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Physicochemical Characterization of *Crithmum maritimum* L. and *Daucus carota* subsp. *gummifer* (Syme) Hook.fil. and Their Antimicrobial Activity against Apple Tree and Grapevine Phytopathogens

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Citation: Sánchez-Hernández, E.; Buzón-Durán, L.; Andrés-Juan, C.; Lorenzo-Vidal, B.; Martín-Gil, J.; Martín-Ramos, P. Physicochemical Characterization of *Crithmum maritimum* L. and *Daucus carota* subsp. *gummifer* (Syme) Hook.fil. and Their Antimicrobial Activity against Apple Tree and Grapevine Phytopathogens. *Agronomy* **2021**, *11*, 886. https://doi.org/10.3390/agronomy11050886

Academic Editors: Célia Cabral and Elisa Julião Campos

Received: 29 March 2021
Accepted: 28 April 2021
Published: 30 April 2021

Abstract: *Crithmum maritimum* and *Daucus carota* subsp. *gummifer* are two species of the Apiaceae family that share multiple characteristics: both are halophitic, live on cliffs in the same geographic habitats, and are edible. While *C. maritimum* is rich in essential oils and flavonoids, *D. carota* is rich in terpenes and a gum producer. In the work presented herein, the biomass of these two wild plants and the bioactive compounds present in their extracts have been studied by elemental and thermal analysis, infrared spectroscopy, and gas chromatography-mass spectroscopy. To explore their bioactivities, both their hydroalcoholic extracts and their major constituents (apiole in *C. maritimum* and geranyl acetate in *D. carota*), either alone or in combination with chitosan oligomers, were assayed in vitro against bacterial and fungal pathogens that affect apple trees (*Malus domestica*) and grapevine (*Vitis vinifera*). Remarkable inhibition was observed against *Erwinia amylovora*, the causal agent of fire blight in apple; *Xylophilus ampelinus* [syn. *Erwinia vitivora*], the causal agent of bacterial blight of grapevine; and *Diplodia seriata*, a virulent pathogen of grapevines that also causes canker, leaf spot and fruit rot of apple. In view of their effectiveness against these three phytopathogens, a potential application of these two medicinal plants in organic farming may be envisaged.

Keywords: antibacterial; antifungal; apiole; chitosan; *Diplodia seriata*; *Erwinia amylovora*; geranyl acetate; Viticulture; *Xylophilus ampelinus*

1. Introduction

Crithmum maritimum L., the sole species of *Crithmum* genus, is a perennial wild plant that is found on cliffs in southern and western coasts of the British Isles, on western and Mediterranean coasts of Europe, in North Africa and the Canary Islands. It is known as *samphire*, *rock samphire*, *sea fennel*, and, in Asturias (Spain), as *cenoyo de mar*. It belongs to the Apiaceae family and is an oleaginous halophyte. It has fleshy, divided aromatic leaves, which have a hot and spicy taste (Figure 1). A detailed morphological description, together with a discussion of its eco-physiological responses to salt stress, may be found in the review paper by Atia, et al. [1].

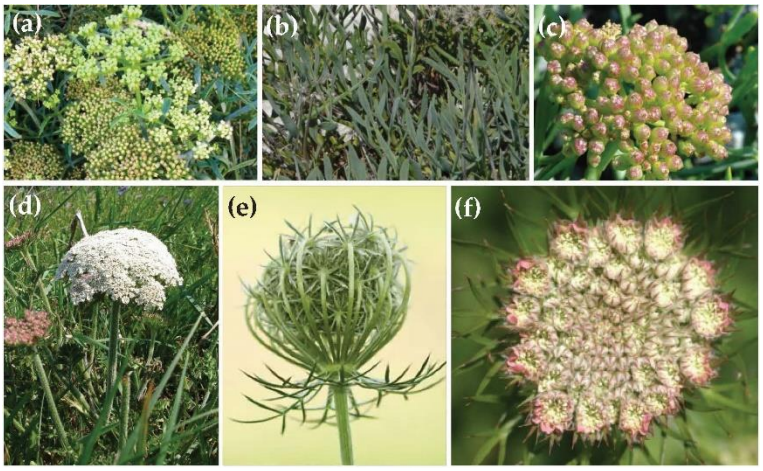

Figure 1. Morphology of *Crithmum maritimum* L. (**a–c**) and *Daucus carota* subsp. *gummifer* Hook.fil. (**d–f**).

Daucus carota subsp. *gummifer* (Syme) Hook.fil. is also a member of the Apiaceae, and is a herb of maritime cliffs, dunes, and grasslands. It is mostly found on the northern coast of Spain, although it may also be found on southern and western coasts of Britain. The common names of this *D. carota* subspecies include *sea carrot*, *wild carrot*, *bird's nest*, *bishop's lace*, and *Queen Anne's lace*. In Spanish language, it is named *zanahoria de acantilado* (tr. cliff carrot). It is hairy, with a stiff, solid stem (Figure 1). The leaves are tripinnate, finely divided, lacy, triangular in shape. Its flowers—small and white, clustered in flat, dense umbels—are sometimes battered and fried. The root is edible while young, but it quickly becomes too woody to consume. The leaves are also edible in little quantities. It contains small amounts of toxicant cyanogenic glycosides [2].

These two medicinal plants have been reported to produce interesting secondary metabolites [3]. Spectrometric analyses of the contents of flavonoids, tannins, and total polyphenols in the aerial parts of rock samphire collected on the Adriatic coast of Croatia in different growth stages were reported by Males, et al. [4], with the highest contents of above components in the samples collected before flowering. Phenolic acids, such as caffeic, chlorogenic, ferulic, p-hydroxybenzoic, p-coumaric vanillic, protocatechuic, and syringic acids were identified by Bartnik, et al. [5]. According to Pavela, et al. [6], the essential oils (EO) of *C. maritimum* show notable variability in chemical composition, being dominated by dillapiole and γ-terpinene (French EO), limonene and γ-terpinene (central Italy EO), and thymol methyl ether and γ-terpinene (Sicilian EO).

In turn, *D. carota* subs. *gummifer* has been reported to contain high contents of monoterpenes (83.9%), the major compounds being geranyl acetate [7] and pinenes. The daucane sesquiterpene, carotol, has also been found in relatively high amounts (11%) [8].

With regard to the potential applications of these bioactive compounds, the antimicrobial activity of the EO of *C. maritimum* has been assayed against common food-borne bacteria, finding significant inhibition against *Escherichia coli*, *Candida albicans*, *Listeria innucia*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* [9,10]. Its antimicrobial activity against a panel of microorganisms, including clinical isolates and food-borne pathogens, has also been studied [11]. The EO obtained from *D. carota* (albeit not for subsp. *gummifer*) has been assayed against *S. aureus*, *E. coli*, *P. aeruginosa*, *Enterobacter aerogenes*, *B. subtilis*, *Campylobacter jejuni*, *Microsporum canis*, and *C. albicans* by Rossi, et al. [12], Ozcelik, et al. [13], and Pavoni, et al. [14]. Only Valente, et al. [8] and Nawel, et al. [15] explored the EO from *D. carota* subs. *gummifer* as a natural source of antifungals against clinical strains of bacteria, yeast, and filamentous fungi.

