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Wissam H. Abdel-Hamid; Sawsan A. H. Al-Hilifi

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Biological Characters and Antioxidant Activity of Hyaluronic Acid Isolated from Some Animal Sources

Wissam H. Abdel-Hamid and Sawsan A. H. Al-Hilifi [a)]

Department of Food Science, College of Agriculture, University of Basrah, Basrah, Iraq

[a)]Corresponding author: sawsan.hameed@uobasrah.edu.iq.

Absract. Hyaluronic acid with unique physical and chemical properties, making it widely used in biomedical, pharmaceutical, cosmetic, and nutritional supplements. Repeating units of glucuronic acid and N-acetyl glucosamine make up hyaluronic acid. Rooster combs and Fish waste, such as eyes and internal viscera, contain chemical compounds rich in antioxidants that contribute to beneficial health effects. Manufacturing processes leave large amounts of waste, which causes the problem of environmental pollution. Nevertheless, these wastes are a rich source of antioxidant compounds. In this study, some rich fish residues were highlighted with hyaluronic acid and the modern techniques used in its extraction and displaying its biological properties and antioxidant effects. This study included the extraction of hyaluronic acid from the manes of roosters and the eyes of some local fish. The extracted acid was identified by FTIR and HPLC. The antioxidant activity of extracted hyaluronic acid was also tested. The acid extracted from rooster combs had higher antioxidant properties compared to the acid extracted from fish eyes. In this study, some fish wastes rich in hyaluronic acid were highlighted, and the modern techniques used in extracting it showed their biological properties and antioxidant activity.

Keywords: Glycosaminoglycans; Hyaluronic acid; antioxidant activity; extraction; Purification.

INTRODUCTION

Biopolymers and their derivatives are various compounds found in abundance in nature that have distinctive characteristics that made them gain great interest in recent times because of their wide applications in various aspects of life. The mono units of biopolymers are sugars, amino acids and nucleotides. Examples of these vital materials are cellulose, starch, pectin, Proteins and Peptides. (Elnashar,2010; Rasel *et al*., 2017).

The use and development of biodegradable natural polymeric materials have made considerable strides in recent years, because these polymers possess important physical, chemical, and mechanical properties as well as their ability to hydrolyse and enzymatically, and among the conditions that must be present in a biopolymer are to be non-toxic, bio-available, bio-network formation during tissue development and building reactions along with properties of mechanical strength, tensile strength, etc. to match synthetic polymers (Thakur et al. 2022; Hassan et al., 2019). Biodegradable polymers have been developed in various biomaterials applications. It gained a lot of attention in the pharmaceutical industry in the 1970s, while polyesters are the first successful biodegradable polymers developed in the textile and sewing industries (Song et al. 2018; Samir et al., 2022). Many biopolymers in nature differ among themselves in the structural unit involved in their structure (Singh 2011; Brigham 2018). Hyaluronic acid HA is an organic chemical hydrocarbon compound that can also be called sulfur-free hyaluronan (Hemshekhar et al., 2016; Zhai et al., 2020). It is a naturally occurring, high molecular weight, bio saccharide viscous substance, in 1934, Karl Meyer and his helper John Palmer saw something interesting in the eyeglasses of bovines. HA, is a natural biopolymer found in the body tissues such as eyes, cartilage, and skin. This substance forms the basis of the connective tissue of the skin. This substance combines with water, as it can bind to water molecules by 1000 times its weight, as it acts as a moisture absorber (Birajdar et al., 2021). It has important biological functions in humans, animals and bacteria alike. It is spread in most of the connective, epithelial and nervous tissues (Khabarov, Boykov, and Selyanin 2015), and has a high concentration in both synovial fluid and the vitreous humor of the eye. It is made up of disaccharide molecules linked together via -1,4 glycosidic bonds and D-glucuronic acid and N-acetyl-D-

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glucosamine units linked via -1,3 glycosidic bonds. Rooster combs are still considered one of the most important traditional sources for extracting HA (Chahuki, *e t al*., 2019). The extraction process to obtain HA, specifically from animal sources, includes: Three main stages: preparation, extraction and purification. HA is a large molecule that has been studied extensively due to its versatility biological roles, potential applications in biomedical engineering such as cancer, wound healing, osteoporosis, in addition to treating skin problems and maintaining its appearance. Antioxidant properties of HA have been demonstrated in vitro and in vivo in recent years (Ke et al., 2011). Because of recent trends in moving away from everything that is manufactured and finding natural alternative sources of antioxidants in the field of food industry and preservation. This study came to shed light on the antioxidant properties Retrieved hyaluronic acid from rooster comb and local fish waste.

MATERIALS AND METHODS

Chemicals

Acetic acid, acetone1, 1-diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid, chloroform, and acetic acid were obtained from Merck Chemicals Co. (Darmstadt, Germany). Butylated hydroxytoluene (BHT), Potassium ferricyanide (K4[Fe(CN)6].3H2O), Sodium Chloride (NaCl), Hydrogen Peroxide (H2O2), Sulphuric acid (H2SO4), Ferric chloride (FeCl3) were purchased from Sigma-Aldrich (Merck).

