

## ANTIOXIDANT EFFECT OF HUMIC SUBSTANCES FROM HUNGARIAN LEONARDITE

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Humic substances are natural substances that are continuously formed from the decay of plant residues. These materials have a very diverse range of properties, making them versatile. According to many new studies, these humic substances also exhibit antioxidant propensities. The aim of this paper was to shed light on whether humic substances really have antioxidant properties.

**Keywords:** humic substances, humic acid, fulvic acid, himatomelanin acid, antioxidant, leonardite

### 1. Introduction

The majority of the population of Hungary suffers from one of the diseases associated with free radicals, e.g. diseases of civilization, obesity, cardiovascular disease, malignant neoplasms, etc. Although it is well-known that our consumption of vegetables and fruit lags far behind those of the other Member States of the European Union, it has been scientifically proven that essential fresh vitamins and provitamins as well as minerals are fundamental not only for the smooth functioning of the body but also for their antioxidant components. Furthermore, they can play a key role in terms of disease prevention. Berries have become a major player in a number of related research papers as they are also essential in the prevention as well as aftercare of cancer and cardiovascular disease. What other options are available and what other substances may still have high antioxidant contents? Based on the aforementioned points, it is also important and justified to study the antioxidant properties of new compounds such as humic substances and determine which of their components play a crucial role in the development of their antioxidant effect, both qualitatively and quantitatively. The aim of this research is to compare the antioxidant properties of humic acids with already well-known natural antioxidants. Firstly, different humic acid

fractions were prepared. The expected results should help to more accurately interpret the complex behavior of humic acids, thereby helping to expand their range of applications. Therefore, if the results are convincing, this could open up new fields with regard to the application of humic acids in the food and cosmetics industries [1].

### 2. Humic substances

Humic substances are a group of naturally occurring macromolecules found throughout nature, that is, in soil, air, water, carbon deposits and peat. Chemically ill-defined humic substances are natural organic colloids comprised of decomposition products of plant-derived biomass as the result of a process called humification. Their conversion into stabilized humic material is one of the most complex and least understood biogeochemical processes of the carbon cycle. Most natural humic acids (HA) are found in older peat, lignite and juvenile lignite. In Hungary, the HA content of the so-called leonardite, a famous source of HA, is close to 70% in its natural form. (A near-surface deposit of leonardite is located in Dudar, where this geological formation can be mined).

In the humification process, dead plant matter that enters the soil is broken down by enzymes found in soil bacteria and fungi, so simple compounds like sugar and ammonia are formed from carbohydrates, fats, proteins and lignin, which on the one hand serve as a source of food for the soil microbes but on the other hand as a source in the formation of humic substances. As a result of biotic and abiotic (condensation and polymerization) processes,

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the decomposition products form high-molecular-weight humus compounds, the presence of which is characteristic of the soil [2].

On the basis of differences in solubility, humic substances can be divided into several groups such as the main fractions humin, HA, and fulvic acids (FA), as well as the alcohol soluble himatomelanic acids (HY). They are a mixture of similarly behaving, yellow-brown-black, acidic, high-molecular, natural organic substances that are operationally defined. Humic substances themselves contain too many kinds of molecules that can be separated by changing the solubility conditions. Even though these molecules behave similarly, each fraction has different properties and their molecular structure is not uniform [2]. The nomenclature of fractions that can be separated according to their solubility is suggested in the guidelines of the International Humic Substances Society (IHSS):

- **Humin** - a black fraction of humic substances that is insoluble in water at any pH.
- **Humic acids** - the fraction of humic substances that is insoluble in water under acidic conditions (pH < 2) but soluble in water at higher pH values. They can be extracted by various alkaline solutions before being precipitated by a strong acid and are dark brown or black in color.
- **Fulvic acids** - the fraction of humic substances that is soluble in water at all pH values. Fulvic acids are light yellow or yellowish brown in color.
- **Himatomelanic acids** - the fraction of humic substances that is soluble in an alcohol.