Nonetheless, to the best of the authors' knowledge, the efficacy of these wild plants extracts has barely been explored against pathogens affecting crop species: *C. maritimum* EO has only been tested against *Erwinia carotovora* (which causes beet vascular necrosis, blackleg of potato and other vegetables, and slime flux on various tree species) by

Ruberto, et al. [16], and against *Mycogone perniciosa* (which causes severe crop losses in common mushroom cultivation) by Glamoclija, et al. [17]. In this work, their application to the control of apple tree (*Malus domestica* Borkh.) and grapevine (*Vitis vinifera* L.) pathogens, in particular against two bacteria, namely *Erwinia amylovora* (Burrill) and *Xylophilus ampelinus* (Panagopoulos, 1969) Willems et al., 1987 [syn. *Erwinia vitivora*], and a fungus, viz. *Diplodia seriata* De Not., is evaluated.

Erwinia amylovora is the causal agent of fire blight, a major global threat to commercial apple and pear production [18]. It is cataloged as a quarantine organism in the European Union, and it has been included in the top 10 plant pathogenic bacteria [19]. A panorama of this pathogen's biology, epidemiology, and control may be found in the recent review by Zhao, et al. [20]. *X. ampelinus* (syn. *Xanthomonas ampelina* and *Erwinia vitivora* [21]), the causal agent of bacterial necrosis of grapevines (known as "maladie d'Oléron" in France and "mal nero" in Italy), severely affects grape crops, resulting in harvest losses as high as 70% of typical yield [22]. The European and Mediterranean Plant Protection Organization (EPPO) categorizes *X. ampelinus* as a quarantine A2 organism, and it is also a quarantine pest for the North American Plant Protection Organization (NAPPO) and the Interafrican Phytosanitary Council (IAPSC). Regarding *D. seriata*, it is a member of the Botryosphaeriaceae family, which are known to be pathogens, endophytes, and saprophytes on a wide range of woody hosts. *D. seriata* is a primary and virulent pathogen of grapevines [23,24], but it also causes frog-eye leaf spot, black rot and canker of apples [25–27].

Taking into consideration that EU regulation (Article 14 in Directive 2009/128/EC, Council Regulation (EC) 834/2007, Commission Regulation (EC) 889/2008, Regulation (EU) 2019/1009, etc.) promotes the use of formulations based on natural products for Integrated Pest Management (IPM), valorization of these two halophytes from the Asturian coast (Spain) as antimicrobial agents for crop protection is proposed. To explore this possibility, a physicochemical characterization of *C. maritimum* and *D. carota* subsp. *gummifer* is first presented, followed by in vitro studies of the efficacy of their hydromethanolic extracts against the above-referred phytopathogens.

2. Material and Methods

2.1. Plant Material and Chemicals

C. maritimum and *D. carota* subsp. *gummifer* samples were collected in the cliffs near the beach of San Antolín (Naves, Llanes, Asturias, Spain—43°26′32.3″ N 4°51′59.6″ W) in early August, in full flowering. Plant parts from different specimens (n = 10 for each species) were thoroughly mixed to obtain separate composite samples for roots, leaves, stems, and flowers.

Chitosan (CAS 9012-76-4; high MW: 310,000–375,000 Da) was supplied by Hangzhou Simit Chem. & Tech. Co. (Hangzhou, China). Neutrase^{TM} 0.8 L enzyme was supplied by Novozymes A/S (Bagsværd, Denmark). Chitosan oligomers (COS) were prepared according to the procedure previously reported in [28].

Apiole (1-allyl-2,5-dimethoxy-3,4-methylenedioxybenzene, CAS 523-80-8) was purchased from Cymit Química SL (Barcelona, Spain). Geranyl acetate (trans-3,7-dimethyl-2,6-octadien-1-yl acetate, CAS 105-87-3), methanol (UHPLC, suitable for mass spectrometry, CAS 67-56-1), TSA (tryptic soy agar, CAS 91079-40-2) and TSB (tryptic soy broth, CAS 8013-01-2) were acquired from Sigma-Aldrich Química (Madrid, Spain). PDA (potato dextrose agar) was supplied by Becton Dickinson (Bergen County, NJ, USA).

2.2. Bacterial and Fungal Isolates

The two bacterial isolates, *Erwinia amylovora* (Burrill) and *Xylophilus ampelinus* (Panagopoulos, 1969) Willems et al., 1987 were supplied by the Spanish Type Culture Collection (CECT), with NCPPB 595 and CCUG 21976 strain designations, respectively. The fungal isolate under study, *D. seriata* (code ITACYL_F098, isolate Y-084-01-01a) was isolated from 'Tempranillo' diseased grapevine plants from protected designation of origin (PDO) Toro (Spain) and supplied as lyophilized vials (later reconstituted and refreshed as

PDA subcultures) by the Agricultural Technological Institute of Castilla and Leon (ITACYL, Valladolid, Spain) [29].

2.3. Preparation of Plant Extracts

C. maritimum and *D. carota* subsp. *gummifer* flowering aerial parts were mixed (1:20, *w*/*v*) with a methanol/water solution (1:1 *v*/*v*) and heated in a water bath at 50 °C for 30 min, followed by sonication for 5 min in pulse mode with a 1 min stop for each 2.5 min, using a probe-type ultrasonicator model UIP1000hdT (Hielscher Ultrasonics, Teltow, Germany). The solution was then centrifuged at 9000 rpm for 15 min and the supernatant was filtered through Whatman No. 1 paper. Aliquots were lyophilized for CHNS analyses.

2.4. Plant Biomass and Extracts Physicochemical Characterization

Elemental analyses were carried out with a LECO (St. Joseph, MI, USA) CHNS-932 apparatus (model No. 601-800-500).

Thermal gravimetric (TGA) and differential scanning calorimetry (DSC) analyses were carried out by means of a simultaneous TG-DSC2 (Mettler Toledo; Columbus, OH, USA), in N_2:O_2 (4:1), with a flow heating rate of 20 °C·min^{-1}.

The infrared vibrational spectra were registered using a Thermo Scientific (Waltham, MA, USA) Nicolet iS50 Fourier-transform infrared spectrometer, equipped with an in-built diamond attenuated total reflection (ATR) system. The spectra were collected with a 1 cm^{-1} spectral resolution over the 400–4000 cm^{-1} range, taking the interferograms that resulted from co-adding 64 scans.

The colorimetric quantification of total polyphenol content (TPC) and total flavonoid content (TFC) was conducted according to the procedures described in [30], using an Agilent (Santa Clara, CA, USA) UV-Vis Cary 100 spectrometer. Contents were expressed in GAE (gallic acid equivalents) and CE (catechin equivalents), respectively. Total carotenoids in *D. carota* subsp. *gummifer* were also determined spectrophotometrically, following the methodology described by Garcia Camacho, et al. [31].

