

Chelating abilities, chemical, spectroscopic, and biological characterization of humic acids derived from solid olive cake compost

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Abstract

Composting of the solid waste generated in large amount in a short time interval from olive industry environmentally very attractive due to increasing its value. On the other hand various authors showed that the characteristics of humic acids depend on the parent material they are extracted of, in addition to many other factors including composting conditions and geographical region. In the present work four samples of pure solid olive mill waste (olive cake) was naturally composted under four different atmospheric conditions without addition of any plant residues or any nutrients, for more than one year. Humic acids (HAs) were extracted from the composts and they were characterized by elemental analysis, FTIR, UV-visible spectroscopy and titration for reactive groups. The results show that the compost prepared as a compact small sphere of diameter 20cm and kept in door at room temperature for more than one year gives a reduced form of humic acids of high maturity, high aromatic content, high carboxylic and phenolic contents and highest chelating ability for Cu⁺² ions when compared with the results of five literature concerning commercial humic acids, The results of biological activity tests indicate *in vitro*, good antioxidant and anti bacterial activity, also exhibited mild antitumer but weak antiviral activity.

Keywords: Cu⁺² ions removal / humic acids / solid olive mill waste / composting / elemental analysis / spectroscopic characterization

Introduction

Humic substances (HS) or humus represent mixture of relatively small organic components, which form supramolecular structures held together by dispersive forces such as π - π and van der Waals interactions De Pasquale et al. (2008). Assessment of the best analytical method for

complete HS characterization is still being discussed, Naděžda and Lubica (2010). They are often, classified into three major groups (fractions) according to their solubility; humic acids (HA) insoluble below pH 2, fulvic acids (FA) soluble at any pH and humins insoluble at any pH. The HA fraction consist aliphatic and aromatic structures with linked peptides, amino

compounds, and fatty acids Schnitzer and Khan (1978).

Humic acids contain large number of functional groups. The commonly encountered ionizable functional groups include carboxylic phenolic, alcoholic groups, ammonium ions, and thiols. To a lesser extent, sulfonic and "active methylene Gyula (2012). Due to poly functionality, humic acids are one of the most powerful chelating agents among natural organic substances. They are ideal as sorption medium for a number of water impurities and especially for heavy metals Chatterjee et al. (2013).

Heavy metals penetrate the soil from various sources and modify the soil properties. Heavy metals are a serious problem for the whole ecosystem particular. They are taken up by plants and then passed on to subsequent links in the food chain. While Cu is an essential micronutrient, exposure to excess Cu has detrimental effects. It has toxic effect on agriculture, environment and human health. In plant, it inhibit growth, interfere with important cellular processes such as photosynthesis and respiration Prasad and Strzalka, (2002), cause oxidative stress in plants and has many other hazard effects García et al. (1999) Humic acids and their derivatives can be used for removing toxic metals and their ion from wastewater and lowers the concentrations of undesirable ions below the permissible limit and in soil remediation at contaminated sites as washing agents for cleaning heavy metal polluted soils Rebhun et al. (1998). It was indicated that humate can enhance removal of phenols, oils and copper in biological system. They also can support growth of the biomass as carbon source and stimulating growth agent Kim et al. (1990).

Various authors showed that the characteristics of humic acids depend on the parent material they are extracted from (Rice and Mac Carthy 1991 and Lobartini et al. 1992) and on the method of extraction, (Yamamoto et al. 1994 and Makarov 1999).

In addition to the above facts, humus regulate the biological activity and fertility of soils (Chouliaras et al. 1998 and Gougoulias et al. 2010). They exert antiviral activities against various DNA as well as RNA viruses. Scientific studies have documented the antiviral effects of humic acid against a multitude of viruses including and influenza, hepatitis, herpes simplex, HIV-1, West Nile, retroviruses, and hemorrhagic fever. Specific properties of humic acid products enable their application in industry, agriculture, environmental

and biomedicine Eladia et. al. (2005) .So They are powerful biologic materials, that will soon Play major role in fixing many environmental problems.

On other hand, very important amounts of olive mill waste are produced in olive cultivation areas. The profitable use of these organic materials are beneficial both to economical value as renewable resources and environmental protection.

According to the statistics of the Egyptian Ministry of Agriculture (2001), the cultivated area of olive trees about 113.080 thousand acres and the average production per acre of 3.8 tons. The pressing of 100 kg of olives (with added water during process extraction) produces on average 60 kg of olive solid waste and 100 to 240 kg of olive mill waste water. These wastes are important source of pollution owing to its relatively high content of organic matter The disposal of these wastes leads to serious environmental problems in olive oil producing areas. The potentially toxic attributes also to the intense of short time interval of production which increases the environmental hazards. In Egypt, olive wastes as other agriculture wastes enormous wealth yet to be exploited.

Composting has been shown to be a suitable method for recycling agriculture wastes, where these organic wastes can be converted into humus, in a matter of a few weeks or months. The naturally occurring microorganisms such as bacteria and fungi, small invertebrates, such as earthworms and millipedes, help to complete the composting process. The produced humus consists of partly decomposed organic materials and newly formed humic like and fulvic like acids. There are many procedures used in composting olive mill solid wastes (Madej et al., 1998; Paredes et al., 2000 and Negro and Solano, 1996). The composting process requires adequate conditions of pH, temperature, moisture, oxygenation and nutrients, to allow the adequate development of the microbial population De Bertoldi (1992). Therefore, changes in these conditions during the process will affect the proliferation of certain microflora, having different enzymatic activities which control the OM degradation. So the quality of humic acids can be greatly affected. Also, some degrees of differences are observed in the physical and structural properties of HA, if geographical origin differs. Barot and Bagla (2009).

