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Practical solutions to the biodeterioration dilemma: activity of *Dittrichia viscosa* extracts and its specialized metabolites on standard organism *Raphidocelis subcapitata*

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***Abstract* – Biodeterioration caused by microorganisms is among the most pressing challenges in the conservation of outdoor stone heritage. In the search for sustainable solutions, the application of plant-extracted bioactive compounds has been gaining increasing attention. This study investigates the biocidal potential of extracts from *Dittrichia viscosa*, a ruderal Mediterranean plant rich in antibiotic and allelopathic compounds. Crude extracts were obtained using solvents of increasing polarity and characterized via NMR and GC-MS to identify major bioactive constituents. Four sesquiterpenoids—α-costic acid and inuloxins A, B and C—were identified as the most promising in terms of biocidal activity and contextually isolated and tested on pure cultures of the green alga *Raphidocelis subcapitata*. Inuloxin A and C significantly reduced algal growth in a dose-responsive manner, while α-costic acid and inuloxin B showed no inhibitory effect. These findings confirm the biocidal effectiveness of these specialized metabolites and support their potential future application in the treatment of biological patinas.**

I. INTRODUCTION

Outdoor stone heritage is subjected to a plethora of spoiling phenomena from both natural and anthropogenic sources. Among the main causes of concern for the conservation of similar artefacts, biodeterioration is widely regarded as one of the most impactful [1]. Biological deterioration may manifest in a variety of ways [2]. Microbial communities colonize stone surfaces, typically developing biofilms rich in EPS (exopolysaccharides), which increase the water retention and the absorptivity of the substrate [3]; such communities can expand and penetrate stone, favoring water infiltration and provoking mechanical stress through freeze/thaw cycles [4], or chemical damage by releasing organic acids [5]. Additionally, aesthetic weathering, often in the form of the chromatic alteration of stone [6], is a problematic phenomenon for the appreciation of artefacts such as paintings and mosaics and usually involves aerial phototrophs such as green algae and cyanobacteria [7,8].

Well thought-out cleaning interventions represent the most effective and lasting approach to keep the growth of microorganisms under control, but similar procedures require a thorough understanding of the artefacts and of the dynamics of biotic degradation to work as intended [9]. Restoration interventions against such organisms must not only be cost-effective and present durable effects, but most importantly they must be suited to the material nature of the artefacts, so as to avoid altering the aesthetic features [10]. For this reason, a wide array of novel eco-sustainable solutions is being investigated right now. Physical methods, like the use of biocidal radiations (UV-C, microwaves, gamma rays) [11, 12, 13] are among the most discussed, as their practicality and non-invasiveness makes them ideal candidates to approach different situations. Unfortunately, the implementation difficulties they often present (i.e. lack of electricity network) restrict their outdoor usage to a specific set of circumstances.

The application of plant-extracted active compounds for cleaning interventions remains one of the most simple, direct, practical and effective solutions to treat biofilms afflicting stone artwork [14]. Study is still mostly focused on what can be extracted and characterized from organisms such as higher plants, which have evolved a complex variety of active compounds to fight infections [15] and compete against other organisms through mechanisms of allelopathy [16]. The plants considered for this approach are usually flowering, aromatic organisms, often belonging to the Lamiaceae family [17].

A promising candidate for the identification of novel potentially active compounds to use in the treatment of biodeterioration comes instead from the Asteraceae family: *Dittrichia viscosa*, formerly classified in the genus Inula, is the ruderal plant chosen in this work for the peculiar chemical properties of its secondary metabolites [18]. *D.*

viscosa is very common, stress-resistant and widespread in all the mediterranean basin, even in profoundly anthropized and degraded environments, making it an ideal source of natural compounds, as it is easy to access and abundant [19].

II. MATERIALS & METHODS

The compounds under investigation were purified from fresh leaves and other aerial portions of the plant, collected before flowering in the Monte Sant'Angelo Complex of the University of Naples Federico II.

