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Alkaline Extraction in Air Enhances Antioxidant and Biological Activities of Humic Acids

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Abstract: Humic acids (HAs) possess diverse functionalities, endowing them with multiple applications as bioactive compounds in agriculture. Alkaline extraction is key to obtaining HAs from their source material. The presence of oxygen during extraction can lead to oxidative changes in the humic structure. The extent of HA transformation depending on their origin remains poorly understood, and the effect of alkaline extraction on the HA biological activities is yet to be estimated. Here, we compare the physicochemical properties of HAs extracted from fresh organic material, compost, in air (HA-O_2) and under nitrogen (HA-N_2). We also assess the antioxidant properties of HAs-O_2 and HAs-N_2 from compost (HAC), Retisol (HAR), and Chernozem (HACh) and relate them to the HA biological activities. Changes in the HAC properties were analyzed using the following techniques: elemental composition, ultraviolet–visible and infrared spectroscopy, ^{13}C nuclear magnetic resonance (^{13}C-NMR), electron paramagnetic resonance (EPR), gel filtration using Sephadex G-75 gel, and potentiometric titration. The HA antioxidant properties were explored using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) assay (antiradical activity) and phosphomolybdenum assay (total antioxidant capacity). The HA biological activity was estimated by priming radish and wheat seeds (0.5 g L^{-1} HAs, 25 °C, 5 h for radish and 14 h for wheat), followed by germination tests. Alkaline extraction of HAC in air vs. nitrogen resulted in a 1.2-fold increase in the O/C ratio and optical density at E_{465}, oxidation of aliphatic fragments, a 2-fold increase in the contents of functional groups, and a 1.2-fold increase in the number of paramagnetic centers. All HA-O_2 preparations have demonstrated an enhanced antiradical activity (1.3–1.6 times) and total antioxidant capacity (1.1–1.3 times) compared to HA-N_2. The Vigor Index of seeds primed with HA-O_2 was 1.1-to-1.8-fold higher than those treated with HA-N_2, depending on the HA origin. We demonstrate that alkaline treatment in air benefits the antiradical and biological activities of HAs, making such preparations more attractive for use as natural antioxidants and priming agents. This opens up new perspectives for using O_2-modified HAs as innovative plant stimulants in agriculture.

Keywords: soil humic acids; compost humic acids; antiradical activity; radish; wheat; seed priming

Academic Editor: Maria Roulia

Received: 8 February 2025
Revised: 6 March 2025
Accepted: 11 March 2025
Published: 13 March 2025

Citation: Zavarzina, A.; Davydova, I.; Kulikova, N.; Nikolaeva, A.; Philippova, O. Alkaline Extraction in Air Enhances Antioxidant and Biological Activities of Humic Acids. *Agronomy* **2025**, *15*, 689. https://doi.org/10.3390/agronomy15030689

1. Introduction

Humic acids (HAs) are polydisperse and heterogeneous products of the decay and transformation of plant and microbial remains, comprising a large proportion (20–50%) of organic matter in soils, peats, and brown coals [1–3]. By their composition and molecular

structure, HAs represent a highly complex system of compounds with amphiphilic properties and varying molecular weights (2 to >100 kDa), containing aromatic rings; aliphatic moieties; and functional groups, such as carboxylic-, phenolic-, and nitrogen-containing, among others [2]. These diverse functionalities determine the HA high reactivity with metal ions and organic compounds [4–6], endowing them with multiple applications in agroecosystems, such as fertilizers [7] and bioactive compounds [8–12]. Parent materials for HAs in agriculture include mainly coal, peat, and compost [9,10]. The manner of application of HAs varies greatly depending on their specific features (i.e., micronutrients added) and crop requirements, including introduction into the soil, foliar, and seed treatment [7,9].

Traditionally, HAs are obtained from parent material with 0.1–0.5 M NaOH, followed by acid precipitation at pH < 2. However, alkaline extraction remains one of the most debatable aspects in humic research [6,13,14]. Phenolic constituents of natural organic matter (NOM), especially *ortho*- and *para*-phenols, are easily oxidized in an alkaline medium due to the ionized state of phenolic groups [11,15]. Hydroxy-radicals and quinones produced may undergo spontaneous coupling to form dark-colored polycondensates [16,17]. In addition, quinones are capable of generating reactive oxygen species (ROS) [18], which may cause depolymerization of high-molecular-weight compounds. Strong alkaline solutions can also hydrolyze esters in organic matter, increasing the concentration of carboxylic acid groups [6]. As a result, "alkali extracts" have long been considered as a laboratory artifact [19–21]. To minimize oxidative changes during alkaline extraction, it is recommended to use inert atmosphere (e.g., argon or nitrogen, www.humic-substances.org (accessed on 6 February 2025)). Extraction under a N_2 gas atmosphere is ignored when HAs are obtained for commercial purposes. Although alkaline extraction in the presence of oxygen may alter the physicochemical properties of HAs, the oxidative changes may be beneficial for their use as soil amendments. For example, an increase in the content of free radicals in HAs during extraction in air or increase in OH group content suggests an enhanced antioxidant capacity of HAs [11]. The presence of quinones that are able to generate ROS is beneficial for the HA bactericidal and fungicidal activities [11,22] and their role as biostimulants for plant growth [23]. An increase in the content of COOH groups may enhance the cation-exchange capability and metal-binding capacity [5,7]. Surprisingly, to the best of our knowledge, the effect of alkaline extraction conditions on HA biological activity has not been studied so far.

Systematic research on the extent of HA transformation depending on its origin is lacking. Only a few studies have investigated changes in the HA physicochemical properties depending on extraction conditions (e.g., Swift and Posner, 1972; cited from [6]). In our earlier work, we compared physicochemical properties of HA preparations from Retisol and Chernozem obtained using 0.1 M NaOH (24 h) in air and nitrogen [21]. While there were hardly any differences observed in Chernozem HA, extraction in air resulted in higher O:C ratios, higher contents of oxygen-containing aromatic fragments, and quinone and carboxyl groups (carbon-13 nuclear magnetic resonance data, ^{13}C-NMR), and a significantly higher content of paramagnetic centers (electron paramagnetic resonance data, EPR) in HA from Retisol. We hypothesize that the stronger the oxidative transformation of organic residues during the natural humification process (as in Chernozem compared to Retisol), or the longer diagenetic alteration of NOM (e.g., as in peat and coal), the lower the extent of HA transformation by alkali in the presence of oxygen. The strongest oxidative changes in HA should occur if fresh organic material (e.g., litter or compost) is used as a source of HA.

Our first objective was to investigate changes in the physicochemical properties of HA preparations obtained from static pile compost in air and nitrogen. We have tested the hypothesis that the most significant oxidative changes during extraction in air occur in HAs obtained from fresh organic material. Our second objective was to explore antioxidant properties of HA preparations of different origins extracted in air (HA-O_2) and nitrogen

($HA\text{-}N_2$) and relate them to HA biological activities during seed priming. Seed priming has been reported to be a cost-effective and quick way to stimulate growth of various crop plants, as it promotes a rapid germination and emergence of seedlings. It is suggested that, during the priming process, metabolic activities necessary for subsequent embryonic growth and radicle protrusion are initiated. As a result, primed seeds achieve higher germination rates as compared to unprimed seeds [24]. Though the beneficial effect of HAs on plants is well-known [9], data on their activity as priming agents are still lacking and mainly focused on different cultivars of rice [25–27]. We hypothesize that the higher the antioxidant activity, the stronger the priming effect.

Our results demonstrate that extraction in air is beneficial for obtaining biologically active HA preparations to be used as plant biostimulants. The established relationship between the oxidation state and biological activities of humic preparations opens up new perspectives for using modified HAs as innovative plant stimulants in agriculture.

2. Materials and Methods

2.1. Humic Acid Preparations

The study was conducted in September 2023–April 2024 and used humic acids (HAs) from static pile compost (HAC), Retisol (Loamic, Cutanic) (HAR) (spruce forest, Moscow region; 56.227922 37.953847), and Vermic Hypocalcic Chernozem (Loamic, Hyperhumic) (HACh) (grass vegetation, Lipetsk region; 53.97153, 37.18133) (Figure 1). Compost was made of grass, spruce needles, oak, maple, and birch leaves and resembled H horizon of forest litter in Retisol. The sample for HA extraction was taken from the bottom of the pile (a 5-year-old material). The ignition loss equaled 37%. Some of the compost and soil properties are listed in Table 1.

