Centaurea acaulis L. Hydrosol Extract as a Biocontrol Agent against P*enicillium italicum* Wehmer, (1894) and *Penicillium digitatum* (Pers.) Sacc. Responsible for Oranges Rot

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Summary

With the aim of finding new natural products for the protection of fruits against the pathogenic species responsible for fruit rot, the essential oil and the hydrosol extract of the root parts of *Centaurea acaulis* L. were tested for their antifungal activities against the species of *Penicillium digitatum* (Pers.) Sacc. and *Penicillium italicum* Wehmer, (1894) responsible for mold on oranges. The GC and GC/MS chromatographic profiles of the essential oil and the hydrosol extract revealed the existence of 15 and 21 components, respectively. Aplotaxene (60.3%) was the dominant compound in the essential oil, while the hydrosol extract was rich in sesquiterpene compounds, including caryophyllene oxide (36.2%) and veridiflorol (12.8%). The hydrosol extract of *C. acaulis* showed good antifungal activity against the two strains of molds *P. italicum* and *P. digitatum* with inhibition percentages of 84.5% and 75.3%, respectively. In addition, the *in vivo* antifungal activity test showed that washing oranges with the hydrosol extract showed a protective effect against orange mold caused by the two fungal strains with a percentage exceeding 93%. According to the results of the present study, the root hydrosol extract of *C. acaulis* can possibly be exploited as a natural agent for the protection of oranges against mold caused by *P. italicum* and *P. digitatum*.

Key words

antifungal activities, *Centaurea acaulis* L., hydrosol extract, protective activity, orange fruits, natural fungicide

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Introduction

In Algeria, fruit farming plays a significant economic role as a commercial activity. The country is renowned for its diverse fruit production, including citrus fruits, apples, pears, figs, and many others. These fruits are cultivated for sale in the domestic market and for export to other countries.

However, despite the prosperity of this activity, fruit producers in Algeria often face a major challenge: issues with mold development on fruits during storage. These molds are caused by microscopic fungi, notably *Penicillium digitatum* (Pers.) Sacc. and *Penicillium italicum* Wehmer, (1894), which are common in the environment. When fruits are harvested and stored for sale or export, they are susceptible to developing green and blue molds on their surface, caused respectively by *P. digitatum* and *P. italicum*. These fungi can rapidly proliferate under conditions of humidity and warmth, leading to the swift deterioration of fruits and significant losses for farmers and producers (Klotz 1930, Dutot et al., 2013).

To mitigate these losses, various strategies have been implemented, including the use of synthetic fungicides sprayed on fruits to prevent or control mold growth. However, it is crucial to consider the aspects related to human health and the environment associated with the use of these chemical products (Schnaubelt et al., 2005; Farag et al., 1989).

It is in this context that the exploration of natural alternatives, such as essential oils and hydrosol extracts from plants, has gained importance (Tabti et al., 2014). These extracts, rich in natural chemical compounds, have demonstrated (Kalemba et al., 2003) antioxidant (Tian et al., 2011) and antifungal (Tabti et al., 2014; Gogoi et al., 2018) properties that could potentially be valuable for fruit preservation and the reduction of post-harvest losses.

However, many studies have been carried out to test essential oils and hydrosol extract of certain plants as a replacement for synthetic fungicides for their protective and curative effects against diseases of fruits infected by species of phytopathogenic fungi. For example, treatment with essential oil and *Thymus capitatus* (L.) Hoffmanns. & Link hydrosol extract on oranges contaminated with *P. italicum* showed 100% fruit protection against development of mold (Tabti et al., 2014). The results of the antifungal activity on the protection of tomatoes with essential oil and hydrosol extract of the *Ballota nigra* L. species artificially infected by *Alternaria alternata* (Fr.) Keissl. (1912) showed a considerable reduction in the disease (Ainseba et al., 2019). In addition, the volatile part and the hydrosol extract of *Calendula arvensis* (Vaill.) L. proved to be protective against the disease of pears caused by fungal strains *Penicillium expansum* Link, (1809) and *Aspergillus niger* van Tieghem 1867 (Belabbes et al., 2017).