The plant material was first ground and subsequently macerated in hydroalcoholic solution of methanol-water 1:1 (24 hours) and the resulting extract was then sequentially fractionated with solvents of increasing polarity (*n*- hexane, dichloromethane, and ethyl acetate). The solvent phases were evaporated under reduced pressure to obtain semi-solid oily residues rich in the compounds of interest. These crude extracts were initially analyzed by Thin- Layer Chromatography (TLC) and characterized by Nuclear Magnetic Resonance (NMR) to preliminary assess the different classes of secondary metabolites present (e.g., terpenoids, phenolics). A further structural investigation coupled to a quantitative analysis of the compound concentrations in the various extracts was then carried out using Gas Chromatography–Mass Spectrometry (GC-MS), which revealed significant concentrations of four major potentially bioactive sesquiterpenoids: α-costic acid and inuloxins A, B and C [20]. A further purification step was performed on organic extracts using column chromatography (CC) and preparative TLC, in order to isolate the four metabolites.

Biological tests were performed according to the OECD 201 guidelines (Freshwater Algae & Cyanobacteria) [21], selecting as a model organism the freshwater green alga *Raphidocelis subcapitata*, once known as *Pseudokirchneriella subcapitata*, because of its documented homogeneous response to stress [22]. The growth inhibition test was thus set up using uni-algal cultures of *R. subcapitata* kindly provided by A.C.U.F. (Algal Collection of University Federico II). The stock culture was obtained using Bold Basal Medium and incubated (20 PAR, 280 rpm, 24±0.1° C) to obtain exponential growth.

The extracts and the pure compounds from *D. viscosa* were dissolved using a 5% DMSO-Water solution. For each of the tested compound or extract, five dosages were inoculated (80, 40, 20, 10 and 5 mg/L respectively) in each of six biological replicates (1 mL of algal culture). Two negative controls (the first using a sterile distilled water inoculum and the second using a 5% DMSO-Water inoculum) and one positive control (3% Biotin T, a known algicide) were set up alongside the others. The inoculated and control cultures were then put inside 4x6 multiwells, every horizontal line representing the six replicates per dosage, and subsequently incubated at 20 PAR, 280 rpm and 24±0.1° C for a total time of 72 h.

Following the endpoint, the algae's main vital parameters (cell count, two peaks for chlorophyll *a* and *b*, and one for total carotenoids) were measured using a Victor spectrophotometer (at respectively 750 nm, 450 nm, 490 nm and 531 nm following 30 s of shaking), with three technical replicate for eac of the samples. Two fluorescence peaks (chlorophyll *a* and *b*) were also measured through the Victor spectrofluorometer function. Average responses were calculated and for each active substance a growth inhibition curve was plotted; regression analysis was then used to calculate IC_{50} (concentration of the inoculated substance necessary to inhibit 50% of the growth).

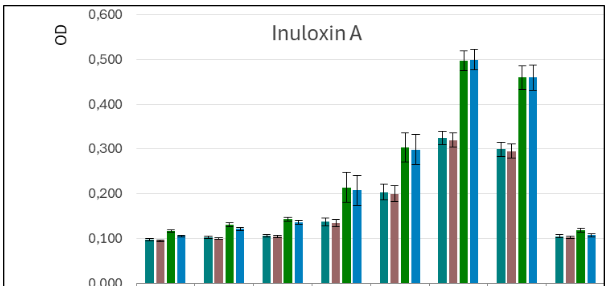
III. RESULTS

The cultures of *R. subcapitata* showed diverse responses to the inoculated substances.

The extracts obtained through solvents of increasing polarity (*n*-hexane, dichloromethane, and ethyl acetate) exhibited a homogeneous trend. Algal cultures exposed to these extracts showed significantly reduced vital parameters compared to negative controls; however, the cellular and pigment-based assays did not show dose-dependent responses in any of the three cases. This may be attributed to early saturation of toxic effects at low concentrations, as well as to potential interference from other compounds present in the crude mixtures. For this reason, the extracts were further purified to isolate the compounds primarily responsible for biocidal activity, thus removing confounding factors and enabling a more direct and proportional assessment of biological activity.

The four isolated compounds selected for testing were identified as the sesquiterpenoids α-costic acid and inuloxins A, B and C. α-Costic acid and inuloxin B induced no growth inhibition and no appreciable deviation in vital parameters compared to the controls. On the other hand, inuloxins A and C proved to be biologically active and noticeably effective in inhibiting the growth of *R. subcapitata* cultures.

