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Identification and antimicrobial activity of most representative secondary metabolites from different plant species

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Abstract

Background: The plant kingdom constitutes an enormous reservoir of bioactive molecules, generally used by plants to prevent or to protect themselves from pathogens' attacks. To date, several primary or secondary plant metabolites have been already proven to exert antibiotic activities; nonetheless, researchers are still continuing to lavish great efforts to identify and characterize new natural molecules one by one. Aiming at the replacement of synthetic chemical products, the bioactivity of plant extracts should be assessed case by case, and active substances should be tested as individuals to obtain accurate information on the real usefulness of plant metabolites. In this work major glycoalkaloids obtained from *Solanum nigrum*, glucosinolates from *Armoracia rusticana,* and cannabinoids from *Cannabis sativa* were identified. The antimicrobial activity of crude extracts and pure components against Gram+ (*Bacillus cereus* (A1I), *Bacillus thuringiensis* (B7I2), and *Bacillus amyloliquefaciens* (A5TI)) and Gram− bacteria (*Pseudomonas orientalis* (A14-1II), and *Stenotrophomonas maltophilia* (B9TIII)), employed as model organisms, was tested.

Result: Major glycoalkaloids, glucosinolates, and cannabinoids were identified in crude plants' extracts using high-resolution LC–ESI-FTICR/MS. From antimicrobial assays useful information towards a few of biological activities of crude extracts and individual components were obtained.

Solanum nigrum extracts revealed inhibition activity on all bacteria tested as well as the main active glycoalkaloids, solamargine and solasonine, which were found to be active even when tested individually. At assayed concentrations, *A. rusticana* extract was active towards a few of the microorganisms tested, confirming that the activity of glucosinolates can be referred only partially to the mother molecules, while biological efficiency of such kind of compounds is mainly due to their enzymatic breaking off, where myrosinase converts them into isothiocyanates and/or thiocyanates. Hemp-type *C. sativa* extract showed antimicrobial activity only against Gram+ bacteria, but the main individual components tested showed always a limited bioactivity.

Conclusion: Promising results were obtained, but tests performed in vitro are only the first step of a wider investigation as required for an extensive application. Further research efforts are necessary to demonstrate the efficiency of natural substances in different target environments.

Keywords: Glycoalkaloids, Glucosinolates, Cannabinoids, *Solanum nigrum*, *Armoracia rusticana*, *Cannabis sativa*, LC–ESI-FTICR/MS, Antimicrobial activity, Gram+ bacteria, Gram− bacteria

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Background

Natural products were developed and used to relieve sickness by the dawn of human history: before the "Synthetic Era", indeed, 80% of medicine, drugs, and pesticides were obtained from roots, barks, and leaves (fluid extracts) of plants, contributing to the diseases' restraint [1, 2]. Despite this success, natural products' research has endured a global decline due to the production difficulties and small quantities obtained [3]. The necessity of using drugs and pesticides in high quantity (linked also to the population growth) has required large amounts of products, which only laboratory synthesis could realize. In this process, however, some negative aspects attributable to a large use of synthetic substances were not considered, such as the organic balance alteration, pollution of various environmental systems, resistance induction, and genetic changes in living beings in a very short period. The current trend is the return, when possible, to the preceding system of diseases' treatment, re-emphasizing the use of metabolic constituents produced by several plant, bacterial, and fungal species [4]. Due to the scientific awareness surrounding the use of natural substances instead of synthetic ones, in recent years many researchers have undertaken studies on the occurrence of secondary metabolites in plants that are widely used in almost all geographical areas and on their possible bioactivity.

Secondary metabolites are organic molecules, not involved in the normal growth and development of an organism, whose functions are largely unknown, although they seem involved in the organism defence [5, 6].

The recognition of the biological properties of thousands of these molecules has increased interest in this field for new drugs, antibiotics, insecticides, fungicides, and herbicides research and brought about a re-evaluation of bacteria, fungi, and plant role, especially in the ecological context. Terpenes (gums, resins, carotenoids, etc.), phenols (lignin, flavones, anthocyanins, tannins, etc.) as well as alkaloids are just some of the substances currently used in different application fields, giving rise to the world's growing attention due to their widespread use and to the concurrent preservation of both human health and environment.

Each family, genus, and species of several plants produce a characteristic mixture of substances that can be present in active form or in prodrug state and are used for taxonomic classification [7]. Normally, they are activated when wounding or infection in the vegetal body occurs [8]. These compounds can be active as single components or strengthen their activity due to synergistic action with other chemical compounds co-synthesized in the plant cells [9].

This work deals with the identification of more representative glycoalkaloids and glucosinolates in crude extracts obtained from *Solanum nigrum* and *Armoracia rusticana*, respectively, and of several cannabinoids extracted from *Cannabis sativa*. Moreover, the antimicrobial activity of crude extracts and some pure components against a limited number of Gram+ and Gram− bacteria, employed as model organisms, was investigated as preliminary assessment of their bioactivity.

Methods

Chemicals

Solamargine and solasonine standards were purchased from Glycomix (UK); sinigrin monohydrate was obtained from Sigma-Aldrich (Steinheim, Germany); Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), cannabinol (CBN), and cannabidiolic acid (CBDa) were purchased from HPC Standards GmbH (Cunnersdorf, Germany). Methanol, acetonitrile, and formic acid were obtained from Carlo Erba (Milan, Italy). Ultrapure water was produced using a Milli-Q RG system from Millipore (Bedford, MA, USA).

Standard preparation

Glycoalkaloids: stock solutions (1 mg L^{-1}) of pure standards in ultrapure water acidified with 1% acetic acid were prepared as reported elsewhere [10, 11] and kept in the darkness at +4 °C. Glucosinolates: stock solutions (1 mg L^{-1}) of pure standards in methanol/water (70/30, v/v) were kept in the darkness at +4 °C [12–15]. Cannabinoids: stock solutions (1 mg L^{-1}) of pure compounds in ethanol were prepared and kept in the darkness at −20 °C. Solutions prepared as above were used for analytical purposes.

