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Review Article

HPTLC Analyses on Different Populations of *Cistus salvifolius* L

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Abstract

HPTLC was used as chromatographic tool to evidence chemical differences in plant extracts used in marketed botanical food supplements, i.e. populations of *Cistus salvifolius* L., collected in different parts of Europe, as case study. The obtained results evidenced either a corresponding fingerprint for the species and several quantitative differences, that were better valued by densitometric analysis. These data are noteworthy considering the actual interest of the aforementioned species by the botanical nutraceutical market and the general need for analytic tools adequate to botanicals contents.

Keywords: *Cistus salvifolius*; HPTLC; Fingerprint; Botanicals; Chlorogenic acid; Caffeic acid

Abbreviations

HPTLC: High Performace Thin Layer Chromatography; NPR: Natural Product Reagent; WRT: Transmition White Light; Rf: Retardation factor defined as the ratio of the distance traveled by the center of a spot to the distance traveled by the solvent front.

Introduction

Actually, new plant raw materials are requested for the expanding botanical food supplements market [1]. Limits concern the identification of the utilized species and natural variability of constituents production among the species. Analytical tools are necessary to define drug identity and test this variability. The tool should evidence the total compostion of the utilized raw material when the phytocomplex is considered the active constituent [2]. This approach is in contrast with current analytical quality control for medicinal plants, as reported in official Pharmacopoeias, based on one substance or single class of natural products, which is missleading in case of taxonomically near species, including cases of presence of poisonous plants. An analytic tool for botanical quality control should be low cost, rapid, reliable, easy to performe and understandable, and contain in information - HPTLC to plant raw material, in order to show the utility of HPTLC fingerprint in botanical nutraceutical quality control [3]. HPTLC is the last evolution of instrumental planar chromatography [4,5], tailored to analyse mixture of organic compounds in pharmaceutical products [6,7]. In HPTLC, different steps are automated, to achieve high resolution and get reliable rapid and low cost quali and quantitative measurements [8]. Allowing to the capacity in evidencing natural products in complex mixtures like plant-borne extracts, including those in very low concentrations, we used HPTLC to perform metabolomic studies, like separation and determination of most of the constituents of an extract. Plates can be visualized and derivatives in several ways, obtaining multiple information, as well as converted in a series of peaks by densitometric treatment. In such way, the comparison between samples is reliable and facilitated by the visual inspection and samples can be analysed side-by-side and exactly in the same conditions. Main product of HPTLC analysis is the metabolic fingerprint, consisting in the individual track typical of the analysed species [9] and its value officially recognized [10-12].

The *Cistus* case

The aim of this work was to test the utilization of HPTLC to monitorate the variation and differences in production of secondary metabolites in samples of *C. salvifolius*, collected in Turkey and in Italy. *Cistus* is a genus of rockrose plants (Cistaceae) containing about 20 species [13] of perennial shrubs. The rockrose name is derived from the great similarity of many characters with *Rosa* species, like *R. canina* L., which counterparts the same habitat. They are mainly present on dry or rocky soils in Europe, with 17 species as typical exaples of the Mediterranean Flora, although several common species can be found in large and abundant populations in every part of Europe wherein the climate conditions are favourable, including the Southern Sveden, Black Sea costs and Northern Danimarca [14]. *C. salvifolius*, commonly known as sage-leaved rock white rose or as salvia cistus, is a common bushy shrub, characterized by five petals with a yellow spot at the basis.

Since ancient times *Cistus* plants have been prized as the source of the substance labdanum, also known as ladanum or ladan, a sticky brown resin. The leaves of several species. i.e. *C. ladanifer* and *C. creticus*, are coated with this highly aromatic resin, whereas other species are devoited of the resin. The use of labdanum was known to the Egyptians, at the times of pharaons [15] and to Greeks, as early as the times of Herodotus and Theophrastus, as an ingredient in perfumes, herbal medicine and incense, and later cited by Dioscorides [16]. Actually, some *Cistus* sp. are used in making cosmetic creams, because of its anti-wrinkle properties and shampoos, as it strengthens the hair follicle, but still prevalent the use in perfumery and many well known perfumes contain *Cistus* labdanum. Labdanum's odour is very rich, complex and tenacious, variously described as amber, animalic, sweet, woody, dry musk, or leather [17]. Labdanum is much valued in perfumery, because of its resemblance to ambergris. Ambergris is a secretion from the digestive system of the sperm whale. Whale produces the ambergris as defence to protect gastroenteric mucoses from the bones of celenterates, that are its main food. But actually

