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# Chemical and Spectroscopic Studies on a Humic Acid Extracted from Clover Straw

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# ABSTRACT



This study investigates the chemical composition and structural characteristics of humic acid (HA) extracted from clover straw using chemical and spectroscopic analyses. The extraction process involved using 0.1N NaOH under nitrogen, followed by acid precipitation, dialysis, and freeze-drying. The isolated HA was analyzed through elemental analysis, functional group quantification, Fourier-transform infrared spectroscopy (FTIR), and proton nuclear magnetic resonance (1H-NMR) spectroscopy. Elemental analysis revealed that carbon (53.9%) and oxygen (36.0%) were the main elements, with smaller amounts of hydrogen (5.2%), nitrogen (4.3%), and sulfur (0.6%). The C/N ratio (12.5) indicated a mature HA structure, while the C/H (10.4) and O/H (6.9) ratios reflected its aromaticity and oxidation levels. Functional group analysis showed high levels of carboxyl (3.82 meq/g), phenolic-OH (1.86 meq/g), and alcoholic-OH (1.83 meq/g) groups, enhancing its reactivity and complexation abilities. FTIR analysis identified major absorption bands related to hydrogen-bonded OH, aliphatic C-H, aromatic C=C, carbonyl (C=O), and polysacchariderelated C-O functional groups, confirming the presence of various oxygen-containing groups. <sup>1</sup>H-NMR spectra displayed signals in the ranges of 6-8.2 ppm (aromatic protons), 3-5 ppm (carbohydrate and oxygenbound protons), and 0.8-3 ppm (aliphatic chains), indicating the presence of polymethylene chains and aromatic rings, suggesting a complex molecular structure. Overall, the study highlights the structural and functional diversity of HA derived from plant residues, with clover straw-derived HA exhibiting rich functional groups and a complex molecular structure.

Keywords: Humic acid, FTIR, 1H-NMR, functional groups

# INTRODUCTION

Humic acids (HAs) are complex organic substances formed during biogeochemical degradation of plant debris and animal residues and condensation of the degraded system. Humic acids often play important roles in environmental processes governing the fate and transport to organic pollutants in natural systems (De-Melo et al. Humic acids are dark-colored. 2016). acidic. predominantly aromatic, hydrophilic, chemically complex, and polyelectrolyte like material that range in the molecular weights from a few hundred to several thousands (Chianese et al. 2020).The chemical composition, structures, and conformation of HA may vary greatly, depending on the origin of its source material (Ukalska-Jaruga et al. 2021).

The elementary analysis of HA<sub>s</sub> provides information on the distribution of carbon, hydrogen, nitrogen, and oxygen. Spectroscopic techniques such as Fourier Transform Infrared Spectroscopy (FTIR) and Proton Nuclear Magnetic Resonance (<sup>1</sup>H-NMR) spectroscopy offer deeper insights into the functional groups, bonding arrangements, and structural features of HA.

The elementary analysis of humic acids (HA<sub>s</sub>) involves determining the elemental composition, which typically includes carbon (C), hydrogen (H), nitrogen (N), sulfur (S) and oxygen (O). This analysis provides valuable insights into the fundamental structure of humic acid and helps in understanding their chemical nature and reactivity.

These elemental data are essential for understanding the role of humic acid in soil chemistry, water retention, and environmental processes (Helal *et al.* 2011).

Infrared (IR) spectroscopy is a valuable technique; hence, it provides key information regarding the nature, reactivity, and structural arrangement of oxygen. Containing functional groups and the occurrence of protein, carbohydrate constituents, and inorganic impurities (metal ions, clay) in the extracted humic acids. Also, infrared (IR) analysis HA contributed substantially to knowledge of the chemistry of humic acid (Terkhi *et al.* 2008; Song *et al.* 2023).

