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Antibacterial and antioxidant properties of humic substances from composted agricultural biomasses

Mariavittoria Verrillo[1], Melania Salzano[2], Davide Savy[1,2*], Vincenzo Di Meo[2], Massimiliano Valentini[3], Vincenza Cozzolino[1,2] and Alessandro Piccolo[1,2]

Abstract

Background: Bioactive components isolated from composted agricultural biomasses have been receiving progressive attention, because they may improve the antibiotic susceptibility of drug resistant bacterial strains. Here, three different humic substances (HS) were isolated from composted artichoke (HS-CYN) and pepper (HS-PEP) wastes, and from coffee grounds (HS-COF), and characterized by infrared spectrometry, NMR spectroscopy, thermochemolysis–GC/MS, and high-performance size-exclusion chromatography. The antibacterial activity of HS was evaluated against some pathogenic bacterial strains, while their bioactivity was determined by a germination assay on basil (*Red–Violet* variety) seeds.

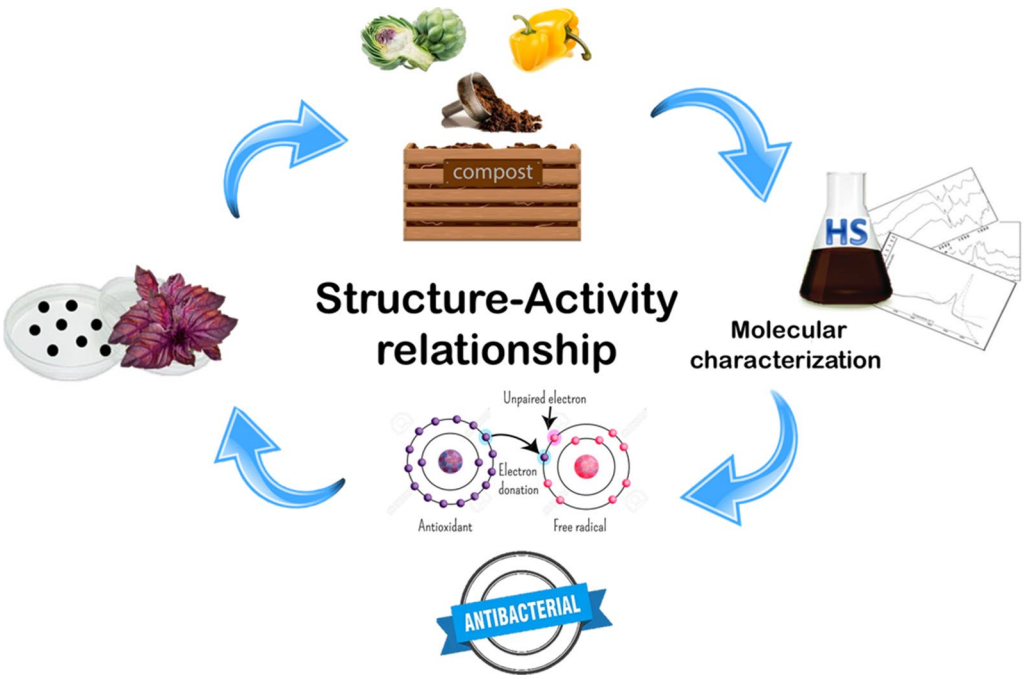
Results: HS-CYN and HS-PEP exhibited the largest antioxidant activity and most significant antimicrobial capacity against some gram-positive bacterial strains, such as *Staphylococcus aureus* and *Enterococcus faecalis*. The same HS determined a significant increase of both root and epicotyls in seed germination experiments. The bioactivity of HS was related not only to their specific molecular composition but also to the conformational stability of their supramolecular structures. Specifically, the greatest bioactive and antimicrobial properties were related to the largest abundance of hydrophobic aromatic and phenolic components and to a more rigid conformational arrangement, that, in turn, appeared to be related to a small fragmentation degree of lignin structures.

Conclusions: Our results showed that extraction of bioactive HS from green composts may be a sustainable and eco-compatible way to valorise agricultural byproducts. HS may be indeed exploited as substrates to produce novel materials not only to improve plant productivity but also for medical applications.

Keywords: Humic substances, Green compost Seed germination, Structure–activity relationship, High-Performance Size-Exclusion Chromatography (HPSEC), ^{13}C-CPMAS NMR, Thermochemolysis, Diffusion disk, Minimal inhibitory concentration, Radical scavenging capacity

*Correspondence: davide.savy@unina.it
[1] Centro Interdipartimentale di Ricerca per la Risonanza Magnetica Nucleare per l'Ambiente, l'Agroalimentare, ed i Nuovi Materiali (CERMANU), Università di Napoli Federico II, Via Università 100, 80055 Portici, Italy
Full list of author information is available at the end of the article

Graphical Abstract



Background

The recent focus on sustainable agriculture tends to support the application of bioactive components derived from recycled biomasses due to their antioxidant and antimicrobial properties [1–3]. Among recycling processes, composting of agri-food or biorefinery wastes can be considered a low-cost and sustainable technology fully integrated into the circular economy concept [4, 5]. In fact, organic isolates from green composts, such as humic substances (HS) and compost tea, may be employed as rhizospheric biostimulants of crops biochemical activities due to their bioactive molecular characteristics [6–9]. Research has been progressively focused not only on the indirect and direct influence of HS on plant growth, but also on their effect on the modulation of the biocontrol of microbial activities [10–12]. Despite their molecular and conformational complexity [13], HS have also been recently applied as starting material in the synthesis of specialized industrial products as medical preparation [14, 15], due to their recognized effective pharmacological properties, such as anti-inflammatory, antioxidant and antiviral activity, as well as immuno-modulatory and anticoagulant properties [16]. However, the literature on the possible antimicrobial activity of HS is limited only to the effect of some oxifulvic acids and compost teas against bacterial strains involved in common human diseases [2, 17].

Concomitantly, there is an increasing attention in developing new potential sources of natural antimicrobials to improve the antibiotic susceptibility of drug resistant bacteria [18]. In fact, many infections are often induced by multi-resistant micro-organisms, resulting in difficult-to-treat diseases, and, consequently, substantial increase in healthcare costs. For example, multiple drug-resistant bacteria such as Methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and Enterobacteriaceae especially *Escherichia coli* and *Klebsiella pneumoniae* are noted as dangerous bacteria of the twenty-first century with an alarming mortality rate of up to 50% leading to an annual death forecast from drug-resistant infections of up to 10 million people by 2050 [19]. The relative accessibility of antimicrobials, as well as the massive use of these compounds for industrial applications, including food production, have both played a major role in the progressive increase of resistant

micro-organisms. Consequently, these multi-resistant micro-organisms represent a progressive global risk to human health [20].

In this context, a search for new compounds with antimicrobial properties in natural materials, such as in complex heterogeneous humic substances isolated from the composting of agricultural biomasses, may become useful to provide new tools against increasingly resistant bacterial pathogens [21]. Hence, the aim of this work was to: (i) molecularly characterize humic substances extracted from different green composts, (ii) investigate the specific antimicrobial capacity of HS against some bacterial strains involved in common human diseases, and (iii) relate their molecular characteristics with their general bioactivity (enhanced seed germination and antioxidant properties).

