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Volatile Components from Aerial Parts of *Centaurea diffusa* and *C. micrantha* ssp. *melanosticta* and Their Biocidal Activity on Microorganisms Affecting Historical Art Crafts

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The chemical composition of the essential oils from aerial parts of two taxa of *Centaurea* belonging to subgenus *Acrolophus*, *Centaurea diffusa* Lam. and *C. micrantha* Hoff. ssp. *melanosticta* (Lange) Dostàl, respectively collected in Croatia and Spain, were evaluated by GC and GC-MS. The main components of *C. diffusa* were hexadecanoic acid (31.1%), (*Z,Z*)-9,12-octadecadienoic acid (10.7%) and damascenone isomers (6.4%), whereas hexahydrofarnesyl acetone (27.8%), hexadecanoic acid (8.3%) and caryophyllene oxide (6.4%) were the most abundant components of *C. micrantha* ssp. *melanosticta*. The oils showed good antibacterial and antifungal activities against some microorganisms that infest historical art works.

Keywords: Centaurea diffusa, Centaurea micrantha ssp. melanosticta, Asteraceae, Hexadecanoic acid, (Z,Z)-9,12-Octadecadienoic acid, Hexahydrofarnesyl acetone, Antimicrobial activity.

The search of new natural biocides that can be used as "green" alternative to synthetic chemicals in order to prevent and reduce the dangerous effects of microorganisms on historical art craft has recently grown up [1a-1f].

of genus Bacillus, Chaetomium, Myrothecium, Species Memnoniella, Stachybotrys, Verticillium, Alternaria, Trichoderma, Fusarium, Penicillium, and Aspergillus frequently found on cellulosic objects stored in archives, libraries, and museums can cause their deep deteriorations. The historical organic materials such as paper, paintings, wood, papyri, incunabula, books, as well as textile and leather are mainly constituted of natural fibres that may offer an environment suitable for the development of several heterotrophic microorganisms (bacteria and fungi). The occurrence of glues residue of vegetable and animal origin can enhance the rate of microbial growth. This phenomenon causes damages at molecular level, such as oxidation, depolymerisation, and breakdown of molecular and supra-molecular structure, with consequences at macroscopic level: loss of strength and elongation, discoloration, changes in appearance. The main responsible for the degradation of cellulosic fibres are cellulases, a group of fibrolytic enzymes which cooperatively hydrolyze cell wall fibers of vegetables into glucose, cellobiose or oligosaccharides [1g]. On the other hand, the degradation of wool is a more complex keratinolysis process that implies an initial denaturation of the substrate by the fissation of disulfide bridges, which are at the origin of the keratin resistance, followed by the hydrolysis of protein linear chains mediated by extracellular proteinases [1h]. Also the stone monuments can be involved in external alteration processes due to fungal activity, such as the production of organic acids and pigments by the hyphal penetrated through the porous stone matrix [1c].

Centaurea diffusa Lam., also known as diffuse knapweed, white knapweed, or tumble knapweed, is a member of the genus *Centaurea* in the family Asteraceae, belonging to subgenus *Centaurea* s.str. [formerly subgenus *Acrolophus* (Cass.) Dobrocz]

[2a]. It is a biennial or short-lived perennial species with flowerheads broadly urn-shaped, 0.6-0.8 in tall, solitary or in clusters of 2-3 at the ends of the branches. Floral bracts are yellowish with a brownish margin, sometimes spotted, fringed on the sides, and terminating in a slender bristle or spine. The heads contain two types of flowers, ray flowers around the edges surrounding tubular disk flowers. The ray flowers are white, rosepurple, to lavender. Basal leaves are stalked and divided into narrow, hairy segments. Stem leaves are smaller, alternate, less divided, stalkless, and become bract-like near the flower clusters. Diffuse knapweed, native to Asia Minor (Turkey, Svria), the Balkans, Ukraine and southern Russia, is a pioneer species that can quickly invade disturbed and undisturbed grassland and shrubland. Once established, diffuse knapweed outcompetes and reduces the quantity of desirable native species such as perennial grasses. Diffuse knapweed contains allelopathic chemicals, which can suppress competitive plant growth and create single species stands. The densities of these stands can range from $1-500 \text{ plants/m}^2$ [2b].