The hydroalcoholic plant extracts were studied by gas chromatography-mass spectrometry (GC-MS) at the Research Support Services (STI) at Universidad de Alicante (Alicante, Spain), using a gas chromatograph model 7890A coupled to a quadrupole mass spectrometer model 5975C (both from Agilent Technologies). The chromatographic conditions were: injection volume = 1 µL; injector temperature = 280 °C, in splitless mode; initial oven temperature = 60 °C, 2 min, followed by ramp of 10 °C/min up to a final temperature of 300 °C, 15 min. The chromatographic column used for the separation of the compounds was an Agilent Technologies HP-5MS UI of 30 m length, 0.250 mm diameter, and 0.25 µm film. The mass spectrometer conditions were: temperature of the electron impact source of the mass spectrometer = 230 °C and of the quadrupole = 150 °C; ionization energy = 70 eV. NIST11 library was used for compound identification.

2.5. In Vitro Antimicrobial Activity Assessment

The antibacterial activity was assessed according to CLSI standard M07-11 [32], using the agar dilution method to determine the minimum inhibitory concentration (MIC). In short, an isolated colony of *E. amylovora* in TSB liquid medium was incubated at 30 °C for 18 h. Serial dilutions were then conducted, starting from a 10^8 CFU·mL^{-1} concentration, to obtain a final inoculum of ~10^4 CFU·mL^{-1}. Bacterial suspensions were then delivered to the surface of PDA plates, to which the bioactive products had previously been added at concentrations ranging from 62.5 to 1500 µg·mL^{-1}. Plates were incubated at 30 °C for 24 h. In the case of *X. ampelinus*, the same procedure was followed, albeit at 26 °C. Readings were taken after 24 h. MICs were determined visually in the agar dilutions as the lowest concentrations of the bioactive products at which no bacterial growth was visible. All experiments were run in triplicate, with three 3 plates per treatment/concentration.

The antifungal activity of the different treatments was determined using the agar dilution method according to EUCAST standard antifungal susceptibility testing proce-

dures [33], by incorporating aliquots of stock solutions onto the PDA medium to obtain concentrations in the 62.5–1500 $\mu g \cdot mL^{-1}$ range. Mycelial plugs (∅ = 5 mm), from the margin of 1-week-old PDA cultures of *D. seriata*, were transferred to plates incorporating the above-mentioned concentrations for each treatment (3 plates per treatment/concentration, with 2 replicates). Plates were incubated at 25 °C in the dark for a week. PDA medium without any amendment was used as the control. Mycelial growth inhibition was estimated according to the formula: $((d_c - d_t)/d_c) \times 100$, where d_c and d_t represent the average diameters of the fungal colony of the control and of the treated fungal colony, respectively. Effective concentrations (EC_{50} and EC_{90}) were estimated using PROBIT analysis in IBM SPSS Statistics v.25 (IBM; Armonk, NY, USA) software.

The level of interaction, i.e., synergy factors, were determined according to Wadley's method [34].

2.6. Statistical Analysis

The results of the inhibition of mycelial growth of *D. seriata* as affected by the different concentrations of the treatments were statistically analyzed using one-way analysis of variance (ANOVA), followed by *post hoc* comparison of means through Tukey's test at $p < 0.05$. IBM SPSS Statistics v.25 software was used.

3. Results and Discussion

3.1. Plant Biomass Characterization

3.1.1. Elemental Analysis of Plant Fractions

The C, H, N, and S percentages of *C. maritimum* components were in the 36.6−40.0%, 6.2–6.3%, 0.7–1.6%, and 0.05–0.1% range, respectively, and those of *D. carota* subsp. *gummifer* in the 39.7−42.8%, 6.3−6.4%, 0.6–2.5%, and 0.0–0.3% range, respectively (Table S1). The distribution of N content showed maximum values in the flowering aerial parts, resulting in C/N ratio values noticeably lower than those found for stems and roots. Regarding the elemental analysis of the gels that resulted from the concentration by vacuum evaporation of the hydroalcoholic extracts of the flowering aerial parts of *C. maritimum* and *D. carota* subsp. *gummifer*, presented in Table S2, slightly higher C/N ratios than those reported in Table S1 were observed.

3.1.2. Thermal Characterization of Flowering Aerial Parts

The DSC curve of the flowering aerial parts of *C. maritimum* (Figure S1) showed exothermic peaks at 290, 330, and 416 °C, in good correspondence with the exothermal effects associated with xylan and lignin [35]. From the TG curve, the ash content was 2.8%. In the case of the umbel of *D. carota* subsp. *gummifer* (Figure S2), exothermal effects occurred at 323, 402, and 444 °C, and the ash content was 2%.

3.1.3. Vibrational Characterization

The FTIR spectra of the various fractions of *C. maritimum* (Table S3) showed the specific bands characteristic of oleaginous plants. In particular, the lipid acyl chains absorb at 2916 and 2848 cm^{-1}, and at 1516 and 1320 cm^{-1}, while at 1732 cm^{-1} the ester carbonyl IR response could be observed. The intensity of these bands was in agreement with the high concentrations of oils that this halophyte can store [36].

The spectra from *D. carota* subsp. *gummifer* (Table S3) featured three specific bands of carotenes at ~1514, ~1147, and ~1009 cm^{-1}. The intensity of the bands at 2360 and 2158 cm^{-1} (attributed to CN stretching) pointed to the presence of appreciable amounts of cyanogen glycosides and anthocyanin. Moreover, the intensity of the amide bands also suggested a significant amount of protein. A notable amount of pectin esters may be inferred from the presence of bands at 2918, 1598, and ~808 cm^{-1}, justifying the ability of this plant to produce gum. With regard to the spectrum from the concentrated gel obtained by evaporation of the hydromethanolic extract of *D. carota* subsp. *gummifer* (Figure S3), the peaks at 2916, 2849, 1732, 1369, 1237, 1144, 1095, and 1015 cm^{-1} were found to be in good

correlation (shifts below 20 cm^{-1}) with those of geranyl acetate (2926, 2858, 1742, 1377, 1233, 1163, 1108, and 1024 cm^{-1}).

3.1.4. On the Usefulness of the Above Physicochemical Techniques

Valuable information may be retrieved from the elemental analysis data: C/N ratios can shed light on the relative presence of carbohydrates and lipids vs. amines, amides, nitriles, and nitro compounds. Hence, the aerial parts, in which the lowest C/N ratios were registered, are to be used if one would like to obtain a high content of bioactive heterocyclic compounds in the hydroalcoholic extracts.

Infrared spectral fingerprinting is useful to identify and/or fingerprint pectins, proteins, aromatic phenolics, cellulose, hemicellulose, etc. without—in most cases—the need for any physical separation [37]. The Apiaceae dicotyledonous herbs *C. maritimum* and *D. carota* are spectroscopically very different from the Gramineae due their higher degree of esterification, which can be crudely assessed by the ratio of the areas of the ester band (at around 1730 cm^{-1}) to the polysaccharides band (at 1170–970 cm^{-1}). Nevertheless, the spectra of *C. maritimum* exhibited five specific bands of cellulose (1472 cm^{-1}, 1320 cm^{-1}, 1104 cm^{-1}, 1074 cm^{-1}, 1034 cm^{-1}), and presence of xylan and lignin could also be inferred from the TG-DTG data for *C. maritimum*. This would support the hypothesis of Abideen, et al. [38], who put forward that the lignocellulosic biomass of this plant could be a potential source of biomass for bioethanol production.