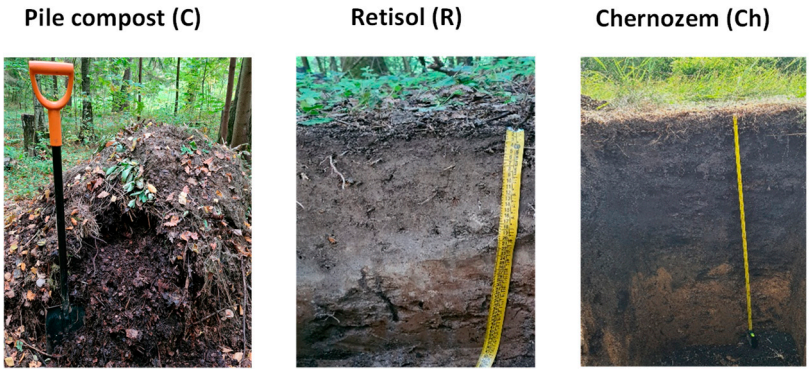

Figure 1. Compost pile and profiles of Retisol and Chernozem soils.

Table 1. Some properties of compost and soil, which served as a source of HA.

HA Source	pH	C, %	N, %	C_{HA}/C_{FA} [1]	C_{HA}/C_{org} [2]
Compost	6.8	15.0	1.6	2.2	0.27 (medium)
Retisol	4.4	5.1	0.3	0.6	0.28 (medium)
Chernozem	5.2	4.7	0.3	2.6	0.65 (very high)

[1] Humic acid to fulvic acid ratio. [2] Humification degree [1].

2.2. Extraction of Humic Acids

Humic acids were extracted using 0.1 M NaOH under air ($HA\text{-}O_2$) or nitrogen ($HA\text{-}N_2$) conditions. Extraction, purification, and characterization of HAR and HACh were as described elsewhere [21]. To obtain HAC, compost was dried and passed through a 2 mm sieve, followed by HA extraction under air or nitrogen conditions for 1 h at a solid/solution ratio of 1:5. The extraction process was repeated three times, and the extracts were pooled.

The pH was adjusted to 7.0 to prevent oxidation of HA, and extracts were centrifuged for 10 min at 10,000 rpm (13,751× *g;* Eppendorf 5804 centrifuge (Eppendorf, Hamburg, Germany)). The supernatants were purified using membrane filtration (0.45 μm and then 0.22 μm), acidified to a pH of 2.0 to obtain HA, and centrifuged (5 min, 5000 rpm, 3438× *g*). Then, the HA pellet was re-dissolved in 0.1 M NaOH (purged by N_2 gas). The procedure was repeated three times, and then the HA pellet was washed with distilled water and dried at 40 °C, after which it was dispersed to a powder in an agate mortar. The ash content of the preparation was determined by heating in an oven at 800 °C for 4 h.

2.3. Physicochemical Properties of HAC

Elemental composition. The elemental composition of HA was analyzed using a Vario LIII CNH analyzer (Elementar Analysensystene GmbH, Langenselbold, Germany). The oxygen content was determined by difference. The analysis was performed in triplicate.

Ultraviolet and visible (UV-Vis) and infrared (IR) spectroscopy. UV-Vis absorption spectra of the HA solutions in 0.05 M NaOH were recorded on a Shimadzu 1800 UV spectrophotometer (Shimadzu Corporation, Kyoto, Japan). IR spectra were recorded using KBr technique on a Bruker Tensor 27 spectrophotometer (Bruker Optik GmbH, Ettlingen, Germany). The UV-Vis analysis was performed in triplicate, and IR spectra were recorded without replication.

13C-NMR spectroscopy. For ^{13}C NMR spectroscopy in the liquid phase, deuterium water (D_2O, 99.95% D) and 40% NaOD in D_2O (99 + % D) (Aldrich, Milwaukee, WI, USA) were used. In total, 40 mg of HAs was dissolved in 0.5 mL of 0.3 M NaOD/D_2O, and then the mixture was homogenized using a Vortex shaker (V-3, ELMI, Riga, Latvia) for 10 min and centrifuged for 5 min at 13,751× *g*. The ^{13}C NMR spectra were recorded using a Bruker Avance 400 spectrophotometer (Bruker Corporation, Billerica, MA, USA), as described in [21]. The distribution of carbon atoms among the main structural fragments of HA was determined by integrating spectral regions using the following assignments (ppm): 220–185 for carbonyl carbon of ketone and quinone groups (C=O); 185–165 for carbon of carboxyl, ester, and amide groups ((C=O)–O, N); 165–145 for aromatic carbon substituted by heteroatoms (C_{Ar}–O, N); 145–108 for unsubstituted or C-substituted aromatic carbon (C_{Ar}–H, C); 108–90 for carbon bonded to two heteroatoms by single bonds (in HA, this is mainly acetal carbon in O–O, N cyclic saccharides); 90–48 for carbon with a single bond to a heteroatom and entering the composition of aliphatic fragments; and 48–0 for carbon of alkyl units not bonded to heteroatoms (CH_n) [21]. The analysis was performed without replication.

Electron paramagnetic resonance (EPR). The content of free radicals in HAC was determined as described in [21]. Briefly, the EPR spectra were recorded using a Radiopan SE/X-2547 spectrometer (Radiopan, Poznan, Poland) in the X-range at room temperature, with a high-frequency power of 1 mW and an amplitude of high-frequency modulation of 0.06 mT. The concentration of free radicals in HA was determined by comparing the areas under the relative integral intensities of the EPR signals of the standard (diphenyl picrylhydrazyl) and the sample. The coefficient of variation for determining the concentration of free radicals in this case does not exceed 5%. The linewidth (in Gauss) was calculated from the distance between the extreme points of the absorption line.

Functional group content. The content of functional groups and the pKa values were determined by potentiometric titration. In total, 50 mg of HA was dissolved in 25 mL of 0.05 M NaOH. Then, 1 M KCl and 0.1 M HCl were added, and the volume of the solution was adjusted to 50 mL using distilled water. The final HA concentration was 1 mg mL^{-1}; the pH of the solution equaled about 11.2, and the ionic strength was 0.1 M. The HAs were first titrated with 0.1 M HCl (backward titration) and then with 0.1 M NaOH (forward

titration), under continuous nitrogen purging, using an automatic titrator Mettler Toledo DG58 (Mettler Toledo, Columbus, OH, USA). The start and end points of HA titration were assessed using Gran functions, pH intervals corresponding to the titration of the groups of a certain type were determined using differential titration curves, and pKa values were determined using Hendersson–Hasselbach equation, as described in [28]. The analysis was performed in triplicate.

Molecular weight (MW) distributions of HAC preparations (0.5 mg mL^{-1}) were obtained by Sephadex G-75 gel filtration using Bio-Rad Econo column (1.0 × 60 cm, Bio-Rad, Hercules, CA, USA) and BioLogic LP chromatographic equipment (BioRad, Hercules, CA, USA). Then, 0.025 M Tris-HCl (pH 8.2) buffer was used, along with 0.05 M NaCl and 0.1% sodium dodecyl sulfate (SDS) to suppress ionic and hydrophobic interactions, respectively. The elution rate was 0.13 mL min^{-1}. Elution profiles were recorded at 280 nm. The void volume of the column (Vo) and the total volume of the mobile phase (Ve) were determined using Blue Dextran 2000 and $(NH_4)_2Cr_2O_7$, respectively. The molecular weights corresponding to the chromatographic peaks were calculated using the Determann empirical formula for globular proteins: log MW = 5.624 − 0.752Ve/Vo. The analysis was performed in triplicate.