For antimicrobial assays, solutions of standard compounds were prepared when necessary before each test using sterilized ultrapure water, which was also employed to dilute them up to the required concentration.

All glass apparatus were heat sterilized by autoclaving for 60 min at 121 °C before use. Aseptic handling materials and laboratory facilities were used throughout the study to maintain sterility.

Plant samples

Black nightshade (*S. nigrum*) unripe berries (glycoalkaloids), horseradish (*A. rusticana*) roots (glucosinolates),

and hemp (*C. sativa*) flowers (cannabinoids) were the vegetal materials used to obtain the extracts.

Green unripe berries of black nightshade (60 berries harvested from 15 different plants in a homogeneous experimental cultivation) and horseradish root (15 plants harvested from a homogeneous experimental cultivation) were supplied, respectively, from a greenhouse located in Metaponto village (Italy) and in a field established at the Institute of Plant Genetics-National Research Council close to Policoro village (Italy). The voucher specimens of both plants were deposited at the Herbarium Lucanum (HLUC in Index Herbariorum) with the ID Code 2320 and the ID Code 9197 for *S. nigrum* and *A. rusticana*, respectively.

Two different flower samples from experimental fields located in southern Italy and derived from the registered *C. sativa* accession "Eletta campana" were supplied with the courtesy of Eletta campana S.r.l. company.

The "Eletta campana" cannabis accession has been bred and grown during the past century both in insular and peninsular areas of Italy, mainly for industrial production purposes [16, 17].

Sample #1: flowers—obtained as representative population—of the "Eletta campana" chemical phenotype, 30 flowers from 30 plants.

Sample #2: field selection of flowers used in varietal improvement schemes to select plants with a higher concentration of THC and CBD compared to the average composition of the "Eletta campana" accession, 30 flowers from 30 selected plants.

Extraction, purification, and preliminary tests

Glycoalkaloids and glucosinolates extractions were made in five replicates following previously published methods [10–15].

Cannabinoids were extracted in five replicates for each sample of hemp flowers crushed using liquid nitrogen, sieved, and lyophilized. Ultrasound-assisted extraction (USAE) was carried out using absolute ethanol as solvent according to published methods [18, 19]. Samples were centrifuged by using the Hettich Zentrifuge, MIKRO220R (Germany) for 12 min at 2400*g* and filtered (PTFE filters 0.20 μm) to clarify the liquid phase.

The replicates were brought together to constitute a representative sample of the total material collected. Subsequently, analytical determinations and antimicrobial assays were conducted.

Aiming at the determination of the minimal inhibitory concentration (MIC), preliminary tests were performed using initial concentration ranges of main representative compounds under investigation: 0.005–1 mM, for black nightshade unripe berries; 1–5 mM, for horseradish extract; 0.001–0.3 mM, for cannabis extracts. MIC assays were performed according to the European Committee for Antimicrobial Susceptibility Testing (EUCAST) and Mann and Markham methods [20, 21].

Analytical procedure

All analytical experiments were performed using a Surveyor LC system coupled to an ESI-FTICR mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with a 20 W CO_2-laser IRMPD (Synrad, Mukilteo, WA, USA), emission wavelength 10.6 μm. Glucosinolates and glycoalkaloids LC separations were performed at ambient temperature by using the same chromatographic conditions reported elsewhere [11, 14, 22, 23]. For separation and identification of cannabinoids, a new optimized LC–ESI-FTICR/MS method was used.

Mass spectrometric conditions were optimized by direct infusion of standard solutions. The instrument was tuned to facilitate the ionization process and to achieve the highest sensitivity. The ESI-FTICR mass spectra obtained were used to characterize the ionization behaviour of the compounds.

Data acquisition and analyses were accomplished using the Xcalibur software package (version 2.0 SR1 Thermo Electron), and total ion current (TIC) acquisition; data were collected in full MS scan mode and processed post-acquisition to identify the compounds of interest. In addition to accurate mass determination and retention times, extensive structural information was obtained by MS/MS fragmentation performance of the compounds investigated (data not shown). The chromatographic raw data were imported, elaborated, and plotted by SigmaPlot 10.0 (Systat Software, Inc., London, UK).

Analytical determination was performed to know, in detail, the composition of crude extracts and subsequently to permit bioactivity testing of pure standards, using about the same concentrations present in the extracts.

Antimicrobial activity assays

The different extracts were tested against five bacterial strains of the culture collection stored in the Department of Sciences, University of Basilicata, Potenza, Italy. Three Gram+ bacteria [*Bacillus cereus* (A1I), *Bacillus thuringiensis* (B7I2), and *Bacillus amyloliquefaciens* (A5TI)], and two Gram− bacteria [*Pseudomonas orientalis* (A14-1II) and *Stenotrophomonas maltophilia* (B9TIII)] were employed as screening microorganisms for this study. All strains were maintained as freeze-dried stocks in reconstituted (11% w/v) skim milk, containing 0.1% (w/v) ascorbic acid, and routinely cultivated in optimal growth conditions.

Antimicrobial activities of all tested extracts were determined by agar well diffusion assay [24].

For each strain, a subculture in a specific broth (PCb) was obtained from the active stock culture by 1% (v/v) inoculum and incubated overnight at 30 °C. A volume of 200 µL for each subculture was used to inoculate the agar media (to achieve a final concentration of 10^9 CFU L^{-1}) and distributed into Petri plates. Each extract (60 µL) was poured into wells (5 mm Ø) bored in the agar plates, and then the plates were incubated at 30 °C. The organic solvent was used as negative control, while the antibiotic was used as positive control. The experiments were performed in triplicate and the antimicrobial activity of each extract was expressed as mean diameter (mm) of the zone of inhibition (ZoI) produced by the respective extract after 24 h of incubation. A value of ZoI < 10 mm was stated to indicate a low antimicrobial activity; 11 < ZoI < 15 mm, a middle antimicrobial activity; and ZoI > 16 mm, a high antimicrobial activity.