It was concluded that humic acids studies are essential for a healthy and productive soil. So, without agriculture learning to accept the use of "qualified and quantified" humic acids, an

essential part of the equation of the biological management plant of soil, food security with our booming population growth will become impossible,

This work aimed to compost solid olive waste without supporting with any plant residues, no additives under uncontrolled atmospheric conditions, followed by extraction and purification of humic acids from these composite, then determine their chemical and spectroscopic characteristics, evaluate their complexing sites contents and their chelating ability for copper ions. This is to introduce the humic acids for studying the possibility of using in treatment of soil and irrigation water in olive cultivation to increase crop production and other environmental applications.

Material and methods

All chemicals used during the investigation were analytical or laboratory grade reagents. Distilled water was used for the whole experiments. A four decimal OHAUS analytical balance, was used. Experiments are made in doublets. Metal ions Solutions was standardized by atomic absorption measurements. Correction for background Cu^{+2} ion was taken into consideration.

Sampling and Composting : Four solid wastes are taken from two different factories in Cairo Egypt. Sample (I) was in form of compact and moist, sphere of about 20cm diameter. It was stored indoor in black plastic bag. Samples II, III, and IV were containing high percentage of water each was about 50 kg and stored indoor, outdoor in closed plastic bucket and in direct sun light, respectively for time intervals of 1-2 years.

Humic acids extraction and purification: Humic acids (HAI, HAI, HAIII, and HAIV are extracted from the composts I,II,III, and IV respectively. The extraction and purification procedure were according to the method developed by the International Humic Substances society (IHSS) which is considered as an acceptable method for the extraction of humic substances from soil Swift (1996) with modification includes the extraction for 6 hours. The precipitated humic acids washed till negative Cl^- test then freeze, humic acid particles were filtered under suction, and then dried in air and kept in desiccators at room temperature.

Humic acids Analysis:

Elemental analysis C, H, and N percents were determined for each humic acids on the Elemental Aero EL 111 Analyzer. The final percentages were calculated on dry ash free base. The oxygen content (%O) strictly speaking (O and S) was calculated by the difference: $\text{O} = 100\% - (\text{C} + \text{H} + \text{N})$. The Atomic ratio were calculated on dry ash free base, The degree of internal oxidation (ω) were calculated according to Maris et al. (2013) by the equation: $\omega = (2\text{O} + 3\text{N} - \text{H}) / \text{C}$ where: O, N, H, and C – are the elemental composition, in atom percentage.

Chemical analysis: Moisture content was measured by drying weighed samples at 105°C for 24 hours. Ash contents was measured by ignition in a muffle furnace at 700°C for 4 hrs, (Black 1965 and Schnitzer 1982).

Complexing sites: The phenolic and carboxylic groups are the most responsible for the development of the pH- dependent electron negative charge which have the ability to bind metal cations. The content of COOH and Phenolic hydroxyl groups is called the total acidity and is determined by the baryta method, while the carboxylic acidity is determined by the Ca-acetate method. The concentration of phenolic groups is calculated as the difference between total and carboxylic acidity the practical procedures were conducted according to Schnitzer and Umesh (1965):

Ultraviolet-Visible spectra: were carried out on potassium humates solution between 190–1100 nm on Perkin Elmer, Precisely Lambda 45, UV/VIS Spectrometer. Humate solution was prepared by dissolving 0.02 g of purified humic acids in 5 ml of 0.1N KOH solution under N_2 the pH was adjusted to 7 by adding 0.1N HCl. KCl solution was added to attain 0.20 M in 25 ml. A blank was set up under the same condition Theng et al. (1967):

Chromaticity Coefficient (E_4/E_6 ratio) The absorbencies of the above solutions at 400 nm (E_4) and at 600 nm (E_6) were recorded. The ratios of the two absorbance (E_4/E_6) were calculated for the four humic acids Iheoma et al. (2007)

FT-IR Spectra These spectra for the isolated humic acids were recorded over a wave number range $400 - 4000\text{ cm}^{-1}$ on a Jasco FTIR 460 plus spectrometer (KBr technique) where the samples were prepared by grinding the solid compound

with anhydrous potassium bromide, then pressed into the form of a disc for convenient sampling.

Metal–Humic Acid Complexation Reactions

In order to study the complexing ability of humic acids (HAI), an aqueous solution of HAI was prepared, by dissolving 0.25 g of the solid in of 0.05 M NaOH. The pH adjusted to 7 with nitric acid and NaOH and the volume was completed to 250 ml. the concentration of this solution equals to 7.31 meq/l of ionizable H^+ ions. A blank was set up in the same manner. Stock solutions of analytical grade purity $CuSO_4 \cdot 5H_2O$ was prepared (0.2 M $CuSO_4$). Two series were set up at the same conditions. In the first series (A) (represent the metal after complexation) various concentrations of Cu^{+2} were injected into 10.0 mL humic acid solution (1.462 meq/L H^+ ions) The volume in each bottle was completed to 50 ml to attain the concentrations 73, 165, 365, 625, 1380 mg /L Cu^{+2} . The second series (B) was set up in the same condition with absence of humic acid. The solutions were mixed and allowed to stand. Following adequate time for reaction and settling of copper humates, the supernatants were centrifuged for five minutes at 5000 rpm and removed. pH, conductivity, copper concentrations of the supernatants were measured with the pH meter (Hanna 210), Conductometer (EYLA), Atomic Absorption Spectrophotometer (AA Analyst 800, Perkin Elmer, USA) respectively. The FTIR spectra of a precipitate is recorded over a wave number range 400 – 4000 cm^{-1} on the Jasco FTIR 460 plus spectrometer.