2.4. Antioxidant Properties of Humic Acids

HA stock solution. For antioxidant assays (except for DPPH assay), a humic acid stock solution (1 mg mL^{-1}, pH ca. 6.0) was used. The stock solution was prepared as follows: 2 mg of HA was dissolved in 0.1 mL of 0.05 M NaOH in ultrasonic bath (50 Hz, 3 min), and then 1.9 mL of H_2O was added.

Antiradical activity of HA was assessed by 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging capacity assay using HA powder. The reaction mixtures contained 1 mL of 0.1 mM DPPH in ethanol and 2.5, 5.0 or 10 mg of HA. In total, 0.1 mM DPPH• was used as a control. To check the HA dissolution in ethanol and subtract the respective absorbance values, the mixtures of HA in ethanol were prepared (HAs–ethanol). After 20 min of incubation in the dark, the mixtures were centrifuged (16,000 rpm, 1 min, CM-50 Centrifuge, Elmi, Riga, Latvia), and the absorbance of the supernatant was measured against ethanol at 517 nm. For each concentration of HA, DPPH• scavenging (%) was calculated as follows:

$$Inhibition,\ \% = \frac{(A_{control} - (A_{HAs-DPPH} - A_{HAs-ethanol}))}{A_{control}} \times 100\% \quad (1)$$

A HA concentration at which 50% DPPH• inhibition was reached (IC-50) was calculated based on the graph: inhibition (%) vs. HA concentration.

Total antioxidant activity was measured by phosphomolybdenum assay [29]. The assay is based on the reduction of molybdenum (VI) to molybdenum (V), with the formation of greenish-blue complex, with the maximum absorbance at 695 nm. Phosphomolybdenum reagent solution was prepared by mixing 0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate [30]. For the assay, HA solutions at concentrations of 0.1, 0.2, 0.3, and 0.4 mg mL^{-1} were prepared. Then, 0.5 mL of HA was mixed with 0.5 mL of phosphomolybdenum reagent solution. Instead of HA, distilled water (0.5 mL) was used as a control. The mixtures were incubated at 95 °C for 90 min. After cooling to room temperature, the absorbance was measured at 695 nm using Shimadzu UV1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) against the control. The coefficient from the linear curve (HA concentration vs. absorbance at 695) was used to obtain the optical density value for antioxidant activity calculations. The results were expressed as ascorbic

acid equivalents (AAEs) per 1 g of HA. For a calibration curve, 0.002–0.1 mg mL^{-1} ascorbic acid solution was used.

Chelating capacity of HA was determined by ferrous ion chelation in the presence of ferrozine [30], as described in [31]. The reaction mixture was as follows: 0.2 mL HAs (0.2, 0.4, 0.6, 0.8, and 1 mg mL^{-1}), 0.74 mL H_2O, 0.02 mL 2 mM $FeCl_2 \times 4H_2O$, 0.04 mL 5 mM ferrozine ($C_{20}H_{12}N_4Na_2O_6S_2$) solution. Instead of HA, distilled water was used as a control. The color control contained 0.94 mL distilled water and 0.2 mL of HA. The reaction was started by adding ferrozine. The absorbance at 562 nm was measured after incubating the mixtures for 10 min at 25 °C. The inhibition of ferrozine reaction with Fe^{2+} in the presence of HA was calculated as follows:

$$Inhibition,\ \% = \frac{(A_{control} - A_{HAs})}{A_{control}} \times 100\% \quad (2)$$

IC-50 was calculated based on the graph of inhibition (%) vs. HA concentration.

All antioxidant property assays were conducted in four replicates.

2.5. Biological Activity Tests

In the experiments, seeds of early radish *Raphanus raphanistrum* ssp. *sativus* L. cultivar Soffit and spring common wheat *Triticum aestivum* L. cultivar Ivolga were used. Biological activity of humic material was evaluated at an early stage of growth under laboratory conditions. The test plants were selected based on different properties of their seeds. Radish is a dicotyledonous crop with small seeds, while wheat is a monocotyledonous plant with a large supply of nutrients in the grain. Comparing the effect of HA on these different seeds will allow us to identify the crop group that would benefit most from HA treatment.

Seeds were immersed in priming media, viz., in HA at 23 ± 1 °C for 5 h or 14 h for radish and wheat, respectively. The concentration of HA was 0.5 g L^{-1}. A ratio of 3 mL of solution per 40 seeds was used for soaking. After soaking, the seeds were rinsed with distilled water five times and dried back to the original weight on filter paper for 48 h. Unprimed seeds were used as a control.

Germination tests were conducted using four replications of 10 seeds in Petri dishes. Each dish received 10 mL of distilled water. The seeds were germinated in the dark at 24 °C for 72 h [32]. Seedlings' root and shoot lengths were measured in each replicate. Seedling Vigor Index (VI) was calculated using the following equation [33]:

$$\mathrm{VI} = \mathrm{Germination} \times \mathrm{Seedlinglength}, \quad (3)$$

where germination is the percentage of germination, and seedling length is the sum of the root and shoot lengths in mm.

In contrast to the separate measurement of germination and length of roots and shoots, VI reflects the ability of seeds to produce normal seedlings under less-than-optimal growing conditions, similar to those that may occur in the field. Higher values of VI indicate a more vigorous seed lot [34].

Malondialdehyde (MDA) concentration in plants is used as a biomarker of oxidative stress, namely lipid peroxidation. Estimation of MDA in plant tissue was performed by thiobarbituric acid (TBA) assay according to [35], with a small modification. Instead of generating a standard curve, the MDA concentration was calculated according to the following formula:

$$\mathrm{MDA} = \frac{A_{523} \times 10^6 \times V \times d}{m \times \varepsilon}, \quad (4)$$

where MDA is the content of MDA, nmol·g^{-1}; A_{523} is the absorbance at 523 nm; V is the volume of aliquot taken to determine the MDA; d is the ratio of the total sample volume to the aliquot taken to determine the MDA; ε is the MDA molar extinction coefficient (155,000 L·cm^{-1}·mol^{-1}); and m is the sample weight.

2.6. Statistical Data Treatment

Data are presented as the mean ± standard deviation (SD). To analyze differences among means, one-way analysis of variance (ANOVA) was applied, followed by a post hoc mean comparison using Fisher's Least Significant Difference (LSD) test at $p < 0.05$. All of the statistical data treatments were carried out using the Statistica 8.0 software package (StatSoft, Dell Inc., Round Rock, TX, USA).

3. Results

3.1. Physicochemical Properties of HAC Extracted in Air and Under Nitrogen

3.1.1. Elemental Composition and Optical Properties

The ash content of HA preparations was about 2% after purification (Table 2). Extraction in air caused higher C and O contents and lowered H content in HAC-O_2 compared to HAC-N_2, which was reflected in a 1.2-fold lower H:C ratio and 1.2-fold higher O:C ratio. HAC-O_2 had both a higher optical density (E_{465}) ($p < 0.05$) and E4/E6 ratio than HAC-N_2 (Table 2).

Table 2. Elemental composition and optical parameters of HAC.

HA	Ash, %	Content, Mass and Atomic, %					Atomic Ratios		E_{465} [3]	E_4/E_6
		C	H	N	S	O	H:C	O:C		
HAC-N_2	1.9	[1] 53.11 ± 0.0 [2] 40.7 ± 0.2	3.6 ± 0.0 33.5 ± 0.0	4.9 ± 0.1 3.2 ± 0.4	1.7 ± 0.0 0.5 ± 0.0	38.5 ± 0.1 22.1 ± 0.2	0.8	0.5	0.03	4.7
HAC-O_2	2.1	[1] 52.8 ± 0.2 [2] 42.7 ± 0.1	3.1 ± 0.1 29.8 ± 0.0	4.5 ± 0.1 3.1 ± 0.1	1.5 ± 0.0 0.5 ± 0.0	40.2 ± 0.1 24.2 ± 0.0	0.7	0.6	0.04	5.5

[1] Mass content, [2] Atomic content; [3] HA concentration 0.001%; ±SD (n = 3).

