A Chemometric Interpopulation Study of the Essential Oils of *Cistus creticus* L. Growing in Crete (Greece)

Costas Demetzos*, Thalia Anastasaki and Dimitrios Perdetzoglou

School of Pharmacy, Department of Pharmacognosy, University of Athens, Panepistimiopolis Zografou, Athens 15771, Greece.
Fax: +3022182210. E-mail: demetzos@galenos.pharm.uoa.gr

* Author for correspondence and reprint requests

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The chemical composition of the essential oils of twenty-five populations of *Cistus creticus* subsp. *creticus* L. from the island of Crete (Greece) and their interpopulation variability were analysed in detail by GC-MS. 142 compounds were identified representing an average of 56.8–89.8% of the oil composition. The components are represented here by homologous series of monoterpenes, oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes, diterpenes, labdane diterpenes, aldehydes, alkanes, esters, fatty acids, ketones, and others. Labdane diterpenes were detected and identified in the essential oils and have been found in high percentage composition. The results from the chemical analysis of the essential oils were submitted to chemometric cluster analysis in order to detect some pattern distribution and to identify which constituents can differentiate the groups of individuals. Two main chemotypes (clusters) were well differentiated; the first deals with eight populations of West Crete and the second with the rest of the populations. Cluster analysis based on labdane type diterpenes patterns, proved to be the best chemotype for the examined populations among the other chemical groups.

Introduction

Sixteen species of the genus *Cistus* (Cistaceae) are known to be native in the flora of the Mediterranean area. (Gultz *et al.*,1996). Leaves of all Cistus taxa are covered with glands secreting resin and essential oil. Plants and resin consist mainly of terpenoids (Gultz *et al.*, 1984; Demetzos *et al.*, 1994c), flavonoid aglycons (Demetzos *et al.*, 1990; Vogt *et al.*, 1987b), and glycosides (Vogt *et al.*, 1987a). Labdane diterpenes, which appears to be the predominant compounds of the essential oils have been studied for their antimicrobial and cytotoxic activity (Chinou *et al.*, 1994; Demetzos *et al.*, 1994a,b; Dimas *et al.*, 1998; Demetzos *et al.*, 1999a; Anastasaki *et al.*, 1999).

Continuing our research on the chemical composition of the genus *Cistus*, we now report the results obtained in an exhaustive investigation of the essential oils of twenty-five wild populations of *C. creticus* growing in Crete (Greece). To study the variability of volatiles among the populations, the essential oils obtained by hydrodistillation from each population were analysed by GC-MS. The results were submitted to chemometric cluster and principal component analysis. Qualitative and quantitative analysis of the essential oils was carried out and the natural chemotypes of the species in this particular phytogeographical region (Crete) is characterised. To our best knowledge no studies have been carried out on labdane diterpenes, as well as on the chemical composition variability of the essential oil of *C. creticus* s.l.(sensu lato) grown in Crete. The aim of this work was to study the interpopulation variability of the volatiles of *C. creticus* subsp. *creticus* and to find out the natural chemotypes of this taxon. In addition the variability of labdanes among the populations studied is of interest, due to their pharmacological properties (Chinou *et al.*, 1994; Demetzos *et al.*, 1999; Dimas *et al.*, 1999; Dimas *et al.*, 2001). The results on their variability will be used as a rule for phytochemical purposes. Chemotaxonomic investigation on this subspecies was also attempted in order to sed light on the origin of variation. There is no published data concerning the study of the variability and chemical composition of the essential oil of Greek populations of *C. creticus* s.l.