HAI solution for the biological tests

0.1g of HAI was dissolved in a minimum amount of 0.05 NaOH. The pH was adjusted to 6-7 by 0.1 N HCl and the volume was completed to 100ml.

Biological Evaluation

Antimicrobial activity Assay The preliminary antimicrobial activity was investigated on the humic acid. All microbial strains were provided from culture collection of the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt. The antimicrobial profile was tested against two Gram-positive bacterial species (*Bacillus subtilis*, *Staphylococcus aureus*), two Gram negative bacterial species (*Escherichia coli*, *Pseudomonas*

aeruginosa), on three fungi (*penicillium italicum*, *Geotrichum candidum* and *Aspergillus funigtus* using a modified well diffusion method (Klančnik et al., 2010).

Antitumor activity assay: Human breast carcinoma (MCF-7), colon adenocarcinoma (HCT-116), cervical carcinoma (HeLa), hepatocellular carcinoma (HepG2), prostate carcinoma (PC-3), and lung adenocarcinoma (A-549) cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The cells were grown on RPMI-1640 medium supplemented with 10% inactivated fetal calf serum and 50 μ g/ml gentamycin. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and were subcultured two to three times a week. For antitumor assays, the work was conducted according to (Mosmann, 1983; Elaasser et al., 2011).

Antioxidant activity assay: The antioxidant activity of extract or purified compound (s) was determined at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University by the DPPH free radical scavenging assay in triplicate and average values were considered. The work was conducted according to Xi and Schaich, (2014)

Antiviral activity assay: The screening antiviral assay system using cytopathic effect inhibition assay at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University. This assay was selected to show specific inhibition of a biologic function, i.e., cytopathic effect (CPE) in susceptible mammalian cells according to Vijayan et al., (2004)

Results and discussion

Humic acids analysis: Elemental analysis: The atomic ratio, H/C: N/C: O/C can be used to identify the type of humic acids source (Alken et al. 1985⁴⁰; Kim 1998⁴¹; Polak and Sułkowski 2006). Also, the atomic ratios C/N, H/C and O/C for soil humic acids give indications about the humic acids nature: C/N (of range 10.1 to 30) is an index of organic material maturity. H/C is a measure of aromatization and condensation (Larger for aliphatic, lower for aromatic and high content of free radical). O/C (of range 0.36-0.68) reflects the increase of oxygen functional groups,

(carboxylic), (Sparks 1999⁴³, Gabbour and Daves 2001 Polak and Sułkowski, 2006). Table (1) shows the ash content, chromaticity ratios and the properties based on the elemental analysis, of humic acids HAI, HAI, HAI and HAI extracted from composts, I,II, III, and IV respectively, in comparison with that of HAsoil and HA_{T12} extracted from an Egyptian soil and

composted olive cake which supported with straw and olive mill waste water after one year respectively. Also, compared with, HA_{LSoilR}, the average elemental analysis of 410 samples extracted from different environments all over the world which were compiled from the literature by James and MacCarthy (1991).

Table (1) Elemntal characteristics of humic acids extracted from composted olive cake under different atmospheric condition (on dry ash free base)

| HA | % | | | | | | Atomic ratios | | | | E ₄ /E ₆ | Empirical formula |
|----------------------|-------|-------|------------|----------|---------|------------|---------------|-----------|-----------|------|--------------------------------|--|
| | ω | Ash | C | H | N | O | C/N | H/C | O/C | N/C | | |
| HAI | -1.5 | 0.39 | 60.70 | 11.70 | 3.28 | 24.32 | 21.59 | 2.30 | 0.30 | 0.05 | 6.04 | C ₂₂ H ₅₀ O ₇ N |
| HAI | -3.22 | 1.05 | 67.00 | 19.42 | 2.64 | 10.94 | 29.61 | 3.48 | 0.12 | 0.04 | 4.91 | C ₂₇ H ₉₂ ON |
| HAI | -3.86 | 0.67 | 69.76 | 22.93 | 2.90 | 4.41 | 28.06 | 3.94 | 0.05 | 0.04 | 4.34 | C ₂₈ H ₁₁₁ ON |
| HAI | -3.11 | 0.89 | 88.16 | 23.47 | 2.10 | 1.27 | 48.98 | 3.20 | 0.01 | 0.02 | 4.34 | C ₁₀₅ H ₃₃₅ O _{N₂} |
| HA _{Soil} | -1.00 | 0.00 | 47.00 | 5.78 | 3.09 | 44.13 | 17.75 | 1.48 | 0.70 | 0.06 | 5.29 | C ₁₈ H ₂₆ O ₃ N |
| HA _{LSoilR} | - | - | 37.18-64.1 | 1.64-8.0 | 0.5-7.0 | 27.1-51.98 | - | 0.08-1.77 | 0.33-0.98 | - | - | - |
| HA _{T12} | 0.071 | 11.94 | 43.13 | 5.15 | 3.6 | 36.18 | 20.50 | 1.43 | 0.5 | 0.06 | 8.00 | C ₁₄ H ₂₀ O ₉ N |

HA_{Soil} extracted from over 2mm sieve soil fraction of the same geographic region **Badr** (2015)⁴⁶. HA_{LSoilR} (410 samples), from environments all over the world were compiled from the literature, James and Maccarthy (1991) HAT12: HA extracted from composted olive cake which supported with straw and olive mill waste water after one year Ghita, et al.(2003).