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ambergris is very difficult to find, being the whale fishing barned from many countries. Therefore, labdanum is the main ingredient used when making the scent of amber in perfumery. Ambergris and labdanum scent similarity is due to the chemical composition of volatile fraction, rich of diterpenic constituents. In ambergris the main compounds, responsible of the fragrance properties, are labdane derivatives, ambrox, amberlyn and ambroxan, derived from (-)-ambrein, a triterpene alcohol, by exposure to air and sunlight. These compounds compart the same labdanum skeleton with the constituents of labdanum [18].

However, these plants are also recorded for the utilization in traditional medicine, as to treat colds, coughs, menstrual problems, rheumatism, as well as for embalming and aphrodisiac purposes [19-22]. *Cistus* family has been reported to exhibit a variety of biological activities such as antialgal, antibacterical, antifungal, antiprotozoal, enzyme inducing, antiinflamatory, modulation of immune cell functions [23-25], as well as cytotossic and cytostatic effects against human cancer cell lines. In ethnobotanical, due to the aroma it contains, *C. salviifolius* has been used against bronchitis and as an expectorant. In addition, it is recorded that it has been used to stop bleeding [26].

Chemical composition of *Cistus* sp. is quite complicated. These plants are well known for a typical plasticidity at the morphological level, that should be present also in the chemical composition of each species. However, composition could change according to the environmental condition, in accordance with the afore mentioned large distribution of *Cistus* sp. Phytochemical reports on *C. salvifolius* concerns the isolation of several phenols, including flavan-3-ols, oligomeric flavan-3-ols, oligomeric anthocianidins, protodelphinins, ellagitannins and the glucoside phenylpropanoid rhododendrin (betuloside) with analgesic and antiinflammatory properties [27-31].

However, biological applications are so far limited. Here, HPTLC was selected to investigate the composition of *Cistus* populations as possible in the total complex secondary products composition and to obtain information about chemical nature of active constituents [32,33].

Experimental

Plant material

C. salvifolius L. leaves were collected in Sardinia (Costa Paradiso) for sample 1 and in Turkey (Saribeyler-Balikesir) at sea level for sample 2 and in another population at 100 m of altitude for sample 3. Collection was performed in the flowering time. Leaves were collected and further immediately exsiccated. A voucher of each collection is deposited, with reference number, at Herbarium of Pharmaceutical Biology, related to the Herbarium Sapientia, University of Rome, Italy, under the herbarium numbers 1-3/CSS/MN. Identification of each species was made by one of us (MN) by comparison with Herbarium samples.

HPTLC analysis

HPLC grade solvents were purchased from Sigma-Aldrich (Milan, Italy) and Carlo Erba (Milan, Italy). The HPTLC system (CAMAG, Muttenz, Switzerland) consisted of Linomat 5 sample applicator using 100 mL syringes and connected to a nitrogen tank; chamber ADC 2 containing twin trough chamber 20 x 10 cm; Immersion device III; TLC Plate Heater III; TLC visualizer linked to winCATS software. Glass plates 20 cm x 10 cm (Merck, Darmstadt, Germany) with glass-backed layers silica gel 60 (2 µm thickness). Before use, plates were prewashed with methanol and dried for 3 min at 100 °C.