On the other hand, given that the fatty acid methyl ester composition of the oils from *C. maritimun* and *D. carota* is comparable to those of other oil crops used for biodiesel production [39], and taking into consideration that their ash contents are not high, their valorization for this application, proposed by Sotiroudis, et al. [36], certainly deserves further attention.

Notwithstanding the above considerations on the utility of thermal and vibrational techniques for plant characterization and applications, they suffer from limitations to identify specific phytochemicals, making it necessary to make use of other more elucidative techniques, such as GC-MS (see below).

3.2. Extracts Characterization

3.2.1. Phenolic Contents

Extracts from *C. maritimum* from the Cantabrian Sea coast showed total phenolic contents (4.6–8.3 mg GAE·g^{-1} dw) and total flavonoid contents (3.0–5.6 mg CE·g^{-1} dw) similar for those reported for Mediterranean origins, such as Tunisia [30,40] (4.1–7.9 mg GAE·g^{-1} dw and 2.9–6.1 mg CE·g^{-1} dw) or the Adriatic coast in Croatia [4] (4.7–9.5 mg GAE·g^{-1} dw and >3.7 mg CE·g^{-1} dw).

For *D. carota* subsp. *gummifer*, the total phenolic content (5.0 mg GAE·g^{-1}) was lower than those found by Ksouri, et al. [41] for *D. carota* L. spp. *carota* extracts (between 7.1 and 13.8 mg GAE·g^{-1}). With regard to the amount of carotenoid components in the umbel extract, by our terpene analyses, it was 81 mg β-car/100 g dw, slightly lower than that reported for *D. carota* leaves (83.5 mg β-car/100 g dw) [42].

3.2.2. Active Components by GC-MS Analysis

GC-MS of *C. maritimum* hydromethanolic extracts (Table 1, Figure S4) allowed the identification of 1-allyl-2,5-dimethoxy-3,4-methylenedioxybenzene (apiole) [m/z = 77, 106, 121, 149, 177, 207, 222], methylthymol [m/z = 91, 119, 149, 164], and 1,2-dimethyl-3-phenylcyclopropene [m/z =129] as major components. Apiole and methylthymol are in correspondence with dill-apiole (an isomer of apiole) and thymol methyl ether, two of the major components of *C. maritimum* essential oil from Kélibia and Monestir reported by Jallali, et al. [30]. Dill-apiole was also referred by Ngom, et al. [43], Houta, et al. [9], and Ben Mustapha, et al. [44]; and thymol methyl eter by Alves-Silva, et al. [45] and Nabet, et al. [10]. The main difference between the composition of our extracts and those reported by some authors [9,10,46] is the absence of γ-terpinene and sabinene in the extracts presented herein. The presence of the polyacetylene falcarinol [m/z = 41, 55, 77, 91, 115, 129, 145,

159, 173] was in correspondence with that of falcarindiol reported by Meot-Duros, et al. [47] and Ngom, et al. [43]. 1,2-dimethyl-3-phenylcyclopropene constitutes a class of mini-tag probes that participate in fast biorthogonal ligations reactions with 1,2,3,4-tetrazines and photoclickable tetrazoles [48].

Table 1. Compounds identified in *C. maritimum* hydromethanolic extract by GC-MS.

Peak	R_t (min)	Area (%)	Tentative Assignments
11	9.842	2.78	benzene, 2-methoxy-4-methyl-1-(1-methylethyl)- (also named methylthymol); 3-methoxy-p-cymene (also named 2-isopropyl-5-methylanisole or tymol methyl ether)
15	11.005	0.88	2-methoxy-4-vinylphenol (or 4-vinylguaiacol); 1-(2-hydroxy-5-methylphenyl)ethanone; 3-methoxyacetophenone
21	14.068	0.80	1,2,3-trimethoxy-5-allylbenzene (or elemicin)
22	15.163	54.58	1-allyl-2,5-dimethoxy-3,4-methylenedioxybenzene (or apiole)
33	18.143	0.92	ethyl 2-(3-hydroxyphenyl)acetate methanol, cyclohexylphenyl-1-(4-hydroxyphenyl)-2-(3-hydroxyphenyl)ethane
49	19.170	3.78	falcarinol; propenoic acid, 3-(cycloheptatrien-7-yl-, methyl ester N,N-dimethyl-1H-inden-2-amine
50	20.499	2.79	1-methyl-4-nitrosobenzene; bicyclo[4.2.0]octa-1,3,5-trien-7-ol
51	20.777	23.83	1,2-dimethyl-3-phenylcyclopropene; α-methyl-2-naphthalenemethanol dimethyl; 1,2-diethenyl tricyclo[3.1.0.0(2,4)]hexane-3,6-dicarboxylate

Major constituents of the hydromethanolic extract of *D. carota* subsp. *gummifer* (Table 2, Figure S5) were: (Z)-3,7-dimethyl-2,6-octadien-1-ol, acetate (or geranyl acetate) [m/z = 41, 69, 80, 93, 107, 121, 136, and 154]), any of the three following: 1,2-dicyclohexyl-1,1-propanedicarbonitrile; 1,6-dibromohexane or 3-methylbut-2-enoic acid, 3,5-dimethylphenyl ester; anhydro-4,6-dimethyl-3-[p-chlorophenyl]-7-hydroxy-1,2,4-triazolo[1,5-a]pyrimidinium-5-one and/or bromocyclohexane; and γ-sitosterol. For comparison purposes, Gil Pinilla, et al. [7] reported the presence of geranyl acetate, linalool, sabinene, terpinen-4-ol, geraniol, α-pinene, and β-pinene in the EO of *D. carota* subsp. *gummifer* from Santander, Cantabria, Spain. The main constituents of *D. carota* subsp. *maritimus* and *D. carota* "Nantes" EOs (from Turkey) reported by Majdoub, et al. [49] and Keser, et al. [50] were geranyl acetate, β-bisabolene, γ-bisabolene, terpinolene, elemicin, myristicin, 5-caffeoylquinic acid, 5-feruoylquinic, and dicaffeic acid. In our study, instead of carotol sesquiterpene, reported by Valente, et al. [8], cariophyllene [m/z = 41, 55, 69, 79, 91, 119, 133, 147, 161, 175, and 189], cariophyllene oxide and farnesene sesquiterpenes were found. Caryophyllene oxide was also reported as a major compound of the hydrosol extract from aerial parts of *Daucus carota* subsp. *sativus* by Tabet Zatla, et al. [51]. Bisabolene was also registered, although as *trans*-Z-α-bisabolene epoxide (R_t = 17.105) and in small amounts. For a thorough comparison of the main components of *D. carota* from different origins, the interested reader is referred to Bendiabdellah, et al. [52].