It is noted that levels of C and H decreased while level of O increased and N remained more or less constant, when comparing, HAI with HAI, HAI and HAI which means that composting progressed well in compost I, So, the C/N ratio decreased while O/C ratio and the internal oxidation parameter, ω, increased. The higher the value of the O/C ratio, the higher ω, the higher the degree of humification and oxidation of humic matter Debska et al. (2012) While the elemental composition of HAI, HAI, and HAI are very far from the literature soil range (HA_{LSoilR}), the elemental composition of HAI lies within the range with hydrogen exception. Also, the atomic ratios H/C: O/C: N/C-which determine humic acids source for HAI and HA_{T12} are not completely identical due to high H content HAI and mixed source of HA_{T12}.

The positive values of degree of internal oxidation are characteristic for soil with aerobic conditions. HAI, HAI, HAI, HAI show negative degrees of internal oxidation, confirming anaerobic conditions prevailing during diagenetic transformation. Accordingly HAI, HAI, HAI and HAI, are in their reduced form Orlov (1985), which is the case as artificially humic acids. However, HAI is the most oxidized i.e, it expected to have the highest carboxylic groups in comparison with HAI, HAI and HAI. Considering the condition of compost I, and in accordance with Kim (2014), the well drained soil,

which is under good aerobic condition can affect humic matter oxidations, it can be reported that solid olive waste with good humidity and under good aerobic condition can affect humic matter oxidation.

E₄/E₆: is an indicator of the degree of humification. A high ratio infers a low molecular weight and a greater level of aliphaticity. A low ratio however indicates a higher molecular weight with greater level of condensation or aromaticity. Sim Siong et al. (2007). E₄/E₆ of HAI can not be compared with HAI, HAI, and HAI as their elemental properties lies far outside the humic acids range. However the molecular weight order can be arranged as: HA_{Soil} > HAI > HA_{T12}.

The van Krevelen diagram is suited to differentiate humic substances. This diagram confirm that some preferred composition, or a relatively narrow range of compositions, exists for HS in nature James and MacCarthy(1991).The diagram is used here to determine the position of HAI in relation to humic acids a from different environments which were studied by other workers. As shown in Fig (1 a, b) HAI tend to occupy the region with higher H/C and lower O/C values. This position of HAI approaches the border range of HAs in thermal waters which are characterized by higher aromaticity and depletion in oxygen than usually experienced for humic materials in soil and fresh water Kovács et al. (2012).

Table (2). Attributions of main infra-red peaks of the humic acids extracted from composted olive mill solid wastes under different conditions. (Jlkovic e t al.. 2001 ; Naidja et al. 2002 Iheoma et al., 2007; Erdogan et al., 2007 ; Giovanela et al.,2010 Peruma et al. ; 2013)

| $\nu \text{ cm}^{-1}$ | assignments |
|-----------------------|---|
| 3404-3383 | Broad and strong band of stretching of alcoholic and phenolic hydroxyl groups, intra molecular H.bonding and probably including those of COOH and N-H |
| 2927 | Aliphatic CH stretch of CH ₂ and CH ₃ also CH ₂ -C=C- in aliphatic or aromatic |
| 2857 | the aliphatic bands C-H |
| 1713 | the C=O stretching vibrations due to protonated carboxylic groups |
| 1654-1636 | C=O stretch, amid I, quinones and aromatic C=C or/and 1-keto-2 hydroxy or amino aryl ketones |
| 1540-1512 | primary amine-NH ⁺ -, symmetric NH ₃ deformation and C=N stretch amide II- aromatic C=C vibration, C-N bend C-CH ₃ |
| 1461-1449 | C-CH ₃ |
| 1376-1380 | B-O stretch (1380-1310), Tertiary butyle group (1374-1366), in plane bend of phenolic OH |
| 1268 | correspond to COO- and to C-O The former peak indicates that part of the humic acid could exist also in the sodium form. |
| 1041 | ortho substitution, C-C-O of primary alcohol |
| 823 | out plane vibration of isolated C-H group found in bituminous brown coal also due to cis cyclic ether, epoxy compound |
| (< 1000) | substituted aromatic rings |

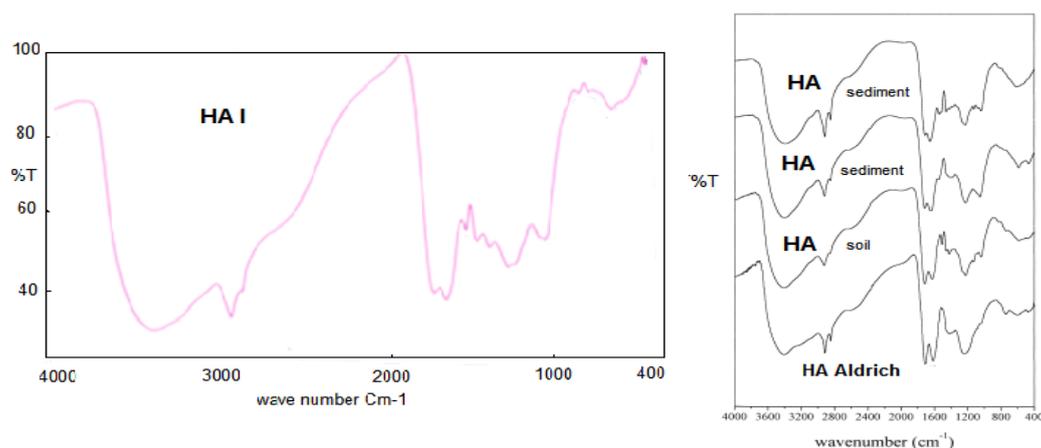


Fig (3): FTIR spectra of HAI in comparison with two sediment , one soil and Aldrich humic acids according to [Giovanela et al.\(2004\)](#):

Similar results were reported, (Ghita, et al 2003 and Senesi et al.2007). In comparison of HAI FTIR with selected spectra in Fig (3) , it is noted that HAI spectra similar to soil and sediment HA, but similar to Aldrich HA in the region from 4000 cm^{-1} to 2000 cm^{-1} which may due to strengthen and broadening of the band at 3383 cm^{-1} indicating higher content of alcoholic and phenolic hydroxyl groups, intra molecular H- bonding probably including those of COOH and N-H.