### 3. Antioxidants

The most important physiological role of antioxidants is to neutralize the free radicals that are continuously formed in the Szent-Györgyi-Krebs cycle and counteract the free radicals with different oxidizing forces that enter the body. An antioxidant is a substance that inhibits oxidation, more broadly speaking, retards or hinders oxidation. They are chemically reducing agents, i.e. electron donors. These materials are usually organic compounds but include metals and organometallic complexes. Many different types of antioxidants exist, which usually work together and do not neutralize free radicals on their own. Although our body itself is able to produce some antioxidants, sometimes it is necessary for us to ingest these substances from external sources. Together, these antioxidants already form a very strong line of defence in our body, e.g. vitamin C, Vitamin A, flavonoids, glutathione, resveratrol, unsaturated fatty acids, etc. [3,4]

### 4. Methods for measuring the antioxidant capacity

The antioxidant capacity is the combined free radical scavenging effect of all antioxidant compounds in the

system studied. Since the need for its accurate numerical determination is growing, a number of analytical procedures and measurement systems have been developed. Given that methodologies are constantly being modified and refined, nowadays, the number of applied methods exceeds one hundred. In the literature, the majority of studies use several methods to determine the antioxidant capacity [5]. The most common methods of measuring the antioxidant capacity can be divided into two main groups: electron transition-based ones (Ferric iron Reducing Antioxidant Power, Total Polyphenol Content, Copper ion Reducing Antioxidant Capacity, Trolox Equivalent Antioxidant Capacity, and 2,2-Diphenyl-1-picrylhydrazyl (DPPH)) as well as those based on hydrogen atom transfer (Oxygen Radical Absorbance Capacity, total peroxy radical-trapping potential, chemiluminescence-based methods, and photochemiluminescence measurements just to mention the most commonly used methods).

Although these two types of measurements determine the antioxidant capacity, the results obtained do not necessarily have to be correlated with each other, since the reducing power of a sample is not necessarily related to its ability to scavenge for the reaction of test compounds.

Electron transfer reactions involve colour changes, from which the antioxidant capacity can be deduced. The essence of these methods is to create a free radical as the result of a reaction. To this free radical, the antioxidant is added at various dilutions leading to a colour change, which is monitored by a spectrophotometer and then the antioxidant capacity of the test substance is calculated from the results obtained.

Methods involving hydrogen atom transfer are based on the kinetics of the reaction. Tests measure how effective a sample is against a given free radical, namely its free radical scavenging capacity [5].

### 5. Antioxidant effect of humic substances

The question may arise as to why humic substances would exhibit an antioxidant effect. Humic acids are chemically very complex mixtures of composite molecules, that is, natural polymers formed during the varying degrees of polymerization of basic building blocks. According to their chemical structure, they are polyhydroxy carboxylic acids with quinone and semiquinone groups. In some respects, they are similar to flavonoids and phenols, in which the so-called flavone skeleton is polysubstituted by hydroxy groups. However, they also have a quinoid structure that is known to be responsible for antioxidant properties. These properties of HA have already been demonstrated in a number of scientific publications by both classical analytical methods (redox titrations) and instrumental analytical measurements (Electron Spin Resonance) [6].

The general structure of HA and the formulae of some well-known antioxidants are presented in Fig. 1 From

