular damage (Singh, Dhanalakshmi, & Rao, 2000). Indeed, a potent antioxidant compound has been isolated from methanolic extracts of *A. barbadensis* with activity similar to α-tocopherol (Botes, Westhuizen, & Loots, 2008; Hu, Hu, & Qiuhui, 2003; Lee, Weintraub, & Yu, 2000).

It has been postulated that flavonoids have a role in the overall radical scavenging activity, while the role of aloin, anthrones and

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chromones has not been fully elucidated yet (Romani et al., 2008). Several factors will affect the antioxidant properties of *Aloe* plants, such extraction and processing methods, raw materials and plant health as well as post-harvest factors (Lucini, Pellizzoni, & Molinari, 2013; Moody, Adebiyi, & Adeniyi, 2004; Pellizzoni, Molinari, & Lucini, 2011; Zhang, Du, Liu, & Liu, 2001). More studies are needed to identify the antioxidant constituents in *Aloe* species and investigate factors affecting radical scavenging activity. Our object was to study the profile of phenolic compounds in *A. barbadensis* and *A. arborescens*, then to investigate the relationship between scavenging activity and secondary metabolites profile, in the main portions of the leaf.

2. Materials and methods

2.1. Plant materials

A. barbadensis Mill. and *A. arborescens* Mill. were supplied by Dester Garden (Brescia, Italy). Three leaves from basal, central and apical positions were harvested from *A. barbadensis*, while three leaves from center and three leaves from lateral stems were taken from *A. arborescens*. This scheme was applied to a minimum of five plants. The leaves of each plant were pooled and homogenised using a blender, in an ice bath, to prepare representative portions of each. The sampling scheme was repeated for split leaf skin and pulp samples. Leaves were selected and the outer green rind separated from the inner parenchyma; each sample was thoroughly homogenised using a blender.

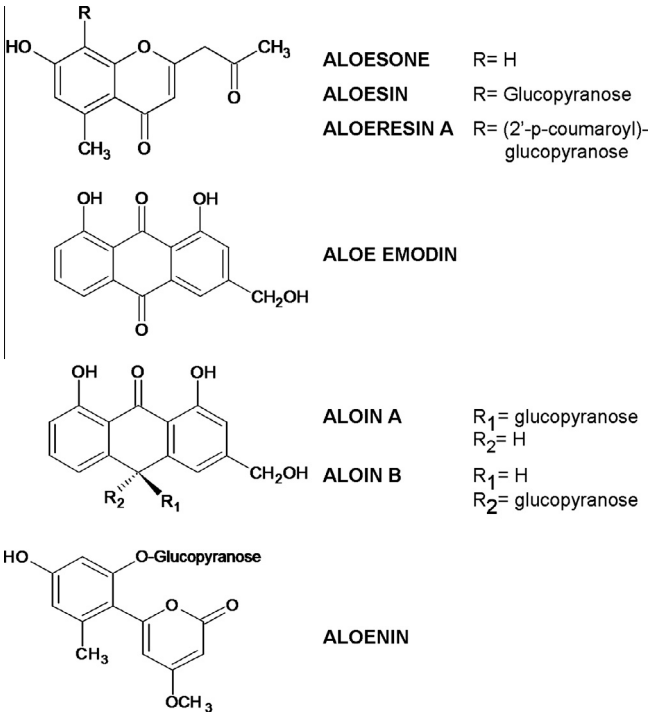

Fig. 1. Structure of the pure Aloe secondary metabolites investigated. Chemical structure of *Aloe* anthrones and chromones on which radical scavenging tests were performed.

2.2. Chemicals and reagents

Ethanol (96%) as well as analytical grade aloin and aloe-emodin were purchased from Sigma Aldrich (St. Louis, MO, USA). The free radical DPPH· (1,1-diphenyl-2-picrylhydrazyl), the azo free radical generator AAPH (2,2′-azobis-2-amidinopropane dihydrochloride), fluorescein, and solvents (methanol and acetonitrile LCMS grade and absolute ethanol) were also purchased from Sigma.

Aloesin, aloeresin A, aloesone and aloenin were a gift from the Department of Industrial and Organic Chemistry, University of Milan. Structures of all pure secondary metabolites tested as radical scavengers are reported in Fig. 1; chromones, anthrones and their glycoside, and phenylpyrones.

2.3. Extraction and preparation for assays

All samples were freeze–dried and then ground into a fine powder for further use. 50 ml of 80% ethanol (v/v) was added to 1 g of lyophilized powder in a round flask, the sample sonicated for 15 min and filtered using GF/A filter paper (Whatman, Buckinghamshire, UK). The residue was washed twice with 10 ml of ethanol. The filtrates were dried at 50 °C using a rotary evaporation. The mass of solids in the different extracts was recorded and the residue was re-suspended in 80% ethanol (v/v) to a final concentration of 100 mg/l immediately before analysis. Solutions of aloin, aloe-emodin, aloesin, aloeresin A, aloesone and aloenin solutions were prepared at the same concentration (100 mg/l) as the plant extracts.

2.4. Total phenolic, flavonoids and flavonols content in leaf portions

The total phenolic content in each extract was determined using the Folin–Ciocalteu assay. Aliquots (1 ml) of each extract were mixed with 2.5 mL of Folin–Ciocalteu reagent (Sigma, diluted fivefold) and with 4 ml (75 g/l) sodium carbonate. Absorbance was recorded at 765 nm, after 40 min at 20 °C in dark. A calibration curve was prepared using gallic acid in ethanol, and the results expressed as gallic acid equivalents (GAE).

Flavonoids content was determined colorimetrically using rutin as the reference compound. Aliquots (1 ml) of each extract (10 g/l) were mixed with 1 ml $AlCl_3$ in ethanol (20 g/l) and diluted with ethanol to 25 ml. The absorption at 415 nm was read after 40 min at 20 °C. Blank samples were prepared from 1 ml plant extract and 1 drop of acetic acid, and diluted to 25 ml. The amount of flavonoids in the extracts was calculated from a rutin calibration curve, and results expressed as rutin equivalents (RE).

Flavonols were determined according to Miliauskas, Venskutonis, and Van Beek (2004). A rutin calibration curve was prepared by mixing rutin in ethanol at different concentrations with 2 ml $AlCl_3$ (20 g/l) and 6 ml sodium acetate (50 g/l). The absorption was read at 440 nm after 2.5 h at 20 °C. The same procedure was carried out with 2 ml of extract (10 g/l) instead of rutin solution. Flavonols content was expressed as RE.

The coefficient of determination R^2 of each calibration curve was calculated for phenolic compounds, flavonoids and flavonols, and accepted when higher than 0.95. Additionally, all dynamic ranges were extended respectively 20% above and 20% below the highest and the lowest absorbance of the extracts.

2.5. Determination of target polyphenols in leaf portions

Selected compounds, chosen as markers of hydroxycinnamic acids (namely cynarin, caffeic, chlorogenic and ferulic acid) and flavonoids (apigenin, luteolin, luteolin-7-*O*-glucoside, myricetin, quercetin and catechin), were determined by liquid chromatography followed by tandem mass spectrometry with an electrospray ionisation source (LC-ESI/MS/MS). A 1200 series liquid chromatograph system, equipped with quaternary pump, electrospray ionisation system and coupled to a G6410A triple quadrupole mass spectrometer detector (all from Agilent technologies, Santa Clara,