The *Centaurea* genus belongs to the Asteraceae family, it is a very popular genus in the world with more than 700 species. Stemless star thistle (*Centaurea acaulis* L.) is a perennial herb widely used by locals for the treatment of colds, dizziness and headaches (De Cássia et al., 2015). The purpose of this work is to report original results of the chemical composition of the hydrosol extract of *C. acaulis* and the *in vitro* and *in vivo* antifungal activities of essential oil and the hydrosol extract in aiming at finding new natural agents for the protection of oranges against molds caused by fungal species *P. italicum* and *P. digitatum*.

Materials and Methods

Plant Material

The collection of *C. acaulis* roots was carried out in the Terny region (Tlemcen, Algeria) in May 2020, at the following geographical coordinates: Latitude 34.7958, Longitude -1.35812 (34° 47' 45" North, 1° 21' 29" West in Algeria). To harvest the roots of *C. acaulis*, a delicate method was employed: we used scissors to cut the roots to avoid completely uprooting the plant. Once collected, these root portions (3 batches of 500 grams each) were immediately placed in a cooler to ensure that the roots remained at an appropriate temperature, thus preventing their deterioration. The samples were transported to the laboratory on the same day and underwent a drying process for a period of ten days, being kept away from light and in a dry location. This pre-drying was carried out before proceeding with the extraction of the samples. The identification of the plant was carried out by professor Bab Ali of the Laboratory of Ecology and Management of Ecosystems of the University of Tlemcen Algeria. Samples were given to the herbarium of Tlemcen (Algeria).

Distillation and Extraction Process

The extraction of the essential oil was carried out by hydrodistillation using a Clevenger-type apparatus for 5 hours at the rate of 500 g of plant material for 5 L of distilled water. The essential oils were recovered using a syringe, in order to eliminate traces of water and stored at a temperature of 4 °C away from light until the moment of their use. During the distillation of the essential oil, one liter of condensed water (hydrosol) was collected in a beaker using the tap of the Clevenger apparatus. This hydrosol (without essential oil) was subjected to a liquid-liquid extraction with 2x200 mL of diethyl ether which gave a yellowish oily extract called hydrosol extract.

Gas Chromatography (GC) and Gas Chromatography/ Mass Spectrometry (GC/MS)

The analysis of the essential oil and the hydrosol extract was carried out using two chromatographic methods, GC and GC-MS. GC analyses were performed using Clarus 500 Perkin-Elmer Auto system apparatus equipped with a fused capillary column (50 m x 0.22 mm I.D; film thickness 0.25 µm). Oven temperature was held at 60 °C for 5 min and then programmed to rise to 250 °C at a rate of 2 °C min^{-1}. The flame ionization detector (FID) temperature was 250 °C and injector temperature was 250 °C. Helium was used as carrier gas with a linear velocity of 0.8 mL min^{-1}. The percentages of compounds were calculated by the area normalization method, without considering response factors. GC/MS analyses were carried with a PerkinElmer Turbo-Mass quadrupole analyzer coupled to a Perkin Elmer Autosystem XL, equipped with two fused silica capillary columns and operated with the same GC conditions described above (Belabbes et al., 2017).

Compounds Identification and Quantification

The retention times (RI) of components of essential oil and hydrosol extract were identified and validated by comparing of their retention indices to those of real substances or to information from the literature (Mc Lafferty et al., 1994; NIST

1999). Their mass spectra were compared with those kept in libraries (Jennings et al., 1980; Mc Lafferty et al., 1988; König et al., 2001; NIST 2008) for further identification. The percentage of abundance of normalization of the peak calculated by integrating the Flame Ionization Detector (FID) response factors with respect to the internal standard tridecane (0.7 g 100 g^{-1}) was used for the quantification of the compounds.