Methods

Compost and humic substances

Green composts were produced in the composting facility of the Experimental Farm of the University of Naples Federico II at Castel Volturno (CE) as reported in Verrillo et al. [17, 22]. Briefly, horticultural residues, such as of artichoke and pepper wastes, and coffee grounds were mixed with corn straw and wood chips from pruning at 70/30 w/w. The three products were placed, as static poles, on perforated rubber tubes through which the air was blown by a rotary pump to ensure an efficient aerobic transformation of the biomass. The composting processes lasted 100 days, including the thermophilic and mesophilic phases and a final maturing period. The mature composts were air-dried, sieved through a 2 mm sieve, and were subjected to extraction of HS [9, 22]. Briefly, compost samples were suspended in a 0.1 M KOH solution and shaken for 24 h. The suspension was then centrifuged at 7000 rpm for 20 min and the supernatant glass-wool filtered. This extraction was repeated twice by a 1 h agitation, the filtrates were combined and acidified to pH 7.4 with 6 M HCl. The HS extracts were dialysed against deionized water through 1 kD cutoff Spectrapore membranes, until the electrical conductivity was lower than 0.5 dS m^{-1} and freeze-dried for further analysis.

Elemental analysis

Elemental content (C, H, N) of different HS (2 mg) were measured by elemental analyzer EA 1108 Elemental Analyzer (Fisons' Instruments). The resulting ash content of all HS was less than 3%.

Diffuse reflectance infrared Fourier transform spectroscopy (DRIFT)

Infrared spectra were recorder by a Perkin Elmer 1720-X FT-IR spectrometer (Waltham, MA, USA), equipped with a diffuse reflectance accessory, and by accumulating up to 8 scans with a resolution of 4 cm^{-1}. DRIFT analysis was performed for all three green composts and HS. Samples and KBr powder were pulverized and mixed in an agate mortar right before spectra acquisition.

Solid-state ^{13}C NMR spectroscopy (^{13}C-CPMAS–NMR)

Solid-state ^{13}C-CPMAS–NMR spectra of green composts and HS isolated were performed on a Bruker AV-300 equipped with a 4 mm wide-bore MAS probe. Samples were placed in 4 mm zirconium rotors with Kel-F caps and the following acquisition parameters applied: 13,000 Hz of rotor spin rate; 2 s of recycle time; 1H-power for CP 92.16 W: 1H 90° pulse 2.85 μs; ^{13}C power for CP 150, 4 W; 1 ms of contact time; 30 ms of acquisition time; 4000 scans. The cross-polarization pulse sequence was applied with a composite shaped "ramp" pulse on the ^{1}H channel to account for the inhomogeneity of Hartmann–Hann condition at high rotor spin frequency. The Free Induction Decay (FID) was converted by a 4 k zero filling and an exponential filter function with a line broadening of 100 Hz.

Interpretation of ^{13}C-CPMAS–NMR spectra was conducted by dividing the spectral range into the six chemical shift regions, related to the main organic functional groups as follows: 0–45 ppm (aliphatic-C), 45–60 ppm (methoxyl-C and N-alkyl-C), 60–110 ppm (O-alkyl-C), 110–145 ppm (aromatic-C), 145–160 ppm (O-aryl-C), and 160–190 ppm (carboxyl-C) [9, 17, 22]. The contribution of each carbon group was estimated by dividing the area of the corresponding spectral interval (Aiabs) by the total spectral area (A0-190abs): Ai% = (Aiabs/A0-190abs) × 100, i = 0–45, 45–60, 60–110, 110–145, 145–160, 160–190 to evaluate the contribution of specific functional group (MestreNova 6.2.0 software, Mestrelab Research, 2010). The structural composition of HS extracts was also estimated by calculating four dimensionless indexes from the relative areas of NMR spectra [23, 24], as follows:

O-Alkyl ratio A/OA = [(0–45)/(60–110)]; Aromaticity index ARM = [(110–160)/(0–190)]; Hydrophobic index HB/HI = ∑[(0–45) + (110–160)]/∑[(45–60) + (60–110) + (160–190)]; Lignin ratio LigR = [(45–60)/(140–160)].

In particular, the LigR ratio is a useful indicator to discriminate between signals from lignin and other phenolic

compounds (lower LigR), and those including peptidic moieties (larger LigR) in the 45–60 ppm range [5, 8].

Offline pyrolysis TMAH–GC–MS

Thermochemolysis was conducted by placing 500 mg of dried humic extracts in a quartz boat incubated with 1 mL of TMAH (25% in methanol) solution for 2 h under a stream of nitrogen. Then, the quartz boat was introduced into a Pyrex tubular reactor (50 cm × 3.5 cm i.d.) and heated at 400 °C for 30 min in a round furnace (Barnstead Thermolyne 21,100). The products released by thermochemolysis were continuously transferred by a helium flow (20 mL min^{-1}) into a series of two chloroform (50 mL) traps kept in ice/salt baths. The chloroform solutions were concentrated by roto-evaporation and the residues were suspended in 1 mL of chloroform, transferred in a glass vial and analyzed by GC–MS with a Perkin–Elmer Autosystem XL using an RTX-5MS WCOT capillary column (Restek, 30 m × 0.25 mm; film thickness, 0.25 µm). The chromatographic separation was obtained with the following temperature program: 60 °C (1 min isothermal), rate 7° min^{-1} to 320 °C (10 min isothermal) applied helium as carrier gas at 1.90 mL min^{-1}, whereas injector temperature was set at 250 °C and split-injection mode had a 30 mL min^{-1} of split flow. Moreover, mass spectra were obtained in EI mode (70 eV), scanning in the range 45–650 m/z, with a cycle time of 1 s. The identification of different organic compounds was performed comparing all mass spectra with the NIST library database, published spectra, and real standards. In addition, external calibration curves obtained by mixing methyl-esters and/or methyl-ethers of the following standards: heptadecane, octadecanoic acid, cinnamic acid, octadecanol, 16-hydroxy hexadecanoic acid, docosandioic acid, and beta-sitosterol was used for quantitative analysis [8, 9, 17, 22].

High performance size exclusion chromatography

For HPSEC analyses we used a PolySep™ GFC-P3000 300 X 7.80 mm (Phenomenex, USA) preceded by a PolySep GFC-P 35 X 7.80 safety guard (Phenomenex, USA) and a 2 mm inlet filter. The elution flow rate was set to 0.6 mL min^{-1}, and the eluent was 0.05 mol L^{-1} NaCl added with 4.6 mmol L^{-1} NaN_3. Both mobile phase and humic solutions were filtered through 0.45 µm Millipore filter prior to the chromatographic analyses. The various HS were dissolved in the eluent solution at a concentration of 0.6 g L^{-1} and 100 µL of this solution were injected into the SEC system. The same humic mixtures were thereafter added with glacial acetic acid (AcOH) to lower their pH to 3.5, filtered through 0.45 µm Millipore filter and analysed again. Eluted peaks were detected by a UV–Vis detector (Perkin Elmer LC295) set at 280 nm, while column calibration was carried out using sodium polystyrene sulfonates of known molecular masses: 123,000, 16,900 and 6780 Da. Furthermore, ferulic acid (194 Da) and catechol (110 Da) served as low molecular weight standards. The obtained relation between molar mass (MM) and elution volume (EV) was:

log MM = 0.1407 * EV + 6.4077 (R^2 = 0.996). Weight Average (Mw) and Number Average (Mn) molecular weights and polydispersity (P) were calculated as described elsewhere [25]. A Unipoint Gilson Software was used to record and elaborate the chromatograms, while the calculations of Mw and P were performed by the Origin software (v. 9.1, Originlab).