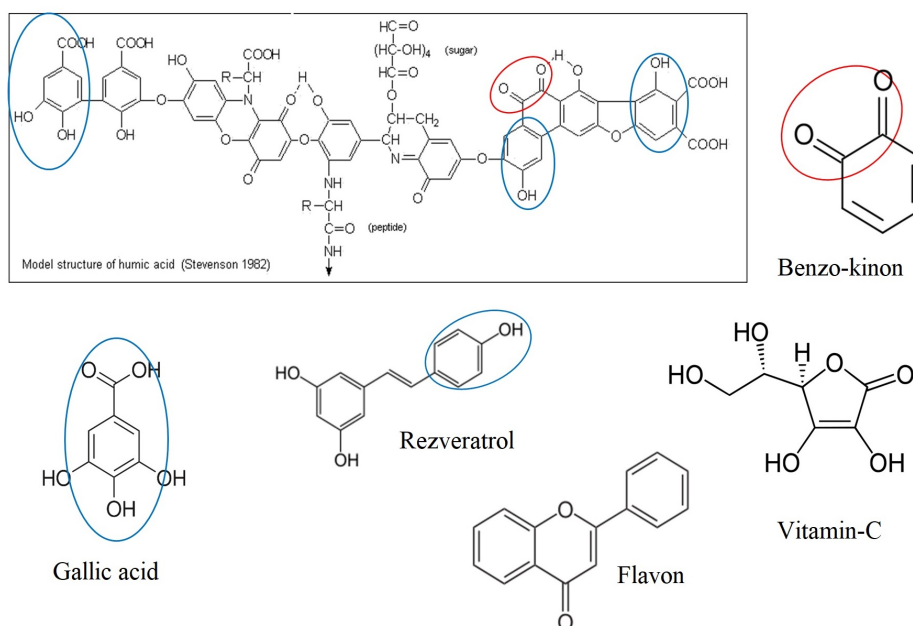


Figure 1: Model structure of HA and identical moieties of known antioxidants [2]

their structure, it is clear that HA are comprised of a number of groups such as already well-known antioxidants, so will exhibit exceptionally high antioxidant capacities [7].

## 6. Results

### 6.1 Measurement of total phenolic content

All measurements of total phenolic content were made according to the method developed by Shetty et al. [8]. During these measurements, only high-quality (a.r.) chemicals were used. The fractions of humic substances were extracted by ourselves from samples of leonardite retrieved from Dudar. Rather than indirectly measuring the antioxidant property of the sample, the method measures its total phenolic content, from which its antioxidant capacity can be deduced. The method consisted of diluting 1 ml of the sample in 5 ml of distilled water and 1 ml of 95% ethanol in a test tube before adding 0.5 ml of 50% Folin-Ciocalteu (FC) reagent (half of distilled water in half FC reagents) to each sample. After being stirred for 5 minutes, 1 ml of 5%  $\text{Na}_2\text{CO}_3$  was added to the reaction mixture and allowed to stand for 1 hour before the absorbance values of the samples were measured at 725 nm. These absorbance values were then converted into  $\mu\text{g}$  gallic acid equivalents in order to compare the measured values with each other and with other data from the literature.

Whilst measuring the total phenolic content of the samples, a series of dilutions was made from our samples of known mass, for which the total phenolic content was measured. A series of dilutions had to be produced in order to measure an absorbance value of approximately one for each sample. This was necessary to be able to

compare the samples because the three different fractions yielded one absorbance value at different concentrations once the reaction was complete. Furthermore, since the samples are coloured (brownish - dark brown), the measured absorbance values had to be calibrated in light of the background of the samples. Therefore, the samples were diluted to the concentration present in the reaction volume. After measuring the series of samples, their total phenolic content was calculated from the calibration curve, which can be seen in Fig. 2, and the results obtained are shown in Table 1.

The results show that different initial concentrations of the various samples are required to achieve a similar absorbance. In the humic acid samples, since the majority of the phenol groups are in this fraction, the lowest initial concentration is required to achieve an absorbance of approximately one. Himatomelanic acid has an average number of phenol groups while FA is comprised of the

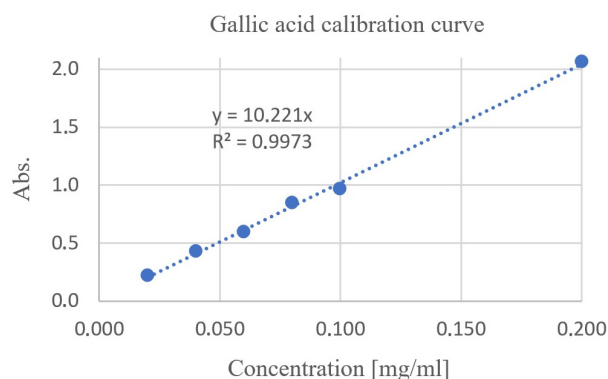


Figure 2: Calibration curve of gallic acid

Table 1: The measured and corrected absorbance of the samples and the gallic acid equivalent (GAE) values

Sample	Corrected concentration [mg/ml]	Abs measured	Abs corrected	GAE [mg/ml] from the calibration curve	GAE [mg/g]
HY	0.59	1.027	0.953	0.103	174.5
FA	1.18	0.999	0.948	0.102	86.8
HA	0.35	1.024	1.024	0.11	310.3
IHSS FA	0.684	-	1	0.107	156.8
IHSS HA	0.212	-	1	0.107	506.1

Table 2: The IC50 readings of the different samples

Name of the sample	Original concentration	IC50 [ $\mu\text{g/ml}$ ]
HY	1.5 mg/ml	200
FA	2 mg/ml	300
HA	3.25 mg/ml	460

fewest. The results suggest that the studied humic fractions are likely to exhibit an antioxidant effect.