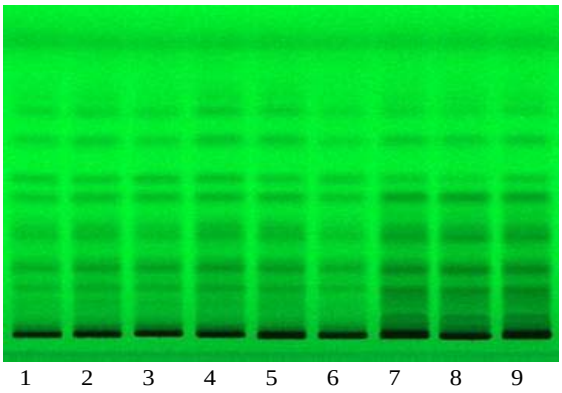


Figure 1: HPTLC plate of *Cistus salvifolius*. extracts. Without derivatization; visualization: UV 254 nm; Tracks: 1-3. Sample 1, collected in Turkey; Tracks 4-6 Sample 2, collected in Turkey; Tracks 7-9 Sample 3, collected in Sardinia (Italy).

The dried extracts of the analysed samples were weighted and dissolved in methanol (30 mg/mL). Filtered solutions were applied with nitrogen flow. The operating conditions were: syringe delivery speed, 10 s μL^{-1} (100 nL s^{-1}); injection volume, 4 µL; band width, 8 mm; distance from bottom, 8 mm. The HPTLC plates were developed in AcOEt:CH_2Cl_2:AcOH:HCOOHH_2O (100:25:10:10:11; *v/v/v/v*) using the automatic and reproducibly developing chamber ADC 2, saturated with the same mobile phase for 20 min at room temperature. The developing solvents (i.e. type of solvents and ratios) were carefully optimized before the analyses. The length of the chromatogram run was 70 mm from the point of application. The developed layers were allowed to dry in air for 5 min and then derivatives with a selected solution, including NPR (1 g diphenylborinic acid aminoethylester in 200 mL of ethyl acetate) and/or anhysaldehyde (1 ml *p*-anisaldehyde, 10 ml H_2SO_4, 20 ml AcOH in 170 ml MeOH), finally, the plates are warmed for 5 min at 120 °C before inspection. All treated plates were then inspected under a UV light at 254 or 366 nm or under reflectance and WRT, respectively, at a Camag TLC visualizer, before and after derivatization. CAMAG digital system win CATS software 1.4.3 was used for the documentation of derivatives plates. Sample solution of the extracts were prepared and stored at room temperature for 3 days and then applied on the same HPTLC plate and the chromatogram evaluated for additional band. Sample solutions of the extracts were found to be stable at 4 °C for at least 1 month and for at least 3 days on the HPTLC plates. Repeatability was determined by running a minimum of three analyses. Rf values for main selected compounds varied ± 0.02 %. The effects of small changes in the mobile phase composition, mobile phase volume, duration of saturation were minute and reduced by the direct comparison. On the contrary, the results were critically dependent on prewashing of HPTLC plates with methanol.

Conclusion

The sequence of the previous plates, reported in Figures 1-4, evidences the necessary treatments to obtain the maximum of the

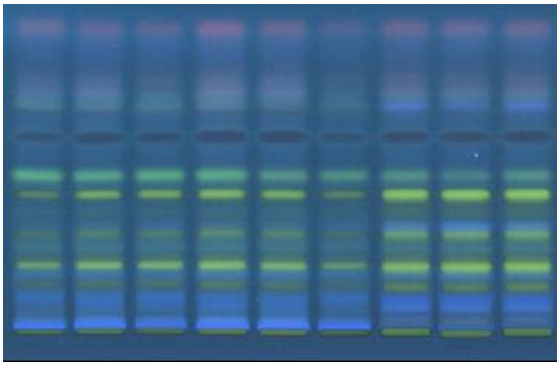


Figure 2: HPTLC plate of *Cistus salvifolius*. extracts. Derivatization: Natural Product Reagent; visualization: UV 366 nm. Tracks as reported in Figure 1.