Nuclear Magnetic Resonance (<sup>1</sup>H-NMR) spectroscopy is a powerful analytical technique widely used to elucidate the molecular structure of organic compounds. It provides detailed information about the hydrogen atoms (protons) in a molecule, including their chemical environment, number, and connectivity. In the context of humic substances. <sup>1</sup>H-NMR allows researchers to identify the presence of various functional groups, such as aliphatic chains, aromatic rings, and carboxylic acids, contributing to a deeper understanding of their structural complexity. The ability of 1H-NMR to distinguish between different proton types makes it an essential tool for characterizing the chemical composition and functional diversity of humic acid derived from plant residues and other organic materials (Machado et al. 2020).

The aim of this study is to analyze the chemical structure of humic acid extracted from clover straw. This will

be achieved through elemental analysis, quantification of functional groups, and the use of FTIR and 1H-NMR spectroscopy.

# **MATERIALS AND METHODS**

#### Humic acid extraction:

The humic acid fraction was obtained from Clover straw. The humic acid fraction was extracted with 0.1N NaOH solution under nitrogen using at a straw-to-solution ratio of 1:5 at room temperature as proposed by Piccolo, (1992). The suspensions were left overnight, then filtered. The filtrate was acidified with pure HCl to pH 2 to precipitate the humic acid. The extracted humic acid was transferred to dialysis cellophane bags and dialyzed against distilled water, changed at frequent intervals, until negative result for chloride were obtained in the water outside the bags.The humic acid fraction was dried at - 40°C under vacuum in a freeze-drier and kept as a powder for analysis. **Ash content:** 

Ash content of the isolated was determined by ignition at 750  $^{\circ}\mathrm{C}$  for 4hrs.

#### Elemental analysis:

Analysis of the humic acid for carbon, nitrogen and hydrogen were performed by the dry combustion method which described by Yonebayashi & Hattori, (1988). Depending on the same reference, sulfur was determined using schoniger–oxygen flask combustion. Oxygen was calculate by difference (C%+N%+H%+S %) from 100.

#### Functional groups analysis:

Total acidity and Carboxyl groups of the humic acid were determined by the Ba  $(OH)_2$  and Ca  $(CH_3 COO)_2$ methods, respectively. Total hydroxyl groups were determined by the acetylation method (Schnitzer and Gupta, 1965). Phenolic OH groups were estimated by the difference between total acidity and carboxyl groups, whereas alcoholic OH groups were due to the difference between total and phenolic OH groups (Kononova, 1966). **Infrared spectra (IR):** 

Infrared spectra (IR) of the studied humic acid is carried out using the KBr pellets technique using a Pye-Unicam Sp-1100 Spectrophotometer. The pellet was prepared by mixing and pressing 1.0 mg of the humic acid with 400 mg KBr (Sardessai & Wahidullah, 1998). The spectrum was recorded over the range of 4000- 2.00 cm<sup>-1</sup>. <sup>1</sup>H-NMR:

<sup>1</sup>HNMR spectra was recorded on Bruker avance III HD FT-high resolution-NMR 400 MHZ at Faculty of Pharmacy, Mansoura University; chemical shifts are expressed in  $\delta$  ppm with reference to TMS.

#### **RESULTS AND DISCUSSION**

#### **Elementary analysis**

Elementary analysis of the studied humic acid is given in Table 1. The results reveal that the percentage value of carbon, nitrogen, hydrogen, sulfur and oxygen are 53.9%, 4.3%, 5.2%, 0.6 and 36.0%, respectively. It was shown that the predominant element of humic acid was carbon (53.9%) followed by oxygen (36%). The importance of element analysis appeared in the calculation of the humic acid empirical formula.

Data in Table 1 also show that C/N ratio of the humic acid is a narrow ratio (12.5), and this ratio indicated

that the extracted humic acid is in a mature state. The C/H ratio of HA (10.4) is considered parallel to the degree of condensation of the aromatic rings in humic substances. The O/H ratio of HA is found to be 6.9. This value indicates more oxidation and hydrogenation of the molecule.