Antioxidant activity and total phenolic content (TPC)

The evaluation of antioxidant activity of three HS from green compost was performed by a spectrophotometric method based on the oxidation of 2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid), diammonium salt (ABTS) by potassium persulfate to form a radical cation ($ABTS^{\bullet+}$) [26]. ABTS was dissolved in distilled water to obtain a 7 mM ABTS stock solution [17, 22, 27].

Then, ABTS• + was generated by reacting ABTS stock solution with 2.45 mM potassium persulfate. The mixture solution stayed in the dark at room temperature for 12–16 h before use. Then, working solution of $ABTS^{\bullet+}$ was prepared by diluting 10 mL of radical cation ($ABTS^{\bullet+}$) solution with 800 mL of water/ethanol (50:50, v/v) mixture with a 0.75–0.80 absorbance at 734 nm. 4 mg of each humic extract were dissolved in 2 mL of water and 100 µl of this solution was added to 1.9 mL of ABTS• + working solution. The mixture was shaken for 2 min at dark to promote the reaction between sample and radical solution and absorbance was measured at 734 nm. The results of antioxidant activity were then expressed as Inhibition percentage (%)0.

Total phenolic content (TPC) of HS was measured by a modified Folin–Ciocalteu colorimetric method [17, 28]. 4 mg of each humic extract were dissolved in 2 mL of methanolic/water solution (50/50). Then, 12.5 µL of each sample was added to 50 µL of Milli-Q water and 12.5 µL of Folin–Ciocalteu reagent was added to the mixture. After 5 min, 125 µL of 7% Na_2CO_3 solution was added to the mixture, samples were incubated for 90 min at 25 °C and the absorbance at 760 nm was measured using Perkin Elmer Lambda 25 UV/Vis Spectrometer. A calibration curve (R^2 = 0.998) was built using increasing amounts of gallic acid (0.1–100 mg L^{-1}) with the same analytical conditions. The results of total phenolic content are expressed as millimoles of gallic acid equivalents (GAE) per mg of dry sample.

Antimicrobial activity

Antibacterial activity assays were carried out by diffusion disk assay (DDK) and broth microdilution method [17, 29, 30]. The bacterial strains used in this study included *Escherichia coli ATCC35218, Staphylococcus aureus ATCC6538P, Pseudomonas aeruginosa ATCC27355, Enterococcus faecalis ATCC29212* and *Klebisella pneumonie ATCC700503*, were kindly provided by Department of Biology of the University of Naples Federico II. Preliminary antimicrobial screening was performed against two Gram positive (*Staphylococcus aureus* ATCC 6538P and *Enterococcus faecalis* ATCC 29212) and two Gram negative microorganisms (*Pseudomonas aeruginosa ATCC27355* and E*scherichia coli* ATCC 35218) applied DDK assay in accordance with the standard method of the National Committee for Clinical Laboratory Standards (NCCLS). This analysis is characterized by different diffusion of each material in agar in presence of a bacterial strain. Actually, when the bacterial cells are sensitive to the substance under study, the bacterial growth is reduced, and an inhibition zone becomes visible as a defined area between the punched-out area and the beginning of the grown bacterium. Conversely, the absence of inhibition zone on agar plate suggests a resistance of bacterial cells to the tested substance [31].

All humic extracts were prepared at a final concentration of 2 mg mL^{-1}. Culture of each bacterial strain was transferred to nutrient agar and incubated at 37 °C for 24 h. The inoculum was standardized by transferring colonies from the nutrient agar to sterile saline solution up to 10^8 CFU mL^{-1} (0.5 McFarland), which is equivalent to 50% transmittance at 580 nm (Coleman model 6120, Maywood, IL). Then, 200 µL of each bacterial suspensions were placed onto the surface of Mueller–Hinton agar, where some disks (6.0 mm diameter) were impregnated with 20 µL of each humic material and incubated at 37 °C for 24 h. In addition, sterile distilled water (20 µL) and ampicillin (30 µg) were used as negative and positive control, respectively. The inhibition zones were measured considering the total diameters and each experiment was performed in triplicate.

The assay for the determination of Minimal Inhibitory Concentration (MIC) was performed by broth microdilution method in accordance with Wiegand et al. [30] with some modifications. The test was carried out in a Mueller–Hinton Broth medium using sterile 96-well polypropylene microtiter plates. Twofold serial dilutions of different HS were carried out in the test wells to obtain concentrations ranging from 10 to 1000 µg mL^{-1}. Then, bacterial cells were inoculated from an overnight culture at a final concentration of ~ 5×10^5 CFU mL^{-1} per well and incubated with a different HS overnight at 37 °C. Finally, the MIC values were estimated measuring the absorbance of microtiter plates at 570 nm. Moreover, the lowest concentration at which no turbidity was observed was considered the MIC value. Three independent experiments were performed for each MIC value. MIC values were measured on *Escherichia coli ATCC35218*, *Staphylococcus aureus ATCC6538P*, *Pseudomonas aeruginosa ATCC27355*, *Enterococcus faecalis ATCC29212* and *Klebisella pneumonie ATCC700503.*

Germination test

The germination assay was conducted in a growth-chamber at 25 °C in the dark by setting relative humidity at 85%. Twenty Basil seeds (*Ocinum basilicum* L, Red–Violet variety) were placed on a filter paper in Petri dishes (9 cm diameter) and moistened with 10 mL of either distilled water (control) or various HS at different concentrations (0, 10, 50, 100 mg L^{-1}). All treatments were carried out in 5 replicates. After 5 days of incubation, germination rate, length of roots and epicotyls were scanned with an Epson Perfection V700 modified flatbed scanner and length measurements were obtained using the WinRhizo Pro software, version 2016 (Regent Instruments, Inc.).

Statistical analysis

The results of the ABTS and Folin–Ciocalteu assays were compared by one-way Analysis of Variance (ANOVA), followed by the Tukey's range test. Furthermore, roots and epicotyl lengths of basil seedlings were compared by performing a one-way ANOVA and the Least Significant Difference (LSD) test. A level of significance of 0.05 was applied for all the tests employed (XLSTAT software, Addinsoft, v. 2014).

Results and discussion

Elemental analysis

The elemental composition of HS is reported in Table 1. A significant variation of carbon content was found for the humic isolates, being largest the value for HS-COF (49.06%), followed in the order by HS-CYN and HS-PEP

Table 1 Elemental composition of humic substances (HS) extracted from artichoke (CYN), coffee grounds (COF), and pepper (PEP) composted residues. Coefficient of variation was always lower than 1%

	C (%)	N (%)	H (%)	C/N	H/C
HS-CYN	41.57	4.27	4.73	11.35	1.36
HS-COF	49.06	5.65	5.07	10.13	1.24
HS-PEP	40.82	4.05	4.38	11.75	1.29

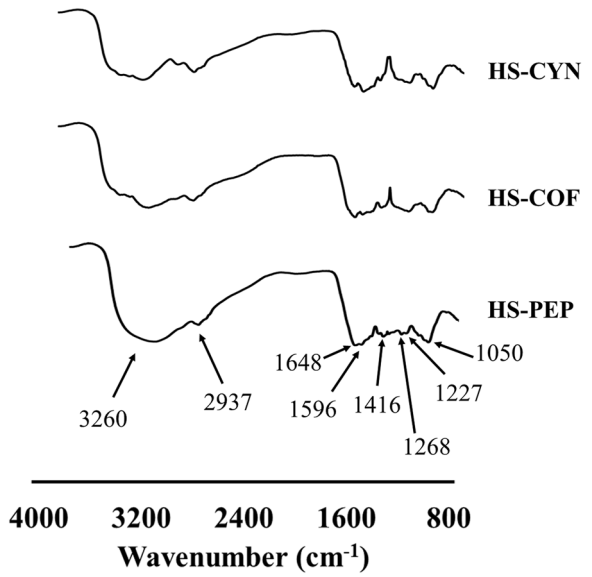


Fig. 1 Diffuse Reflectance Infrared Fourier Transform spectroscopy (DRIFT-IR) spectra of humic substances (HS) extracted from artichoke (CYN), coffee grounds (COF), and pepper (PEP) composted residues