Pathogenic Fungal Strains

The technique used in this test and that of Tabti et al. (2014) was that *P. italicum* and *P. digitatum* were isolated from rotten oranges. The latter were identified on the basis of cultural and morphological characteristics. Fungal isolates were stored for 14 days in a dark room in dextrose agar medium at a temperature of 23 °C. Conidia were removed from the agar surface and suspended in 5 mL of sterile distilled water containing 0.1% v/v Tween 80. The spore suspensions were filtered through two layers of sterile cheesecloth to eliminate the mycelial fragments. For the calculation of the number of spores an automated cell counter (TC20 from BioRad) was used and spore count was adjusted to $1x10^6$ spores mL^{-1} using a hemocytometer (Barnett et al., 2006; Samson et al., 2001).

'In vitro' Antifungal Assay

The protocol used in this experiment was based on the method published by Tabti et al., 2004. The essential oil and hydrosol extract were tested for their effects against infections caused by *P. italicum* and *P. digitatum* fungi. The extracts were dissolved in 0.5 mL of 10% (v/v) DMSO and poured into 9 cm glass Petri dishes containing 9.5 mL of molten potato dextrose agar to have final concentrations of 50 and 100 µL mL^{-1} for each extract. Petri dishes were wrapped with waterproof film and incubated at a temperature of 27 °C. The inhibition of mycelial growth was estimated by measuring the average of two perpendicular diameters of the colony for 7 days. Control plates (without essential oils and hydrosol extracts) were inoculated following the same procedure. Three repetition tests were carried out. The percentage of inhibition by the essential oil and the hydrosol extract of the radial growth of *P. italicum* and *P. digitatum* was evaluated on the 7th day using the formula of Pandey et al. (1982):

$$(PI\%) = [Con–Tre)/Con] \times 100;$$

where Con = the number of colonies counted on plate of control and Tre = the number of colonies counted on the plate treated with the extracts.

'In vivo' Antifungal Assay

The protocol of Ainseba et al. (2019) was used to determine the protective effect of essential oil and hydrosol extract on oranges artificially inoculated with the two fungal strains. During this experiment, five (5) healthy oranges, uniform and showing no infection, were chosen. The oranges were cleaned with distilled water and soaked in 70% ethanol for 2 min. After rinsing, the oranges were pierced with a sterilized rigid rod 1 cm in diameter to a depth of 3-4 mm. The fungal inoculum containing 10^6 spores/mL was prepared by scraping spore material from the surfaces of the colonies with a wet cotton swab and re-suspending it in distilled water containing 0.5% Tween 80. In a 1 L plastic boxes container, the 5 oranges were placed together with a disc of filter paper 4 cm in diameter soaked with 2 mL of essential oil. For the protective effect of the hydrosol extract, the test was carried out in a 1 L plastic box. The oranges were washed with a hydrosol extract at a concentration of 0.1 mL L^{-1} and placed in the box. Control experiments were performed in which the essential oil and hydrosol extract treatment were removed. The experiments with the two extracts were stored at 24 ± 1 °C for 15 days. Three repetition tests were carried out. The percentage of rotting incidence (RI) of oranges was determined using the formula:

$$RI\ (\%) = (RO / N) \times 100$$

where, RO = Number of rotten oranges; N = Number of oranges used in the experiment.

Statistical Analysis

An analysis of variance (ANOVA) was used to statistically evaluate the means of the data. Significant values determined were based on p-values ($P \leq 0.05$). Each test was performed three times.

Results

Chemical Analyses

The extraction from the root parts of *C. acaulis* resulted in a light-yellow essential oil, with a yield of 0.2% based on the dry plant material. The analysis of the chemical composition of the essential oil of the root part of *C. acaulis* allowed the identification of 15 compounds: nine (9) non-terpene compounds, five (5) sesquiterpenes and one (1) monoterpene (Table 1). The essential oil was dominated by non-terpene compounds (70.7%), followed by hydrocarbon sesquiterpenes (10.5%) and oxygenated sesquiterpenes (7.6%). The main components were aplotaxene (60.3%), (E)-β-caryophyllene (6.5%), caryophyllene oxide (6.5%) and hexadecanoic acid (5.3%). The hydrosol extract gave a yield of 0.1%. The chemical composition of the hydrosol extract of *C. acaulis* enabled us to identify 21 compounds representing 92.9% of the total extract. The analysis showed the presence of oxygenated compounds dominated by sesquiterpenes (74.1%) and diterpenes (15.3%). The main compounds were caryophyllene oxide (36.2%), veridiflorol (12.8%), (E)-phytol (8.5%) and (Z)-phytol (6.8%) (Table 1).