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Experimental

Sampling

Collection sites

The aerial fruiting parts from *C. creticus* subsp. *creticus* were collected in July 1997 on the island of Crete. Dr Perdetzoglou and Dr Demetzos (P& D) are the collectors of the samples from the following regions: North Perivolia (C1) (P&D, no 1623, **AT**hens **H**erbarium (**ATH**)); South Perivolia (C2) (P&D no 1624, ATH); North Fournes (C3) (P&D, no 1625a, ATH); South Fournes (C4) (P& D, no 1625b, ATH); Xamoudochori (C5) (P&D, no 1627, ATH); Tafoi Venizelon (C6) (P&D, no 1628, ATH); Kounoupidiana (C7) (P&D, no 1629, ATH); Xorafakia (C8) (P&D, no 1630, ATH); Akrotiri (C9) (P&D, no 1633, ATH); Ag. Triada (C10) (P&D, no 1634, ATH); Polyrinia (C11) (P& D, no 1636, ATH); Keramoti (C12) (P&D, no 1638, ATH); Malaksa (C13) (P&D, no 1641, ATH); Ormos Stomiou (C14) (P&D, no 17, ATH); Sarakina (C15) (P&D, no 46, ATH); Ammoudara (L1) (P&D, no 1646, ATH); Gournia (L2) (P&D, no 1647, ATH); Thrypti (L3) (P&D, no 1648, ATH); Ferma (L4) (P&D, no 1651, ATH); Ahlia (L5) (P&D, no 1653, ATH); Maronia (L6) (P&D, no 1658, ATH); Vai (L7) (P&D, no 1661, ATH); Vai (L8) (P&D, no 1662, ATH); Tourloti (L9) (P& D, no 1663, ATH); Roussa Eklisia (L10) (P&D, no 78, ATH). The samples of the plants were identified by one of us (D.P) and voucher specimens have been deposited in the Museum of Natural History Goulandris (**ATH**) in Athens, as well as in the Laboratory of Pharmacognosy, School of Pharmacy, University of Athens. The above abbreviations, i.e. C1-C15 and L1-L10 indicated on the map of Crete (Fig. 1) represented the localities of collections.

Analysis of essential oils

GC-MS analysis

Aerial fruiting parts from ten individual plants of each studied population were collected and used as homogenous samples. All the samples were collected at the same time, in order to avoid the diurnal variability. The essential oils for the were obtained by hydro distillation of 100 g air-dried material, for 3 h to 130 °C. All the oils were dried over anhydrous Na_2 SO_4, stored under refrigeration and were studied by GC-MS.

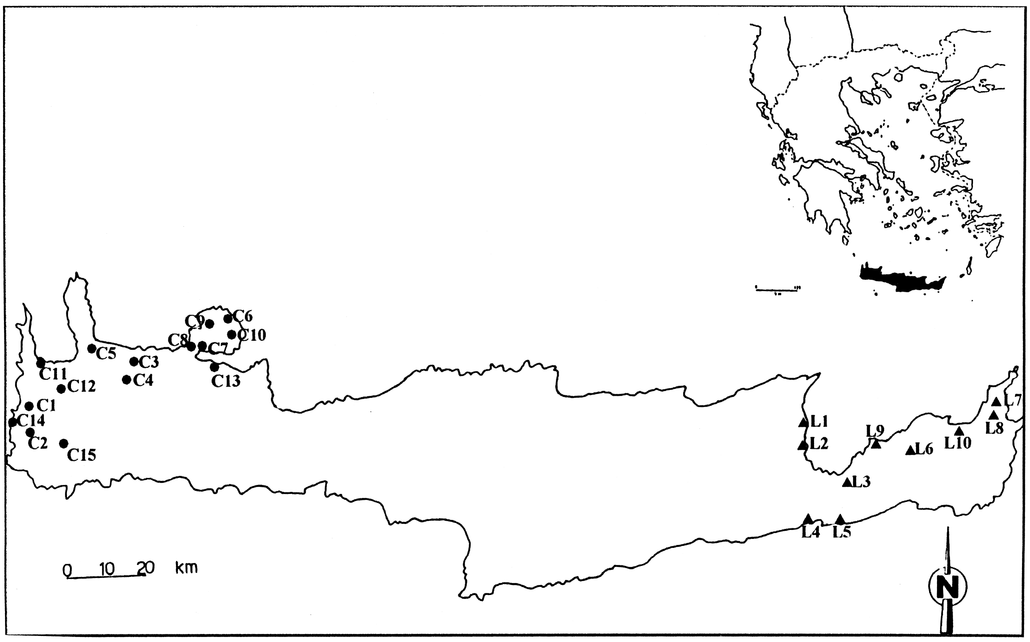

Fig. 1. Localities of collection of twenty-five populations of *C. creticus* in Crete; C1 -C15 and L1 – L10 (see text).