Extracts producing an inhibition zone were screened to determine the minimum inhibitory concentrations and evaluate the antimicrobial effectiveness of each extract against different bacterial strains by means of the agar well diffusion method [24]. The medium inoculated with the strain subculture was distributed into Petri plates, and different concentrations of extracts, ranging from 1 to 100 mg L^{-1}, were poured into wells bored in the agar plates and the plates were incubated for 24 h. After incubation, the MIC was determined as the lowest concentration of the extract inhibiting the growth of bacterial strains. The MIC values were obtained in triplicate tests.

Results were compared by analysis of variance (ANOVA) and Bonferroni post hoc test using GraphPad Prism 6 software, version for Windows.

Results

Analytical outcomes

Identification of main glycoalkaloids

Figure 1 shows the LC–FTICR/MS separation in positive ion mode of an aqueous extract of black nightshade berries. Analysis of the extracts revealed the presence of two main glycoalkaloids identified by accurate *m/z* values of protonated species, comparison with authentic standard, and on the basis of IRMPD fragmentation in the ICR cell of precursor ions $[M+H]^+$. In the insets, the mass spectra of two main peaks corresponding to solasonine found at *m/z* 884.50079 ($C_{45}H_{74}NO_{16}$, exact *m/z* 884.50021) and solamargine found at *m/z* 868.50476 ($C_{45}H_{74}NO_{15}$, exact *m/z* 868.50530) are reported. Both compounds were identified with a mass error lower than 1 ppm, which indicates a very good mass accuracy. In the IRMPD MS spectra (data not shown), several common loss from sugar moiety and product ions were observed. Ions generated from fragmentation of B-ring or E-ring of aglycons were diagnostically useful for establishing their membership in the general family of glycoalkaloids [25]. The other intense peak in the TIC (Fig. 1) can be due to a derivative

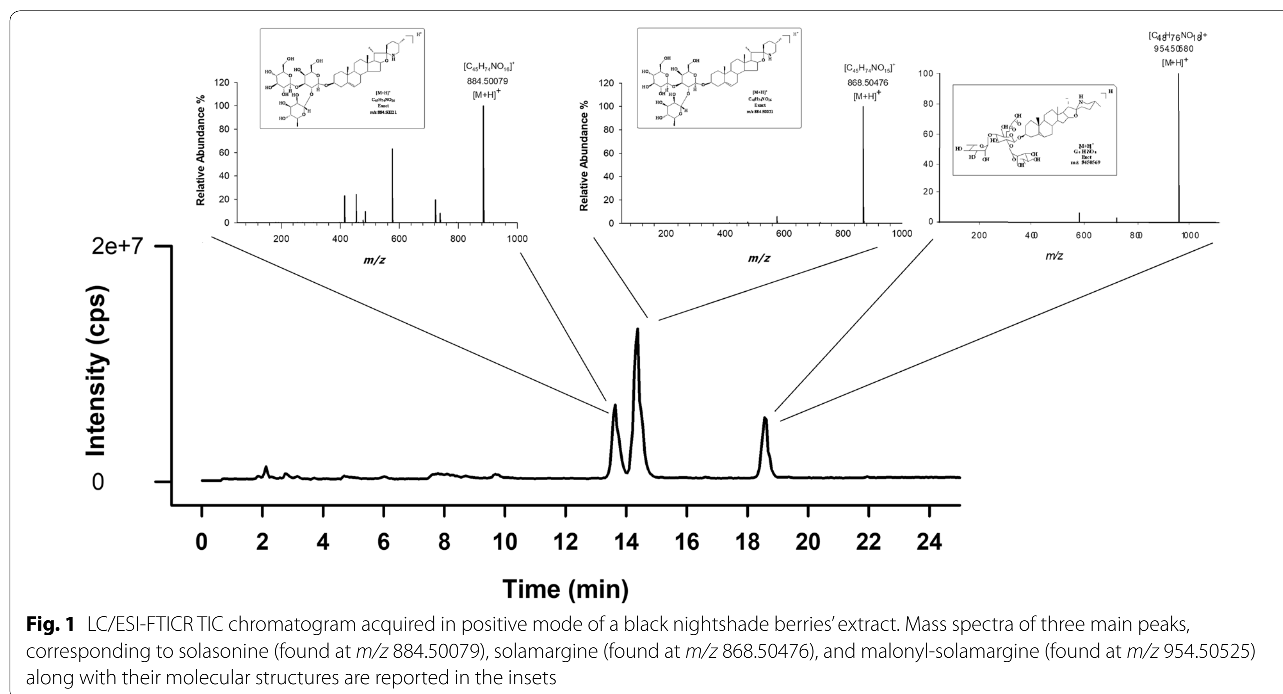

Fig. 1 LC/ESI-FTICR TIC chromatogram acquired in positive mode of a black nightshade berries' extract. Mass spectra of three main peaks, corresponding to solasonine (found at *m/z* 884.50079), solamargine (found at *m/z* 868.50476), and malonyl-solamargine (found at *m/z* 954.50525) along with their molecular structures are reported in the insets