'In vitro' Evaluation of Antifungal Activity

Antifungal activity against *P. italicum* and *P. digitatum* causing orange rot showed that the percentage of inhibition increased with increasing concentration. At a concentration of 100 µL/mL, the hydrosol extract showed very good activity with inhibition percentages of 84.5% against *P. italicum* and 75.3% for *P. digitatum* ($P \leq 0.05$) (Table 2), whereas the essential oil showed weak inhibitory activity against the fungi tested with a percentage inhibition of 20% and 35% for *P. italicum* and *P. digitatum* at the same concentration (100 µL mL^{-1}), respectively (Table 2).

Table 1. Chemical compounds identified in the essential oil and the hydrosol extract of the root part of *C. acaulis*

No.[a]	Components	lRI$_a$[b]	RIa[c]	RI$_p$[d]	EO[e]	HE[f]	Identification[e]
1	Hex-3-en-1-ol (E)	812	791	1966	0.4	-	RI, MS
2	Hex-3-en-1-ol (Z)	831	825	1380	0.2	-	RI, MS
3	(Z)-hex-3-en-1-ol	851	834	1380	0.2	-	RI, MS
4	β-Pinene	978	970	1110	3.9	-	RI, MS
5	Linalool	1081	1088	1544	-	1.2	RI, MS
6	Terpinen-4-ol	1164	1162	1590	-	0.2	RI, MS
7	Methyl-salicylate	1173	1170	1731	0.7	0.1	RI, MS
8	Decanol	1185	1185	1498	-	0.2	RI, MS
9	Dec-3-en-2-one	1219	1221	1601	1.3	-	RI, MS
10	Dodecanal	1389	1390	1695	2.5	-	RI, MS
11	(E)-β-Caryophyllene	1424	1418	1591	6.5	-	RI, MS
12	α-Humulene	1456	1450	1665	0.5	-	RI, MS
13	Germacrene-D	1480	1478	1704	3.5	-	RI, MS
14	4-epi-Cubebol	1487	1487	1870	-	0.2	RI, MS
15	Bicyclogermacrene	1494	1491	1727	-	-	RI, MS
16	α-Muurolene	1496	1493	1719	-	-	RI, MS
17	E-E-α-Farnesene	1498	1497	1744	-	-	RI, MS
18	β-Cadinene	1507	1506	1752	-	-	RI, MS
19	δ-Cadinene	1516	1515	1752	-	-	RI, MS
20	3-(Z)-Hexenyl-benzoate	1554	1550	2088	-	0.9	RI, MS
21	Germacrene-D-4-ol	1573	1567	2025	-	3.6	RI, MS
22	Caryophyllene oxide	1576	1571	1980	6.5	36.2	RI, MS
23	Veridiflorol	1591	1584	2089	-	12.8	RI, MS
24	Humulene epoxide II	1601	1599	2044	1.1	3.9	RI, MS
25	Y-Eudesmol	1619	1617	2197	-	2.3	RI, MS
26	epi-Cubenol	1624	1623	2059	-	1.9	RI, MS
27	τ-Muurolol	1634	1630	2103	-	2.1	RI, MS
28	β-Eudesmol	1644	1640	2232	-	3.7	RI, MS
29	α-Cadinol	1645	1641	2232	-	2.3	RI, MS
30	α-Eudesmol	1653	1649	2220	-	1.4	RI, MS
31	(Z,Z)-Farnesol	1653	1653	2163	-	1.4	RI, MS
32	Aplotaxene	1661	1661	2226	60.3	-	RI, MS