3.1.2. Infrared Spectra

Differences in the extraction conditions did not cause any changes in the IR spectra of HA (Figure 2). Both preparations contain a wide band attributed to the stretching of OH and NH groups linked by intermolecular hydrogen bonds (3300 cm^{-1}) and a shoulder in the region of 3050 cm^{-1} attributed to aromatic C–H groups stretching [36]. The weak intensity of this band may be due to the band overlapping from the broad band of the OH stretching [36]. A pronounced peak at 2930 cm^{-1} and a shoulder at 2845 cm^{-1} are attributed to aliphatic CH_2 and CH_3 group stretching, and deformation vibrations of these groups are denoted by a weak peak at 1450 cm^{-1} (Table 3). The region of 1710–1030 cm^{-1} is characterized by a range of absorption bands of varying intensity. The C–O stretching of the COOH group (1710 cm^{-1}) is characteristic of the H-form of the preparation.

HAs contain a strong band of amide groups (amide I, 1650 cm^{-1}) and narrow bands of weak intensity at 1535 (amide II) and 1420 cm^{-1} (amide III), indicating the presence of N-containing components. The bands at 1260–1220 cm^{-1} are due to C–O and O–H stretching in COOH. Weak narrow peaks at 1080–1030 cm can be attributed to C–O stretching of polysaccharides. Thus, the data from IR spectroscopy indicate the presence of aliphatic and nitrogen-containing, as well as polysaccharide, components in the HAC, for which the relative content does not differ between HAC-O_2 and HAC-N_2 (Table 3).

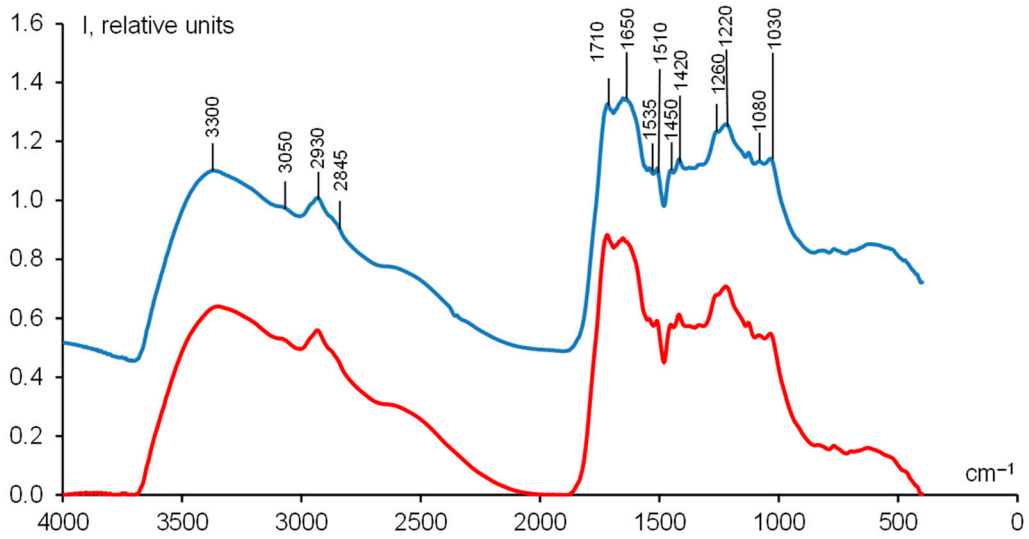

Figure 2. IR spectra of HAC-N_2 (blue line) and HAC-O_2 (red line).

Table 3. Absorption bands in the IR spectra of HAC.

Absorption Maxima, cm^{-1}	Group and Oscillations
3300 w [1]	O–H stretching, N–H stretching (trace), intermolecular hydrogen bonds
3050 sh [2]	Aromatic C–H stretching
2930 m [3], 2845 sh	C–H stretching in aliphatic CH_2, CH_3 groups
1710 m	C=O stretching of COOH, aldehydes and ketones
1650 s [4]	C=O stretching of amide groups (amide I), C=O of quinones and/or H–bonded conjugated ketones
1535 w	N–H deformation and C=N stretching (amide II)
1510 w	aromatic C=C stretching
1450 w	C–H in CH_2 (or CH_3) deformation
1420 m	C=N stretching of primary amides (amide III)
1260 sh, 1220 m	C–O stretching of COOH, O–H deformation of COOH C–O stretching of aryl ethers and phenols
1080, 1030 w	C–O stretching of polysaccharides

[1] w—weak; [2] sh—shoulder; [3] m—medium; [4] s—strong.

3.1.3. ^{13}C-NMR and EPR Spectroscopy Data

The distribution of carbon atoms among structural fragments in HAs (^{13}C-NMR spectroscopy) showed a 1.3-fold decrease in the relative content of unoxidized CH_n groups in HAC-N_2 compared to HAC-O_2 and a 1.2-fold increase in the content of oxidized aliphatic fragments and OC–O,N structural units in HAC-O_2 (Table 4). The relative content of unoxidized aromatic carbon (C_{ar}) was slightly higher in HAC-O_2, which is consistent with the elemental composition and spectroscopy data in the visible region (Table 2).

The content of paramagnetic centers in HAC preparations (Table 4) was one order higher than in their source material (4.05×10^{15}). Extraction in air resulted in a 1.3-fold increase in the radical content in HAC-O_2 compared to HAC-N_2. The width of the spectral line showing the scatter of the energy characteristics of free radicals was noticeably lower in HA preparations (Table 4) than in the parent compost (6.10).

Table 4. ^{13}C NMR spectroscopy and EPR data of HAC.

HA	Distribution of Structural Groups by Spectral Region (ppm), % of the Total Area of NMR Spectrum							Paramagnetic Properties, EPR Data	
	Aliphatic Fragments		Sugars	Aromatic Fragments		Carboxyls, Ketones, Quinones		Intensity, Spin g^{-1}	Line-Width, Gauss
	CHn 5–48	CH_3–O, N CH_2-O 48–92	OC–O, N 92–110	Car 110–144	CarO 144–164	COO 164–185	C=O 185–220		
HAC-N_2	22.9	16.7	5.9	22.8	9.4	17.6	4.7	7.08×10^{16}	4.02
HAC-O_2	17.3	20.1	8.2	23.6	9.3	17.3	4.2	9.11×10^{16}	4.87

3.1.4. Potentiometric Titration Data

The backward potentiometric titration of HA preparations showed considerable differences in the content of functional groups titrated in the pH ranges of 10.5–7.7 and 7.7–5.1: it was 4.10- and 1.9-fold higher in HAC-O_2 compared to HAC-N_2 (Table 5). The total content of functional groups in HAC-O_2 (16.5 mmol g^{-1}) was 2.1-fold higher than in HAC-N_2 (7.75 mmol g^{-1}) ($p < 0.05$). There were no such differences observed between HA preparations during forward titration (Table 5).

Table 5. Functional group content and pKa values of HA preparations.

HA Preparation	Backward Titration (0.1 M HCl), pH Intervals			Forward Titration (0.1 M NaOH), pH Intervals	
	10.5–7.7	7.7–5.1	5.1–3.7	3.8–6.4	6.4–10.4
	Functional group content, mmol g^{-1}				
HAC-N_2	4.55 ± 0.25	1.65 ± 0.34	1.55 ± 0.44	1.90 ± 0.10	10.4 ± 0.10
HAC-O_2	8.47 ± 0.50	6.73 ± 1.15	1.30 ± 0.26	1.67 ± 0.10	10.5 ± 0.10
	pKa values				
HAC-N_2	9.4 ± 0.0	6.4 ± 0.2	4.4 ± 0.1	4.9 ± 0.1	9.6 ± 0.1
HAC-O_2	9.5 ± 0.3	6.5 ± 0.5	4.4 ± 0.2	5.0 ± 0.1	9.7 ± 0.1

±SD (n = 3).

3.1.5. Molecular Weight Distributions

The gel filtration data showed the presence of two fractions in HAC: a high-molecular-weight fraction with a peak center molecular weight (MW) > 75 kDa and low-MW fraction with a MW of about 30 kDa and relative content of 70% (Figure 2). The extraction in air hardly resulted in any changes in the MW distributions of HAs; thus, neither notable depolymerization nor polymerization reactions took place (Figure 3).