 Table 1. Elemental composition and ratios of humic

 acid

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Element	Va	lue	Atomic ratio	Value
Carbon	53	3.9	C/N	12.5
Nitrogen	4.	30	C/H	10.4
Hydrogen	5.	20	O/H	6.90
Sulfur	0.	60	Ash,%	1.12
Oxygen	30	5.0		

High carbon content suggests a structure rich in aromatic and aliphatic components, indicating a complex and condensed molecular network typical of well-humified organic matter. Elevated oxygen percentage reflects a significant abundance of oxygen-containing functional groups such as carboxyl (-COOH) and hydroxyl (-OH), which are critical for cation exchange capacity and nutrient retention. Nitrogen content points to the partial preservation of proteinaceous or amine-based residues, enhancing HA's potential as a source of organic nitrogen. The C/N ratio (12.5) indicates a stable and mature humic substance, typical of well-decomposed organic material with a reduced microbial degradability. A high C/H ratio (10.4) implies a greater degree of aromaticity over aliphatic character, associated with higher chemical stability and resistance to microbial degradation. The O/H ratio (6.90) also highlights the richness in polar, oxygenated functional groups, contributing to HA's reactivity and environmental functionality. These findings suggest that the HA extracted from clover straw possesses chemical features that make it highly suitable for agricultural and environmental applications.

According to De-Melo *et al.* (2016), high carbon and oxygen contents are indicative of a structurally complex humic material with high reactivity and potential for improving soil chemical and physical properties. Moreover, the C/N ratio falling within the range of 10-15is often considered a hallmark of stable humic substances, as noted by Chianese *et al.* (2020), which supports their role in long-term soil fertility. The abundance of oxygenated functional groups also enables HA to chelate metal ions and enhance nutrient availability, a property empHAized in the work of Rigobelo, (2024), who demonstrated the positive influence of humic substances on root growth and nutrient uptake in plants.

#### Functional groups

As shown in Table 2, the studied HA contains some groups in relatively high content *i.e.*, carboxyl, phenolic OH and alcoholic OH. This indicates that the extracted humic acid was in a mature state, as reported by Ukalska-Jaruga *et al.* (2021). High total acidity (5.68 meq/g) suggests a strong potential for cation exchange and buffering capacity, making the HA highly reactive in soil environments. Carboxyl groups (3.82 meq/g) dominate the acidity profile, confirming their key role in the acidic behavior of HA. These groups are primarily responsible for metal ion complexation and nutrient binding. Total OH groups (3.69 meq/g) indicate a substantial presence of hydroxyl functionalities, contributing to the reactivity and solubility of the HA. Phenolic OH (1.86 meq/g) and alcoholic OH (1.83 meq/g) are almost equally represented, suggesting a balanced distribution of polar hydroxyl groups. Phenolic OH groups, in particular, contribute to antioxidant activity and redox interactions in the rhizosphere.

These data highlight the chemical versatility of the studied HA, with high concentrations of carboxylic and hydroxyl groups that enhance its functionality in soil amendment and plant growth promotion. These findings are in harmony with those of De-Melo *et al.* (2016); Chianese *et al.* (2020); Ukalska-Jaruga *et al.* (2021).

 
 Table 2. Oxygen-containing functional groups in the studied HA (meq/g HA)

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Group (meq/g HA)	Value	
Total acidity	5.68	
СООН	3.82	
Total OH	3.69	
Phenolic OH	1.86	
Alcoholic OH	1.83	
On the basis of dry ash free HA		

#### FTIR spectroscopic analysis of humic acids

FTIR spectra of the studied humic acid showed to contain various bands that were characterized for specific molecular structures as mentioned by Bagherifam *et al.*, (2023) and Song *et al.*, (2023). The IR spectrum of humic acid under investigation is shown in Table 3 and Fig 1.