## 6.2 DPPH method

Measurement of the antioxidant capacity based on stable DPPH radical scavenging is one of the first methods. The reaction proceeds as follows: the dark purple radical formed in the reaction mixture loses its colour when it reacts with antioxidants.

This method is widely used because the radical-forming molecule DPPH is commercially available, stable as well as not particularly reactive nor aggressive, which is beneficial in the reactions that take place as taking measurements is simple. On the other hand, a stable radical that is not found in the living organism is used instead of a radical formed during the normal metabolism in the cell. Using this method, it is not possible to estimate how effective the sample is as an antioxidant with regard to biological radicals [8].

During the measurements, 6 ml of a DPPH working solution (0.1 mg/ml) was added to one ml of the sample, vortexed and stored in the dark for 30 minutes until the colour reaction began. After 30 minutes, the absorbances were measured at 517 nm. From the measured values that had previously been corrected, the percentage of inhibition (Inhibition%, Inhib.%) and the value corresponding to 50% inhibition (IC50 value) were calculated. While the measurements were taken, in the same way as when the total phenolic content was measured, a series of dilutions was made from a sample of known concentration. From the measured absorbances, after background correction the percentages of inhibition of the samples were calculated and plotted as a function of the concentration in order to determine the IC50 values of the samples (Fig. 3).

From the data, it can be concluded that the materials prepared and tested exhibit antioxidant properties because they inhibit the decomposition of the DPPH radical.

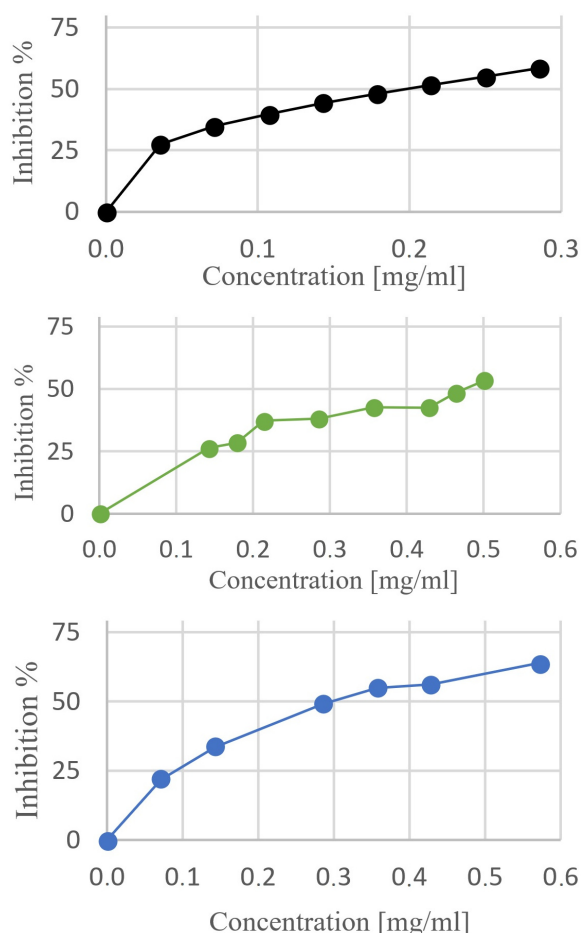


Figure 3: Percentage of inhibition of himatomelanic acid (top), humic acid (middle), and FA (bottom) as functions of the concentration to determine the IC50 values

Their IC50 values (Table 2) are promising and comparable with other data in the literature [9].

## 7. Conclusions

Although the results revealed that the samples made from domestic raw materials exhibit antioxidant properties, further studies are needed to gain a complete picture of the antioxidant capacity of these substances. Furthermore, in the future, it is our intention to confirm the results collected so far with in-vitro and in-vivo experiments.

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