3.2. Antioxidant Properties of HAs

The antioxidant properties of HAs differed substantially (Table 6). The antiradical activity of HAs, measured by the DPPH assay, increased from Retisol to Chernozem (the lower the IC-50 value, the higher the antiradical activity). The antiradical activities of preparations extracted in air were 1.5- (HAC), 1.6- (HAR), and 1.3-fold (HACh) higher than those extracted under nitrogen (statistically significant at $p < 0.05$).

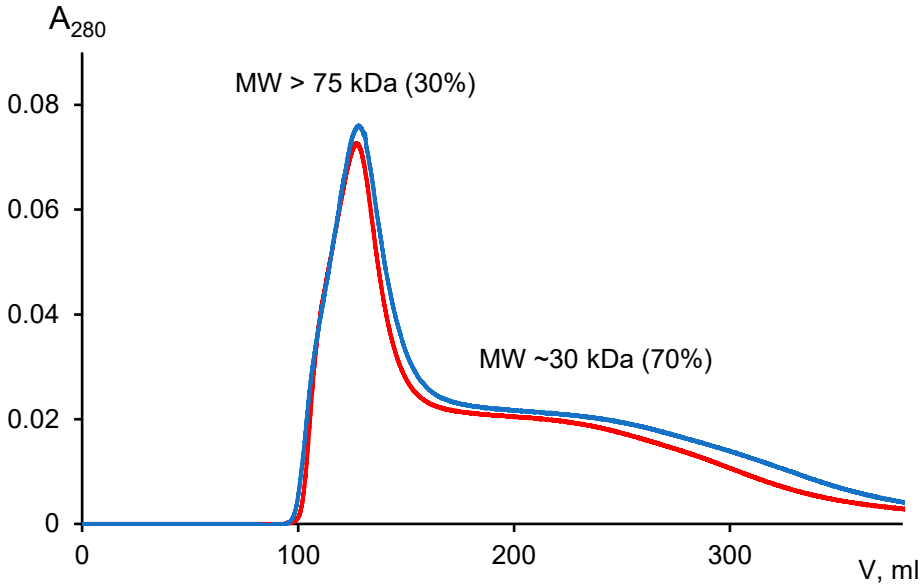

Figure 3. Molecular weight distributions of HAC-N_2 (blue) and HAC-O_2 (red) using Sephadex G-75 gel in 0.025 M Tris-HCl (pH 8.2) buffer with an addition of 0.05 M NaCl and 0.1% SDS. The first peak corresponds to the void volume (Vo) of the column, MW > 75 kDa. The molecular weight and the relative contents of the fractions are shown above the corresponding peaks.

Table 6. Antioxidant properties of humic acids.

Compost		Retisol		Chernozem	
HAC-N_2	**HAC-O_2**	**HAR-N_2**	**HAR-O_2**	**HACh-N_2**	**HACh-O_2**
Antiradical activity (DPPH assay), IC-50					
4.55 ± 0.39	3.05 ± 0.03	7.33 ± 0.33	4.65 ± 0.15	3.42 ± 0.05	2.73 ± 0.03
Total antioxidant capacity (phosphomolybdenum assay), mg AAE g^{-1}					
166.6 ± 6.5	170.4 ± 1.0	115.7 ± 0.4	139.1 ± 9.4	25.5 ± 0.6	31.9 ± 1.4
Chelating capacity (ferrous ion chelation), IC-50					
1.00 ± 0.14	1.04 ± 0.05	1.38 ± 0.08	1.40 ± 0.05	1.37 ± 0.09	1.39 ± 0.02

±SD (n = 4).

Total antioxidant capacity of HAs assessed by the phosphomolybdenum method showed a 5-fold decrease from HAC to HACh (Table 6). The antioxidant properties of the preparations extracted in air were 1.2-fold higher than those of HAs extracted under nitrogen, except HAC.

Unlike antiradical and antioxidant properties, there were no differences between the chelating capabilities of HAs extracted in air and under nitrogen. Chelating capacity (assessed at pH 5.8) was lowest in HAC and identical in HAR and HACh.

3.3. Biological Activity of HAs

Averaged over treatments, priming did not affect seed germination, with germination percentages ranging from 93% to 100% and from 95% to 100% for radish and wheat seeds, respectively (Table 7). However, priming resulted in enhanced growth of radish seedlings. Among the studied humic materials, the HAR-O_2 had the most pronounced activity, the priming of which led to an increase in the length of the shoot to 155%, and the length of the roots to 229% (Table 7). In general, HAs obtained in a nitrogen atmosphere demonstrated a less pronounced effect on shoot and root lengths compared to HAs isolated in air.

Table 7. The effect of different priming treatments on germination and length of shoots and roots of radish and wheat seedlings.

Variant	Germination, %	Shoots	Roots
		Length, % of Control	
	Radish		
Control	93 a [1]	100 ab	100 a
HAC-N_2	95 a	77 a	88 a
HAC-O_2	93 a	103 ab	195 ab
HAR-N_2	100 a	129 bc	177 ab
HAR-O_2	100 a	155 c	229 b
HACh-N_2	100 a	109 ab	93 a
HACh-O_2	100 a	121 bc	105 a
	Wheat		
Control	100 a	100 ab	100 a
HAC-N_2	95 a	81 a	111 a
HAC-O2	99 a	124 b	140 a
HAR-N_2	98 a	96 ab	99 a
HAR-O_2	100 a	94 ab	115 a
HACh-N_2	98 a	87 a	102 a
HACh-O_2	95 a	97 ab	104 a

[1] The values denoted with different letters within a column are significantly different at $p < 0.05$, according to Fisher's LSD test.

In contrast to separate measurements of germination and length of roots and shoots, VI reflects the ability of seeds to produce normal seedlings under less-than-optimal growing conditions, similar to those that may occur in the field. Primed seeds produced more vigorous seedlings than the unprimed ones in the case of radishes, but not in the case of wheat (Figure 4). Radish seed priming with HAs isolated in air (HAR-O_2, HACh-O_2, and HAC-O_2) resulted in a 1.1–2.1-fold increase in VI. For HAs isolated in a nitrogen atmosphere (HAR-N_2, HACh-N_2, and HAC-N_2), the beneficial effect was less pronounced, and the VI increase above the control value did not exceed 1.7 times (HAR-N_2). The VI of seeds primed with HAs in air exceeded that of the ones primed with HAs obtained in a nitrogen atmosphere 1.3, 1.1, and 1.8 times for humic materials from sod–podzolic soil, Chernozem, and compost pile, respectively. After treatment of wheat seeds with HAs, the VI did not statistically differ from the control.

In contrast to VI, priming with HAs affects the MDA level both in radish and wheat seedlings (Table 8). In most cases, treatment with HA increased the MDA content. The exception was the radish seedlings grown from seeds primed with HA isolated from compost (HAC-N_2 and HAC-O_2). Contrasting with the growth indicators, the method of isolating HA had hardly any effect on the content of MDA in radish and wheat seedlings.

A comparison of data on antioxidant activity of the studied humic materials and their biological activity toward radish showed a tendency to a higher biological effect as DPPH• radicals scavenging activity increases (Figure 5a). Spearman rank order correlation coefficients between the DPPH• IC_{50}, the length of shoots and roots, and the VI of radish seedlings were −0.943, −0.829, and −0.943, respectively. Additionally, a statistically significant correlation (r_S = −0.886) was observed between the AOC of HA and MDA contents in shoots of radish seedlings (Figure 5b). No correlation was found between the HA antioxidant activity and growth parameters of wheat seedlings.