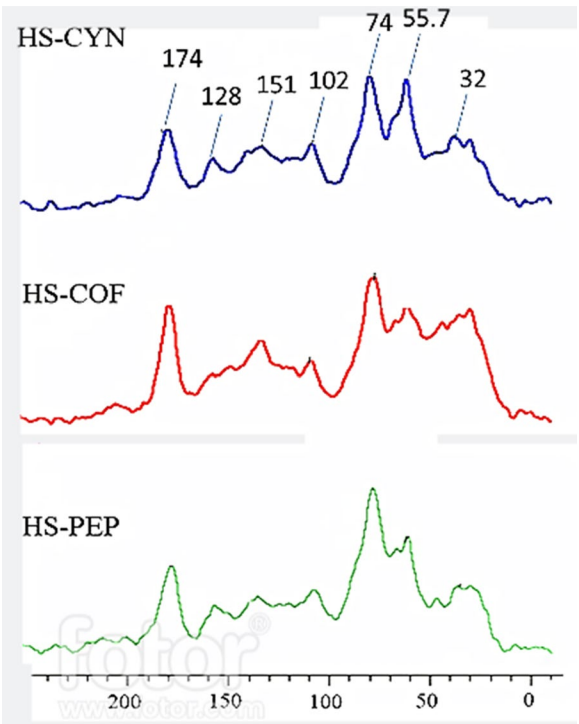


Fig. 2 ^{13}C CPMAS NMR spectra of humic substances (HS) extracted from artichoke (CYN), coffee grounds (COF), and pepper (PEP) composted residues

(41.57 and 40.82, respectively). The greater nitrogen percentage of HS-COF, in comparison to other tested materials, was related to the abundance of N content in the coffee residues employed to the composting process [32]. Furthermore, the analysis of C/N ratio in all samples tested exhibited for HS-PEP a larger value than for other extracts related to a slower microbial degradation in composting process. Conversely, a smaller C/N ratio found in HS-COF may be related to an abundance of low molecular-weight N-rich materials. In addition, a small H/C value obtained for HS-COF and HS-PEP samples, suggest a predominance of aromatic compounds over aliphatic components [8, 33].

Infrared spectroscopy

DRIFT spectra of HS samples showed a similar distribution of main functional groups (Fig. 1). The broad absorption band around 3000–3500 cm^{-1} is attributed to the OH stretching vibrations in alcohols, phenolic and carboxylic acids, while the bands at 2937 cm^{-1} are referred to symmetric and asymmetric C–H stretching of methyl and methylene groups in aliphatic chains.

In the central region, the signals around 1900 cm^{-1} are related to −C = O deformation of acidic group [34], while the bands around 1620 cm^{-1} and 1590 cm^{-1} may be related to either the amide I and amide II bonds of peptides [8], as well as to ring vibrations of aromatic moieties. The peak at 1514 cm^{-1} is assigned to the ring stretching vibrations in aromatic moieties. The bending of C–H and C–O bonds at 1457 cm^{-1} indicates the presence of alkyl chains and carboxylates functions in aliphatic acids [8, 17]. In addition, the signals showed in the region between 1419 and 1220 cm^{-1} is assigned to vibration of carboxylic acid groups. Particularly, the broad around 1419 cm^{-1} was connected to $–COO^-$ stretchings, whereas the signal around 1220 cm^{-1} was associated to –CH stretch and –OH bending of COOH [35]. Finally, the peak around 1220 cm^{-1} was related to the vibrations of alcoholic moieties [34].

^{13}C-CPMAS–NMR spectroscopy

The ^{13}C-CPMAS–NMR spectra of HS from green composts (Fig. 2) showed an abundance of O-alkyl carbons (60–110 ppm) in mono-, oligo-, and polysaccharides [36], whose signals were more intense for HS-PEP followed by HS-CYN and HS-COF, accounting for 39.75, 29.78 and 24.69%, respectively (Table 2). In particular, the intense signal at 72 ppm is attributed to the overlapping of C-2, C-3, and C-5 carbons in the pyranosidic structures of cellulose and several hemicelluloses, whereas the 104 ppm signal is commonly attributed to the anomeric carbons. The similarly large NMR resonances between 0 and 45 ppm are assigned to methylene and methyl groups in alkyl chains deriving from

Table 2 Percent of relative abundance of chemical shift intervals (ppm) of ^{13}C-CPMAS–NMR spectra of humic substances (HS) extracted from artichoke (CYN), coffee grounds (COF), and pepper (PEP) composted residues

	190–160	160–145	145–110	110–60	60–45	45–0	HB/HI[a]	LigR[b]	A/AO[c]	ARM[d]
HS-CYN	11.54	5.51	16.26	29.78	15.98	20.93	1.4	2.9	0.7	0.3
HS-COF	12.85	3.42	15.33	24.69	13.07	30.63	1.7	3.8	1.2	0.2
HS-PEP	11.08	4.49	14.40	35.75	14.22	20.06	1.1	3.2	0.6	0.2

[a] Hydrophobic index (HB/BI): [(0–45) + (45–60) + (110–145) + (145–160)]/[(60–110) + (160–190)]

[b] Alkyl ratio (A/AO): [(0–45)/(60–110)]

[c] Lignin ratio (LigR): [(45–60) 145–160)]

[d] Aromatic index (ARM) = [(110–160)/(0–190)]

lipids, such as plant waxes and polyesters [9]. The broad signal in the 45–60 ppm region is assigned to methoxyl carbons, in both guaiacyl and syringyl units of lignin fragments, and Cα in oligo- and polypeptides [37]. Furthermore, the 110–140 ppm range refers to unsubstituted and C-substituted phenyl carbons, while the 140–160 ppm interval shows the O-bearing C in hydroxyl- and methoxy-groups of aromatic rings in polyphenol compounds and lignin fragments, whose carbons in 3 and 5 positions are linked to methoxyl carbons [9, 37]. The intense signal centred at 174 ppm in all spectra (Fig. 2) corresponds to carbonyl carbons of aliphatic acids, amide functional groups and carboxylate derivatives of pectin components [5]. The NMR results were agreed with those from IR spectroscopy, suggesting a large heterogeneity of compost-extracted HS. However, NMR spectroscopy proved to be more powerful tool, enabling to dig into specific organic groups present in the humic extracts.

The molecular differences among HS and their biochemical stability may be inferred by the dimensionless structural parameters calculated from the spectral areas, such as aromaticity (ARM), and hydrophobic (HB/HI), alkyl (A/OA) and lignin (LigR) ratios (Table 2). This indexes may be used as indicators to evaluate the biochemical stability of organic matrices [17, 22, 38, 39]. The hydrophobicity was largest for HS-COF, followed in the order by HS-CYN and HS-PEP (Table 2). HS-COF also showed the greatest A/OA value with a prevalence of Alkyl-C in respect to O-alkyl derivatives, thus revealing a more abundant contribution of apolar alkyl functionalities than the other two HS (Table 2). The values of LigR in all extracts (Table 2) highlighted a close correlation of spectral intensities associated to methoxyl groups (45–60 ppm) and O-aryl-C molecules (140–160 ppm). The greatest value for HS-COF suggested a preferential accumulation of lignin-derived phenolic during composting of coffee grounds residue that is reflected in the corresponding HS isolates [9]. The aromaticity index exhibited greater values for HS-CYN than for other humic samples suggesting an abundance of lignin and aromatic compounds (Table 2).