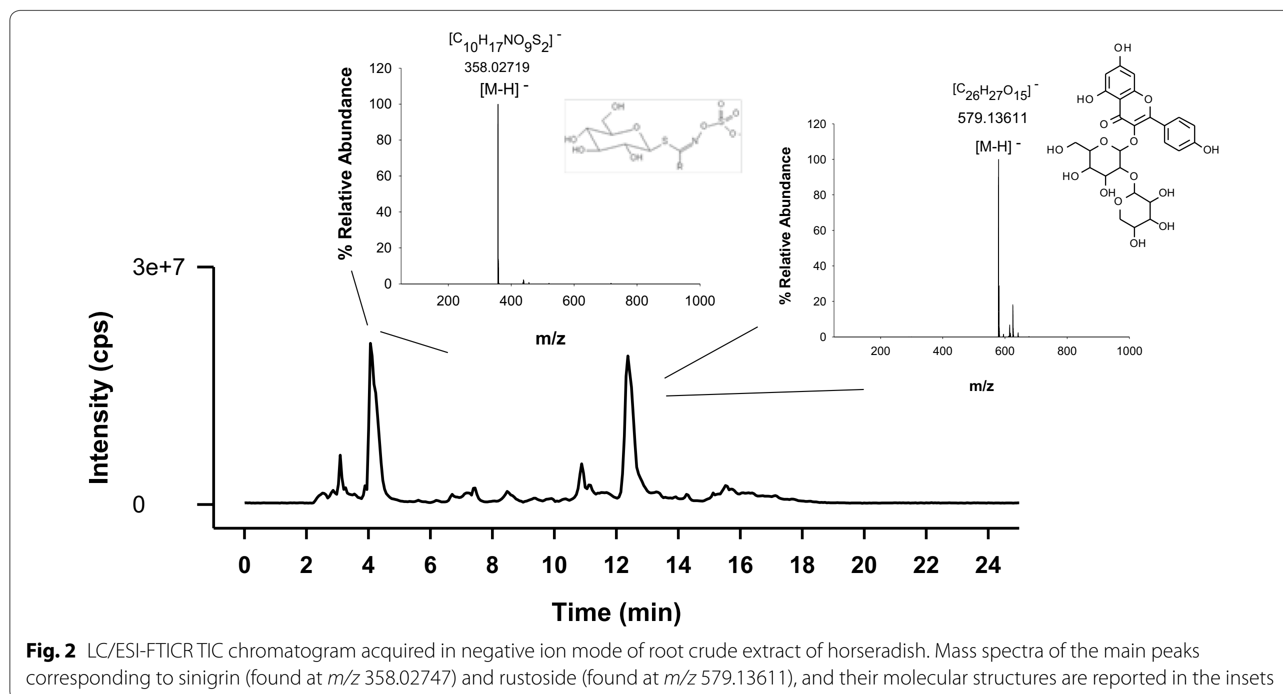

Fig. 2 LC/ESI-FTICR TIC chromatogram acquired in negative ion mode of root crude extract of horseradish. Mass spectra of the main peaks corresponding to sinigrin (found at *m/z* 358.02747) and rustoside (found at *m/z* 579.13611), and their molecular structures are reported in the insets

compound of solamargine, the malonyl-solamargine at *m/z* 954.50525 ($C_{48}H_{75}O_{18}N$, exact *m/z* 954.50569).

Quantitative analysis revealed that black nightshade berries extract contains a high amount of solamargine and solasonine (1.35 and 1.52 g kg^{-1} dry weight, respectively) and a small concentration of other minor known glycoalkaloids, confirming results obtained by Ventrella et al. [13].

Identification of glucosinolates

The identification of GLSs was based on the study of characteristic fragments of these compounds in IRMPD MS/MS spectra, and on the measure of accurate masses observed using LC/ESI-FTICR/MS, according to Agneta et al. [12, 22]. In Fig. 2, the total ion chromatogram (TIC) acquired in negative ion mode of a horseradish root extract is shown. The qualitative and quantitative analyses of this extract confirmed the occurrence of a high amount of sinigrin (2.04 g kg^{-1} dry weight), which accounts for more than 90% of the total GLS, and of the other 16 GLSs in trace quantity [12, 22]. In the inset of Fig. 2, the mass spectrum of the peak corresponding to sinigrin, found at *m/z* 358.02747 ($C_{10}H_{17}NO_9S_2$, exact *m/z* 358.02720, error 0.8 ppm) is shown. By accurate high-resolution mass analysis, the peak eluting at 12.5 min was excluded to be a glucosinolate, but was found to be rustoside, also known as kaempferol 3-lathyroside, which is a member of the class of compounds known as flavonoid-3-*O*-glycosides normally derived from horseradish.

GLSs exhibited $[M-H]^-$ as the precursor ion that corresponds to easy deprotonation of the sulphate group. Moreover, the dissociation of $[M-H]^-$ precursor ion yielded abundant product ions, which gave much information on the structure of the side chain and were of great value for a correct assignment of known and unknown GLSs. Typical fragments of GLS with nominal *m/z* 97, 195, 241, 259, and 275, which correspond to the fragment ions $HSO4^-$, $C_6H_{11}O_5S^-$, $C_6H_9O_8S^-$, $C_6H_{11}O_9S^-$, and $C_6H_{11}O_8S_2^-$, respectively, were found in the spectrum examined (data not shown). Other characteristic fragments, such as $[M\text{-}80\text{-}H]^-$, $[M\text{-}162\text{-}H]^-$, $[M\text{-}178\text{-}H]^-$, $[M\text{-}196\text{-}H]^-$, and $[M\text{-}242\text{-}H]^-$, were very informative for correct molecular identification of GLSs [15].