The possibility of exploitation of the two studied plants for agricultural chemicals industry applications is supported by above GC–MS results: apiole and dill-apiole, major constituents of *C. maritimum*, have been shown to be a good insecticide when they were isolated from the roots of *Anethum graveolus* L. [53], whereas geranyl acetate, the major component of *D. carota*, has antifungal and anti-inflammatory properties, referred in the studies by Gonçalves, et al. [54] and by Khayyat and Sameeh [55]. Thymol, a phytochemical from *C. maritimun*, interferes with the formation and viability of hyphae and induces morphological alterations in the envelope (i.e., the plasma membrane and the mannoproteins, enzymes, beta-glucans, and chitin of the wall) of *C. albicans*, and it also exhibits anti-inflammatory effects by reducing the production and gene expression of the pro-inflammatory mediators [56]. Falcarinol has also been identified as an important

antifungal compound, inhibiting spore germination of various fungi in concentrations ranging from 20 to 200 μg·mL^{-1} [57].

Table 2. Compounds identified in *D. carota* subsp. *gummifer* hydromethanolic extract by GC-MS.

Peak	R_t (min)	Area (%)	Tentative Assignments
6	6.219	1.12	1,6-anhydro-2,4-dideoxy-β-D-ribo-hexopyranose; propanoic acid, 2,2-dimethyl-, hexyl ester; 2-methylbutanal
20	11.925	22.73–39.68	(Z)-3,7-dimethyl-2,6-octadien-1-ol, acetate (or geranyl acetate)
22	12.519	2.70	caryophyllene; bicyclo[7.2.0]undec-4-ene, 4,11,11-trimethyl-8-methylene-,[1R-(1R*,4Z,9S*)]
26	13.254	1.87	2,6-dimethyl-3,5,7-octatriene-2-ol; geranyl acetate, 2,3-epoxy-
28	13.756	1.49	(E,Z)-α-farnesene; 6-epi-shyobunol; epiglobulol
34	14.569	1.30	caryophyllene oxide; cyclohexaneethanol, 2-methylene-
40	15.528	1.35	1,2,3,5-cyclohexanetetrol, (1α,2β,3α,5β)-; 4-methyl-5-propyl-nonane; trichloroacetic acid, 4-methylpentyl ester
55	19.418	2.61	4-hydroxy-4-(4,6-dimethylcyclohex-3-enyl)butan-2-one; 3-buten-2-one, 4-(3-hydroxy-6,6-dimethyl-2-methylenecyclohexyl)-; 7,8-epoxy-α-ionone
59	19.920	0.65	spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-; biciclo[4,1,0]heptan-3-ol,3,7,7-trimethyl-, [1S-1α,3α,6α]-
62	20.163	1.23	3-carene; tricyclo[2.2.1.0(2,6)]heptane, 1,3,3-trimethyl-
63	20.431	1.16	5-ethyl-2,4-dimethyl-2-heptene; hexan-3-yl (E)-2-methylbut-2-enoate
84	23.201	1.33	hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester
86	23.566	2.42	anhydro-4,6-dimethyl-3-[p-chlorophenyl]-7-hydroxy-1,2,4-triazolo[1,5-a]pyrimidinium-5-one
91	24.593	2.38	(9Z,12Z)-1,3-Dihydroxypropan-2-yl octadeca-9,12-dienoate (or β-monolinolein)
97	25.299	5.50	1,2-dicyclohexyl-1,1-propanedicarbonitrile; 1,6-dibromohexane; 3-methylbut-2-enoic acid, 3,5-dimethylphenyl ester
99	25.480	4.92	3-ethyl-2-butenoic acid, phenyl ester; bromocyclohexane
103	25.947	1.71	3-methyl-but-2-enoic acid, 1,7,7-trimethyl-bicyclo[2.2.1]hept-2-yl ester
107	30.192	2.52–6.95	γ-sitosterol

3.3. In Vitro Antimicrobial Activity

3.3.1. Antibacterial Activity

The inhibition of both *C. maritimum* and *D. carota* extracts against *Erwinia amylovora* and *Xylophilus ampelinus* were similar (Table 3), although it was slightly higher against *X. ampelinus* in the case of *C. maritimum*. As regards the activities of the two main active principles, viz. pure apiole (an essential oil) and pure geranyl acetate (a monoterpene), the obtained results were comparable to or lower than those of the plant extracts. Another was the case for the conjugate complexes, in which a synergistic behavior was observed among COS and the phytochemicals in all cases. The best results against *E. amylovora* were attained with the COS-*C. maritimum* complex (MIC = 187.5 μg·mL^{-1}), while against *X. ampelinus* the lowest MIC (125 μg·mL^{-1}) corresponded to the COS-geranyl acetate complex, followed by the COS-*C. maritimum* complex (MIC = 250 μg·mL^{-1}).

The above results of antibacterial effect of chitosan-phytochemical conjugates against *Erwinia* spp. were in accordance with the previous reports [58,59], in which the chitosan-phytochemical conjugates exhibited higher antimicrobial activity than that of unmodified chitosan. For instance, Kim, et al. [60] reported that the MICs of chitosan-phytochemical

conjugates ranged from 32 to 512 μg·mL^{-1} against foodborne pathogens, while the MICs of the unmodified chitosan were in the 128−1024 μg·mL^{-1} range.

Table 3. Antibacterial activity of chitosan oligomers (COS), *C. maritimum* and *D. carota* subsp. *gummifer* extracts, pure apiole and geranyl acetate, and their corresponding conjugate complexes (COS−*C. maritimum*, COS−*D. carota*, COS−apiole and COS−geranyl acetate) against the two phytopathogenic bacteria under study at different concentrations (expressed in μg·mL^{-1}).

Pathogen	Compound	62.5	93.7	125	187.5	250	375	500	750	1000	1500
E. amylovora	COS	+	+	+	+	+	+	+	+	+	-
	C. maritimum	+	+	+	+	+	+	+	+	+	-
	D. carota	+	+	+	+	+	+	+	+	+	-
	Apiole	+	+	+	+	+	+	+	+	+	-
	Geranyl acetate	+	+	+	+	+	+	+	+	+	+
	COS-apiole	+	+	+	+	+	+	+	-	-	-
	COS-geranyl acetate	+	+	+	+	+	+	+	+	-	-
	COS-*C. maritimum*	+	+	+	+	-	-	-	-	-	-
	COS-*D. carota*	+	+	+	+	+	+	-	-	-	-
X. ampelinus	COS	+	+	+	+	+	+	+	+	+	-
	C. maritimum	+	+	+	+	+	+	+	-	-	-
	D. carota	+	+	+	+	+	+	+	+	-	-
	Apiole	+	+	+	+	+	+	+	+	+	-
	Geranyl acetate	+	+	+	+	+	+	+	+	+	-
	COS-apiole	+	+	+	+	+	+	+	-	-	-
	COS-geranyl acetate	+	+	+	-	-	-	-	-	-	-
	COS-*C. maritimum*	+	+	+	+	+	-	-	-	-	-
	COS-*D. carota*	+	+	+	+	+	+	-	-	-	-

"+" and "-" indicate presence and absence of bacterial growth, respectively.