Table 3 Relative yield (%) of main thermochemolysis products released from humic substances (HS) extracted from artichoke (CYN), coffee grounds (COF), and pepper (PEP) composted residues. The coefficients of variation were invariably smaller than 5%

Thermochemolysis products	HS-CYN	HS-COF	HS-PEP
Lignin	65.1	55.7	68
Lig G6/G4	5.8	6.7	13
Lig S6/S4	8.9	21.2	16.6
Aromatic (*non*-lignin)	4.6	9.1	5.9
N derivatives	12.7	20.2	13.9
FAME	10.1	10.3	9.2
Carbohydrates	3	–	2.2
Sterols	1.9	2	0.3
Alcohols	0.4	2	0.3
Alkanes	0.1	–	0.2

Offline TMAH–Pyr–GC–MS

The main thermochemolysis products of HS samples were methyl ethers and esters of alkyl and aryl compounds of plant and microbial origin (Table 3, Additional file 1: Fig. SF1), as well as compounds related to lignin (Lig), methyl esters of linear fatty acids (FAME), nitrogen-containing (N), alicyclic compounds (e.g., sterols) and derivates of polysaccharides. Lignin monomers were identified by the following symbols: P = *p*-hydroxyphenyl, G = guaiacol (3-methoxy, 4-hydroxyphenyl), and S = syringyl (3,5-dimethoxy, 4-hydroxyphenyl) [40].

The largest percentage of lignin compounds was found for HS-PEP (68% of the Total Ion Chromatogram—TIC) followed by HS-CYN and HS-COF (65.1 and 55.7% of the TIC, respectively). The most abundant lignin derived molecules were oxidized products of both di- and trimethoxyphenylpropane, such as benzaldehyde (G4 and

S4), acetophenone (G5 and S5), and benzoic acid (G6 and S6) (Additional file 1: Table S1, Fig. S1). Other identified lignin thermochemolysis products were *cis* and *trans* isomers of 1-(3,4-dimethoxyphenyl)-2-methoxyethylene (G7 and G8) and 1-(3,4, 5-trimethoxyphenyl)-2-methoxyethylene (S7 and S8), as well as the enantiomers of 1-(3,4-dimethoxyphenyl)-1,2,3-trimethoxypropane (G14 and G15) and the 1-(3,4,5-trimethoxyphenyl)-1,2,3-trimethoxypropane (S14 and S15). The 3,4-dimethoxyphenyl-2-propenoic acid (G18) compounds originated from either lignin guaiacyl units or suberin aromatic domains. In addition, other aromatic compounds were identified, such as phenols, methyl-phenols, and alkyl-benzenes derivatives, which may have multiple origins (polysaccharides, proteins, lignin) or may derive from secondary reactions during the thermochemolysis.

Specific parameters may be obtained from some lignin-derived compounds, which provide helpful indications on lignin decomposition [40]. Specific indexes are calculated by dividing the area of 3,4-dimethoxylbenzoic acid and 3,4,5-trimethoxylbenzoic acids (G6 and S6, Additional file 1: Table S1) over the corresponding aldehydic forms (G4 and S4, Additional file 1: Table S1). The larger the values of these two parameters, the more advanced is the lignin decomposition [41]. HS-PEP had the greatest G6/G4 ration, whereas similar were the values for HS-COF and HS-CYN. Conversely, the largest S6/S4 ratio was shown by HS-COF, followed by HS-PEP and HS-CYN (Table 3). For both these rations the smallest values were observed for HS-CYN, thus suggesting the occurrence of a larger fraction of unaltered lignin, whereas the other humic samples contained a more fragmented lignin (Table 3).

The HS extracts also showed differences in their relative content of the N-containing compounds (Table 3). The largest amount of nitrogenated molecules was found for HS-COF (20% of the TIC), followed by HS-PEP (14% of the TIC) and HS-CYN (13% of the TIC), and were identified as residues of amino acids, and peptides [5, 22]. Moreover, the humic samples had similar content of methyl esters of fatty acids (FAME) with the presence of pentadecanoic (C15), hexadecanoic (C16) and octadecanoic (C18) acids, deriving from plants and microbe cell walls (Table S1) [42]. A relatively smaller amount of carbohydrates was found in all pyrograms (Table 3), this result being well in line with previous literature [8, 9]. Such an outcome is related to the low efficiency of thermochemolysis in detecting polyhydroxy molecules in complex matrices [5].

High performance size exclusion chromatography

The HPSEC profiles of HS are shown in Fig. 3, while the resulting Weight Averaged (Mw) and Number Averaged (Mn) molecular weights, as well as the polydispersity (P), are reported in Table 4. The exclusion chromatograms were composed of two peaks with elution volumes of 6.0 ad 7.2 mL, respectively, and suggested that HS had similar hydrodynamic *radius*, this hypothesis being confirmed by the comparable Mw, as calculated over the entire chromatogram. Furthermore, the polydispersity (≤ 2.0) pointed out the relatively monodisperse size distribution of the humic materials (Table 4). The conformational stability of our materials was assessed by adding the humic solutions with acetic acid (AcOH) to lower the pH from 7 to 3.5 and injecting them again into the SEC system [27, 43]. AcOH is indeed able to disrupt the metastable arrangement of humic supramolecular structures stabilized at pH 7 mainly by weak hydrophobic interactions (van der Waals, π–π, CH–π), while it cannot affect that of covalently linked polymers [44]. The protonation of carboxyl functional groups in humic molecules

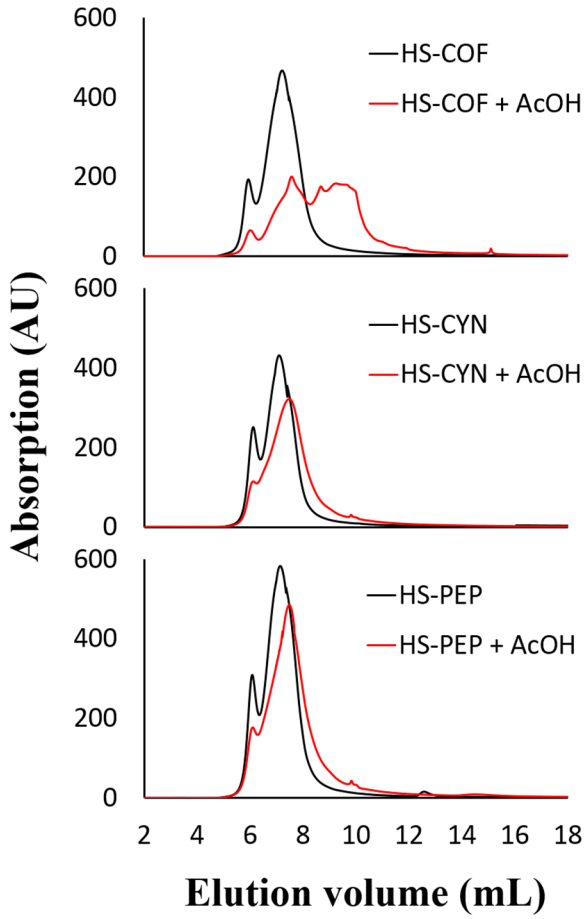


Fig. 3 HPSEC chromatograms of humic substances (HS) extracted from artichoke (CYN), coffee grounds (COF), and pepper (PEP) composted residues, before and after addition of acetic acid (AcOH) to adjust sample pH from 7 to 3.5