Identification of cannabinoids

Using optimized reversed-phase liquid chromatography (RP-HPLC) coupled to electrospray ionization in positive mode (ESI^+) and Fourier transform ion cyclotron resonance (FTICR)/MS, together with tandem mass spectrometry (MS^n) studies performed using IRMPD and collisional induced dissociation (CID), it was possible to separate and quantify four known cannabinoids (THC, CBD, CBDa, and CBN), useful for the chemotype definition and the classification of *C.*

Abbreviation	Name	Molecular structure
CBD	Cannabidiol	
CBDa	Cannabidiolic acid	
CBDV	Cannabidivarin	
CBN	Cannabinol	
THC	Δ9-tetrahydrocannabinol	

Scheme 1 Common names and molecular structures of cannabinoids detected

sativa (Scheme 1). The total ion current (TIC) chromatogram (Fig. 3) revealed the occurrence of three main cannabinoid peaks assigned to CBD, THC, and CBDa; in the insets, the mass spectra of these peaks are shown: CBD, found at *m/z* 315.23159 ($C_{21}H_{30}O_2$, exact *m/z* 315.23184, error −0.8 ppm); THC, found at *m/z* 315.23148 ($C_{21}H_{30}O_2$, exact *m/z* 315.23186, error −1.2 ppm); CBDa, found at *m/z* 359.22126 ($C_{22}H_{30}O_4$, exact *m/z* 359.22169 error −1.2 ppm). CID and IRMPD fragmentation of precursor ions $[M+H]^+$ generates several common species that are diagnostically useful for establishing their identity (data not shown). The wide peak at 7.4 min corresponds to cannabidivarin (CBDV, $C_{19}H_{26}O_2$, *m/z* $[M+H]^+$ 287.20056), a non-psychoactive cannabinoid homologue of CBD with the side chain shortened by two methylene bridges; it is not useful to determine the chemotype in *Cannabis* plant destined for human consumption or industrial transformation (Scheme 1).

The quantification of secondary metabolites (THC, CBD, CBN, CBDa) was performed in parallel through low-resolution mass spectra, selected reaction monitoring (SRM), and high-resolution total ion current (TIC).

As described in Table 1, the chemovar analysed as sample #1 does not exceed the THC limit (0.2%) recommended by the European Union regulations [26, 27], confirming previous findings [28].

The ([THC] + [CBN])/[CBD] ratio (phenotypic index) of samples was used to assess the chemical phenotype (chemotype) of the specific accession [29].

The high content of cannabidiol (CBD) suggests that the "Eletta campana" accession can be defined as an industrial hemp having a ratio [CBD]/[THC] > 10 (CBD-prevalent chemotype) [30]. In our case, the CBN

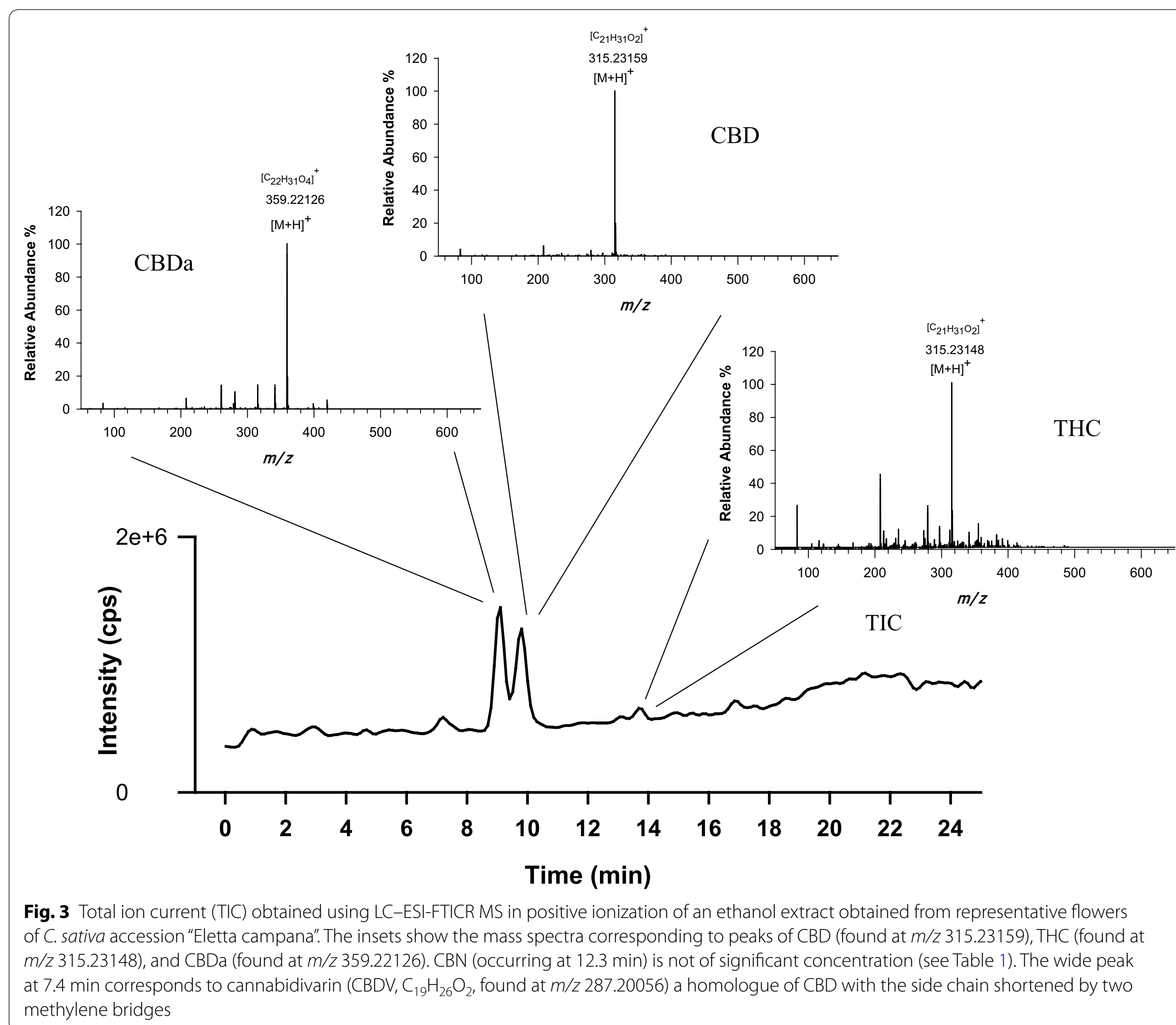

Fig. 3 Total ion current (TIC) obtained using LC–ESI-FTICR MS in positive ionization of an ethanol extract obtained from representative flowers of *C. sativa* accession "Eletta campana". The insets show the mass spectra corresponding to peaks of CBD (found at *m/z* 315.23159), THC (found at *m/z* 315.23148), and CBDa (found at *m/z* 359.22126). CBN (occurring at 12.3 min) is not of significant concentration (see Table 1). The wide peak at 7.4 min corresponds to cannabidivarin (CBDV, $C_{19}H_{26}O_2$, found at *m/z* 287.20056) a homologue of CBD with the side chain shortened by two methylene bridges