In line with Kim, et al. [60], it may be speculated that the mechanism of action behind this enhanced behavior operates via multiple mechanisms: positively charged chitosan can interact with the negatively charged bacterial cell surface, which leads to a weakening of the cell wall, either by cell wall damage alone or accompanied by cell lysis. Conjugation with phytochemicals may increase the osmotic pressure-induced disruption and shrinkage of the bacterial membrane because of a reduction in the permeability of the membrane to intracellular components, and the conjugates may also form a barrier on the bacterial surface and prevent the entry of nutrients. It may also be hypothesized that conjugation with phytochemicals increases the affinity of chitosan for the bacterial cell envelope because of an enhanced lipophilicity (conferred—in the case of apiol—by the allyl side chain bonded to the aromatic ring; and, in the case of geranyl acetate, by the presence of two double bonds in the unsaturated chain). In any case, it should be taken into consideration that further research is required to support aforementioned hypotheses.

3.3.2. Antifungal Activity

Diplodia seriata mycelial growth inhibition results are presented in Figure 2 and Figrue S6. The preconized antifungal activity of *D. carota* [8], based on its relatively high content of terpenes, was not observed in our assays. That of *C. maritimum* was also low, with EC_{50} and EC_{90} values of 832 and 2933 μg·mL^{-1}, respectively. Even when its main component, apiole (whose antifungal effect has been referred to the presence of two methoxyl groups in positions 2, 3 of their benzene ring, optimum to gain a correct balance of hydrophilicity-lipophilicity [61]), was assayed as a pure substance, the results were moderate, with EC_{50} and EC_{90} values of 333 and 822 μg·mL^{-1}, respectively.

These results are in line with the low activity of *D. carota* EO against certain *Candida* spp. and *Aspergillus* spp. reported by Valente, et al. [8], and with the lack of activity of apiole against *C. acutatum*, *C. fragariae*, *C. gloeosporioides*, and *F. oxysporium* reported by Meepagala, et al. [62].

Another was the case for geranyl acetate, the main component of *D. carota*: when it was assayed as a pure substance, it led to EC_{50} and EC_{90} values as low as 147 and 172 μg·mL^{-1}, respectively.

Regarding the activity of the conjugate complexes with COS, an enhancement in the antifungal activity was registered in all cases. The lowest EC_{50} and EC_{90} values were obtained for COS-geranyl acetate (68 and 113 μg·mL^{-1}, respectively) and for COS-*C. maritimum* extract (75 and 331 μg·mL^{-1}, respectively), for which a synergy factor above 5 was obtained (Table 4).

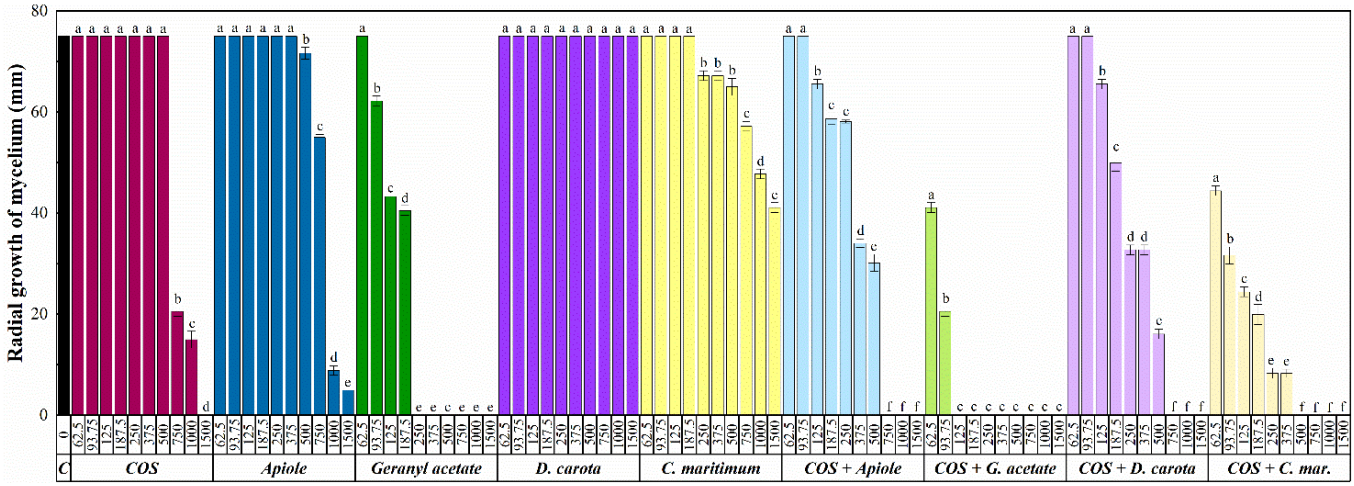

Figure 2. Radial growth of the mycelium for *D. seriata* in in vitro tests conducted in PDA medium with different concentrations (62.5, 93.75, 125, 187.5, 250, 375, 500, 750, 1000, and 1500 μg·mL^{-1}) of chitosan oligomers (COS), *C. maritimum* extract and pure apiole, *D. carota* extract and pure geranyl acetate, and their respective conjugate complexes. The same letters above concentrations mean that they are not significantly different at $p < 0.05$. Error bars represent standard deviations.

Table 4. EC_{50} and EC_{90} effective concentrations for the different treatments, expressed in μg·mL^{-1}, and synergy factors estimated according to Wadley's method.

Effective Concentration	COS	Apiole	Geranyl Acetate	*D. carota*	*C. maritimum*	COS-Apiol	COS-Geranyl Acetate	COS-*D. carota*	COS-*C. maritimum*
EC_{50}	744	807	147	–	832	333	68	269	75
EC_{90}	1180	1353	272	–	2933	822	113	633	331
SF						1.53	3.91	–	5.08

SF = synergy factor.

The molecular mechanisms behind chitosan interactions with fungi have been recently discussed in a review paper by Lopez-Moya, et al. [63]. Nonetheless, the information available on the mechanism of synergistic action of COS-phytochemical conjugates is not well-established yet. It has been hypothesized that it may be the result of an enhanced additive fungicidal effect *per se*, and/or via a concurrent action on diverse fungal metabolic sites. The conjugation with phytochemicals may increase the cationic surface charge of COS, enhancing the linkage (through electrostatic interactions) to the negatively charged site-specific binding receptors on the fungal membrane [28,64–66].