Table 4 Weight Average (Mw) and Number Average (Mn) molecular weight, and Polydispersity (P), as calculated from UV-detected HPSEC chromatograms for humic substances (HS) extracted from artichoke (CYN), coffee grounds (COF), and pepper (PEP) composted residues, before and after addition of acetic acid (AcOH). The coefficients of variation were invariably smaller than 5%

	Peak interval (mL)	Mw	Mn	*P*
HS-CYN	4.5–14.21	50,373.8	34,510.4	1.5
HS-CYN + AcOH	4.9–16.1	40,690.5	16,999.7	2.4
HS-COF	4.68–16.2	47,454.5	23,253.8	2.0
HS-COF + AcOH	4.68–16.2	26,333.1	9344.3	2.8
HS-PEP	4.6–14.8	49,024.9	29,794.5	1.6
HS-PEP + AcOH	4.7–17.0	40,910.4	13,304.4	3.1

with pH lowering, drives the formation of new hydrogen bonds among the complementary oxygen-containing molecules, thereby forcing the humic supramolecular associations established at pH 7 into smaller but thermodynamically more stable conformations [45]. Contrary to the invariance of the chromatographic behaviour of real polymers, the pH change from neutral to acidic of the humic supramolecular structures upon AcOH addition results is an increase in the molecular sizes distribution over the HPSEC chromatogram and a reduction of the absorbance of the new peaks due to the phenomenon of hypochromism [13, 43, 46].

As compared to the chromatograms obtained at pH 7, the lowering of solution pH of the HS studied here altered the elution profiles and showed both a hypochromic effect and a peak shift to larger elution volumes (Fig. 3). However, the effect of the AcOH treatment depended on the HS composition. In fact, the pH change determined a larger alteration of the elution profile for HS-COF than for the other two HS, that was reflected by a 44.5% drop of Mw for the former, in respect to a Mw decrease of 19.2 and 16.6% observed for HS-CYN and HS-PEP, respectively (Fig. 3; Table 4). These differences revealed that HS-COF had a conformational structure stabilized by only small molecular mass molecules and easily disrupted by the pH change, while the more stable conformations of two other HS were presumably due to the presence of less fragmented molecular structures. The low molecular mass compounds in HS-COF may derive from the fragmentation of lignin structures into single or oligomeric phenols, as suggested by the thermochemolysis results that indicated a more extensive lignin fragmentation for HS-COF than for HS-CYN and HS-PEP (Table 3). Possible reasons for the different lignin degradation among the HS samples may reside in either a more efficiently degrading microbial consortium developed in the composting of coffee grounds or in a more easily decomposable lignin originally present in the coffee residues. In this respect, it is to be noted that humic acids isolated from an artichoke-derived compost was found to display a HPSEC behaviour similar to that reported here, again possibly due to large fragments of plant biopolymers that survived the microbial decomposition during the composting process [47].

Antioxidant activity

Modern drug development frequently exploits natural compounds as therapeutic agents for disease prevention [48]. Natural substances exhibit different chemical structures with a large applicability, for example, as antioxidant agents in different metabolic processes [49]. In particular, antioxidant medications provide a significant protection against various diseases that are related to oxidative stress generally induced by free radicals, such as reactive nitrogen species (RNS) and reactive oxygen species (ROS) [50]. Moreover, the antioxidant and scavenger activities of phenolic/quinonic components in natural extracts with their electron donors/acceptors behaviour, have been extensively studied [51]. Humic substances can also be used as natural antioxidants to neutralize free radicals due to their heterogeneous composition and supramolecular structure, thus offering different antioxidant sites capable of environmental or medical applications [52].

Here we found that the ABTS assay showed larger values of percent inhibition for HS-CYN (68%) than for HS-PEP and HS-COF (43 and 39%). In addition, similar results were obtained expressing the antioxidant ABTS results as TEAC (mmol of Trolox equivalent·kg^{-1} of sample) with values equal to 383.8 for HS-CYN followed, in the order, by HS-PEP and HS-COF (290 and 227.5, respectively) to compare the antioxidant capacities of humic extracts with a standard antioxidant compound. In line with previous studies [17, 22], the antioxidant activity of HS extracts from green composts showed a direct correlation with their total phenolic content (TPC) obtained by the Folin–Ciocalteu assay (Fig. 4), as well as with their aromatic composition.

The similar trend observed in the case of ABTS assay and total phenolic content determined by the Folin–Ciocalteu method could be explained considering the prominent role of phenolic moieties for the determination of antioxidant capacities of humic or humic-like materials [17, 22, 27]. Although the Folin–Ciocalteu reagent is not considered to be specific for polyphenols [53], our findings are in line with previous studies [17, 22, 27, 54], suggesting a strong correlation between the total phenolic content of HS samples and their antioxidant capacities to development of novel and sustainable antioxidant systems [55]. In this work, the antioxidant activities of

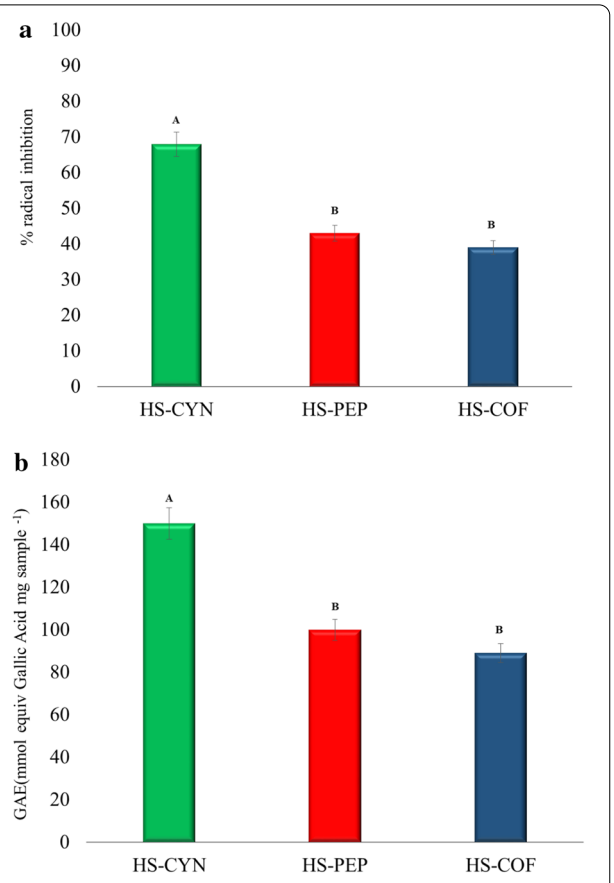


Fig. 4 Antioxidant activity of humic substances (HS) extracted from artichoke (CYN), coffee grounds (COF), and pepper (PEP) composted residues, as measured by ABTS assay (**a**), and Folin–Ciocalteu method (**b**). Vertical bars represent the standard deviation. Different capital letters indicate significant differences according to Tukey test ($p \leq 0.05$)

HS-CYN and HS-PEP were well related with their content in lignin fragments, as shown by both NMR and thermochemolysis results (Tables 2 and 3), being in line with previous works that described the great antioxidant power of mono- and oligo-hydroxylated benzene units [56, 57]. Furthermore, the significant antioxidant activity of HS-CYN may be also related to its conformational rigidity as assessed by HPSEC (Fig. 3, Table 4), that was related to a greater preservation of lignin-derived polyphenols, which are commonly held responsible for radical scavenger activity [58]. This combination beetween a rigid conformational structure and a small degree of lignin fragmentation in HS-CYN (Tables 3 and 4, Fig. 4) may also reflect a recalcitrance of this sample to microbial degradation and a longer persistence of its antioxidant capacity.