The yield of the essential oils varies from 1.6 to 37.2% (w/w) (Table I). The essential oils were analysed using capillary GC-MS spectrometry system operating in the EI mode. An HP-5 MS fused silica capillary column of 30 mx0.25 mm (0.25 μm film thickness) was used for the analysis. The carrier gas was helium, at a pressure of 53.1 kPa. Mass unit conditions: ion source 230 °C, ionisation energy 70 eV and electron current 1453 μA. The column was temperature programmed as follows: 50 °C for 5 min and the temperature was increased to 280 °C at a rate of 3 °C/min (split ratio 20:5). The identification of the chemical constituents was based on their retention indices (Adams, 1995; Anastasaki *et al.*, 1999), on the basis of their mass spectra fragmentation, using the Wiley 275/ NBS GC-MS library, (>96% match) and/or comparison with authentic samples previously isolated in our laboratory (Demetzos *et al.*, 1999a; Anastasaki *et al.*, 1999).

Chemometric statistical analysis

The first part of chemometric statistical analysis (Fig. 2), was based on the whole set of data (Table II). The second part of the cluster analysis (Fig. 3), was based on the labdane diterpenes set of data. Prior to cluster analysis, the variables were standardized for a normalized procedure. The set of data was processed through NCSS and STATISTICA commercial statistical packages. City-block (Manhattan) (method to measure the distance among the individuals i.e. sample of plants) was used to measure the similarity between samples (localities) and Ward's linkage method was used as an agglomerative algorithm.

Results and Discussion

The essential oils were obtained by hydrodistillation and yield 2.0% (C5 population) to 37.2% (L1 population) on dry weight basis (Table I). The different yield obtained from the twenty-five populations of various geographical sites may be due to different ecological (*i.e.* improved defence against microbial or insect attack) and geographical factors such as the soil type (Robles and Garzino, 2000) as well as local microenviromental conditions. The qualitative and quantitative analysis of the essential oils was made using GC-MS. Between the 236 detected compounds, 142 were identified and characterized on the basis of their mass spectra fragmentation pattern and with authentic samples reaching an average of 56.8–89.9% of the essential oil of each population. The results are represented here as homologous series of monoterpenes, oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes, diterpenes, labdane diterpenes, alkanes, aldehydes, ketones, fatty acids, esters and others. The oxygenated monoterpenes were detected in the most of populations, ranging from 0.8% in population L2 to 9.8% in population C4. Abundant oxygenated monoterpenes were carvacrol varying from 0.2% in population L9 to 9.8% in population C4, desmethoxy ensecalin varying from 0.8% in population C8 to 5.1% in population C12 and *trans*-ambrinol varying from 0.4% in population C12 to 2.7% in population L10. Cineole 1.8, *cis*-thujone, *trans* pinocarveol, camphene-hydrate and terpin-4-ol are not detected at all in eastern Crete, while eugenol and β-ionone are not detected in western Crete. The respective monoterpene hydrocarbons were totally absent in most of the populations *i.e.* C1, C5,

Table I. Yield% (w/w) of essential oil from the leaves of *C. creticus*.
Population (East Crete)

	*C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15
Yield%	13.7	10.6	18.5	5.9	2.0	14.4	21.3	14.5	27.1	14.1	4.2	13.8	10.2	6.7	2.2

*C: Populations from various localities of Crete (see Experimental part).
Population (West Crete)

	*L1	L2	L3	L4	L5	L6	L7	L8	L9	L10
Yield%	37.2	23.6	15.2	11.6	17.2	12.0	18.2	29.1	24.7	22.2

*L: Populations from various localities of Crete; (see Experimental part)