3.3.3. Comparison with Efficacies Reported in the Literature

Results from studies on the antimicrobial activity of the specific bioactive substances under study (*C. maritimum* and *D. carota* extracts, apiol, and geranyl acetate) against diverse foodborne and clinical bacteria and fungi are summarized in Table 5. The reported MICs and IC_{50} values are generally lower than those reported herein (in this work, the lowest MIC values were 125 and 187.5 μg·mL^{-1} against *X. ampelinus* and *E. amylovora*, respectively, and the lowest EC_{50} and EC_{90} values against *D. seriata* were 68 and 113 μg·mL^{-1}, respec-

tively), but it is worth noting that there are certain pathogens for which no inhibition could be attained, and that there is a large variability in the reported values depending on the bioactive product (and its provenance) and even as a function of the strain/isolate for the same pathogen. A comparison with the values reported for other phytopathogens was not possible, given that no inhibition could be attained using a hexane extract of *C. maritimum* leaves against *Erwinia carotovara* subsp. *carotovora*, and the minimum inhibitory quantity (MIQ = 1 µL/disc) reported using *C. maritimum* roots essential oil against *Mycogone perniciosa* was not expressed in standard units.

A comparison can instead be made with the efficacy of other natural products reported in the literature against the actual phytopathogens under study. To the best of the authors' knowledge, no assays with plant-derived products have been conducted against *X. ampelinus*, but *E. amylovora* has the subject of several studies, summarized in Table 6. In this work, the lowest MIC value against *E. amylovora* was 187.5 µg·mL^{-1}, better than those attained with the extracts from Damask rose and golden wreath wattle flowers, *Conocarpus lancifolius* leaves and different phenolic extracts from clove, oregano, artichoke, or walnut shells. Nonetheless, lower MICs have been reported for the resinous exudates from *Adesmia boronioides* and alkaloids from African rue seeds.

Table 5. Antibacterial and antifungal activities of *C. maritimum* and *D. carota* extracts, apiol, and geranyl acetate reported in the literature.

Phytochemical	Product Type	Microorganisms	Effectiveness	Ref
Apiole	EO from rhizomes of *Athamanta turbith* 33–49% apiole	Bacteria:	MIC (mg·mL^{-1})	[67]
		E. coli ATCC 25922	43.3	
		P. aeruginosa ATCC 27853	>86.6	
		S. aureus ATCC 25923	43.3	
		S. epidermidis ATCC 12228	86.6	
		M. luteus ATCC 10240	43.3	
		K. pneumoniae NCIMB 9111	>86.6	
		Fungi:		
		C. albicans ATCC 10259	>86.6	
	EO from aerial parts of *Piper holtonii* 57% apiole	Fungi:	IC_{50} (µg·mL^{-1})	[61]
		Colletotrichum acutatum	<50	
		Botryodiplodia theobromae	36.16	
Geranyl acetate	EO of lemongrass varieties 0.5–1% geranyl ac.	Bacteria:	MIC (µg·mL^{-1})	[55]
		P. aeruginosa	4.5–9	
		S. aureus	4.5–18	
	EO from aerial parts of *Thapsia minor*: 83% geranyl acetate	Fungi:	MIC (µL·mL^{-1})	[54]
		C. albicans ATCC 10231	>20	
		C. tropicalis ATCC 13803	>20	
		C. krusei H9	10–20	
		C. guillermondii MAT23	1.25	
		C. parapsilosis ATCC 90018	2.5–5	
		T. rubrum CECT 2794	0.32	
		M. gypseum CECT 2905	0.64	
		M. canis FF1	0.32–0.64	
		C. neoformans CECT1078	0.32	
		E. floccosum FF9	0.16	
		A. flavus F44	>20	
		A. niger ATCC16404	>20	
		A. fumigatus ATCC 46645	10–20	

Table 5. *Cont.*

Phytochemical	Product Type	Microorganisms	Effectiveness	Ref
D. carota subsp. *gummifer*	EO of aerial parts, 37% geranyl acetate	Fungi: *C. albicans* ATCC 10231 *C. tropicalis* ATCC 13803 *C. krusei* H9 *C. guillermondii* MAT 23 *C. parapsilosis* ATCC 90018 *T. rubrum* CECT 2794 *M. gypseum* CECT 2908 *M. canis* FF1 *E. floccosum* FF9 *A. flavus* F44 *A. niger* ATCC 16404 *A. fumigatus* ATCC 46645	MIC ($\mu L \cdot mL^{-1}$) >20 10 >20 1.25 >20 0.32 0.64 0.64 0.32 >20 10 2.5	[8]
	EO of aerial parts 52–77% geranyl ac.	Bacteria: *E. coli* ATCC 25922 *P. aeruginosa* ATCC 27853 *S. aureus* ATCC 25923 *B. cereus* ATCC 9634 *E. faecalis* ATCC 29212 *K. pneumoniae* ATCC 10031	MIC ($mg \cdot mL^{-1}$) >6.0 >6.0 5.1 3.8 4.3 >6.0	[15]
D. carota subsp. *hispidus*	EO of aerial parts	Bacteria: *E. coli* ATCC 35218 *S. aureus* ATCC 25923 *E. faecalis* ATCC 29212	MIC ($mg \cdot mL^{-1}$) 1.25 2.5 1.25	[68]
C. maritimum	Plant extract and EO of aerial parts	Bacteria: *E. coli* ATCC 10536 *P. aeruginosa* ATCC 9027 *S. aureus* ATCC 6538 *B. cereus* ATCC 11778	IC_{50} = 0.47 $mg \cdot mL^{-1}$ (Kélibia) and 3.3 $mg \cdot mL^{-1}$ (Monastir)	[30]
	Hydromethanolic extract of aerial parts	Fungi: *E. coli* ATCC 25922 *C. albicans* ATCC 10231	MIC ($\mu g \cdot mL^{-1}$) 0.11 0.11	[10]
	Hexane extract of leaves	Bacteria: *E. coli* BCC 3.08.001 and ATCC 4157 *B. cereus* BCC 3.05.002 *M. luteus* ATCC 10240 *E. carotovora* BCC 3.08.031 Fungi: *C. albicans* BCC 3.08.036.	MIC ($\mu g \cdot mL^{-1}$) - 50 50 - -	[47]
	Volatile oils of leaves	Fungi: *C. albicans* ATCC 10231 *C. guillermondii* MAT23 *C. neoformans* CECT 1078 *E. floccosum* FF9 *T. rubrum* CECT 2794 *M. gypseum* CECT 2908 *M. canis* FF1	MIC ($\mu g \cdot mL^{-1}$) 2.5–5 0.32–2.5 0.32–0.64 0.08–0.32 0.08–0.32 0.08–1.25 0.08–0.64	[69]
	Essential oil of roots	*M. perniciosa*	MIQ = 1 µL/disc	[17]

In relation to the antifungal activity against *D. seriata*, the lowest EC_{50} and EC_{90} values for the products assayed herein were 68 and 113 $\mu g \cdot mL^{-1}$, respectively. These were substantially lower than those attained with other natural compounds. For instance, a concentration of 1000 $\mu g \cdot mL^{-1}$ was required to completely inhibit the mycelial growth of *D. seriata* for chitosan oligosaccharides (molecular weight < 3000 Da) [80]; and only

96.8% growth inhibition was reached for chitosan at 25 mg·mL^{-1} [80]. Growth inhibition percentages of 20.6, 90.5, 47.7, 68.2, and 77.8% were reported by Cobos, et al. [80] for *Evernia prunastri* lichen extract (4%), garlic extract (10%), lemon peel extract (10%), propolis (10 mg·mL^{-1}), and vanillin (5 mg·mL^{-1}), respectively. If COS-conjugate complexes are considered instead, the EC_{90} values attained with a COS–ε-polylysine conjugate (580 µg·mL^{-1}) [28], and a COS–tyrosine conjugate (672 µg·mL^{-1}) [65] were substantially higher than those obtained for COS-geranyl acetate and COS-*C. maritimum* extract, and comparable to those of COS-apiol and COS-*D. carota* subsp. *gummifer* extract.