Table 5 Inhibition zones (mm) of humic substances (HS) extracted from artichoke (CYN), coffee grounds (COF), and pepper (PEP) composted residues, against *S. aureus*, *E. faecalis*, *E. coli* and *P. aeruginosa* by disk diffusion method (DDK). The coefficients of variation were invariably smaller than 6%

Bacterial strain	Reference BSA	Reference AMP	HS-CYN	HS-COF	HS-PEP
S. aureus	n.i	8.5	10.1 ± 0.3	7.3 ± 0.3	9.4 ± 0.2
E. faecalis	n.i	7.2	8.7 ± 0.1	5.2 ± 0.3	7.4 ± 0.04
E. coli	n.i	6.8	7.4 ± 0.2	6.8 ± 0.2	5.2 ± 0.1
P. aeruginosa	n.i	5.1	5.4 ± 0.1	6.6 ± 0.02	5.1 ± 0.3

n.i. no inhibition, overgrowth of bacterial cells on the platelets

BSA albumin serum bovine

AMP ampicilline

Antimicrobial activity

Bacterial infections pose a direct threat to human health due to the progressive resistance to drugs and insurgence of novel pathogenic strains [59]. Natural products represent a specific source of bioactive metabolites, which can be exploited for their therapeutic effects [60, 61]. In this context, the investigation of antimicrobial properties of compost derivatives can be applied in different fields, such as agriculture or medicine as a sustainable alternative to the use of industrial synthetic products for the control of human or plants diseases [62–64]. In the case of green compost, only few reports describe the antimicrobial effect of their humic extracts, such as fulvic acids or compost tea, against bacterial strains involved in common human diseases [2, 17].

In this work, we attempted to fill this gap by evaluating the antibacterial activity of different HS isolated from green composts through the application of the diffusion disk (DDK) method [65]. In the preliminary screening, HS were tested against some Gram-positive (*S. aureus* and *E. faecalis*) and Gram-negative bacterial strains (*P. aeruginosa* and *K. pneuomoniae*) (Table 5), while albumin serum bovine–BSA was used as reference negative control. An improved antimicrobial activity was found against Gram-positive bacterial strains by all HS samples. The best performance against *S. aureus* was observed for HS-CYN and HS-PEP followed by HS-COF, with inhibition zones equal to 10.1, 9.4 and 7.3 mm, respectively (Table 5).

A similar trend was observed for the minimum inhibitory concentration assay (MIC), that revealed that all HS were more effective than control against *Staphylococcus aureus* and *Enterococcus faecalis.* Conversely, a lesser susceptibility was found for gram negative bacterial strains,

Table 6 Antibacterial activity against Gram-positive and Gram-negative bacterial strains of humic substances (HS) extracted from artichoke (CYN), coffee grounds (COF), and pepper (PEP) composted residues, as determined by MIC (Minimal Inhibitory Concentration)

MIC ($\mu g \cdot mL^{-1}$)									
	HS-CYN			HS-COF			HS-PEP		
Bacterial strain	Replicates[a]			Replicates[a]			Replicates[a]		
Staphylococcus aureus	1.2	1.5	1.2	10.5	10	10	1.9	1.9	2
Enterococcus faecalis	2	2	2	14	14.5	14.5	2.3	2.6	2.5
Escherichia coli	1.7	1.7	1.6	2.5	2.5	2	1.5	1.5	1
Pseudomonas aeruginosa	1.8	1.8	2	1.9	1.8	1.9	2.5	2.5	3
Klebsiella pneumoniae	2	2.3	2.5	8.5	8.5	8	2.5	2.5	2.5

[a] Replicates were performed by three independent experiments

such as *Escherichia coli* and *Klebsiella pneumoniae*, which generally showed larger MIC values (Table 6). In particular, HS-CYN had the best antibacterial performance, with a small MIC value against *Staphylococcus aureus* and *Enterococcus faecalis* (1 and 1.50 μg mL^{-1}, respectively) followed by *Escherichia Coli* (MIC value of 1.75 μg mL^{-1}), and *Klebsiella pneumoniae* (2.0, 2.3 and 2.5 μg mL^{-1}).

While previous studies pointed out that humic matter isolated from compost owns antimicrobial activity [3, 17, 18, 22, 66], here we find that HS isolated from composted agricultural biomasses not only reduce the growth of microbial pathogens causing common human diseases, but that such bioactivity is also related to the HS molecular composition. The humic extracts employed here have revealed an effective antimicrobial activity which may be associated to the abundance of aromatic and phenolic fractions, as found in HS-CYN and HS-PEP. In fact, the observed order of HS antimicrobial activity: HS-CYN > HS-PEP > HS-COF well correlates with the content in oxidized lignin, as indicated by both NMR spectra (Table 2) and thermochemolysis analysis (Table 3). A similar relation between the phenolic content and antimicrobial activities against human pathogens was previously reported [67].

However, the mechanism of antibacterial activity exerted by phenolic compounds is not yet fully understood. It is hypothesized that phenolics interaction with active sites of different enzymes may induce irreversible changes in cell membrane permeability or wall integrity with a consequential bacterial cell death [68]. In this study, the HPSEC chromatographic behaviour shown by HS-CYN may be related to its greater antibacterial efficacy. In fact, the observed conformational stability of HS-CYN, presumably provided by the presence of unfragmented polyphenolic structures, may induce a modification in the permeability of cell bacterial membranes, thereby reducing the growth of pathogenic microbial strains [69]. While no univocal structure–activity relationship has been yet produced to explain the molecular bases of HS bioactivity against human bacterial diseases, a working hypothesis for such effects may refer to the combination of conformational stability and molecular composition that humic matter can concomitantly exert on different cell types [10]. Furthermore, another controversial issue resides in the different susceptibility of Gram-positive and Gram-negative bacteria to polyphenols. Several works suggested that the antibacterial activity of polyphenols is generally more effective against Gram-positive than Gram-negative bacteria, due to cell walls linked to a molecularly complex outer membrane, that slows down the passage of chemicals [70]. This seems to be in line with our findings that showed the largest antibacterial performance of HS-CYN, owing to its great abundance of preserved unfragmented polyphenols (Fig. 3, Tables 3 and 4). However, the outer membrane of Gram-negative bacterial strains was also found to be altered by some specific phenolic compounds [71], as it is in fact observed in the case of HS-COF. This behaviour may be in turn explained by invoking the metastable conformation shown by HS-COF in the HPSEC profile (Fig. 3), that may easily be disrupted when in interaction with gram negative bacterial strains and induced to release small bioactive molecules with antibacterial activity (Tables 5 and 6). Again, this phenomenon suggests a close correlation between molecular composition and antibacterial activity of HS.