The UV- vis spectra of the humic acids solutions were recorded from 190 nm to 1100 nm. The four

Humic acid give absorption spectra with absorption increasing at lower wavelengths as the case with natural humic acids (Kim 1998 ; Giovanela et al 2010 Barison et al. 2010). The four spectra show humps at about 280 nm and maxima at 208 nm. regions This is similar with the UV-vis spectra of humic acids previously extracted from soil, Nile water hyacinth, sewage sludge, and cattle manure composites (Badr, 2003 and Ebtehar, 2011). These similarities may due to the same climate conditions, as it was confirmed by others Helena et al, (1994).

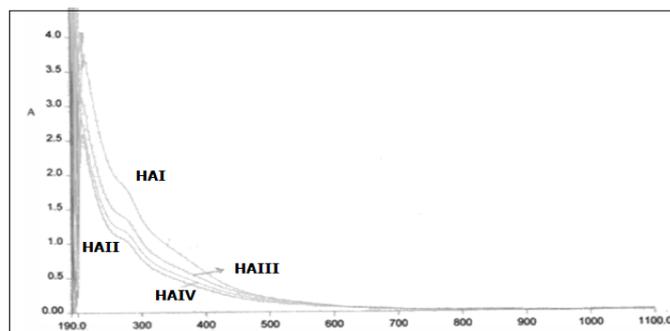


Fig. 4: UV-Vis spectra of four humic acids extracted by 0.1N NaOH and 6 hrs extraction time from olive mill solid waste composted for about 18 month under four different atmospheric conditions. (Concentration= 200mg/L in 0.2KCl,pH= 7)

Table (3) Infrared and UV-vis absorbance values belonging to wave numbers 2925 cm⁻¹, 1620 cm⁻¹ and 254nm respectively compared with the degree of internal oxidation ω

| HAs | A _{240nm} | A _{2925cm⁻¹} aliphatic | A _{1620cm⁻¹} aromatic | aromatic aliphatic | ω |
|-----|--------------------|---|--|-----------------------|-------|
| HAI | 2.05 | 0.489 | 0.444 | 0.933 | -1.5 |
| HAI | 1.18 | 0.294 | 0.116 | 0.395 | -3.22 |
| III | 1.52 | 0.498 | 0.308 | 0.618 | -3.86 |
| IV | 1.31 | 0.268 | 0.107 | 0.399 | -3.11 |

The four spectra differed in the optical densities, absorbance, at 254 nm in the order HAI> HAI> HAI> HAI which indicate increasing in the concentration of the absorbing moieties in this order. Also UV- absorbance at 254 nm, has been shown to increase with increasing molecular weight, degree of condensation, and the ratio of aromatic to aliphatic moieties . Table (3) shows the aromatic / aliphatic ratios calculated from absorbance at 2925 cm⁻¹and 1620 cm⁻¹ according to Lis et al. (2005). From these results, it is observed that the order of increasing the aromatic / aliphatic ratios parallel to the order of increasing absorbance at 254 nm. This reported by Stevenson (1994). Accordingly HAI extracted from compost I is the highest humified and characterized by highest carboxylic groups as confirmed by elemental analysis.

Chemical evaluation of complexing sites

Table (4) presents the acidic functional groups contents of the extracted humic acids as determined by barium hydroxide and calcium acetate methods. The carboxyl contents of HAI and HAI cannot be determined because of their high buffering capacity at the pH of the titration end point in case of the calcium acetate procedure. It is noted that, the condition of compost I, which permit to the highest oxidation and condensation resulted in the highest total acidity of HAI, which confirm the results elemental and spectroscopic analysis. Total acidity of HAI approaches the higher values within the total acidity range of soil humic acids means that HAI has a high ability to bind positively charged ions.

Table (4) Total acidity (T.A.), contents of COOH and OH groups of the four extracted humic acids

| HA | H ₂ O% | Functional group in meq/ g | | |
|-----------------|-------------------|----------------------------|----------|----------|
| | | T.A. | COOH | OH |
| HA _I | 9.03 | 7.31 | 3.11 | 4.20 |
| HAI | 8.12 | 2.81 | 1.87 | 0.94 |
| HAI | 8.89 | 1.73 | - | - |
| HAI | 10.95 | 2.43 | - | - |
| Soil Ref. range | -- | 5.5-8.3 | 2.4 -3.9 | 2.8 -4.4 |

Interaction between Cu(II) and olive cake humic acids

Table (5) shows the results of complexation reactions of HAI under the effect of varying copper ion concentration. Fig (5) shows the FTIR

spectra of HAI- Cu humate complex. It is observed that at the concentration of copper 73 mg/L, the pH of the solution was 4.66 and the conductance of the solution was 39.4 mS/cm. However, after complex formation, the concentration of copper in the supernatant was reduced to 57 mg/L.