Several papers have been published on the chemical investigations of *C. diffusa* indicating the occurrence, as secondary metabolites, of sesquiterpene lactones [2c], polyacetylenes [2d-2e], triterpenes [2f], and 8-hydroxyquinoline, found to be a good herbicide [2g]. On the other hand, to the best of our knowledge, only one manuscript has been published on the essential oil of *C. diffusa*, collected in Turkey [2h].

C. micrantha Hoff. ssp. *melanosticta* (Lange) Dostàl (syn. *C. langei* Nyman, *C. melanosticta* (Lange) Franco) belongs to subgenus *Centaurea* s.str. [formerly subgenus *Acrolophus* (Cass.) Dobrocz], sect. *Centaurea*. It is a biennial plant with erect, much-branched,40-90 cm highstem, forming a lax panicle. Leaves are green above, lanate beneath; lowerones are 2-pinnatifid. Capitula are solitary. Involucre is 5-6 mm in diameter, narrowed atbase, while appendages are black whit 0.5-0.7 mm long apex and 0.5 mm longfimbriae. Florets are purple; pappus – *c.* 1/5 as long as achene.

The species is distributed in northwestern Spain and north and central Portugal [3]. To the best of our knowledge no investigations have been reported on this subspecies.

Consequently, in the context of our on-going research on *Centaureae* Mediterranean taxa [4], we were interested to investigate the chemical composition of the essential oils obtained from *C. diffusa* and *C. micrantha* ssp. *melanosticta*, as well as their biological properties against several microorganisms, including *Bacillus subtilis, Staphylococcus aureus, Fusarium oxysporum* and *Aspergillus niger*. These biological targets were chosen for their known ability to infest historical art craft materials [1].

Hydrodistillation of C. diffusa aerial parts gave a yellow oil (CD). Forty-eight compounds were identified in CD, representing 92.5% of the total components (Table 1). The oil was rich in fatty acid and esters (46.3%). Hexadecanoic acid (31.1%) and (Z,Z)-9,12octadecadienoic acid (10.7%) were, by far, the main components of this class as well as of the oil. It is worthy of mention also the presence of good amount of sesquiterpene hydrocarbons (12.2%) with calarene (3.1%) as the main component and carbonylic compounds (9.8%) rich of damascenone isomers (6.4%). Monoterpenes were present in very low quantity (2.8%) and diterpenes totally absent. Hexadecanoic acid, the main compound of C. diffusa, was shown to be the most abundant component also of essential oil of C. aladagensis [5a], C. luschaniana, C. tossiensis, C. wagenitzii [5b], C. saligna [5c], and C. paphlagonica [5d] from Turkey and of C. nicaeensis, C. parlatoris, and C. solstitialis ssp. schouwii (D.C), species growing wild in Sicily [5e]. The comparison of the composition of our oil with that of C. diffusa collected in Turkey [2g] pointed out some remarkable differences. In fact, eudesmol (45.4%), absent in CD, was the main compound whereas hexadecanoic acid, the principal metabolite in CD (31.1%) was present in the Turkish population to a lesser amount (4.5%).

Hydrodistillation of *C. micrantha* ssp. *melanosticta* aerial parts gave a yellow oil (**CD**). Therty-six compounds were identified in **CD**, representing 90.7% of the total components (Table 1). The main class was represented by carbonylic compounds with hexahydrofarnesyl acetone (27.8%), by far, the main component of the class and of the oil. Oxygenated sesquiterpenes were quite abundant (20.0%) with caryophyllene oxide (6.4%) and spathulenol (4.2%) as main metabolites. It has to be highlighted the presence of diterpenes (14.7%), a feature quite rare in genus *Centaurea*, and a minor amount of fatty acid and esters (12.9%) with respect to **CD**.