C9, C10, C13, C15, L1, L2, L3, L4 and L5. Their percentage content in the rest of populations varies from 0.2% in population L6 to 11.4% in population C4. Among monoterpenes santolinatriene exist only in population L8 of west Crete, while *p*-cymene and γ-terpinene exist only in population C4 of east Crete. The oxygenated sesquiterpenes were detected in higher percentages than the respective sesquiterpenes varying from 2.8% in population C13 to 44.2% in population C5, while in the sesquiterpenes the percentages varies from 2.1% in population C13 to 20.4% in population C12. Important oxygenated sesquiterpenes were guaiol varying from 0.4% in population L2 to 10.8% in population C5, viridiflorol varying from 1.1% in population C8 to 9.0% in population C12, β-eudesmol varying from 0.4% in population L10 to 5.4% in population C4 and selin-11-en-4α-ol varying from 0.8% in population L6 to 5.2% in population C12. The essential oils were characterized by high percentages of diterpenes, especially of labdanes (Table II). Within the class of labdanes manoyl oxide and 13-*epi*-manoyl oxide were abundant in all samples. The percentage content of manoyl oxide varies from 0.9% in population L1 to 20.4% in population C13, and in the case of 13-epi-manoyl oxide from 2.5% in population C5 to 36.8% in population C10. 3β-acetoxy manoyl oxide is well distributed within labdanes and was detected in 23 among the 25 populations in a range between 0.4% in population C14 to 12.8% in population L1. Sclareol a well know labdane diterpene having antimicrobial activity (Demetzos *et al.*, 1999) and labd-7,13-dien-15-ol were both detected in 20 populations (Table II) account for 0.6% in population C11 to 5.1% in population C13 and 0.7% in population L3 to 5.0% in population C3 respectively. Data on labdane type diterpenes by GC-MS is limited by the lack of authentic samples and most of them have never been analyzed by this method. That is why many of them are refered here as Not Identified (NI) (Table II). Several of them have recently been analyzed by our laboratory and identified in essential oils and extracts (Angelopoulou *et al.*, 2001a; Angelopoulou *et al.*, 2001b; Anastasaki *et al.*, 1999). The variation of labdane diterpenes, which was found to be the major class of components in all populations, may be due to several microenvironmental parameters, which are probably caused by a combination of irradiance, climate, nutrients, water availability, etc. (Robles and Garzino, 2000). Since labdane diterpenes are interest compounds having biological activities (Chinou *et al.*, 1994; Demetzos *et al.*, 1994a,b; Dimas *et al.*, 1998; Demetzos *et al.*, 1999a; Anastasaki *et al.*, 1999) their

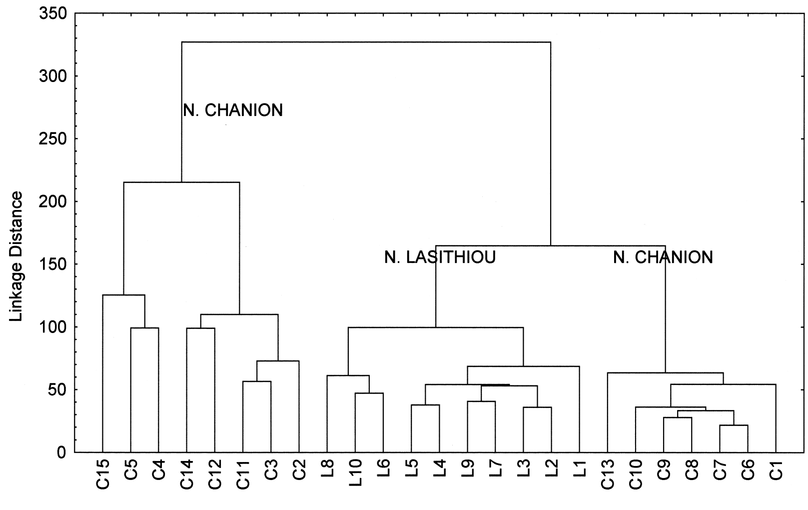

Fig. 2. Two-dimentional dendrogram obtained in the cluster analysis of the essential oils of twenty-five populations of *C. creticus* based on the whole set of data (Table II; see note at the end of the Ren. + Disc. chapter for this Table). Horizontal: samples-populations analysed; vertical: differentiation level between samples and populations. N. CHANION is the western part of Crete island, while N. LASITHIOU is the eastern (see Fig. 1).
Linkage Distance is the distance of each individual (sample of the plant) from all the others.