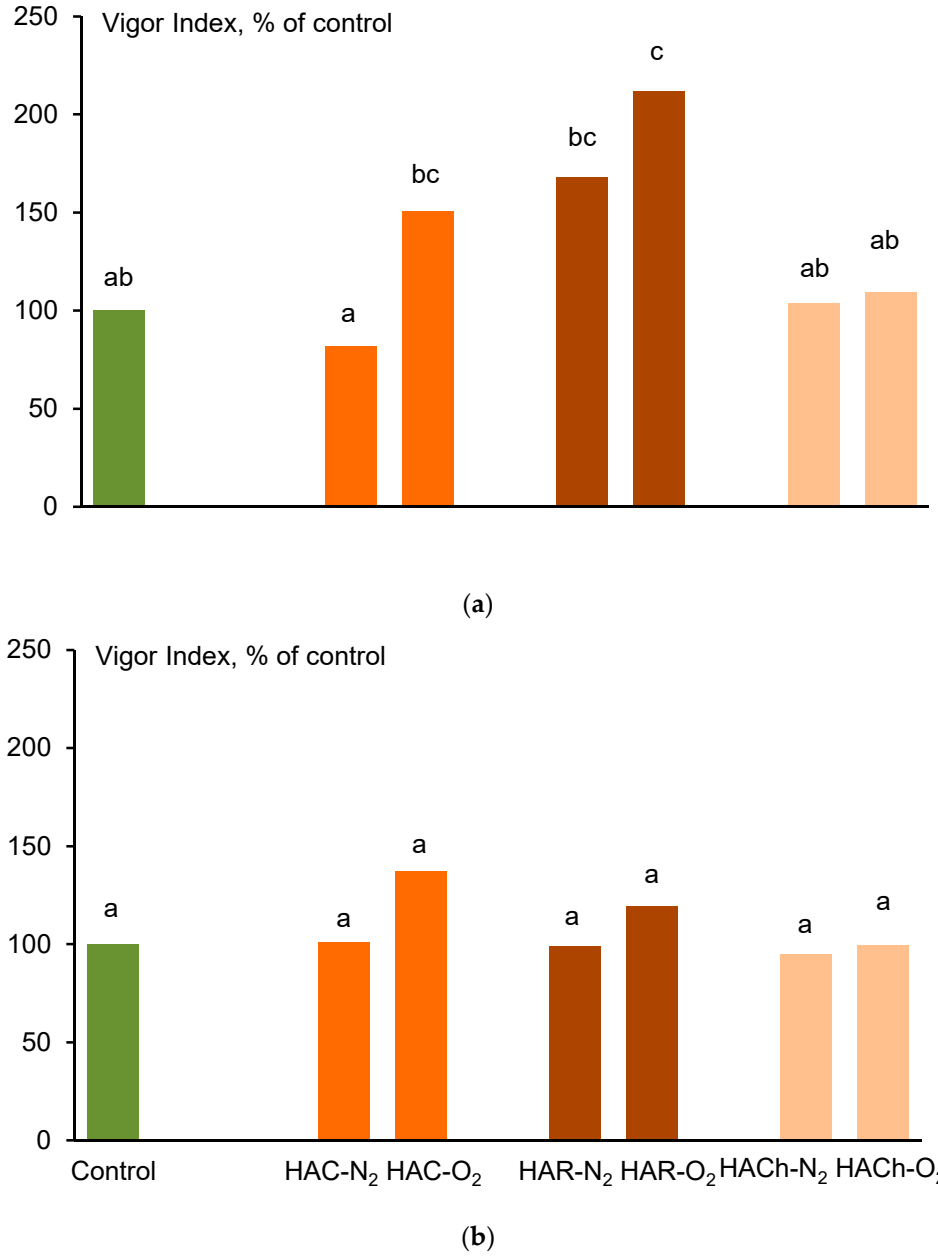

Figure 4. The effect of different priming treatments on the Vigor Index of radish seedlings (**a**) and wheat seedlings (**b**). The values denoted by different letters within a column are significantly different at $p < 0.05$, according to Fisher's LSD test.

Table 8. The effect of different priming treatments on MDA content (average ± SD) in shoots and roots of radish and wheat seedlings.

Variant	Shoots	Roots
	MDA, nmol g^{-1}	
	Radish	
Control	21.1 ± 1.0 c [1]	26.2 ± 0.3 b
HAR-N_2	23.7 ± 0.2 d	26.8 ± 2.3 bc
HAR-O_2	24.2 ± 0.8 de	31.7 ± 1.2 d
HACh-N_2	24.4 ± 1.0 de	31.8 ± 0.4 d
HACh-O_2	25.2 ± 1.3 e	29.1 ± 0.8 c
HAC-N_2	12.4 ± 0.3 a	18.8 ± 1.3 a
HAC-O_2	17.5 ± 0.1 b	20.5 ± 1.6 a

Table 8. *Cont.*

Variant	Shoots	Roots
	MDA, nmol g^{-1}	
	Wheat	
Control	33.1 ± 1.3 a	35.7 ± 1.7 ab
HAR-N_2	41.2 ± 0.1 bc	45.4 ± 7.6 c
HAR-O_2	41.2 ± 1.9 bc	43.3 ± 2.4 bc
HACh-N_2	39.1 ± 0.6 b	39.1 ± 1.1 abc
HACh-O_2	43.2 ± 0.3 c	34.5 ± 1.8 a
HAC-N_2	40.0 ± 2.1 b	34.6 ± 0.5 a
HAC-O_2	40.4 ± 1.0 b	41.2 ± 8.9 abc

[1] The values denoted with different letters within a column are significantly different at $p < 0.05$, according to the Fisher LSD test.

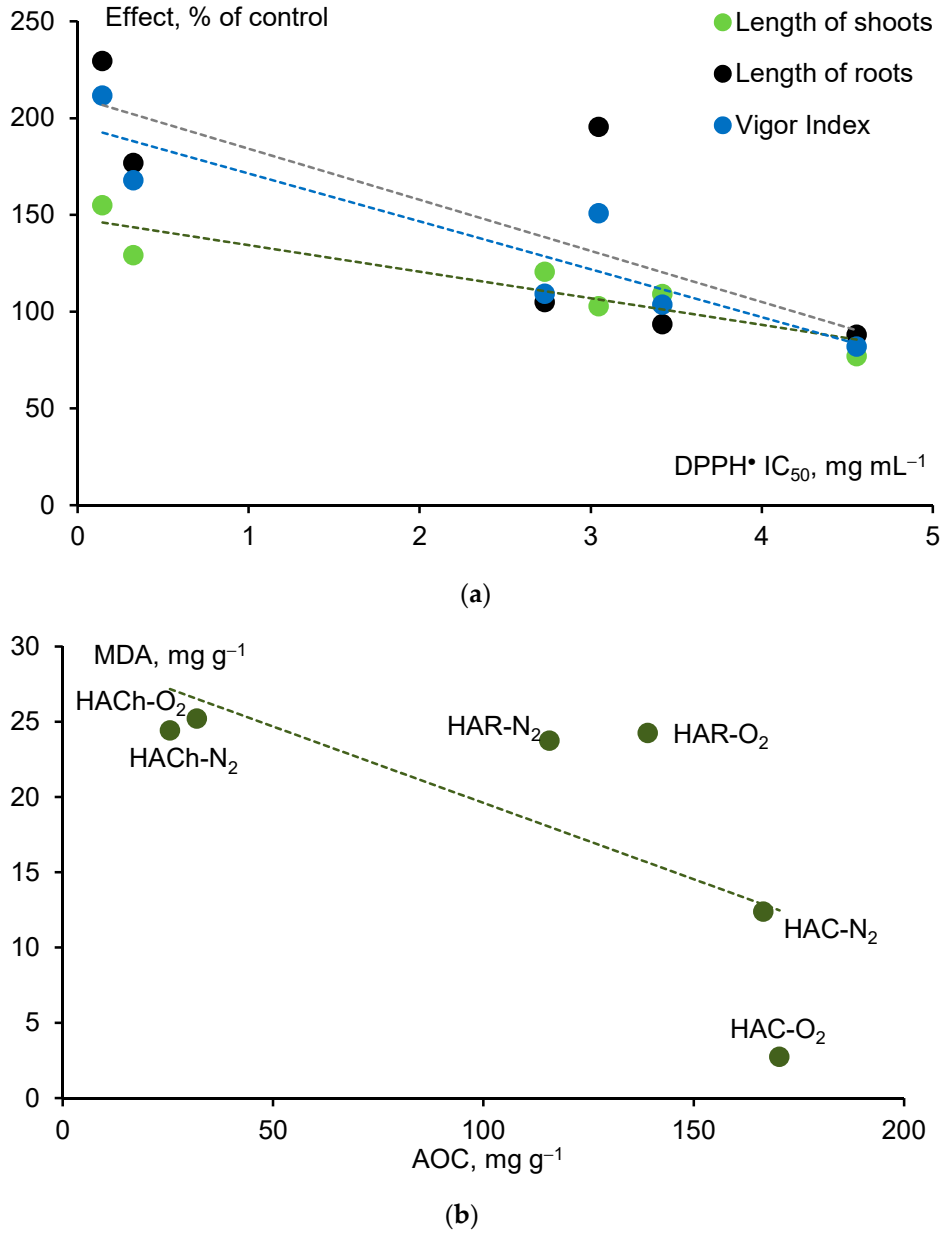

Figure 5. The relationship between antioxidant properties of HAs and their biological activity: DPPH radical scavenging activity vs. length of shoots and roots, and Vigor Index of radish seedlings (**a**); AOC vs. MDA content in shoots of radish seedlings (**b**).