Table 2 reports the antimicrobial activity of the essential oils (**CD** and **CM**). The oil of *C. diffusa* showed significant results both towards bacteria (*Bacillus subtilis, Staphylococcus aureus*) and toward moulds (*Fusarium oxysporum* and *Aspergillus niger*). These findings can be related to the occurrence in the oil of high levels of fatty acids.

 Table 1: Percent composition of the essential oils of C. diffusa Lam. and C. micrantha

 Hoff. ssp. melanosticta (Lange) Dostàl aerial parts (subgenus Acrolophus).

RI ^a	RI ^b	Component	CD ^e	CM ^d	Ident. ^e
		Monterpene hydrocarbons	1.5	0.0	
1025	11278	p-Cymene	0.8		1, 2, 3
1030	1203	Limonene	0.7		1, 2, 3
		Oxygenated monoterpenes	1.3	0.0	
996	1195	1,8-Dehydrocineole	0.5		1, 2
1201	1613	Safranal	0.8		1, 2
		Sesquiterpene hydrocarbons	12.2	0.7	
1373	1493	a- Ylangene	2.3		1, 2
1377	1497	a-Copaene		t	1, 2
1380	1547	α-Funebrene	1.9		1, 2

187 β58 β-Elemene 0.5 1,2 144 1554 as-Intalicene 1.4 1,2 1423 1654 Calarane (β-Gujuncne) 3.1 t 1,2 147 1076 Calarane (β-Gujuncne) 3.1 t 1,2 147 1076 Calarane (β-Gujuncne) 1.1 0.1 1,2 149 1074 Calarane (β-Gujuncne) 1.1 0.1 1,2 1495 1740 Valuescence 1.1 0.1 1,2 1495 1740 Valuescence 0.3 1.2 1498 1744 Valuescence 0.3 1.2 1542 1918 a-Cadnal (sequiterpenes 3.3 2.0.0 1578 2150 Spathuleno (a 4.2 1,2 1580 2107 Guaiol 0.4 1,2 1,2 1580 2108 A-Cadnal (Teuchan) 0.7 1,2 1,4 1590 2143 a-Cadnal (Teuchan) 0.7 1,2 1,4 1502 2167 Guaiol 0.5						
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$\begin{array}{c c c c c c c c c c c c c c c c c c c $	1870	2822	Pentadecanoic acid	0.2		1, 2, 3
$\begin{tabular}{ c c c c c c c c c c c c c c c c c c c$	1972	2931	Hexadecanoic acid	31.1	8.3	1, 2, 3
$\begin{tabular}{ c c c c c c c } \hline Phenolic compounds & 4.1 & 0.0 \\ \hline 1091 & 1835 & Guaiacol & 0.6 & 1, 2 \\ \hline 1312 & 2180 & 4-Vinylguaiacol & 2.7 & 1, 2 \\ \hline 1353 & 2186 & Eugenol & 0.8 & 1, 2 \\ \hline & Diterpenes & 0.0 & 14.7 & t & 1, 2 \\ \hline 1671 & 2832 & (Z)-Cniferyl alcohol & t & 1, 2 \\ \hline 1950 & 2622 & (Z)-Phytol & 2.1 & 1, 2 \\ \hline 2345 & >3000 & Sandaracopimar-15-en-8.\betayl & 1.2 & 1, 2 \\ \hline acctate & & & & & & & \\ \hline Hydrocarbons & 13.2 & 12.9 & & \\ \hline 1353 & 1721 & 1, 2-Dihydro-1, 1.6- & 1.5 & 1, 2 \\ \hline 1731 & 2193 & 2,6-Diisopropylnaphtalene & 3.1 & 1, 2 \\ \hline 2300 & 2300 & Tricosane & 1.6 & 3.5 & 1, 2, 3 \\ \hline 2400 & 2600 & Hexacosane & 0.4 & 1, 2, 3 \\ \hline 2500 & 2500 & Pentacosane & 0.4 & 1, 2, 3 \\ \hline 2600 & 2600 & Hexacosane & 0.3 & 1, 2, 3 \\ \hline 2700 & 2700 & Heptacosane & 0.3 & 1, 2, 3 \\ \hline 2800 & 2800 & Octacosane & 1.8 & 3.2 & 1, 2, 3 \\ \hline 1002 & 1243 & 2-Pentylfuran & 0.4 & 1, 2 \\ \hline 102 & 1243 & 2-Pentylfuran & 0.4 & 1, 2 \\ \hline 103 & 100 & Hentriacontane & 0.2 & 1, 2, 3 \\ \hline 1002 & 1243 & 2-Pentylfuran & 0.4 & 1, 2 \\ \hline 1002 & 1243 & 2-Pentylfuran & 0.4 & 1, 2 \\ \hline 1002 & 1243 & 2-Pentylfuran & 0.4 & 1, 2 \\ \hline 102 & 1243 & 2-Pentylfuran & 0.4 & 1, 2 \\ \hline 103 & 100 & Hentriacontane & 0.$	2104	3160	(Z,Z)-9,12-Octadecadienoic acid	10.7		1, 2, 3
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a: HP-5 MS column;
 b: HP Innowax column ^c: CD = Centaurea diffusa Lam. aerial parts
 d: CM = C. micrantha Hoff. ssp. melanosticta (Lange) Dostàl;
 c. 1, retention index, 2: mass spectrum, 3: co-injection with authentic compound;
 c: t: trace, <0.05..

