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Correlation between the antibacterial activity and the composition of extracts derived from various Spanish *Cistus* species

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ABSTRACT

Cistaceae is a large family of shrubs commonly distributed in the Mediterranean ecosystem. The aim of this study was to explore the potential antimicrobial properties against *Escherichia coli* and/or *Staphylococcus aureus* of different extracts obtained from four *Cistaceae* species that are especially abundant in Spanish semi-arid regions. MIC_{50} values of the extracts of *C. salviifolius* exhibited potent bacteriostatic effects against *S. aureus* compared with the other *Cistus* species tested. Spray-drying had less impact on the antimicrobial activities and polyphenolic contents than did evaporation followed by freeze-drying. When *C. salviifolius* extract was concentrated and the polar fraction was removed, its bacteriostatic and bactericidal activities against both strains were significantly enhanced. Seasonal influences on the composition have also been found. Up to 48 compounds were found in the aqueous extract of *C. salviifolius* using RRLC–ESI–TOF–MS. The analysis of the composition of the extracts revealed that the inhibitory activity against *E. coli* may be related to the presence of galloylated flavanols and specific flavonols, whereas the inhibitory capacity against *S. aureus* may be related primarily to polar compounds and to other flavonols. Potential synergistic effects among polyphenols may deserve further studies. These extracts may serve as an alternative source of antimicrobial ingredients focused on medical devices or cosmetics.

1. Introduction

The *Cistaceae* family (also known as rock-rose) is a large family of perennial shrubs that grow mostly in the Mediterranean semi-arid ecosystem. Some of the species of this family are rich in flammable resins, and their overgrowth lead to environmental problems and wildfires (Ferrandis et al., 1999). Therefore, this vegetable waste may serve as a source of potential ingredients for further industrial applications. This family includes the *Cistus* genus, which is divided into three subgenera: *Cistus*, *Leucocistus* and *Halimioides*. Some species are much appreciated in the perfume industry, and others have been used since ancient times in traditional folk medicine. Among these species, *C. ladanifer*, *C. salviifolius*, *C. clusii*, *C. albidus*, and *C. populifolius* are especially abundant in the Iberian Peninsula (Stübing and Peris, 1998).

C. ladanifer, or "'sticky' or 'common' rockrose" belongs to the *Leucocistus* subgenus. It produces a sticky resin, the labdanum, which has been used as a sedative, hemostatic and anti-infective agent. *C. salviifolius* also belongs to the *Leucocistus* subgenus. It has traditionally been utilized as an astringent and cicatrizing agent in some countries of the Mediterranean area in addition to being used as a tea substitute (Quer, 2005; Stübing and Peris, 1998). *C. clusii* that belongs to the *Halimioides* subgenus, has been used in folk medicine for different purposes (Guinea et al., 1990). *C. albidus*, or white rockrose, is a shrub belonging to the *Cistus* subgenus and it has traditionally been utilized for a variety of illnesses (Quer, 2005; Stübing and Peris, 1998).

Most studies of *Cistus* species have focused on the essential oil compositions of these shrubs, but a few studies have attempted to elucidate the complex polyphenolic profile of this family. The following polyphenolic compounds have been identified in various

Abbreviations: FD, freeze drying; FRAP, ferric-reducing ability power; GAE, gallic acid equivalents; MBC, minimum bactericidal capacity; MIC_{50}, minimum inhibitory concentration required to inhibit 50% of bacterial growth; ORAC, oxygen radical absorbance capacity; RRLC–ESI–TOF–MS/MS, rapid-resolution liquid chromatography coupled with electrospray ionization time-of-flight mass spectrometry; SDY, spray drying; TBARS, thiobarbituric acid-reactive substances; TEAC, Trolox equivalent antioxidant capacity.

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Mediterranean *Cistus* species: apigenin and kaempferol derivatives in *C. ladanifer* (Chaves et al., 1997; Ramalho et al., 1999; Saracini et al., 2005), ellagitannins and glycosylated quercetin or myricetin derivatives in *C. salviifolius* (Saracini et al., 2005), oligomeric proanthocyanidins in *C. albidus* (Qa'dan et al., 2003), flavanols *in C. clusii* (Gallucci et al., 2009), diterpenes and flavonoids in *C. creticus* (Skorić et al., 2012) and flavanols, gallic acid and rutin in *C. incanus* and *C. monspeliensis* (Santagati et al., 2008). We have previously reported the comprehensive composition of the aqueous extracts of *C. ladanifer* and *C. populifolius* using a highly resolved technique such as high-performance liquid chromatography with diode array detection coupled to electrospray time-of-flight and ion-trap tandem mass spectrometry (HPLC–DAD–TOF–IT) (Barrajon-Catalan et al., 2010; Fernández-Arroyo et al., 2010). Moreover, we recently reported the relationship between the polyphenolic chemotype and the taxonomical classification of up to ten species of the *Cistus* subgenus (*Leucocistus*, *Cistus* or *Halimioides*) (Barrajon-Catalan et al., 2011).

The ancient ethnobotanical use of various *Cistus* species reveals that these plants are a good remedy for several microbial disorders and infections (Bassolé and Juliani, 2012; Salin et al., 2011). The antimicrobial activity of the essential oil of *C. salviifolius* (Gertsch, 2011; Güvenç et al., 2005) and that of extracts obtained by the use of organic solvents from *C. creticus* (Güvenç et al., 2005) and *C. ladanifer* (Ferreira et al., 2012) have been reported. Aqueous extracts derived from *C. ladanifer* and *C. populifolius* and containing ellagitannins and flavonoids also possess significant antimicrobial activity (Barrajon-Catalan et al., 2010) against both Gram-positive and Gram-negative bacteria.