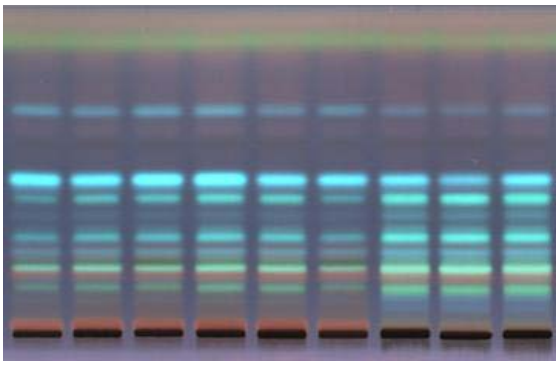


Figure 3: HPTLC plate of *Cistus salvifolius*. extracts. Derivatization: Natural Product Reagent and Anisaldehyde-sulfuric acid; visualization: UV 366 nm. Tracks as reported in Figure 1.

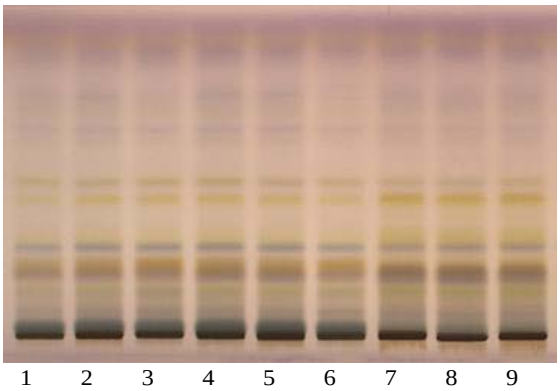
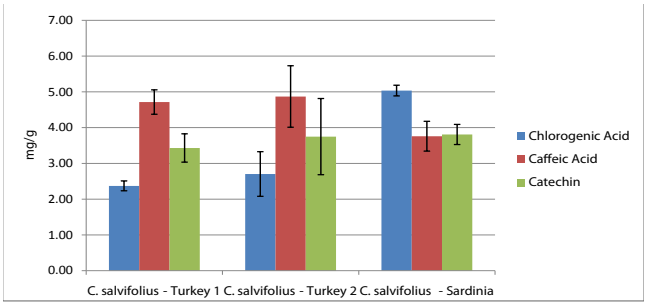


Figure 4: HPTLC plate of *Cistus salvifolius*. extracts. Derivatization: Natural Product Reagent and Anisaldehyde-sulfuric acid; visualization: White light (WRT). Tracks as reported in Figure 1.

information. The different derivatization was also utilized to evidence the standards, which were also useful to quantify these compounds inside the extracts, as reported in Figure 5. The comparison of the fingerprints of plants collected in Turkey, reported in tracks 1-3 and 4-6 with Italian sample, reported in tracks 7-9, allows evidencing a similar composition, with little difference on the quantity of few spots.

Therefore, we can produce the following conclusions:



Sample	Chlorogenic Acid	Caffeic Acid	Catechin
C. salvifolius - Turkey 1	2.37 ±0.14	4.71 ±0.34	3.43 ±0.40
C. salvifolius - Turkey 2	2.70 ±0.62	4.87 ±0.86	3.75 ±1.07
C. salvifolius - Sardinia	5.04 ±0.15	3.76 ±0.42	3.81 ±0.28

Figure 5: Quantification of markers presence in *Cistus salvifolius* populations. Each datum represents the mean of 3 replicates, whose tracks are reported in Figure 1-4. Means are followed by the standard deviations.

a) All the fingerprints are similar, albeit quantitative differences are present, and therefore useful as species fingerprint. This is against the information reporting a high plasticity in *Cistus* species. The absence of labdanun production is confirmed by the absence of the spots of labdane terpenes, usually Rf = 0.7-0.8. In particular, the fingerprints of samples from Turkey appeared very similar against the other ones from Sardinia.

b) The absence of any ibridation with other *Cistus* sp., that can be evidenced by HPTLC fingerprint that in case of ibridation reports the mix of the spots of the two ibridized species.

c) A difference between the two samples of *C. salvifolius* from Turkey concerns the quantities of chlorogenic acid. The standards of constituents were selected on the basis of their key influence in the same metabolic pathways, avoiding the use of selected specific markers. The minor production of caffeic acid could be referred to environment effects.

The reported data evidence the possibility of the utilization of HPTLC in biological studies and are noteworthy and useful for the development of new botanical nutraceuticals from *C. salviflolius* and related species.

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