Table 1 Composition of the two samples of *C. sativa* analysed: Δ9-tetrahydrocannabinol (THC), cannabidiol (CBD), and (CBN + THC)/CBD ratio identifying the chemotype

Samples	THC	CBD	CBN	(CBN + THC)/CBD
1	0.021% (2.1 ± 0.5 g kg^{-1} dw)	0.781% (78.1 ± 1.3 g kg^{-1} dw)	0.008% (0.81 ± 0.12 g kg^{-1} dw)	0.037
2	0.050% (5.0 ± 0.8 g kg^{-1} dw)	0.967% (96.7 ± 1.7 g kg^{-1} dw)	0.010% (0.98 ± 0.28 g kg^{-1} dw)	0.061

concentration is not significant for the chemical definition of cannabis quality.

Data reported in Table 1 for the sample #2 indicate that the field selection of plant flowers was able to discriminate a group of plants with a higher content of analysed cannabinoids.

Antimicrobial activity assays

The antimicrobial activities and MICs were evaluated against selected bacterial strains giving different results depending on the type of plant under observation.

Statistics of the antimicrobial activity data (diameters of ZoI) confirmed that the diameter ranges chosen

Table 2 Antimicrobial activity and MIC of *S. nigrum*, *A. rusticana*, and *C. sativa* extracts tested

Bacteria	*S. nigrum*		*A. rusticana*		*C. sativa* Sample #1[a]		*C. sativa* Sample #2[b]	
	ZoI (mm)[c]	MIC (mg L^{-1})	ZoI (mm)[c]	MIC (mg L^{-1})	ZoI (mm)[c]	MIC (mg L^{-1})	ZoI (mm)[c]	MIC (mg L^{-1})
Gram+								
Bacillus cereus (A1I)	15.2 ± 1.0^{A}	5 ± 1^{H}	NI	–	37.0 ± 1.0^{C}	5 ± 1^{H}	36.5 ± 1.0^{C}	5 ± 1^{H}
Bacillus thuringiensis (B7I2)	13.5 ± 0.8^{B}	10 ± 1^{K}	NI	–	37.5 ± 0.9^{C}	10 ± 1^{K}	37.5 ± 0.7^{C}	5 ± 1^{H}
Bacillus amyloliquefaciens (A5TI)	15.0 ± 0.9^{A}	10 ± 1^{K}	NI	–	34.5 ± 0.9^{D}	5 ± 1^{H}	34.0 ± 0.9^{D}	5 ± 1^{H}
Gram−								
Pseudomonas orientalis (A14-1II)	13.5 ± 1.0^{B}	10 ± 1^{K}	NI	–	NI		NI	
Stenotrophomonas maltophilia (B9TIII)	15.0 ± 1.0^{A}	10 ± 1^{K}	NI	–	NI		NI	

Assays were performed in triplicate and results are the mean of three values ± standard deviation

NI no inhibition zone was observed

Different letters in superscript to numerical data indicate significant differences (p value < 0.05)

[a] Sample #1 is composed of flowers representative of the "Eletta campana" cannabis accession

[b] Sample #2 was obtained from a field mass selection of "Eletta campana" flowers having a higher THC and CBD content

[c] ZoI: diameter of inhibition zone obtained after 24 h of incubation in agar well diffusion assays. ZoI < 10 mm: low antimicrobial activity; 11 < ZoI < 15 mm: middle antimicrobial activity; ZoI > 16 mm: high antimicrobial activity

Table 3 Antimicrobial activity and MIC of pure compounds as components *of S. nigrum*

Bacteria	Solamargine		Solasonine		Solamargine/solasonine (1:1 v/v)	
	ZoI (mm)[a]	MIC (mg L^{-1})	ZoI (mm)[a]	MIC (mg L^{-1})	ZoI (mm)[a]	MIC (mg L^{-1})
Gram+						
Bacillus cereus (A1I)	15.0 ± 1.0^{A}	5 ± 1^{H}	14.0 ± 0.9^{A}	5 ± 1^{H}	15.0 ± 1.0^{A}	5 ± 1^{H}
Bacillus thuringiensis (B7I2)	12.0 ± 0.9^{B}	20 ± 1^{L}	12.0 ± 0.8^{B}	40 ± 1^{M}	13.0 ± 1.0^{B}	40 ± 1^{M}
Bacillus amyloliquefaciens (A5TI)	12.0 ± 0.9^{B}	20 ± 1^{L}	12.0 ± 1.0^{B}	40 ± 1^{M}	14.5 ± 1.0^{A}	20 ± 1^{L}
Gram−						
Pseudomonas orientalis (A14-1II)	13.0 ± 0.8^{B}	40 ± 1^{M}	13.0 ± 0.7^{B}	40 ± 1^{M}	13.5 ± 1.0^{B}	40 ± 1^{M}
Stenotrophomonas maltophilia (B9TIII)	15.0 ± 0.9^{A}	10 ± 1^{K}	14.0 ± 0.8^{A}	10 ± 1^{K}	15.0 ± 1.1^{A}	10 ± 1^{H}

Assays were performed in triplicate and results are the mean of three values ± standard deviation

Different letters in superscript to numerical data indicate significant differences (p value < 0.05)

[a] ZoI: diameter of inhibition zone obtained after 24 h of incubation in agar well diffusion assays. ZoI < 10 mm: low antimicrobial activity; 11 < ZoI < 15 mm: middle antimicrobial activity; ZoI > 16 mm: high antimicrobial activity

(< 10 mm; 11–15 mm, > 16 mm) were able to well discriminate significant differences among the antimicrobial activities (Tables 2, 3, 4).

