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The chemical composition of the flowers essential oil of *Inula crithmoides* (Asteraceae) growing in aeolian islands, Sicily (Italy) and its biocide properties on microorganisms affecting historical art crafts

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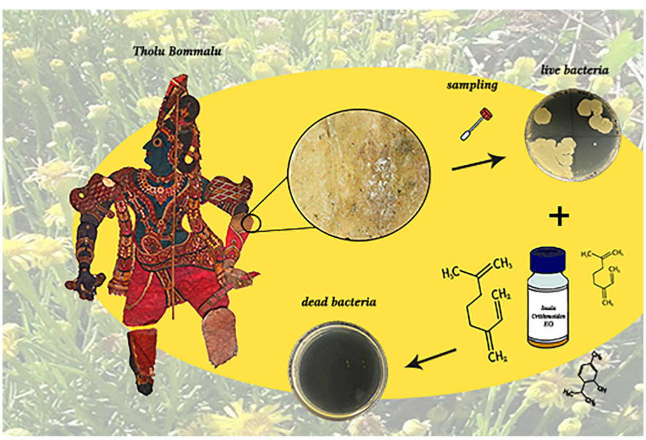
ABSTRACT

Essential oils have been used for a long time in several fields of interest. Recently, they have also been applied in the conservation of Cultural Heritage to contrast biodeterioration replacing the most current biocides toxic for humans and environment. *Inula crithmoides* L. (syn. *Limbarda crithmoides* (L.) Dumort) is a halophyte species distributed along the Mediterranean coasts and it is used as an edible vegetable since the young leaves or shoots are eaten raw or cooked. Several biological properties have been determined for this plant including antimicrobial activities. In this study the volatile composition of the aerial part of an accession from the Aeolian Islands, Sicily (Italy) is described. Furthermore, the *in vitro* antibacterial assay against four species of bacteria isolated from a XX century *Tholu Bhommalu*, a leather painted puppet from Andhra Pradesh (India), was showed by the Agar disc diffusion method.



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1. Introduction

Inula crithmoides L., golden samphire, synonyum of *Limbarda crithmoides* (L.) Dumort, (Theplantlist) is a perennial halophyte plant belonging to genus *Inula* (tribe *Inuleae* of the Asteraceae family) growing up to 1 m. It is in flower from July to August, and the seeds ripen from August to September. The flowers are hermaphrodite (have both male and female organs) and are pollinated by bees, flies, beetles, etc. It grows along the coasts of Europe, including Britain, and western Asia, in saline soils and it can tolerate maritime exposure (Tutin and Heywood 2006). It is often used as food, both cooked and raw, especially in Lebanon, Spain and Italy (Facciola 1990; Zurayk and Baalbaki 1996; Guarrera et al. 2006; Tardio et al. 2006).

Previous phytochemical investigations on *I. crithmoides* showed the presence of triterpenoids, (Belloum et al. 2013), flavonoids (El-Lakany et al. 1996; Males et al. 2004), butyl glycosides (El-Lakany et al. 2003), sesquiterpenes (Mahmoud et al. 1981), quinic acid derivatives (Aboul Ela et al. 2012), diterpenes, thiophenes and several thymol derivatives (Metwally and Dawidar 1985; Marco et al. 1993; Adorisio et al. 2020). Four previous papers have been published on the composition of the essential oils of *I. crithmoides* collected in Sicily (Fontana et al. 2014), Central Italy (Giamperi et al. 2010), Tunisia (Jallali et al. 2014) and Spain, Malta and Greece (Tsoukatou and Roussis 1999). For another hand, the biochemical potential of other species belonging to the Fagaceae family has been demonstrated. The extracts of *Castanea sativa* (Genovese et al. 2020) and *Quercus incana* (Sarwar et al. 2018), in fact, have shown antimicrobial and antitumor activity. In particular, 4-hydroxy decanoic acid, extracted in ethyl acetate from *Quercus incana*, was significantly active against *Bacillus subtilis*, *Staphylococcus aureus* and *Micrococcus luteus* (Gram-positive) (Zone of inhibition: 8.0, 16.0 and 11.0, respectively).