In fact, palmitic acid (the main component of **CD**) has been shown to have an excellent antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus* [6a] and remarkable anti-fungal properties against *Fusarium oxysporum* and *Aspergillus niger* [6b]. Furthermore (Z,Z)-9,12-octadecadienoic acid (linoleic acid) has been proved to be very active against *Bacillus subtilis* and in lesser amount against *Staphylococcus aureus* [6c] and to have antifungal activity against *Fusarium oxysporum* [6b].

The oil of *C. micrantha* ssp. *melanosticta*, on the other hand, is active only on Gram+ bacteria (*Bacillus subtilis* and *Staphylococcus aureus*) probably due to the occurrence, in its composition, of several metabolites whose antimicrobial activities against these bacteria have been well stated: hexahydrofarnesyl acetone [6d], phytol [6e], caryophyllene oxide [6f, 6g] and spathulenol [6g].

The relevant biocidal activity of *C. diffusa* and *C. micrantha.* ssp. *melanosticta* essential oils is worthy of interest considering that the microorganisms affected are recognized to infest archives, libraries, and historical textile objects. In light of this, preparation based on **CD** and **CM** could find useful applications in the protection and disinfestation of museum objects.

Experimental

Plant material: Aerial parts of *C. diffusa* Lam. (**CD**) were collected at the full flowering stage 3 km south of Nerežišća, (43°19'35" N; 16°35'12" E; 457 m s/l) Brac (Croatia), at the end of June 2015. Aerial parts of *C. micrantha* Hoffmanns subsp. *melanosticta* (Lange) Dostal (CM) were collected at the full flowering stage near Les Planes, (41°42'28" N; 02°46'54" E; 106 m s/l), 25 km north of Blanes, Costa Brava (Spain), at the end of June 2016. Typical specimens (PAL 15/90MB and PAL16/97MB, respectively), identified by Prof. Svetlana Bancheva, have been deposited in the Department STEBICEF, University of Palermo, Palermo, Italy.

Isolation of the essential oil: The air-dried samples (200 g) were ground in a Waring blender and then subjected to hydrodistillation for 3 h according to the standard procedure described in the European Pharmacopoeia [7a] using a Clevenger-type apparatus. One mL of *n*-hexane was used to improve the phase separation. The oils were dried over anhydrous sodium sulfate and then stored in sealed vials, at -20°C, ready for the GC and GC-MS analyses. The sample yielded 0.21% of **CD** and 0.17% of **CM**, respectively, of yellow oil (w/w).