Table 3. FTIR spectra of the studied humic	acid	
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Wave length (cm <sup>-1</sup> )	Functional group
3400	Hydrogen-bonded OH
2927.92	C-H Stretching (aliphatic)
1646.74	C=O Stretching x Aromatic C=C
1217.40	C-O Stretching and OH deformation of COOH
1121.93	C-O Stretching of polysaccharide
527.92	C-Cl ( chlorine group)



Fig.1. FTIR spectra of the studied humic acid

The results revealed five distinct peaks of the FTIR spectrum that appeared at several wavelengths lying between (3000-2850 cm<sup>-1</sup>), (1760 1650cm<sup>-1</sup>) (1320-1210cm<sup>-1</sup>), (1150-1100cm<sup>-1</sup>) and (690-515cm<sup>-1</sup>). The presence of two distinted relatively near infrared bands with wavenumber of 3400 and 2900 cm<sup>-1</sup>. These absorption bands may be due to hydrogen-bonded OH and aliphatic C-H stretch, respectively.

The infrared spectrum revealed a sharp peak at region 2927.92 cm<sup>-1</sup> which corresponded to C-H aliphatic

stretching groups. Another band accurred at wavelength 1646.74 cm<sup>-1</sup>. This band could be assigned as (C=O aromatic stretching group) of conjugated quinines (Bagherifam *et al.* 2023). A noticeable peaks were observed at 1217.40 and 1121.93 cm-1 regions which attributed to C-O aliphatic stretching ether group related to carbohydrate structure (Song *et al.* 2023). The emergence of the peak at region 527.92 could be related to chlorine C-Cl group.

### <sup>1</sup>H-Nuclear Magnetic Resonance (<sup>1</sup>H-NMR)

The <sup>1</sup>H-NMR spectra of the studied humic acid is given in Fig. 2. The spectrum shows chart lack of aromatic protons and many aliphatic protons of the sugar moiety with their characteristic splitting patterns, in addition to lacking formyl and acid protons also. The peaks are very broad, not sharp.

As shown in Fig. 2, it is clear that the spectrum consists of three broad regions at chemical shifts; 6-8.2 ppm, 3-5.0 ppm and 0.8- 3ppm. In addition, there are many sharp resonances at 8.2, 6.8, 3.76, 2.2, 1.9, 1.2 and 0.8 ppm.



Fig. 2. <sup>1</sup>H-NMR spectrum of the studied humic acid

The signal from 6.0\_8.2 ppm arises from aromatic protons, including phenols, oxygen-containing heteroaromatics aromatics. Wilson, (1983) reported that protones of aromatic rings are usually present in humic materials. The fact that the aromatic signal extends as low as 6.0 ppm suggests that phenols are present. As shown in Fig. 2, many polycyclic aromatics can be characterized by a resonance of 8.1 ppm due to sterically hindered periproton (Wilson, 1983). The signal from 3.0-5.0 ppm arises from aliphatic protons, including protons to carboxylic acid groups; protons of methyl groups and methylene groups; protons of carbon attached to oxygen groups and sugars of carbohydrates. No aromatic hydrogen was identified in this signal. The sharp resonance at 3.76 and 2.19 ppm may arsie from protons of methyl groups to protons-x of carbon attached to oxygen groups, respectively.

The sharp signal at 1.25 ppm (Fig. 2) appears to be a singlet and thus may arise from methyl group\* to unsaturated groups. The resonance of 0.83 ppm indicates methyl groups of alkyl chains at least three carbons removed from deshielding groups. Newman *et al.* (1980) suggested that polymethylene protons, and protons attached to carbons B to aromatic rings, are the main contributors to this resonance of H-spectra

The chemical shifts of protons, were used to determine the structure of the humic acid sample, revealing the presence of polymethylene terminal methyl

groups in polymethylene chains. This indicates that polymethylene chains play a significant role in humic acids. Additionally, aromatic ring protons are commonly found in isolated humic acid samples.

# CONCLUSION

The humic acid extracted from clover straw is chemically mature and structurally diverse, with a high content of oxygenated functional groups, aromatic rings, and aliphatic chains. Elemental analysis confirmed its high carbon and oxygen content. FTIR and 1H-NMR spectroscopy showed a complex mixture of functional groups associated with aromatic and aliphatic structures. These properties indicate potential applications in soil amendment, metal chelation, and interacting with organic pollutant interaction in environmental and agricultural applications.