Moreover, the pH of the supernatant solution became 5.63 and the conductivity was also increasing to 166.4 mS/cm. These changes occur systematically with increasing copper ions. This implies that the complex formation between Cu(II) and humic acid was occurred at the slightly acidic solution. These results are in agreement with results obtained for reaction of Fe (III) with humic acid of the Buriganga River, Mohammad et al. (2010). The increase of conductivity observed can be attributed to liberation of Na⁺ ions from humate molecules because the mobility of Na⁺ ions is much higher than any other molecule in the system.

FTIR analysis of HAI –Cu humate complex showed changes in the phenolic –OH related peaks (3383 cm⁻¹), the acid related peaks 1713, 1638 and 1265 cm⁻¹ and the amide related peaks in the region 1511 cm⁻¹. The strengthening of the peak at 3383 cm⁻¹, with the remaining of the band at 1376 cm⁻¹ suggest that the phenolic OH not participate in Cu chelation by HAI. The complete disappearance of the bands at 1713, 1265 and 1511 cm⁻¹ and weakening at 1638 cm⁻¹ suggested a Cu-N coordination and an extensive decarboxylation in the humic acid structure.

Table (5) Variation of pH and conductivity with the change of concentration of Copper (II) in HAI solution(1.452meq/l).

| Before complexation | | | After complexation | | | |
|---|------|----------------------|---|------|----------------------|---------------------------|
| Concentration Of Cu(II) Solution (mg/L) | pH | Conductivity (mS/cm) | Concentration Of Cu(II) in supernatant (mg/L) | pH | Conductivity (mS/cm) | complexed Cu ions in mg/L |
| 73 | 4.66 | 039.4 | 57.0 | 5.63 | 166.4 | 16 |
| 165 | 4.33 | 070.2 | 140 | 5.46 | 196.2 | 25 |
| 365 | 4.04 | 120.1 | 317 | 5.20 | 240.0 | 48 |
| 625 | 3.77 | 205.0 | 565 | 4,74 | 319.0 | 60 |
| 1380 | 3.47 | 352.0 | 1370 | 3.70 | 472.0 | 10 |

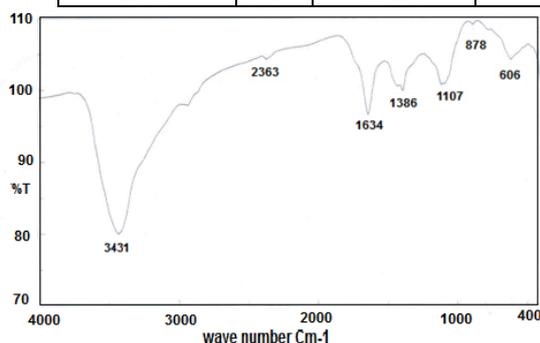


Fig. 5 FTIR spectra of HAI-Cu chelate

Determination of conditional stability constant β for the Cu(II) humate complex

The interaction of the Cu(II) ion with humic acid can be written as



the conditional stability constant β for the Cu(II) humate complex is given by

$$\beta = \frac{[\text{CuHAI}]}{[\text{Cu}^{2+}]_{\text{free}}[\text{HAI}]_{\text{free}}} \quad (2)$$

Where [CuHAI] is the concentration of the complexed Cu(II) ion, and equals to the total Cu(II) ion concentration (initial Cu(II) ion concentration) minus the non-complexed Cu(II) ion, $[\text{Cu}^{2+}]_{\text{free}}$, since hydrolysis of the Cu(II) ion at slightly acidic pH is negligible. $[\text{HAI}]_{\text{free}}$ is defined as the concentration of active sites

available for Cu(II) ion complexation and is calculated according to

$$[\text{HAI}]_{\text{free}} = [\text{HAI}]_{\text{tot}} - [\text{CuHAI}] \quad (3)$$

As is customary, the total acidity was considered to constitute the metal complexing centers of humic substances, so the total acidity content was used in place of the ligand concentration (Stevenson 1982; Senkyr et al.; 1999 Choppin, 1999 Kolokassidou et al. 2009), so

$$[\text{HAI}]_{\text{tot}} = \text{total acidity in meq/L}$$

The value of the formation constant for the Cu⁺² humate complex can be calculated by linear regression analysis of the corresponding data using equation (2) in its logarithmic form:

$$\log \frac{[\text{CuHAI}]}{[\text{HAI}]_{\text{free}}} = \log \beta - \log [\text{Cu}^{2+}]_{\text{free}} \quad (4)$$

The intercept of the line described by equation (4) corresponds to the logarithmic value of the conditional constant ($\log \beta$) and the value of the slope to the stoichiometric factor of Cu²⁺ in the equation of the complexation reaction (Kolokassidou et al. 2009).

Fig (5) gives a representation of the experimental data obtained from the above measurements which are presented as $\log ([\text{CuHAI}]/ [\text{HAI}]_{\text{free}})$ vis $\log [\text{Cu}^{2+}]_{\text{free}}$. The data results in slope of the curve, $n=1.0$, indicating that the complexation scheme suggested is correct and $\log \beta$ for the Cu(II) humate complex = 6.9. However, Table (6) gives the values of $\log \beta$ obtained in comparison with

log β for Cu²⁺ ions binding to humic acids from different sources and Table (7) gives maximum amounts Cu +2 ions removed by HAI in comparison with five commercial humic acids according to the given literatures.