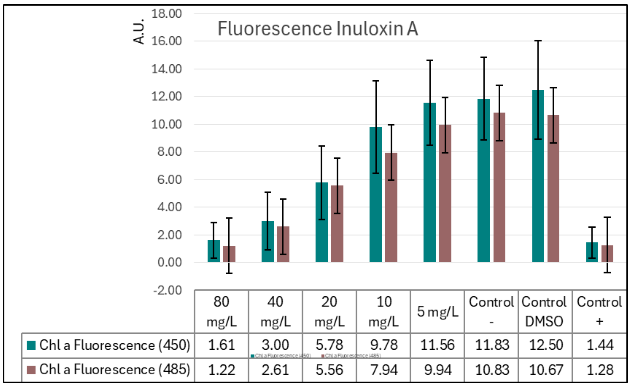
Algal cultures inoculated with Inuloxin A showed a marked reduction in growth rates between the 5 and 10 mg/L dosages, after which the response plateaued up to the maximum tested concentration of 80 mg/L, as shown in Fig. 1.



	80 mg/L	40 mg/L	20 mg/L	10 mg/L	5 mg/L	Control	Control DMSO	Biotin T
Cell density	0,097	0,102	0,106	0,137	0,203	0,324	0,299	0,105
Carotenoids	0,095	0,100	0,104	0,135	0,200	0,319	0,295	0,102
Chlorophyll a	0,116	0,130	0,143	0,214	0,303	0,498	0,460	0,118
Chlorophyll b	0,106	0,121	0,136	0,208	0,299	0,499	0,460	0,108

Fig. 1. Optical densities for each of the selected biological parameter (cell density and three wavelengths associated to the concentration of photosynthetic and accessory pigments), grouped for growing concentrations of Inuloxin A. The growth inhibition is visible at higher dosages.

Fluorescence assays, the results of which are visible in Fig. 2, highlight an even more conspicuous reduction in pigment activity along the curve, with the 40 and 80 mg/L inoculums of Inuloxin A showing a reduced activity comparable to the one obtained with the addition of the established algicide (positive control, i.e. 3% Biotin T).



	80 mg/L	40 mg/L	20 mg/L	10 mg/L	5 mg/L	Control -	Control DMSO	Control +
Chl a Fluorescence (450)	1.61	3.00	5.78	9.78	11.56	11.83	12.50	1.44
Chl a Fluorescence (485)	1.22	2.61	5.56	7.94	9.94	10.83	10.67	1.28

Fig. 2. Fluorescence peaks of chlorophyll a and b after a three-day exposure to Inuloxin A, grouped for each concentration of the inoculated metabolite.

Notably, as previously stated, the effects at the highest dosage for both spectrophotometric and fluorometric assays was comparable to that observed within the positive control, the Biotin T (cell density of 0.105 OD for Biotin T vs. 0.097 OD for inuloxin A). To quantify this saturation trend, which was particularly highlighted within the 10 to 5 mg/L margin, the IC_{50} of inuloxin A was calculated and found to be 6,28 mg/L (Fig. 3).

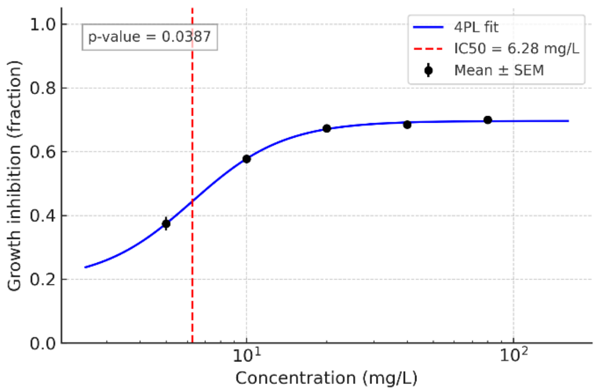
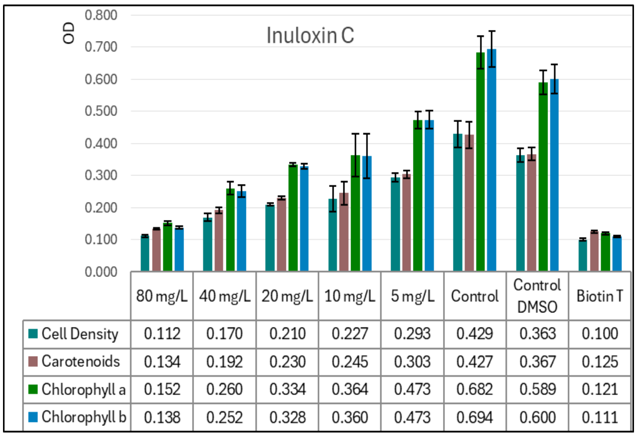


Fig. 3. Dose-response curve for Inuloxin A. For this plot, growth inhibition (G.I.) was normalized against negative controls (water inocula).