4. Discussion

4.1. Physicochemical Properties of HAC and Antioxidant Activities of HAs

Extraction in air compared to nitrogen atmosphere caused significant oxidative changes in HA from compost: an increase in the oxygen content and optical density of HAC (Table 2), oxidation of aliphatic fragments (^{13}C-NMR data), and an increase in the content of free radicals (EPR data) (Table 4) and the content of functional groups titrated at pH over 7.7 (Table 5). Similar to HAR and HACh [21], no changes occurred in the IR spectra (Figure 2) or molecular weight distributions of HA (Figure 3), suggesting that IR spectroscopy and gel filtration are not sensitive enough for monitoring oxidative changes in HAs during alkaline extraction.

Changes in physicochemical properties of HAC suggest its oxidative and structural rearrangements. The dark color of HA is attributed to the system of conjugated double bonds in its aromatic structures [1]. The higher the absorbance at 465 nm and the lower the E4/E6 ratio, the higher the aromaticity of HAs [2]. An increase in optical density and decrease in the E4/E6 ratio in HAC-O_2 suggests the development of electron-conjugated aromatic systems possibly due to oxidative condensation reactions between phenolic units [17]. This is consistent with an increase in the relative content of aromatic C in HAC-O_2 according to ^{13}C-NMR data (Table 4). Extraction in air caused oxidation of aliphatic fragments in HAC, while aromatic ones remain unaffected (Table 4). This is possibly due to a short extraction time of HAs (1 h) in the present work. Both aromatic and aliphatic structures were oxidized in HAR when a 24 h alkaline extraction was used [21].

Unlike HAR and HACh with functional groups unaffected during extraction in air [21], the presence of oxygen caused a 2-fold and 4-fold increase in the content of groups titrated within the pH ranges of 7.7–10.5 and 5.1–7.7 in HAC (Table 5). The pH range over pH 8.0 is attributed to titration of phenolic groups; the pH range of 3.7–5.0 is attributed to carboxylic groups; and the intermediate pH range of 5.1–8.0 is attributed to weak carboxylic, N-containing groups, and strong phenolic ones [28]. Higher content of groups titrated in the pH range of 5.1–7.7 in HAC-O_2 may be due to hydrolysis of ether bonds in the presence of oxygen [6] or formation of weak carboxylic groups. An increase in the number of groups titrated in the "phenolic" pH region may be due to the formation of radicals increasing the ionization state of phenolic hydroxyl and its ability for dissociation, as shown for lignin [37]. The EPR data (Table 4) support this hypothesis. The lower amounts of functional groups titrated by 0.1 M NaOH (forward titration) are usually attributed to conformational changes in HAs [6,28]. A considerably higher content of functional groups in HA from compost compared to soil (4.6 mmol g^{-1} in HAR and 5.4 mmol g^{-1} in HACh) [21] suggests compost as a promising source material for obtaining high reactive HAs for commercial use.

EPR spectroscopy was used to estimate the concentration of free radicals in HA (Table 4). The paramagnetism of HAs is explained by radicals of the semiquinone type stabilized in aromatic structures [38,39] and by generation of unpaired π-electrons due to defects in the polyconjugation systems [40]. The concentration of free radicals in HAs (by EPR) correlates with the absorbance at 465 nm, O and H contents, C/H ratio, and aromatic C content [38]. An increase in the number of paramagnetic centers in HAC-O_2 vs. HAC-N_2 (Table 4), coupled with an increase in the optical density, suggests structural rearrangements, leading to a more pronounced electron-conjugated system in HAC-O_2, helping to stabilize the radicals produced by the oxidation of phenolic units. Free radicals in HAs are also localized in aliphatic chains [39]. A higher free radical content in HAC-O_2 can result from the oxidation of aliphatic units such as aryl ether linkages [41]. This is in agreement with the higher relative content of oxidized aliphatic structures in HAC-O_2 (^{13}C-NMR data; Table 4). The free radical content in HAC-N_2 (7.1×10^{16} spin g^{-1}) is within the values reported for soils (from 0.3×10^{16} to 2.4×10^{18} spin g^{-1}) [38], being comparable

to Retisol HA-N_2 (6.9×10^{16} spin g^{-1}) but was two orders lower than in Chernozem HAs ($1.3–1.5 \times 10^{18}$ spin g^{-1}) [21]. The origin of HA has a significant impact on the parameters of their EPR spectra [38,42]. The electron-conjugated systems develop during the progress of humification with an increase in the aromatic C content [38]. Thus, the HA samples isolated from soils have a higher spin concentration in comparison to those incubated from plant material [42].

In summary, physicochemical characterization of HAC supports our hypothesis that the younger the material used for HA isolation, the stronger the oxidative changes during alkaline extraction in air. Taken together, alkaline treatment leads to changes in physicochemical properties of HAs, accompanied by structural rearrangements, and an increase in the oxygen content and the content of free radicals.

4.2. Antioxidant Properties of HAs

The antioxidant activity of HA is generally attributed to the presence of phenolic hydroxyl, quinoid, and other chemical groups with a highly delocalized molecular orbital. Due to the presence of these functional groups, HAs can donate protons, catch free radicals, and chelate reactive ions [43]. The paramagnetic centers occurring in HAs, namely peripheral aliphatic groups with unpaired electrons, defects in highly condensed core aromatic regions, and transition metal complexes, were also suggested to be responsible for their antioxidant and biological activity [39].

Numerous studies have demonstrated that antioxidant activity depends substantially on the test system used, and so any conclusions are recommended to be based on at least two different test systems [30]. In our work, the antiradical, antioxidant, and chelating activities of HAs were measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) antiradical test, phosphomolybdenum assay, and iron (II)-binding capacity, respectively. The antiradical activity of HAs (DPPH• radical scavenging, Table 6) is consistent with EPR data being higher in preparations extracted in air and increasing in the following order: HAR < HAC < HACh. The total antioxidant activity (phosphomolybdenum assay) was also generally higher in preparations extracted in air (except for HAC) and was significantly lower in HACh compared to HAR and HAC (Table 6). A weak correlation between antiradical and total antioxidant activities of HAs is usually reported based on free radical scavenging assays, namely DPPH [30], as DPPH can detect antioxidants such as flavonoids and phenols, while phosphomolybdenum generally detects only some phenolics, mainly ascorbic acid, tocopherol, and carotenoids, as the method is based on the reducing potential of the hydroxyl group of the 6-hydroxychroman ring, which is shared by all vitamin E isomers [29]. Preparations extracted in air and under nitrogen showed no difference in chelating capacity measured by binding ferrous ions at pH 5.8 (Table 6). It is consistent with potentiometric titration data showing no difference between the number of groups titrated in the pH range 5.6–8.7 for HAR and HACh [21] and pH range 3.8–6.4 for HAC (Table 5). A significant correlation between ferrous ion chelation (IC-50) and HAs' acidic groups titrated in the pH range 5.6–8.7 was found ($r = -0.79$).