Several studies showed the antioxidant and antifungal properties both of the extracts and the essential oils of *I. crithmoides* (Abdel-Wahhab et al. 2008; Giamperi et al. 2010; Bucchini et al. 2013, 2015, Jallali et al. 2014, 2020). Furthermore, the herbicidal (Omezzine et al. 2011), antileishmanial (Oliveira et al. 2018), antiproliferative (Adorisio et al. 2020) and antibacterial (Aboul Ela et al. 2011; Assi et al. 2014; Jallali et al. 2014) properties of this species have also been determined. It should also be noted that other species belonging to these taxa, such as *Inula viscosa* (L.) Aiton and *Inula cappa*, have shown good antibacterial activities. The essential oil of *I. viscosa,* dominated by the presence of polygodial (19.8%), phytol (12.3%), fokienol (6.0%) and intermedeol neo (5.1%), had moderate activity against *S. aureus* and *E. enterica* (Inhibition Zone: 26.8 and 24.9 mm, respectively) (Ounoughi et al. 2020); In turn, the essential oil of *Inula cappa* (Buch-Ham. ex. D. Don) DC, tested against ten bacterial strains, showed significant activity against *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Xanthomonas phaseoli*, and *Bacillus subtilis* (Minimum inhibitory concentration (MIC) of 16.0, 14.0, 15.0 and 14.0, respectively) (Priydarshi et al. 2016).

Biological colonisation represents one of the most dangerous degradation processes of Cultural Heritage. Several microbial taxa, such as fungi and bacteria, are capable to colonise objects of cultural interest, sometimes endangering their integrity inducing irreversible damage (Leplat et al. 2017). Microorganisms can cause several types of degradation and alteration processes, also leaving pigments staining the artwork surface. In order to minimise the deterioration of colonised substrate, conservators need to perform specific treatments. Moreover, bacteria and fungi can produce acids corroding and changing the surface color (Caneva et al. 2007). Nowadays, the most used method to eradicate microbial colonisation is based on application of biocidal products, detrimental for conservator health and environment, such as Benzalkonium chloride, toxic and not degradable, being persistent in the environment and contaminating areas far from the site of application (Arias-Estévez et al. 2008). Currently the 'green conservation' is preferred and many chemical products are not used anymore (Casiglia et al. 2019).

Leather artefacts are especially affected by biological colonisation when they are kept in unfavorable conditions, far from the international standards (El-Shamy 2019).

Consequently, as continuation of our research on essential oils of Sicilian plants (Gagliano Candela et al. 2020; Ilardi et al. 2020; Catinella et al. 2021), on their biological properties (Badalamenti et al. 2020a, 2020b; Napoli et al. 2020) and as natural biocides for the conservation of the artistic heritage (Rotolo et al. 2016; Casiglia et al. 2019; Gagliano Candela et al. 2019; Palla et al. 2020), we decided to investigate the chemical composition of *I. crithmoides* the essential oil, obtained from plants collected in Aeolian Island (Sicily, Italy), and its biocidal properties on microorganisms, isolated from a leather historical art crafts, comparing them to the commercial biocide benzalkonium chloride (BAC).

2. Results and discussion

2.1. Chemical composition of essential oil

Hydrodistillation of *I. crithmoides* aerial parts gave a pale-yellow oil. Overall, 40 compounds were identified, representing 90.1% of total components, listed in Table S1 according to their retention indices on a HP-5 MS column and classified into five classes on the basis of their chemical structures. Monoterpene hydrocarbons formed the main class, representing 45.2% of the oil, with β-myrcene (13.7%) as the most abundant component. In the same class, *p*-cymene (11.7%), β-phellandrene (6.6%), α-pinene (5.5%) and camphene (5.2%) were also present in good amounts. Oxygenated monoterpenes were the second most abundant class (39.9%), with thymol acetate (14.4%) as the main component of this class; instead sesquiterpenes hydrocarbons (1.9%) and the oxygenated sesquiterpenes (0.5%) were present in very poor amount. Table 1 reports the main constituents of the oil.

Every *I. crithmoides,* essential oil obtained from plants collected in Sicily (Fontana et al. 2014), Central Italy (Giamperi et al. 2010), Tunisia (Jallali et al. 2014), Spain, Malta and Greece (Tsoukatou and Roussis 1999), were dominated by monoterpene hydrocarbons (32.1–87.4%): α-phellandrene (2.2–26.2%), *p*-cymene (trace-53.8%), β-phellandrene present only in Greek (30.7%) and Sicilian samples (8.5%), limonene (3.2–24.0%) in Tunisian samples. On the other hands, β-myrcene, main monoterpene hydrocarbon of *I. crithmoides* EO, was practically absent in all oils analysed so far. Similarly, to all the other oils, with the exception of the accessions from Sicily (Fontana et al. 2014) and Malta (Tsoukatou and Roussis 1999), Lipari EO sample is devoid of sesquiterpene derivatives. Finally, in Sicilian plants it is noteworthy the presence of thymol (2.1%) and the absence of scopoletin (Tsoukatou and Roussis 1999; Giamperi et al., 2010), 1-methyl

Table 1. Main components (%) of the essential oil of aerial parts of *I. crithmoides* collected in Aeolian Islands.