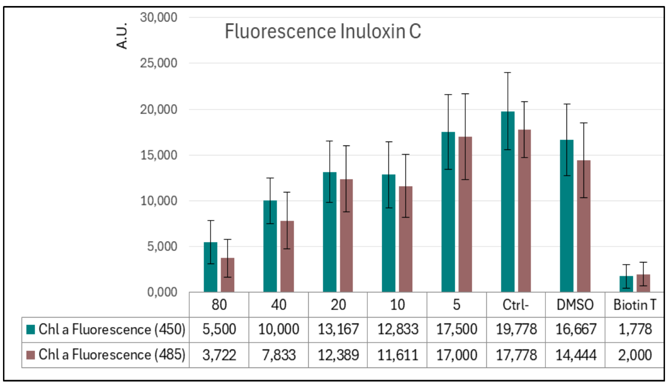
The same tests, performed with differential inocula of Inuloxin C, highlighted a trend comparable to the Inuloxin A inoculated cultures (Figg. 4 and 5). Both spectrophotometric and fluorometric assays showed a distinct reduction in growth between inoculated and control cultures, and a very distinct similarity between the effects of the highest dosage (80 mg/L) of Inuloxin C and the positive control. In both cases, the growth inhibition was dosage-dependent, although with slightly higher chlorophyll A activity at the 80 mg/L dosage compared to the ones measured for Inuloxin A.



	80 mg/L	40 mg/L	20 mg/L	10 mg/L	5 mg/L	Control	Control DMSO	Biotin T
Cell Density	0.112	0.170	0.210	0.227	0.293	0.429	0.363	0.100
Carotenoids	0.134	0.192	0.230	0.245	0.303	0.427	0.367	0.125
Chlorophyll a	0.152	0.260	0.334	0.364	0.473	0.682	0.589	0.121
Chlorophyll b	0.138	0.252	0.328	0.360	0.473	0.694	0.600	0.111

Fig. 4. Optical densities for each of the selected biological parameter (cell density and three wavelengths associated to the concentration of photosynthetic and accessory pigments), grouped for growing concentrations of Inuloxin C. The growth inhibition is visible at higher dosages.

These results indicate that both inuloxin A and inuloxin C, present high inhibitory activity on the selected model organism and, therefore, hold strong potential for the treatment of biological patinas. To identify and optimize a suitable carrier for delivering high concentrations of this active compound, carrier solvents— such as triglyceride-based mixtures of capric and caprylic acids—are being presently tested. Preliminary assays suggest higher, dose-proportional biocidal activity when compared to the previously tested crude mixtures.



	80	40	20	10	5	Ctrl-	DMSO	Biotin T
Chl a Fluorescence (450)	5,500	10,000	13,167	12,833	17,500	19,778	16,667	1,778
Chl a Fluorescence (485)	3,722	7,833	12,389	11,611	17,000	17,778	14,444	2,000

Fig. 5. Fluorescence peaks of chlorophyll a and b after a three-day exposure to Inuloxin C, grouped for each concentration of inoculated metabolite.

IV. CONCLUSIONS

The results of this study highlight the remarkable potential of *Dittrichia viscosa* extractable pure compounds, specifically the sesquiterpene lactones inuloxins A and C, which demonstrated significant inhibitory effects on the growth of *Raphidocelis subcapitata* in liquid culture. The isolation of pure compounds and their subsequent characterization allowed for a more reliable assessment of their biological activity, eliminating the confounding effects observed with crude solvent mixtures.

Given the pronounced efficacy of these specialized metabolites and the promising performance of oily carriers in enhancing its activity, future investigations will focus on the development of solid-phase testing protocols, initially to be carried out on environmental isolated strains typically involved in the biodeterioration of stone artefacts – among which green algae, diatoms and cyanobacteria - and subsequently extended to mixed microbial consortia. Ultimately, these experimental approaches aim to simulate real-world conditions, by testing the formulations on biofilms developed over lithic artefacts collected from the Archaeological Park of the Baia's Baths.

This transition from liquid-phase inhibition tests to solid-phase applications represents a crucial step towards validating the use of *D. viscosa*-derived compounds as environmentally sustainable alternatives for the treatment and prevention of biodeterioration in cultural heritage conservation.

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