4.3. Biological Activity of Humic Acids in Relation to Their Antioxidant Properties

The most common priming treatments include hydropriming, osmopriming, and priming with biostimulants [24]. Sheteiwy et al. demonstrate that priming with humic materials is a promising tool for improving nano-ZnO tolerance in the rice Zhu Liang You 06 and Qian You No. 1 [25]. Priming of rice seeds with HA resulted in the improved seed germination and seedling growth. It has been recently found that application of HA significantly increases growth of the FARO-44 [26,27] and Giza 177 rice plants [44]. Along with rice, lentil seed priming with HA has been also shown to be effective in

modifying seedling vigor [45,46]. Overall, depending on the crop and priming conditions, the authors reported a 15–36% increase in VI, which is within the range of the values observed in our study for radish (9–112%) and wheat (1–37%). High values of VI indicate a vigorous seed lot [34]. The observed differences are probably explained by the fact that the effectiveness of priming depends on the species and even genotype of the crop. In addition, the concentration and timing of the treatment are also important, which is considered to be a hindrance to a wide application of the priming methodology [47]. Our findings indicate a higher sensitivity of small-seeded radish to humic material compared to wheat seeds rich in organic substances and minerals. Future research should focus on investigating the possible uptake of HA by seeds, as well as a detailed assessment of the effect of HAs on seed metabolism and early stages of crop development. Identifying specific molecular pathways that HA-O_2 activates to enhance seedling vigor would provide further insights into beneficial effect of humic materials on crop growth at the early stages.

According to the current view, seed priming activates pre-germination metabolism in seeds, triggering various biosynthesis processes, including antioxidants, which help in radical protrusion and enhance the antioxidant defense system against DNA damage [48]. Thus, applying exogenous antioxidants during priming has been shown to be effective in increasing seedling vigorousness [49]. The observed relationship between the antiradical activity of HAs and their effect on radish growth parameters is well consistent with this hypothesis (Figure 5a). The lack of a similar relationship for wheat can be attributed to the unique metabolic processes of germinating seeds of these crops. The imbibition stage was shown to be an active period for phenolic antioxidant synthesis in most seeds. However, Cevallos-Casals and Cisneros-Zevallos [50] reported no increase in both total phenolics and radical activity toward DPPH• radicals during imbibition of wheat seeds compared to dry seeds. In contrast, for radish seeds, total phenolics and radical activity were significantly higher in imbibed seeds compared to dormant seeds, indicating that phenolic compounds with more DPPH• reactive hydroxyl groups have been synthesized during water imbibition [50]. On the other hand, the initial quality of seeds affects their response to priming [51]. The germination rate of unprimed wheat seeds in our study added up to 100%, while it added up to only 93% for radish (Table 7). Therefore, one can expect a greater sensitivity of radish seeds to various treatments, as observed in this study.

Our findings demonstrate the growth parameters of radish seedlings, related to antioxidant activity, as determined by their antiradical activity toward DPPH•, but not the total antioxidant capacity, derived from the phosphomolybdenum method. Therefore, the biological activity of HAs in terms of priming agents is likely to mainly depend on their antiradical activity due to the presence of phenolic moieties [30], rather than on strong reducing agents usually detected by phosphomolybdenum assays [29].

The AOC of HAs negatively correlates with the MDA content in the shoots of radish seedlings (Figure 5b). However, the data analysis presented in Figure 5 shows that a marked decrease in the content of MDA was observed only when the AOC exceeded 150 mg g^{-1}, while at lower values, the effect was less noticeable. In addition, the treatment of seeds with Retisol or Chernozem HAs with AOC in the range of 25.5–139.1 mg g^{-1} resulted in an increase in the MDA content compared to the control. The latter contradicts data on the decreased content of MDA due to priming rice and lentil seed with humic materials, which is usually interpreted as a lowered level of oxidative damage since MDA is a secondary oxidation metabolite derived from lipid hydroperoxide products [27,44–46]. The observed contradiction is most likely to be associated with the fact that a decrease in the level of MDA was noted in older seedlings (over 10 days), while we analyzed 3-day-old ones in our work. It is known that MDA content often relates to stress acclimation rather than damage, since MDA can exert a positive role by activating regulatory genes

involved in plant defense and development [52]. Therefore, a reduction in MDA levels in seedlings primed with HA at later stages may be due to a defense signaling after transient MDA accumulation; otherwise, the sustained accumulation of MDA may trigger cell death [ibid.]. The use of humic materials as priming agents has been reported as a tool for plant hormesis management: they stimulate cellular stress response, including secondary metabolite production, in order to help organisms to establish adaptive responses [53]. Further research would be beneficial to better understand the complex relationship between HA priming and oxidative stress.

In summary, our findings demonstrate a positive relationship between the biological activity of HAs and their antioxidant activity due to the presence of phenolic moieties possessing antiradical activity. The main directions of future research should focus primarily on identifying the molecular mechanisms of the beneficial effect of humic substances as priming agents on a wide range of crops. Special attention should be paid to assessing the possibility of enhancing the humic acid activity through targeted oxidation to increase the content of phenolic groups. Since it is difficult to reproduce laboratory conditions in real-world settings, it is essential to conduct field experiments to evaluate the effect of seed priming with humic materials on yield and quality.

5. Conclusions

Alkaline extraction in air vs. under nitrogen resulted in significant changes in the properties of HA preparations from compost: a 1.2-fold increase in the oxygen content, optical density, and relative content of oxidized aliphatic fragments (^{13}C-NMR data); elevated levels of paramagnetic centers (1.3-fold); and a 2-fold increase in the content of functional groups. Extraction in air caused an increase in antiradical and total antioxidant activities of all HAs under the study and enhanced biological activities during seed priming. Radish-seed priming with HAs-O_2 resulted in a 1.1–2.1-fold increase in VI. The VI of radish seeds primed with HAs-O_2 exceeded that after treatment with HAs-N_2 1.3, 1.1, and 1.8 times for HAR, HACh, and HAC, respectively. No priming effect was found for wheat. Thus, our findings show a higher sensitivity of small-seeded crops to humic material compared to large-seeded ones rich in organic substances and minerals. Overall, alkaline treatment in air enhances antiradical and biological activities of HA, making such preparations attractive for use as natural antioxidants and priming agents. To go deeper inside, further research is needed to reproduce the observed beneficial effect of HA priming on a wide range of crops, both in laboratory and field studies. In addition, a detailed study of the processes occurring in seeds due to seed treatment with HA will allow us to develop ways to increase effectiveness of biologically active humic substances through their targeted modification via oxidation.

Author Contributions: Conceptualization, A.Z. and N.K.; methodology, A.Z. and N.K.; validation, A.Z. and N.K.; formal analysis, I.D., A.Z., A.N., O.P. and N.K.; investigation, I.D., A.Z., A.N., O.P. and N.K.; resources, A.Z. and N.K.; writing—original draft preparation, A.Z. and N.K.; writing—review and editing, A.Z. and N.K.; visualization, I.D., A.Z. and N.K.; project administration, A.Z. and N.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Russian Science Foundation grant number 23-14-00152 (extraction of HA from compost and its physicochemical characterization) and by the Federal budget of the Russian Federation, grant numbers CIT&S no. 116020110002-8 (biological activities of humic acids and their action as priming agents) and CIT&S no. 121040800154-8 (antioxidant activity assays).

Data Availability Statement: *Raphanus raphanistrum* ssp. *sativus* L. cultivar Soffit (variety code in the Russian State Register 8902208, originator is the Federal State Budgetary Scientific Institution "Federal Scientific Center for Vegetable Growing", Russia) and spring common wheat *Triticum aestivum* L.

cultivar Ivolga (the variety code in the Russian State Register: 8801193, the originator is the Russian State Agrarian University, Moscow Timiryazev Agricultural Academy). Radish and wheat seeds were kindly provided by Dr. T.I. Khusnetdinova (Department of Soil Science, Lomonosov Moscow State University, Moscow, Russia) and by Prof. D.V. Vinogradov (Ryazan State Agrotechnological University named after P.A. Kostychev, Ryazan, Russia).

Acknowledgments: The EPR analysis data of HAC provided by S.N.Chukov and ^{13}C-NMR analysis performed by A.I.Konstantinov are gratefully acknowledged. The authors acknowledge support from Lomonosov Moscow State University Program of Development (gel-filtration analysis using Bio-Rad chromatographic equipment (Biologic LP, USA)).

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

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