Compounds	LRI[a]	LRI[b]	%	Identification
α-Pinene	932	1019	5.5	LRI, MS, Co-I
Camphene	944	1060	5.2	LRI, MS, Co-I
β-Myrcene	981	1150	13.7	LRI, MS, Co-I
α-Phellandrene	998	1166	0.9	LRI, MS, Co-I
p-Cymene	1013	1257	11.7	LRI, MS, Co-I
β-Phellandrene	1022	1201	6.6	LRI, MS, Co-I
Thymol	1267	2185	2.6	LRI, MS, Co-I
Thymol acetate	1352	1813	14.4	LRI, MS, Co-I
2,3,4-Trimethylacetophenone	1548	1882	5.3	LRI, MS

Percentages (%), in elution order, are given on apolar column (HP-5 MS). Linear retention indices LRI[a] and LRI[b] are given, respectively, on the apolar (HP-5 MS) and the polar column (DB-Wax). All compounds are identified by LRI and MS from laboratory libraries and by Co-elution (Co-I) with authentic standard (Sigma-Aldrich).

Table 2. The antibacterial activity of *Inula crithmoides* essential oil by the diffusion method on disc.

Biocide products	Conc. (%)	Inhibition zone diameter (mm)[a]			
		Bacillus sp.	*Georgenia* sp.	*Streptomyces* sp.	*Ornthinibacillus* sp.
Inula crithmoides EO	50	14	18	16	16
	100	17	22	19	21
benzalkonium chloride	3	7	9	8	6
pentane	100	0	0	0	0

aDiameter of the inhibition halo, including the disc diameter, outlined as: sensitive strain = i.h > 9 mm; resistant strain < 9 mm.

ethyl-trimethyl benzene (Giamperi et al., 2010) and (12*Z*)-abienol (Tsoukatou and Roussis 1999), quite abundant in other oils.

2.2. Antimicrobial activity

Table 2 shows results of antibacterial activity using Agar disc diffusion method. Specifically, *Georgenia* sp. isolated colonies are the most susceptible to *I. crithmoides* essential oil. Relevant sensitivity is highlighted for both EO solutions (50%, 100%), able to produce inhibition halos up to 18 mm; *Bacillus* sp. seems to be less sensitive, although i.h. diameter is > 9 mm.

Antimicrobial activity of *I. crithmoides* EO, displayed against all isolated colonies (Figure S1), is clearly greater than controls: (BAK) benzalkonium chloride, the reference biocide, and pentane.

The biocide properties of *I. crithmoides* EO are related to with its chemical composition. In fact, several microbiological studies evidenced that β-myrcene possesses antibacterial properties on *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* (Wang et al. 2019) as well as on plant pathogenic bacteria *Agrobacterium tumefaciens* and *Erwinia carotovora* var. *carotovora* (Abdel Rasoul et al. 2012). On the other hand, several studies on essential oils containing high amounts of *p*-cymene and thymol (Bukvički et al. 2014; Yang et al. 2014) have shown excellent antimicrobial activities against some *Gram*-positive (*Listeria monocytogenes*) and *Gram*-negative (*Salmonella typhimurium* and *Escherichia coli*) bacteria and yeast strains (*Pichia membranaefaciens*, *Zygosacharomyce bailii* and *Aureobasidium pullulans*).

However, although the thymol derivatives (thymol acetate and thymol methyl ether, 15.6% of the essential oil), do not have a free phenolic hydroxyl group, which it has been shown to be essential for the antimicrobial activity of similar compounds (Ben Arfa et al. 2006), they can have also a positive contribute to the antimicrobial properties.

3. Experimental

3.1. Plant material

Aerial parts from *Inula crithmoides* L. (**Ic**) were collected in 'Acqua Calda' site in Lipari, one of Aeolian Islands, Sicily (Italy) (38°31′11″ N, 14°56′00″ E, 2 m a.s.l), in August 2020 and stored at the Department STEBICEF, University of Palermo, Sicily, Italy (voucher MB 65/2020).

3.2. Isolation of volatile components

Air-dried samples were ground in a Waring blender and then subjected to hydro-distillation for 3 h, according to the standard procedure described in European Pharmacopoeia (2020). The oil was dried over anhydrous sodium sulphate and stored in sealed vial under N_2, at −20 °C, ready for the GC and GC-MS analyses; The sample yielded 0.25% of oil (w/w).