Solanum nigrum and *C. sativa* extracts demonstrated a certain antimicrobial activity, while *A. rusticana* did not reveal any activity against bacteria in this research.

The Gram− bacteria, *P. orientalis* and *S. maltophilia*, were sensitive only to the *S. nigrum* extract, showing a middle inhibition diameter of 13.5 and 15 mm, respectively; moreover, this extract proved a middle antimicrobial activity against all Gram+ bacteria (inhibition zone ranging from 13.5 to 15.2 mm) (Table 2).

Both flower samples of *C. sativa* showed a similar effect on Gram+ bacteria with a high antimicrobial activity; these extracts were more effective against *B. thuringiensis* and *B. cereus* with 37.5 and 37.0 mm diameter of inhibition zone, respectively, while *B. amyloliquefaciens* was slightly less sensitive (Table 2).

The active extracts of *S. nigrum* and *C. sativa* were subjected to determine MIC by the agar well diffusion method against the respective susceptible bacterial species (Table 2). The results obtained indicated that Gram+ and Gram− bacterial species tested were sensitive to different extracts in a similar way with an MIC of 5–10 mg L^{-1}. The more effective extracts were the

Table 4 Antimicrobial activity and MIC of standard pure compounds as components *of C. sativa*

Bacteria	THC		CBD		CBD/THC	
	ZoI (mm)[a]	MIC (mg L^{-1})	ZoI (mm)[a]	MIC (mg L^{-1})	ZoI (mm)[a]	MIC (mg L^{-1})
Gram+						
Bacillus cereus (A1I)	8.5 ± 1.2^{A}	> 60	12.0 ± 1.0^{B}	> 60	14.5 ± 1.4^{C}	> 60
Bacillus thuringiensis (B7I2)	9.5 ± 1.3^{A}	> 60	13.0 ± 1.1^{B}	> 60	14.5 ± 1.3^{C}	> 60
Bacillus amyloliquefaciens (A5TI)	8.0 ± 0.8^{A}	> 60	11.0 ± 0.9^{B}	> 60	15.0 ± 1.3^{C}	> 60
Gram−						
Pseudomonas orientalis (A14-1II)	NI	–	NI	–	NI	–
Stenotrophomonas maltophilia (B9TIII)	NI	–	NI	–	NI	–

Assays were performed in triplicate and results are the mean of three values ± standard deviation

NI no inhibition zone was observed, *THC* Δ9-tetrahydrocannabinol, *CBD* cannabidiol

Different letters in superscript to numerical data indicate significant differences (p value < 0.05)

[a] ZoI: diameter of inhibition zone obtained after 24 h of incubation in agar well diffusion assays. ZoI < 10 mm: low antimicrobial activity; 11 < ZoI < 15 mm: middle antimicrobial activity; ZoI > 16 mm: high antimicrobial activity

two samples of *C. sativa* with the higher antimicrobial ability and a low inhibitory concentration (Table 2).

The antimicrobial activity of standard pure components of the plants was investigated to understand whether the activity observed in our experiments was due to the synergistic action of more than one constituent in the extracts [31].

In the case of *S. nigrum*, solamargine, solasonine, and the solamargine/solasonine mixture (1:1 v/v) were tested. All bacteria were sensitive to both components with a middle antimicrobial activity ranging from 12 to 15 mm (Table 3).

Among Gram+ bacteria, *B. cereus* was the most sensitive (with an MIC of 5 mg L^{-1}) compared to the other two species, *B. thuringiensis* and *B. amyloliquefaciens*, which were inhibited at higher concentrations (ranging from 20 to 40 mg L^{-1}). Gram− bacteria, instead, showed the same behaviour in the presence of standard pure compounds (Table 3).

The *C. sativa* components were able to inhibit only the Gram + bacteria tested; THC showed a low antimicrobial activity, while CBD and the CBD/THC mixture (1:1 v/v) proved a middle activity, underlining a stronger effect when the mixture was used (Table 4); nevertheless, the bacterial species appeared not very sensitive to the standard pure components, requiring an inhibitory concentration of > 60 mg L^{-1}.

Discussion

The antimicrobial activity and MIC were evaluated against selected bacterial strains of significant environmental and health concern, used as model of target organisms. *B. cereus* is an endospore-forming Gram+ bacterium that can cause food poisoning. Capable of adapting to a wide range of environmental conditions, it is distributed widely in nature and is commonly found in the soil as a saprophytic organism. As a soil bacterium, *B. cereus* can spread easily to many types of foods such as vegetables, eggs, meat, and dairy products, and is known to cause 2–5% of food-borne intoxications due to its secretion of emetic toxins and enterotoxins. Food poisoning occurs when food is left without refrigeration for several hours before it is served. The remaining spores of contaminated food from heat treatment grow well after cooling and are the source of food poisoning. In addition, *B. cereus* is an opportunistic human pathogen and is occasionally associated with infections, causing periodontal diseases and other more serious infections [32]. The availability of natural substances active towards this microorganism, but well tolerated by the human body, could be useful to increase the food storage time. *B. thuringiensis* is a Gram+, rod shaped, and aerobic spore-forming soil bacterium producing crystalline proteins (endotoxins) that have insecticidal properties; on the other hand, this bacterial species synthesizes several enzymes and toxins that give them a wide adaptation to natural habitats [33]. The intrinsic resistance and adaptability of this bacterium makes it an ideal model for the tests performed in this research. *B. amyloliquefaciens* is a non-pathogenic Gram+ soil bacterium. Similar to other *Bacillus* species, it is capable of producing endospores allowing it to survive for extended periods of time. The species also shows some antifungal properties, which are influenced by environmental nitrogen availability. It synthesizes a natural antibiotic protein active against other photogenic *Bacillus* spp. and is used in agriculture, aquaculture, and hydroponics to fight root pathogens [34]. For this reason, it was important to verify if it was inhibited by secondary metabolites produced by plants. *P. orientalis* is a Gram−, rod-shaped bacterium placed