Continued. Table 1

No.[a]	Components	lRI$_a$[b]	RIa[c]	RI$_p$[d]	EO[e]	HE[f]	Identification[e]
33	Eudesm-4(15)-7-dien-1β-ol	1672	1670	2347	-	2.3	RI, MS
34	Pentadecanol	1998	1696	2456	-	0.9	RI, MS
35	Hexadecanoic acid	1954	1958	2458	5.3	-	RI, MS
36	(Z)-Phytol	2080	2081	2572	-	6.8	RI, MS
37	Heneicosane	2100	2099	2101	0.5	-	RI, MS
38	(E)-Phytol	2114	2113	2591	-	8.5	RI, MS
	Total %				**93.4**	**92.9**	
Hydrocarbon monoterpenes					3.9	-	
Oxygenated monoterpenes					0.7	1.4	
Hydrocarbon sesquiterpenes					10.5	-	
Oxygenated sesquiterpenes					7.6	74.1	
Oxygenated diterpenes					-	15.3	
Non-terpene compounds					70.7	2.1	

Note:
[a] Order of elution is given on apolar column (Rtx-1);
[b] Retention indices from literature on the apolar column (RILit);
[c] Retention indices on the apolar Rtx-1 column (RIa);
[d] Retention indices on the polar column RI$_p$;
[e] EO: Essential oil;
[f] HE: hydrosol extract;
[g] RI: Retention Indices; MS: Mass Spectra in electronic impact mode.

Table 2. *In vitro* antifungal test of essential oil and hydrosol extract against *P. italicum* and *P. digitatum*

Microorganisms	Essential oil (µL mL^{-1})		Hydrosol extract (µL mL^{-1})	
	50	100	50	100
	Percentage of inhibition			
P. italicum	16 ± 0.6^a	20 ± 0.2^a	48 ± 1.2^b	84.5 ± 2.2^d
P. digitatum	25 ± 0.1^a	35 ± 0.4^b	52 ± 1.6^c	75.3 ± 1.8^d

Note: Values with different letters are significantly different according to test at $P \leq 0.05$ significance level

Protective Effect of Essential Oil and Hydrosol Extract on the Development of Mold Caused by the Two Fungi

Given the good *in vitro* antifungal activity shown by the hydrosol extract, an experiment on oranges infected with *P. italicum* and *P. digitatum* was carried out to evaluate the protective effect. The results of the *in vivo* treatment of orange rot by *P. italicum* and *P. digitatum* with the hydrosol extract are presented in Tables 3 and 4. Based on the results of the rot incidence (RI) on the fruits, the treatment of oranges with the hydrosol extract of the roots of *C. acaulis* showed a protective activity against *P. italicum* and *P. digitatum* of oranges until the end of 15th day, compared to the control (oranges without treatment). The hydrosol extract used at a concentration of 0.2 mg L^{-1} showed a significant protective effect ($P \leq 0.05$) on oranges up to 15th day of storage against rot caused by *P. digitatum* (93.5%) and *P. italicum* (93.7 %). No rotting or development of mycelium was observed compared to the controls, which were largely damaged (90%) on the 15th day by both *P. digitatum* and *P. italicum* (Table 3 and 4, Fig. 1 (c)).

Table 3. Effect of essential oil and hydrosol extract on the protection of orange fruits against the infections caused by *P. italicum*

Days of storage	Rotting incidences (%)		
	Positive control	Essential oil	Hydrosol extract
1th	0.0 ± 0.0 a	0.0 ± 0.0 a	0.0 ± 0.0 a
6th	20 ± 1.6 b	15 ± 0.2 b	0.0 ± 0.0 a
10th	60 ± 2.4 c	45 ± 2.3 c	0.0 ± 0.0 a
15th	90 ± 3.5 d	80 ± 2.6 d	6.3 ± 1.2 a

Note: Values with different letters are significantly different according to test at $P \leq 0.05$ significance level. RI: Rotting Incidence

Table 4. Effect of hydrosol extract on the protection of oranges against the infections caused by *P. digitatum*