3.3. GC, GC-MS analysis of essential oil

GC-MS analysis was performed according to Rigano et al. (2020) procedure, using an Agilent 7000 C GC system, fitted with a fused silica Agilent HP-5 MS capillary column (30 m × 0.25 mm i.d.; 0.25 μm film thickness), coupled to an Agilent triple quadrupole Mass Selective Detector MSD 5973 (ionisation voltage 70 eV; electron multiplier energy 2000 V; transfer line temperature, 295 °C Solvent Delay: 4 min). GC analysis was performed with a Shimadzu QP 2010 plus equipped with a AOC-20i autoinjector (Shimadzu, Kyoto, Japan) gas chromatograph equipped with a FID, a capillary column (DB-Wax) 30 m × 0.25 mm i.d., film thickness 0.25 μm and a data processor. The oven program was as follows: temperature increase at 40 °C for 5 min, at a rate of 2 °C/min up to 260 °C, then isothermal for 20 min. Helium was used as carrier gas (1 mL min^{-1}). The injector and detector temperatures were set at 250 °C and 290 °C, respectively. 1 μL of oil solution (3% EO/Hexane v/v) was injected with split mode. Linear retention indices (LRI) were determined by using retention times of *n*-alkanes (C8-C40) and the peaks were identified by comparison with mass spectra and by comparison of their relative retention indices with WILEY275, NIST 17, ADAMS, and FFNSC2 libraries.

3.4. Add control solutions

Benzalkonium chloride (3% vol/vol), the reference biocide and pentane (100%), the solvent used in 50% EO solution, were controls used in Agar disc diffusion (ADD) essays.

3.5. Microbial taxa

Microbial patina on leather substrata (*Tholu Bommalu*, a cultural heritage puppet from Andhra Pradesh, India, exposed in the International Puppet Museum 'Antonio Pasqualino', Palermo, Italy), was sampled by sterile stubs. Distinctive colonies, in morphology and/or pigmentation, were isolated on Nutrient-Agar *in vitro* culture. Taxonomical identification was performed by molecular analysis of 16S rDNA gene or ITS1-2 containing DNA region (Rotolo et al. 2016; Palla et al. 2020). Specifically, *Bacillus* sp., *Georgenia* sp., *Ornithinibacillus* sp. and *Streptomyces* sp. were identified as prevalent genera (Gram + bacteria).

3.6. Antibacterial activity assays

Agar disc diffusion *in vitro* method was performed to evaluate antibacterial activity of EO and BAK solutions.10 μL of each bacterial broth culture (normalised to the concentration of 1×106 CFU/mL) was uniformly spread on Nutrient-Agar surface (by sterile Drigalsky spatula) allowing surface to dry (1 h at 30 °C). Sterile paper disc (6 mm diameter, Dutscher papier, FR) imbibed by 10 μL of *I. crithmoides* essential oil (100% or 50%) or Control solutions (BAK 3% v/v, pentane 100%,) was leaned on agar medium (9 cm Petri dishes) surface. 50% EO solution was carried out by pentane, due to non-miscibility of essential oils in water. After incubation at 30 °C for 24 h, inhibition halos (i.h.) different in diameter (mm) were revealed, reflecting the antimicrobial activity. outlined as: sensitive strain = i.h. $>$ 9 mm, resistant strain = i.h. $<$9 mm. *Georgenia* sp. colonies were the most susceptible isolated bacteria, showing a relevant sensitivity to the essential oil with an inhibition zone up to 18 mm; on the other side, *Bacillus* sp. were the most resistant with i.h of 14–17 mm diameters. Table 2 shows the results of the antibacterial activity using Agar disc diffusion method (Figure S1). Generally speaking, the inhibitory effect of the EO of *I. crithmoides,* in both concentrations, is greater than the reference antibiotic (BAK). Pentane, used to carry out 50% EO solution, was applied as control to evaluate the real biocide power of the EO diluted and it did not show inhibition zone.

4. Conclusions

The present work has focused on determining the yield, chemical composition and antimicrobial properties of the *Inula crithmoides* essential oil, showing a good antibacterial activity compared to the commercial biocide benzalkonium chloride. The bioactivity of Aeolian *I. crithmoides* essential oil observed is mainly due to the richness in thymol derivatives (thymol, thymol acetate and thymol methyl ether) and monoterpene hydrocarbons (β-myrcene and *p*-cymene). Since this species is largely distributed in the Mediterranean coastline, these finding suggest its usage as 'green' and cheap alternative to the more toxic and expensive commercial biocides (such as BAC) for the conservation of cultural heritage.

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