Germination test

Different studies have suggested the relationship between the biostimulation activity of humic materials on plant growth and their molecular structure [9, 72]. In this work, the biological effect of different HS from green composts at increasing concentrations was evaluated by

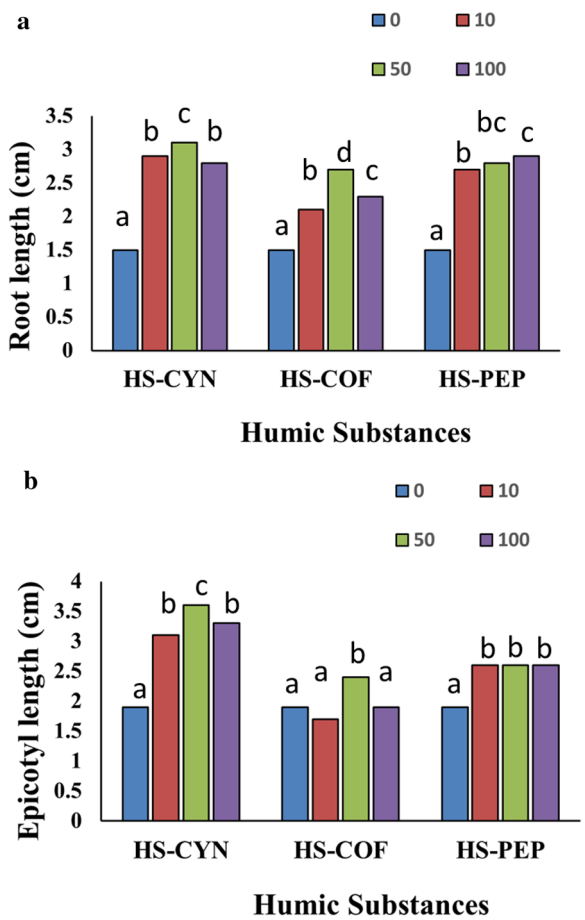


Fig. 5 Length of root **A** and epicotyl (**B** of basil seedlings treated with increasing doses of aqueous solutions of humic substances (HS) extracted from artichoke (CYN), coffee grounds (COF), and pepper (PEP) composted residues. Different letters indicate significant differences from the control at 0.05 probability level, as revealed by the LSD test

a germination test on basil seeds (Fig. 5). The application of both HS-CYN and HS-COF progressively increased root length until the 50 mg L^{-1} concentration, while at larger concentration a reduction in root elongation greater than for control was observed. HS-PEP also promoted root development in a dose-dependent manner, but the greatest root elongation was observed for seedlings treated with 100 mg L^{-1} (Fig. 5). A hormetic effect was noticed for HS-CYN regarding the epicotyl development, with the applied intermediate concentration being the most bioactive. Conversely, the addition of HS-COF significantly enhanced epicotyl elongation only at 50 mg L^{-1}, whereas the other doses were ineffective. Finally, also HS-PEP stimulated the epicotyl growth in a significant manner, although without any difference among the applied concentrations (Fig. 5).

Our results indicated HS-CYN as the most positively bioactive substrate towards plant development, followed by HS-PEP and HS-COF. Moreover, the application of HS-CYN and HS-PEP resulted in a similar root development (Fig. 5), thus suggesting a common molecular-based mechanism to be responsible for the observed comparable bioactivity towards plant growth. Indeed, the two HS materials were found by solid-state NMR spectroscopy to possess a similar relative amount of alkyl and both lignin-derived and *non*-lignin aromatic components, as well as comparable values for Alk/O-Alk and LigR ratios and similar conformational distribution (Tables 2, 3, 4, Fig. 3). Furthermore, the differences in the epicotyl development shown by these two materials may be also accounted to some chemical dissimilarities, such as a significantly smaller lignin fragmentation degree and relatively larger amount of sterols found for HS-CYN than for HS-PEP (Table 3). On the other hand, the smaller bioactivity of HS-COF shown in the germination test may be attributed to its specific structural features, such as the largest relative content of alkyl and nitrogenous components, the most advanced fragmentation of lignin, and the most unstable conformational structure (Tables 2, 3, 4).

Our results generally highlighted the positive role of aromatic and phenolic moieties in stimulating plant growth, and such outcome is well in line with previous literature. In fact, Aguiar et al. [38] found that the content of aromatic compounds was positively related to the emergence and physiology of maize (*Zea mays* L.) roots. Moreover, alkyl compounds have been recently found to depress the bioactivity of humic-like materials towards maize root development and to moderately stimulate that of maize coleoptile [41]. Overall, our data indicated that HS with a small degree of lignin fragmentation, poor amount of alkyl molecules and rigid conformational behaviour showed a larger promoting effect during the early growth of basil plantlets. Interestingly, the biological effect of polyphenols might be associated to their antioxidant properties. Indeed, HS-CYN showed the largest relative content of unfragmented lignin, and both the largest plant bioactivity and radical scavenging properties, while HS-COF had the least (though still positive) biological and antioxidant activities, and HS-PEP revealed an intermediate behaviour (Figs. 4 and 5). The antioxidant capacity of the contained polyphenols has been claimed to be responsible, at least partially, for the HS bioactivity since the complex polyphenolic systems of humic materials may interact with plant redox components. Previous works have shown that the biostimulation of HS is related to their capacity in influencing the redox system of oat (*Avena sativa* L.) roots [73, 74]. Furthermore, the HS ability to modulate the ROS signalling

pathways of plants has been recently suggested as one mechanism underlying their biological effect [75]. The relevant antiradical capacity of polyphenolic-rich humic bioactive materials was also accounted to significantly stimulate root development in maize plants [27, 76, 77].

Conclusions

In this study, we showed that residues from agricultural biomasses may be composted to provide humic extracts with relevant biological activities towards plant development and against microbial pathogens responsible of human diseases. We found that the observed HS bioactivity depended on the content of specific molecular components, such as aromatic compounds and polyphenolic lignin fragments. Furthermore, the observed biological effects were related to HS size and conformational stability in water, as assessed by HPSEC.

Overall, our work suggests that agricultural residues can become a source of bioactive humic compounds, with high antibacterial and plant growth promoting role. Such products may, therefore, be used in the production of novel materials to be employed as antimicrobial agents. However, their properties may also exert a role in the fields of material chemistry or environmental sciences.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-022-00291-6.

Additional file 1. Table S1. List of the main products released by the thermochemolysis from humic substances from artichoke (HS-CYN), coffee grounds (HS-COF) and pepper (HS-PEP). **Fig. S1.** Total ion chromatograms of thermochemolysis products of humic substances from artichoke (HS-CYN), coffee grounds (HS-COF) and pepper (HS-PEP).

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Not applicable.

Authors' contributions
MV carried out part of the experiments and wrote the manuscript. MS characterised the materials and drafted the manuscript. DS carried out part of the experiments and wrote and revised the manuscript. VDM provided the compost and contributed to HS extraction. VC conceptualized the work and supervised the biological experiments. MV carried out part of the experiments and wrote and revised the manuscript. AP acquired funding and supervised and revised the work. All authors read and approved the final manuscript.

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Availability of data and materials
The data sets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Centro Interdipartimentale di Ricerca per la Risonanza Magnetica Nucleare per l'Ambiente, l'Agroalimentare, ed i Nuovi Materiali (CERMANU), Università di Napoli Federico II, Via Università 100, 80055 Portici, Italy. [2]Present Address: Dipartimento di Agraria, Università di Napoli Federico II, Via Università 100, 80055 Portici, Italy. [3]Department of Chemistry, Sultan Qaboos University, P.O. Box 123, Muscat, Oman.

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