Days of storage	Rotting incidences (%)		
	Positive control	Essential oil	Hydrosol extract
1th	0.0±0.0 a	0.0±0.0 a	0.0±0.0 a
6th	30.5±1.2 b	16.5±0.2 a	0.0±0.0 a
10th	60.5±2.2 c	35±1.3 b	0.0±0.0 a
15th	90.2±3.6 d	75±1.6 c	6.5±1.2 a

Note: Values with different letters are significantly different according to test at $P \leq 0.05$ significance level. RI: Rotting Incidence

Figure 1. *In vivo* test of the effect of *C. acaulis* hydrosol extract against *P. digitatum* and *P. italicum*. (a) untreated control oranges infected with fungal strains (left and right). (b) oranges infected with *P. digitatum* and treated with the hydrosol extract. (c) oranges infected with *P. italicum* and treated with the hydrosol extract.

Discussion

According to the bibliographic research that we conducted, only one study on the chemical composition of the essential oil of the aerial and root parts of *C. acaulis* was realized. The chemical composition of essential oil of *C. acaulis* collected from Western Algeria in this study was found to be similar to that reported by Benhamidat et al. (2022). The essential oil of the root parts was dominated by the presence of aplotaxene (40.6-61.6%) and caryophyllene oxide (7.2-14.7%) (Benhamidat et al., 2022). Previous work on species of the genus *Centaurea* has shown that essential oils of *C. paphlagonica* (Bornm.) Wagenitz, *C. wagenitzii* Hub.-Mor., *C. tossiensis* Freyn et Sint and *C. luschaniana* Heimerl are characterized by the presence of monoterpene constituents (aspinene, terpinene and carvacrole), sesquiterpenes (caryophyllene, eudesmol and germacrene), hydrocarbons (tricosane, pentacosane and heptacosan) and fatty acids (hexadecanoic acid, tetradecanoic acid and dodecanoic acid) (Kose et al., 2008; Kose et al., 2009). However, the chemical composition of *C. acaulis* root hydrosol extract has been studied for the first time. The hydrosol extract consisted mainly of oxygenated compounds; no hydrocarbon compounds were detected. The protective activity of this extract can be explained by the presence of components capable of stopping the growth of mold phytopathogenic fungi. The antifungal property of *C. acaulis* hydrosol extract is probably associated with the large amount of oxygenated sesquiterpenes, in particular the main components of caryophyllene oxide and veridiflorol. Caryophyllene oxide is an oxygenated terpenoid known for its antifungal properties against dermatophytes and used as a preservative in foods, drugs and cosmetics (Yang et al., 1999). Viridiflorol has been proven to possess cytotoxic, anti-inflammatory and antimicrobial activities (Trevizane et al., 2016). The antifungal activity of the hydrosol extract of *C. acaulis* may be due to synergism between major compounds and some other compounds present in the hydrosol extract (Belabbes et al., 2017).

Conclusion

The present study allowed us to identify thirty-eight (38) chemical species present in the essential oil and the hydrosol extract of the roots of *C. acaulis*. Aplotaxene was the main compound of the essential oil, while the hydrosol extract was mainly composed of a large group of oxygenated sesquiterpenes and diterpenes. The results of the tests of the antifungal activity of the hydrosol extract against *P. italicum* and *P. digitatum* under *in vitro* conditions proved to be very promising, whereas the essential oil presented a weaker activity. The *in vivo* tests of the effect of hydrosol extract on the incidence of *P. italicum* and *P. digitatum*, agents that cause penicillium rot on oranges, have recorded promising results. Therefore, we can conclude that *C. acaulis* root hydrosol extract can be exploited as a natural fungicide for the treatment of diseases of oranges, however, further work is needed to confirm its use in agricultural field.

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CRediT authorship contribution statement

Mohammed El Amine Dib: Conceived the project and supervised the work. **Amina Soulimane, Nabila Ainseba:** Conceptualization, Investigation, performed most of the experiments, Data analysis. Manuscript draft preparation. **Nassim Djabou, Alain Muselli:** Performed some of the experiments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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