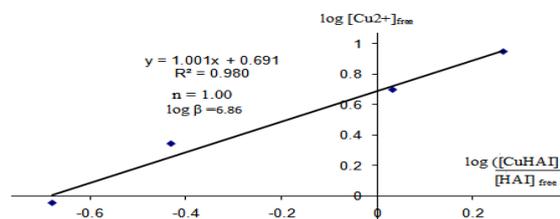


Fig 6 Representation of the experimental data given in Table (5) in terms of meq /L of the reacting species

According to these comparisons the conditional stability constant found for Cu HAI olive cake complex studied is quite comparable to those reported in the literature. In the same time it has the highest ability to remove Cu +2 ions from aqueous solution when compared with five commercial humic acids. Considering these results, it can be said that natural produced olive cake humic acids can absorb high concentrations of Cu²⁺ions, making it difficult to be liberated to the soil environment, which is very promise for use as adsorbents in the removal of heavy metals in wastewater treatment processes as natural, safe and gainful compounds.

Table (6) log β of Cu-HAI humate obtained in comparison with Log β or log K of Cu²⁺ ions binding to humic acids from some sources

| log β | pH | I in M | Method | source | Ref |
|---------|---------|--------------|------------------------------|--------------------|----------------------------|
| 8.3 | 4 | 0.01 | potentiometric Titration | soil | Stevenson and Chen(1991) |
| 5.2 | 6 | Not reported | fluorescence quenching model | Soil | Diana et al.(2006) |
| 6.9 | 3.7-4.7 | 0.002 | Cation exchange method. | olive-cake compost | Present work |
| 2.33 | 4.0 | 0.01 | exchange-resin | Aldrich | Ivana ,et al. (2011) |
| 4.9-5.7 | 6.0 | 0.1 | ion selective electrode | olive-cake compost | Kolokassidou et al. (2009) |

Table (7) Table Maximum Cu²⁺ ions removed by HAI in comparison with five Commercial humic acids according to cited literatures

| Source | Fluka AG& Aldrich Chemica I Co | Commercial origins | Commercial | HAI | Soil |
|---------------|---------------------------------|----------------------|----------------------|--------------|---------------------|
| Cu +2 in mg/g | 51.47 & 33.68 | 281 & 102 | 100 | 300 | 180 |
| ref | Beveridge and Pickering, (1980) | Jordão et al. (2001) | Barbosa et al.(2010) | Present work | Diana et al. (2006) |

Antimicrobial Evaluation

Humic acids extracted from compost I (HAI) was evaluated for their *in vitro* antibacterial activity against *Staphylococcus aureus* and *Bacillis subtilis* as examples of Gram-positive bacteria and *Pseudomonas aeruginosa* and *Escherichia coli* as examples of Gram-negative bacteria. They were also evaluated for their *in vitro* antifungal potential against representative fungal strains i.e. *Aspergillus fumigatus*, *Penicillium italicum* and *Geotrichum candidum*. The organisms were tested against the activity of solutions of concentration (1 mg/mL) and using

inhibition zone diameter in mm as criterion for the antimicrobial activity (agar well diffusion method) as summarized in Table 8. As shown by these results, the humic acid preparation displayed good *in vitro* antibacterial and antifungal action but still lower than the reference drug tested. *Bacillis subtilis* was the most susceptible microorganism tested. From the screening results, the compound exhibited the highest antibacterial activity measured in 21.9, 21.3, and 9.2 mm against *Bacillus subtilis* *Staphylococcus aureus*, and *Escherichia coli*, respectively. Interestingly, significant antifungal activity was measured against *Penicillium italicum*, *Geotrichum candidum* and *Aspergillus fumigatus*.

Table (8) *In vitro* antimicrobial activity of the tested HAI by well diffusion agar assay expressed as inhibition zone diameter (mm) in the form of mean ± SD from three replicates.*

| Tested compounds | Gram-positive bacteria | | Gram-negative bacteria | | Fungi | | |
|-----------------------|--------------------------|------------------------------|-------------------------|-------------------------------|------------------------------|-----------------------------|----------------------------|
| | <i>Bacillus subtilis</i> | <i>Staphylococcus aureus</i> | <i>Escherichia coli</i> | <i>Pseudomonas aeruginosa</i> | <i>Aspergillus fumigatus</i> | <i>Penicillium italicum</i> | <i>Geotrichum candidum</i> |
| Humic acid | 21.9±0.37 | 21.3± 0.44 | 9.2± 0.25 | NA | 17.2±0.58 | 20.9±0.44 | 20.3±0.58 |
| AmphotericinB* | - | - | - | - | 23.7±0.10 | 21.9±0.12 | 28.7±0.22 |
| Ampicillin* | 32.4±0.10 | 27.4±0.18 | - | - | - | - | - |
| Gentamicin* | - | - | 22.3±0.18 | 17.3±0.15 | - | - | - |

* Amphotericin B; Ampicillin and Gentamycin were used as reference standard; NA: No antimicrobial activity detected under these experimentation conditions; - : Not analyzed