Gas chromatography-mass spectrometry: Analytical gas chromatography was carried out on a Perkin-Elmer Sigma 115 gas chromatograph equipped with a HP-5MS capillary column (30 m x 0.25 mm, 0.25 µm film thickness), a split-splitless injector heated at 250 °C and a flame ionization detector (FID) at 280°C. Column temperature was initially kept at 40°C for 5 min, then gradually increased to 250°C at 2°C/min, held for 15 min and finally raised to 270°C at 10°C/min. The injection volume was 1.0 µL (split ratio 1:20). A fused silica HP Innowax polyethylenglycol capillary column (50 m x 0.20 mm, 0.25 µm film thickness) was also used for

References

analysis. In both cases helium was the carrier gas (1 mL/min). GC-MS analysis was performed on an Agilent 6850 Ser. II apparatus, fitted with a fused silica DB-5 capillary column (30 m x 0.25 mm, 0.33 μ m film thickness), coupled to an Agilent Mass Selective Detector MSD 5973; ionization voltage 70 eV; electron multiplier energy 2000 V; source temperature 250°C. Mass spectra were scanned in the range 35-450 amu, scan time 5 scans/s. Gas chromatographic conditions were the same as those for GC; transfer line temperature, 295°C.

Identification of components: Most of the constituents were identified by GC by comparison of their retention indices (R_i) with either those in the literature (7b, 7c) or with those of authentic compounds available in our laboratories or purchased from the Sigma-Aldrich Co. Retention indices were determined in relation to a homologous series of *n*-alkanes (C_8 - C_{30}) under the same conditions. Further identification of oil components was achieved by comparing their mass spectra on both columns, either with those stored in NIST 02 and Wiley 275 libraries or with mass spectra from the literature (7c-7e) and our personal library. Component relative concentrations were calculated based on GC peak areas without using correction factors. These results are shown in Table 1.

Antimicrobial assay: The antimicrobial activities of the essential oil were tested against a panel which included 8 bacterial species, selected as representative of Gram-positive: Bacillus subtilis (ATCC 6633), Staphylococcus aureus (ATCC 25923), S. epidermidis (ATCC 12228), Streptococcus faecalis (ATTC 29212); and Gram-negative: Proteus vulgaris (ATCC 13315), Pseudomonas aeruginosa (ATCC 27853); two moulds, Fusarium oxysporum (ATCC 695) and Aspergillus niger (ATCC 16401). The strains were grown on Tryptone Soya Agar (Oxoid, Milan, Italy) for the bacteria, Saboureaud Dextrose Agar (SDA) with chloramphenicol for yeasts and SDA for moulds. For the antimicrobial tests, Tryptone Soya broth (Oxoid, Milan, Italy) for bacteria and Sabouraud dextrose broth (SDB) for yeasts and fungal strains were used. The antimicrobial activity was evaluated by determining the minimum inhibitory concentration (MIC) and the minimum microbiocidal concentration (MMC), which includes minimum bactericidal (MBC) and minimum fungicidal concentrations (MFC), using the broth dilution method [7f]. Oil samples were tested in triplicate.

Table 2: MIC (μg/mL) and MMC^{*} (μg/mL) of essential oil from *Centaurea diffusa* (CD) and *C. micrantha.* ssp. *melanosticta* (CM).

()()-						
Strain	CD	СМ	Ch	Ke		
Bacillus subtilis ATCC 6633	12.5 (25)	12.5	12.5	NT		
Staphylococcus aureus ATCC 25923	25 (50)	25	25	NT		
Staphylococcus epidermidis ATCC 12228	25	50	3.12	NT		
Streptococcus faecalis ATCC 29212	50	50	25	NT		
Proteus vulgaris ATCC 13315	100	100	25	NT		
Pseudomonas aeruginosa ATCC 27853	100 (>100)	100 (>100)	100	NT		
Fusarium oxysporum ATCC 695	6.25 (12.5)	25	NT	3.12		
Aspergillus niger ATCC 16401	6.25	25	NT	3.12		

* MBC are reported in brackets when different from MIC; NT: not tested; Ch: Chloramphenicol; Ke: Ketoconazole.

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