The aim of the present study was to evaluate the antibacterial activities of the hydroalcoholic or aqueous extracts, which were obtained via different drying methods, and derived from four species of the *Cistus* genus that are especially abundant in the Iberian Peninsula. The most active extract was further fractionated. A tentative correlation between the detailed polyphenolic compositions, using RRLC–ESI–TOF–MS, and the antibacterial activity of *C. salviifolius* aqueous extract was achieved for the first time.

2. Materials and methods

2.1. Plant collection and sample preparation

Specimens of different native *Cistus* plants were obtained from various areas of Spain. The *Cistus ladanifer* L. samples were obtained from a mountain area close to Puertollano in Ciudad Real province, near Andalucía, *Cistus albidus* L., *C. salviifolius* L. and *C. clusii* Dunal samples were collected from a semi-arid area close to Monóvar in the Alicante province. All of the samples were collected between June 2010 and November 2011. The plant material was properly identified and labeled by the authors and qualified personnel. Prior to processing, the samples were washed with water to remove residues of dust and arthropods.

2.2. Extract processing and fractionation

The botanical samples were processed within 24 h after collection and were crushed with a grinder to obtain particles between 3 and 5 mm in size. The samples were then subjected to aqueous or hydroalcoholic maceration for 4 h with gentle stirring at a temperature no higher than 65 °C. The liquid extracts were filtered to remove suspended solids and rotavapored to concentrate the samples (or to eliminate ethanol in the case of hydroalcoholic extraction) to approximately 8°Brix before drying. The extracts were then subjected to different drying procedures, such as lyophilization, freeze drying (FD; Telstar Cryodos 80, Spain) or spray drying (SDY; Büchi Mini Spray Dryer B-290, Spain) to obtain powdered extracts. The powdered extracts were dissolved in the appropriate buffer for each test and filtered through 0.2 μm filters.

The selected extract was fractionated as previously described (Beltrán-Debón et al., 2010; Herranz-Lopez et al., 2012) using affinity chromatography with an Amberlite FPX66 resin (The Dow Chemical Co.), which shows high affinity for polyphenolic compounds. The selected extract was dissolved in distilled water and centrifuged at 3000 rpm. The supernatant was then loaded onto the Amberlite chromatography column (2 × 25 cm) at a flow rate of 4 mL/min, washed with 30 volumes of distilled water and eluted at a rate of 2 mL/min with 3 volumes of ethanol. The flow-through, washed and eluted fractions were collected separately and freeze-dried for further analysis.

2.3. Phenol and flavonoid quantitation

The total flavonoid content was quantified according to a previously described method (Pourmorad et al., 2006) using quercetin (SIGMA–ALDRICH, Europe) as a standard. The total polyphenolic content was determined using the Folin–Ciocalteu method (Huang et al., 2005) with gallic acid (SIGMA–ALDRICH, Europe) as a standard. The absorbance measurements were performed using a UV–VIS spectrophotometer (Cecil 2041 2000 Series, UK).

2.4. RRLC–ESI–TOF–MS

The mass spectrometry analysis was essentially performed as previously described (Fernández-Arroyo et al., 2010). The analytical separation was achieved using a RRLC (rapid-resolution liquid chromatography) 1200 Series instrument from Agilent Technologies (Santa Clara, CA, USA) equipped with a G1312B binary pump and a diode array detector (DAD) G1315C. A Zorbax Eclipse Plus (Agilent Technologies) RP-C18 column (4.6 × 15 mm, 1.8 μm particle size) was used. The injection volume was 15 μL, and the flow rate was 0.8 mL/min. The mobile phase consisted of 0.5% acetic acid (A) and acetonitrile (B). The following gradient was used: 0 min, 0% B; 20 min, 20% B; 30 min, 30% B; 40 min, 50% B; 50 min, 75% B; 60 min, 100% B. The compounds were ionized and transferred from the RRLC (instrument separation) to the mass time-of-flight (TOF) spectrometer using an electrospray ionization source (ESI). The temperature was 190 °C, the nebulizer gas pressure was 2 bars, the nebulizer gas flow was 9 L/min, the voltage in the spray chamber was −500 V and the input voltage was +4500 for capillary V. Details for TOF analysis are described in the Supplementary information.

2.5. Antioxidant capacity measurements

The Trolox equivalent antioxidant capacity (TEAC), the ferric ion reducing antioxidant power (FRAP), the oxygen radical absorbance capacity (ORAC) and determination of the inhibition of lipid peroxidation by the thiobarbituric acid reactive species (TBARS) assays were performed as previously described (Barrajon-Catalan et al., 2010) and are briefly described in the Supplementary information.

2.6. Growth inhibition and bactericidal assays on Staphylococcus aureus and Escherichia coli

E. coli (CECT 515) and *S. aureus* (CECT 59) were used as models for Gram-negative and Gram-positive bacteria, respectively. Both strains were obtained from the Spanish Type Culture Collection (Colección Española de Cultivos Tipo, CECT, Universitat de Valencia, Spain). MIC_{50} determination assays were performed in 96-well plates using an adapted microdilution assay based on previously reported methods (Eloff, 1998; Iscan et al., 2002). To obtain the minimum bactericidal concentration (MBC), a modification of a