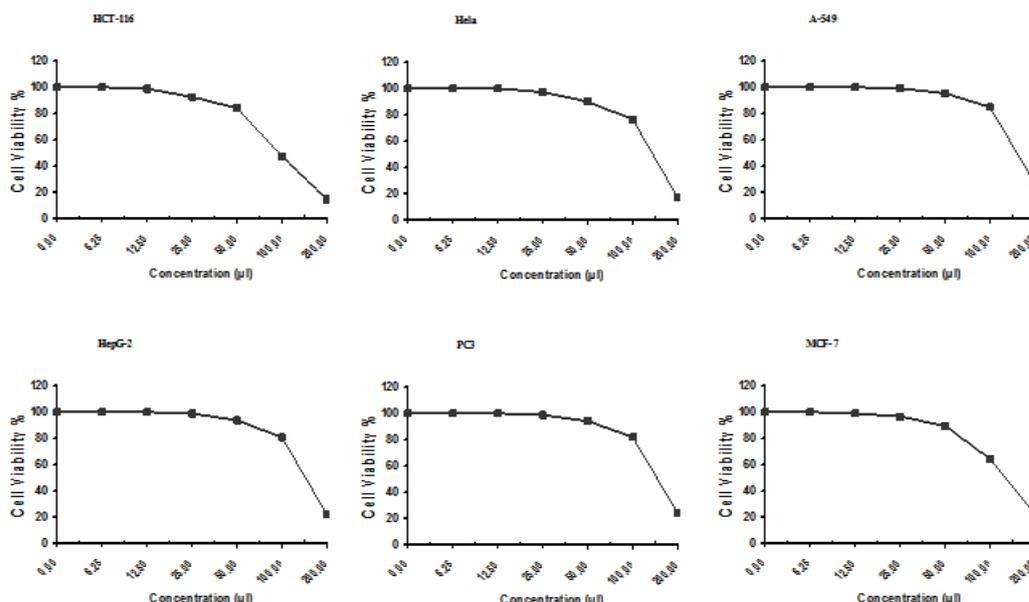


Fig 7: The dose response curve showing the *in vitro* cytotoxic effect of the humic acid (HAI) against six carcinoma cell lines.

Cytotoxic activity:

The *in vitro* growth inhibitory activity of the compound was investigated using MTT assay. Data generated were used to plot a dose response curve of which the concentration of test compounds required to kill 50% of cell population (IC₅₀) was determined and the results revealed that all the tested compounds showed inhibitory activity to the six tumor cell lines in a concentration dependent manner. The humic acid compound exhibited the highest inhibitory effect against colon adenocarcinoma (HCT-116) cell line by 85.37, 52.49, 16.05, 7.33 and 1.21% when the cells were treated with 200, 100, 50, 25, and 12.5 µg/ml, respectively (Fig. 6) with IC₅₀ value of 96.6 µg/ml. Similarly, the compound showed the inhibitory effect against breast carcinoma (MCF-7), cervical carcinoma (HeLa), hepatocellular carcinoma (HepG2), prostate carcinoma (PC-3), and lung adenocarcinoma (A-549) cell lines showing IC₅₀ values of 134, 144, 152, 155 and 160 µg/ml, respectively

Antioxidant Evaluation

Also, the synthesized humic acid exhibited good antioxidant activity with IC₅₀ value of 23.2 µg/ml when tested for *in vitro* DPPH scavenging activity (Fig. 7).

Antiviral Evaluation

The synthesized humic acid exhibited weak antiviral activity when tested against herpes simplex virus type 1 & 2 at 200 µg/ml.

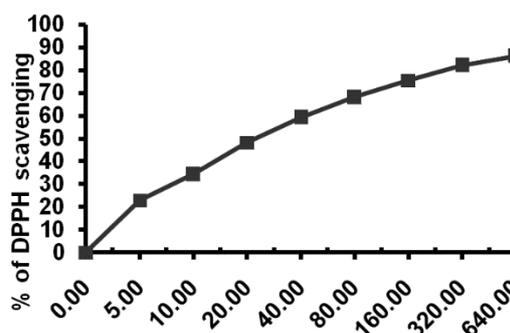


Fig 8: The dose response curve showing the *in vitro* DPPH Scavenging activity of HAI

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المخلص العربي

عنوان البحث: القدرة الكلايية والخواص الكيميائية والطيفية و البيولوجية لأحماض الهيوميك المستخلصة من كومبوستات المخلف الصلب الناتج من آلة عصر الزيتون

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لقد خلص الى ان الابحاث و الدراسات على احماض الهيوميك ضرورية لتربة صحية ومنتجة ولا بد للمختصين من فحص هذه الاحماض كيميائيا وتقدير الكميات اللازمة منها للتسميد ، حتى تتحقق الوفرة في انتاج الطعام الجيد. في هذا البحث تم استخلاص أحماض الهيوميك من كومبست تم تحضيره من

المخلفات الصلبة الناتجة من صناعة زيت الزيتون تحت ظروف طبيعية مختلفة ، وبعد تنقية الأحماض تم عمل التحليل العنصرى لها وقياس الأطياف الالكترونية واطياف الأشعة تحت الحمراء و تقدير تركيز المجموعات الفعالة وقياس قدرة الأحماض على التفاعل مع العناصر الثقيلة . وقد وجد ان الأحماض المستخلصة تشبه الأحماض المستخلصة من التربة الزراعية إلا انها تحتوي على نسبة أعلى من الهيدروجين ، وان ذلك يعود الى جاحة الكومبست -اثناء تحضيره- الى نسبة اعلى من الاكسجين (والرطوبة) . وأن هذه الأحماض تحتوى على تركيز عالى من المجموعات الفعالة التى تجعل لها قدرة مخلبية قوية . وقد تم تأكيد ذلك بدراسة تفاعلها مع ايونات النحاس ووجد أن ثابت التراكب اعلى من القيم المنشورة وان قدرتها على ازالة أيونات النحاس الثنائية من المحاليل المائية تفوق قدرة كل حمض من خمسة احماض هيوميك تجارية طبقا للقيم المنشورة فى الأبحاث ، يعنى هذا إمكانية دراسة استعمالها فى معالجة المياه وإزالة العناصر الثقيلة كمواد طبيعية آمنة ومفيدة للتربة وزيادة الانتاج فى مجال الزراعة فى مصر . و قد اوضحت الأختبارات أن لهذه الأحماض أيضا نشاط بيولوجي ضد بعض انواع البكتيريا والفطريات ونشاط ضعيف ضد الفيروسات والاورام كما أن لها قدرة مؤثرة كمضادة للأكسدة.