in the *P. fluorescens* group. It shows antagonistic activity against several phytopathogenic bacteria [35] and as *B. amyloliquefaciens* can be inhibited by secondary metabolites produced by plants. *S. maltophilia* is an aerobic, non-fermentative, Gram− bacillus possessing flagella in a multitrichous formation, and is found naturally in the rhizosphere. However, it is also the third most common nosocomial pathogen with multi-drug resistance that targets immune-compromised patients in hospitals, making it important in medical bacteriology [36].

Antimicrobial trials have demonstrated that black nightshade extract is active on assayed microorganisms. Solamargine and solasonine, main components of black nightshade extract, were very active also when tested individually or as binary mixture. Glycoalkaloids, which are produced by widely cultivated Solanaceae plants, are confirmed to be bioactive substances useful for different applications acting as cellular membrane disrupting factors or inhibitors of acetylcholinesterase activity [6, 11, 13].

Horseradish (*A. rusticana*) extract was not active towards any of the tested microorganisms at assayed concentrations as expected for glucosinolates in the absence of the enzymatic reaction needed for the production of active derivatives [37].

Hemp (*C. sativa*) extract showed antimicrobial activity only against Gram+ bacteria, as Gram− bacteria seem to be more resistant to the secondary metabolites contained in the extract [38, 39]. The different compositions of the samples analysed did not influence their bioactivity.

The main components tested, either as individual compounds or as CBD–THC mixture, showed a bioactivity about three times lower compared to the raw extract; the rationale behind such a behaviour could be that antibacterial properties were due to the synergistic effect of many components (such as terpenoids, carboxylic moieties, and simple or complex phenols) present in the extract even if the prenyl moiety of cannabinoids has been highlighted as effective in antimicrobial activity [40–43].

The results of the antimicrobial activity of natural extracts against both Gram+ and Gram− microorganisms of this study are in agreement with the research work of Tajkarimi et al. [44]. Gram+ bacteria tend to be more sensitive to the antimicrobial properties of natural extracts [45, 46], while Gram− are less susceptible to the antibacterial action of natural substances since they possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through their lipopolysaccharide covering. In addition, Gram− microorganisms generally present higher MICs than the Gram+ ones. Numerous researchers investigating the same topic agree that these compounds are, in most cases, slightly more active against Gram+ than Gram− bacteria. However, Wilkinson et al. [47] remarked that some exceptions may occur, since *Aeromonas hydrophila* (Gram−) appears to be one of the most sensitive species to the action of essential oils obtained from different natural essences (thyme, cinnamon, bay, clove, almond, etc.). Moreover, the authors have postulated that individual components of natural extracts exhibit different degrees of activity due to their chemical composition, which can vary according to the geographical origin and harvesting period.

It should also be emphasized that biopharmaceuticals and biopesticides may have different action mechanisms than those of the conventional synthetic products, even if the corresponding compounds are similar. A low or inadequate dosage could cause failure of protection, which could lead to the abandoning of natural products in favour of conventional methods. Therefore, using bioactive substances efficiently requires specific knowledge of the agent and the target disease for optimizing their application time, doses, and rates.

Conclusion

In conclusion, the bioactivity of plant extracts to replace synthetic chemical products should be assessed case by case, and active substances should be tested as individuals to obtain more extended information on the real applicability of plant metabolites against pathogens. Promising results were obtained, and glycoalkaloids antimicrobial activity was confirmed herein, in line with previous reports against insects [6, 11, 13]. But tests performed in vitro are only the first step of a deeper research aimed at extending the use of natural substances to combat plant or animal diseases. Further research efforts are necessary to demonstrate plant secondary metabolite efficiency in the target environments, to better understand their biological activities and to develop actions strategies of such complex mixtures usage.

Abbreviations

CBD: cannabidiol; CBDa: cannabidiolic acid; CBN: cannabinol; CID: collisional induced dissociation; ESI: electrospray ionization; EUCAST: European Committee for Antimicrobial Susceptibility Testing; GLS: glucosinolate; IRMPD: infrared multiple photon dissociation; LC–FTICR/MS: liquid chromatography–Fourier transform ion cyclotron resonance/mass; MIC: minimal inhibitory concentration; PCb: potato and carrot broth; RP-HPLC: reversed-phase liquid chromatography; SRM: selected reaction monitoring; THC: Δ9-Tetrahydrocannabinol; TIC: total ion current; USAE: ultrasound-assisted extraction; ZoI: zone of inhibition.

Authors' contributions

All authors of this research paper have directly participated in the planning, execution, or analysis of this study and read and approved the final manuscript. FL performed analytical MS determinations and wrote the relative

paragraphs in "Methods". MGB carried out microbial assays and wrote the relative paragraphs in "Methods". SM prepared crude extracts and surrogates and wrote the relative paragraphs in "Methods". SDeF selected all plant species tested for the experiments and provided the registration of plants' materials. GS discussed the bioactivity results. LM, SAB, and LS planned this research and discussed a part of the results. LS and SAB revised the whole manuscript. All authors read and approved the final manuscript.

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