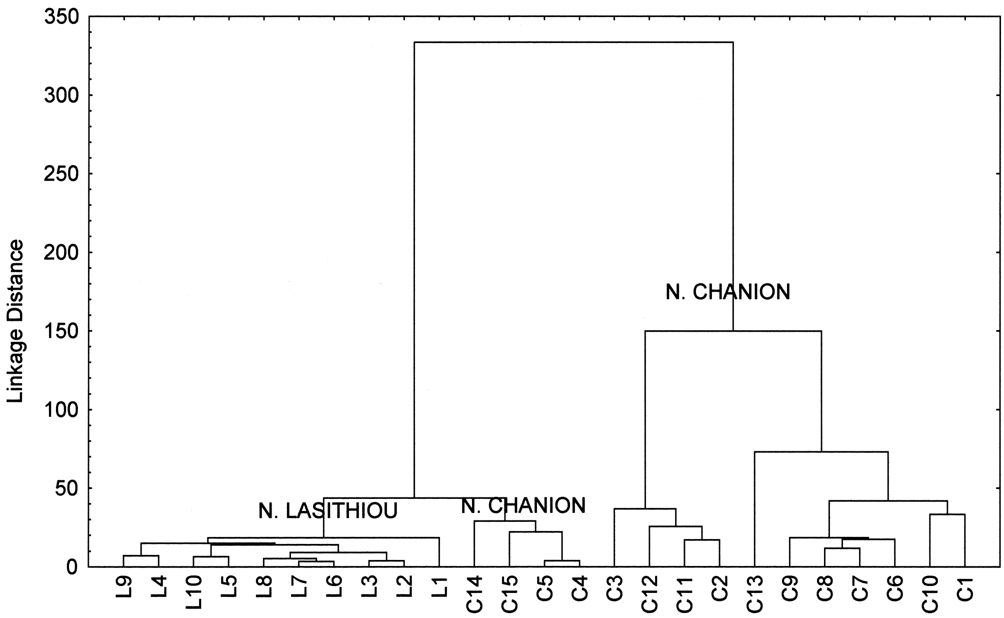

Fig. 3. Two-dimentional dendrogram obtained in the cluster analysis of the essential oils of twenty-five populations of *C. creticus* based on the labdane diterpenes set of data (Table II). Horizontal: samples-populations analysed; vertical: differentiation level between samples and populations.

variability has a possible ecological role, of improved the defence of plants against microbial attack or insect and animal predation.

Using cluster analysis the number of samples studied can be classified into number of groups (natural chemotypes), according to their chemical composition by 'magnifying' their similarities.

Results obtained from cluster analysis, showed the existence of a high interpopulation variability within the essential oils of *C. creticus*. From those twenty-five populations-samples submitted to multivariate analysis, two well-defined groups of essential oils were differentiated by cluster analysis; (Figs. 2 and 3). Concerning Fig. 2 that was based on the whole set of data we can observe two subclusters; the first one deals with eight populations found of the West part of Crete [NOMOS (= prefecture) CHANION; Fig. 1], and the second deals with two well differentiated geographically subsets of data: the first one of seven populations of peninsula Akrotiri, except the population C1 finding outside the peninsula (Fig. 1), and the second one the whole set of data belonging to the East Crete (NOMOS LASITHIOU; Fig. 1). According to Fig. 3 based on the labdane diterpenes set of data, we can observe two subsets; one deals with eleven populations of NOMOS CHANION, six of them finding on the peninsula Akrotiri (West Crete), except the population C1 finding outside of peninsula (Fig. 1), and the second is divided in two subgroups; the first one deals with the populations of East part of Crete (NOMOS LASITHIOU) and the second deals with the four populations of NOMOS CHANION (West part of Crete) found outside of peninsula Akrotiri. We applied the test of correlation coefficient to the Table II in order to clarify the possible linear correlation between studied populations. According to that procedure there is a strong positive linear correlation between the constituents of populations C7 and L9 ($R = 0.95$; significant at $p<0.05$).

On a phytochemical basis, the observed differentiation in the frame of NOMOS CHANION, are mainly due to the different percentages of Manoyl oxide and 13-epi-manoyl oxide (labdane diterpenes) (Figs. 1, 3). Concerning the morphological variability in *C. creticus* s.l. (sensu lato), there is no published data. Our personal repeated fieldwork in Crete (1996–1999) does not reveal any morphological variability within this species. Moreover there are no available data concerning the relationship of the chemical variability documented here to the variability of the essential oils of related *Cistus* species, neither data on the phytogeographic variation in morphology or chemical characters of the studied species or related *Cistus* species on Crete or elsewhere. Detailed data on the constituents are gathered in a Table II, which will be supplied by the authors upon request.

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