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دراسة كيميائية وطيفية لحمض الهيوميك المستخلص من تبن البرسيم

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#### الملخص

هدفت هذه الدراسة إلى تحديد التركيب الكيميائي والخصائص التركيبية لحمض الهيوميك المستخلص من تبن البرسيم باستخدام تحاليل كيميانية وطيفية. تم الاستخلاص باستخدام محلول هيدروكسيد الصوديوم ( ١, • عياري ) تحت جو من النيتروجين، تلاه الترسيب الحمضي، ثم التنقية بالغسيل والتجفيف بالتجميد. تم تقييم حمض الهيوميك الناتج من خلال التحليل العنصري، وتقدير المجاميع الفعالة، وأطيف الأشعة تحت الحمراء(FTIR) ، والرنين المغاطيسي النووي للبروتون (H-NMR). أظهرت نتائج التحليل العنصري أن الكربون ( ٥,٣٠٪) والأكسجين (٣,٦٠٪) كانا العنصرين السائدين، بينما وُجد الهيدروجين ( ٥,٢٪)، والنيتروجين ( ٤,٣٪)، والكربيت ( ٢,٠٪)، المعربي أقل ، وأشار معامل أن الكربون ( ٥,٣٠٪) والأكسجين ( ٣,٦٠٪) كانا العنصرين السائدين، بينما وُجد الهيدروجين ( ٥,٢٪)، والنيتروجين ( ٤,٠٪)، والكربيت ( ٢,٠٪) بنسب أقل. وأشار معامل ( ١٢. ( ١2.5)) إلى نضج التركيب العضوي، في حين تعكس نسب ( ١٥.4) ٢/ و ( 6.6) C/H ( 6.9) مال ( وهاتية و الأكسدة. كشفت نتائج تحليل المجاميع الفعالة عن احتواء الحمض على نسب عالية من المجاميع الكربوكسيلية ( ٣,٨٠ ميلي مكافئ/جم)، والهيدروكسيل الفينولي ( ١,٥٠٪)، والكسدة. كشفت نتائج تحليل المجاميع الفعالة عن احتواء ما يكسبه قدرة عالية من المجاميع الكربوكسيلية ( ٢,٨٠ ميلي مكافئ/جم)، والهيدروكسيل الفينولي ( ١,٥٠٠ ميلي مكافئ/جم)، والهيدروكسيل الكحولي ( ٢,٥٠ ميلي مكافئ/جم)، مالي مكافئ/جم)، والهيدروكسيل الكحولي ( ١٩٨٠ ميلي مكافئ/جم)، ما يكسبه قدرة عالية على التفاعل والتعقيد مع العناصر أظهرت أطياف FTIR وجود نطاقات امتصاص رئيسية تعود لمجمو عات المروبينية، وروابط ٢ مالاليفاتية، وروابط C=C الأروماتية، و ٣٦،٨٠ ميلي مكافئ/جم)، ومركبات عديدات السكاريد.(-O). أما ملياف المروبي الورات المريبول المدى ٦ - ٢٠ من ألمدى ٦ - ٢٠ من المون (بروتونات أروماتية)، و ٣–٥ حار (C=C) ، ومركبات عديدات السكاريد.(-O). أما أطياف رسيل الوراتية، ما يشرر المدى ٦ - من ميلي بولي ميثيلين وحلقات أروماتية)، و حرات مرتبطة بالأكسجين)، و٨,٠ حار من ألمياون (سالسل اليفاتية). ما يون تضم سلاسل بولي ميثيلين وحلقات أروماتية، وعماه النتات مرتبطة بالأكسجين)، و٨,٠ -٢ جزء في المليون (سالسل المخافات النباتية، ويمكن الاستنتاج أن تضم سلاسل بولي ميثيلين وحلقات أروماتية)، وعماء المينيوي و النوطي ي والوطيفي لمن الهيوميك الس