Table 6. Natural products assayed against *Erwinia amylovora*.

Phytochemical	Effective Dose	Ref.
EO of *Rosa damascena* flowers	MCB = 1386.5 µg·mL^{-1}	[70]
Water extract (7.4% *w*/*w*) of *Acacia saligna* flowers	MIC = 300 µg·mL^{-1}	[71]
Alkaloids extract from *Conocarpus lancifolius* leaves	MIC > 200 µg·mL^{-1}	[72]
Phenolic extracts from: *Syzygium aromaticum* *Origanum vulgare* *Cynara cardunculus* var. *scolymus* stem *Juglans regia* shells	MIC (mg·mL^{-1}) 10.2 91% inhibition at 41.0 48% inhibition at 41.0 No inhibition	[73]
Exudate from *Adesmia boronioides* (8.5% resin/fresh plant)	MIC = 64 µg·mL^{-1}	[74]
Alkaloids extr. from *Peganum harmala* seeds	MIC = 50 µg·mL^{-1}	[75]
Extracts from *Coccoloba uvifera* leaves: Aqueous Acetone Ethanol	Diam. inhib. zone (mm) at 2500 µg·mL^{-1} 8 ± 1 10 ± 1 14	[76]
EO from: *Cinnamomum zeylanicum* *Laurus nobilis* *Thymus vulgaris* *Syzygium aromaticum* *Pinus* spp. *Cymbogon citratus* *Mentha spicata* *Melaleuca alternifolia*	Diam. inhib. zone (mm), concentr. N/A 31.2 22 20.6 18 17 13 13 12	[77]
EO from aerial parts of flowering: *Thymus vulgaris* *Satureja hortensis*	Diam. inhib. zone (mm), concentr. N/A 25 25	[78]
EOs extr. by steam or hydrodistillation from: *Melissa officinalis* flowers/leaves *Mentha arvensis* aerial part *Nepeta cataria* flowering tops *Origanum compactum* aerial part *Origanum vulgare* aerial part *Thymus vulgaris* aerial part	Diam. inhib. zone (cm), concentr. N/A 6.17–8.7 7.67–12.7 12.1–24.00 21.33–29.3 14.50–25.5 14.33–37.0	[79]

MIC = minimum inhibitory concentration; MBC = minimum bactericidal concentration; EO = essential oil; N/A = not available.

4. Conclusions

The hydromethanolic extract of the aerial parts of *C. maritimum* was found to be rich in apiole (55%) and that of *D. carota* in geranyl acetate (40%). In the in vitro assays, a strong

synergistic behavior was observed upon conjugation of the bioactive constituents of plant extracts with chitosan oligosaccharides, with synergy factors in the 3.9–5.1 range. For the COS-*C. maritimum* complex, MIC values of 187.5 and 250 $\mu g \cdot mL^{-1}$ were obtained against *E. amylovora* and *X. ampelinus*, respectively; and EC_{50} and EC_{90} values of 75 and 331 $\mu g \cdot mL^{-1}$ were found against *D. seriata*. For COS-*D. carota* extract, a MIC value of 375 $\mu g \cdot mL^{-1}$ was observed against the two bacterial phytopathogens; and an EC_{90} of 633 $\mu g \cdot mL^{-1}$ was attained against *D. seriata*. Taking into consideration that the conjugate complexes of both halophyte extracts showed a better performance than other natural compounds reported in the literature against *E. amylovora* and *D. seriata*, they may be put forward as promising antimicrobial treatments, either in organic agriculture or as a substitute for treatments based on chemical synthesis fungicides in conventional management.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11050886/s1, Table S1: Elemental (CHNSO) composition (wt.%) of *C. maritimum* and *D. carota* fractions; Table S2: Elemental composition (wt.%) of *C. maritimum* and *D. carota* flowering aerial parts concentrate hydromethanolic extracts; Table S3: Main bands in the ATR-FTIR spectra of various *C. maritimum* and *D. carota* subsp. *gummifer* fractions and their assignments; Table S4: GC/MS analysis of *C. maritimum* hydromethanolic extract; Table S5: GC/MS analysis of *D. carota* subsp. *gummifer* hydromethanolic extract; Figure S1: TG, DSC and DTG curves for *C. maritimum*; Figure S2: TG, DSC and DTG curves for *D. carota* subsp. *gummifer*; Figure S3: ATR-FTIR spectrum of *D. carota* subsp. *gummifer* hydromethanolic extract; Figure S4: GC-MS spectrum of *C. maritimum* hydromethanolic extract; Figure S5: GC-MS spectrum of *D. carota* subsp. *gummifer* hydromethanolic extract; Figure S6: Sensitivity test for *D. seriata*.

Author Contributions: Conceptualization, J.M.-G., C.A.-J. and P.M.-R.; methodology, B.L.-V.; validation, C.A.-J., B.L.-V., J.M.-G. and P.M.-R.; formal analysis, P.M.-R.; investigation, E.S.-H., L.B.-D., C.A.-J., B.L.-V., J.M.-G. and P.M.-R.; resources, J.M.-G. and P.M.-R.; writing—original draft preparation, E.S.-H., L.B.-D., C.A.-J., B.L.-V., J.M.-G. and P.M.-R.; writing—review and editing, P.M.-R.; visualization, E.S.-H. and L.B.-D.; supervision, L.B.-D. and P.M.-R.; project administration, J.M.-G. and P.M.-R.; funding acquisition, J.M.-G. and P.M.-R. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Junta de Castilla y León under project VA258P18, with FEDER co-funding; by Cátedra Agrobank under "IV Convocatoria de Ayudas de la Cátedra AgroBank para la transferencia del conocimiento al sector agroalimentario" program; and by Fundación Ibercaja-Universidad de Zaragoza under "Convocatoria Fundación Ibercaja-Universidad de Zaragoza de proyectos de investigación, desarrollo e innovación para jóvenes investigadores" program. Instituto Universitario de Investigación en Ciencias Ambientales de Aragón (IUCA) is gratefully acknowledged for covering the APCs.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to their relevance to be part of an ongoing Ph.D. Thesis.

Acknowledgments: The authors gratefully acknowledge the support of Pilar Blasco and Pablo Candela at the Servicios Técnicos de Investigación, Universidad de Alicante, for conducting the GC-MS analyses.

Conflicts of Interest: The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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