Raw Materials

Rooster comb was collected from the local slaughterhouses as a by-product of cleaning the chickens in the city of Basrah, while the eyes of the fish were obtained from the local markets, where the animal tissues were cleaned with water several times to get rid of dirt and blood residues resulting from the cleaning operations. The samples were then rinsed in distilled water, then it was poured into petri dishes and dried at 40 °C for 48 h, depending on the type of animal tissue being used. The fine powder was stored in light-permeable containers at -3 ± 18 °C before the next analysis.

Extraction of Hyaluronic Acid From Animal Sources

HA was extracted using a method similar to that reported previously Kang et al. (2010) with some minor modifications. First, 500 g of rooster combs and fish eyes of frozen rooster combs were ground in an electric grinder, to around 0.5 cm in size, and then Acetone was added 1:2 (w/v) of three times within 24 h r to remove fat from the samples, then filtered the solution using filtered (Whatman No. 1 paper) and then heated to 45 °C in a dryer, then 1 L of sodium acetate 0.05M was added to the samples at every 2-h interval. Each time the viscous fluid was collected by squeezing through a cotton cloth. After the extraction, the rooster comb and fish eyes residue were discarded. The aqueous extracts were mixed with 1.5 L of ethanol. The centrifuged was then performed for 30 min. at 3000 rpm, the precipitate was collected and washed four times in 100 ml chloroform to remove the remaining proteins, The solution was then deposited with ethanol once more for the gel no longer formed., and the precipitate was collected, dried, and the final dried HA material stored at 40 °C.

Determination of Hyaluronic Acid By Carbazole Method

HA was determined in the extracts using the carbzole method, which involved taking 0.1 mg ml of each sample and adding 0.025 M of sodium tetra borate solution (in sulfuric acid sp. gr.1.84), heating in a water bath at 100 ° C for ten minutes, cooling the tubes in an ice bath for 10 minutes, and then adding 0.2 ml of carbzole reagent (in 95% ethanol) and boiling for 15 minutes in at 530 nm, the absorbance was measured.

FTIR Spectroscopy

Fourier-Transform Infrared (FTIR) spectroscopy of composite HA samples was done by (Perkin Elmer Spectrum One Nicolet 520, Japan, JASCO). The experiment was conducted at laboratory temperatures by mixing HA samples with KBr and was evaluated at 4000–400 cm^{-1} (Alizadeh-Sani et al., 2018).

High-Pressure Liquid Chromatography (HPLC)

HPLC was used to analyze HA components. Lyophilized HA extracts were diluted in 1 mL of distilled water and filtered before HPLC analysis. The injected sample volume was 10 µL and commercial HA as a standard was used to analyze. HPLC system implemented using a column C18 (260 x 4.5mm, 5 µm, Thermo Fisher Scientific, Japan). The gradient was 30 minutes at 205 nm and the solvent flow rate was 10 ml min-1.

Antioxidant Activity of HA

DPPH Radicals Scavenging Activity

The free radical DPPH (2, 2-diphienyl-1-picrylhydrazile) was determined using the method reported by Wang et al. (2019), with some modifications, by mixing 1 ml HA (0-125) mg/ml with 1 ml DPPH (0.01M ethanol). The combination was incubated in the dark for 30 minutes at laboratory temperature before being measured at 517 nm. BHT was utilized as a comparative sample, and the control sample was made in the same way as the sample except for the inclusion of methanol instead of the sample. For the following equation:

$$\textbf{DPPH scavenging activity} \quad \frac{A_C - A_S}{A_C} \times 100$$

$A_{Control\ is}$ the absorbance value of the DPPH, as is the sample's absorbance

Reducing Power

The reduction power was calculated using the approach proposed by Zhang et al. (2022). To summarize, 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 0.1% (w /v) K3Fe (CN) were added to each sample with various adjustments. After 30 minutes in the dark at 50°C, 2. 5 mL of 10% (w/v) trichloroacetic acid solution was added to the mix. After 10 minutes, the absorbance at 700 nm was measured using the supernatant 2.5 mL combined with 2 mL distilled water and 0.1 mL ferric chloride (0.1% w/v).

Ferrous in chelating activity

The chelation of Ferrous ion was assessed using the Kosnett (2013), which was some modified by Halfi (2009). Briefly, 0.4 mL of 2 mM FeCl2 was added to 0.4 mL of different concentrations HA (1-5mg/ml). The reaction was initiated by the addition of 0.4 mL of 5 mM 8-hydroxy quinolone. The reaction mixture was then kept at laboratory temperature for 10 min in the dark. The absorbance of the solution was thereafter measured at 562 nm. The control sample was prepared in the same way as described above except for sample addition. EDTA was used as a reference standard for the assay.According to the following equation

% of the chelating activity = $[1-A_{c}\backslash A_{S}]\times 100$

The absorbance of the control is Acontrol, and AS is the absorbance of the samples.

Assay for hydrogen peroxide (H_2O_2) scavenging activity

The ability of HA to scavenge (H2O2) was estimated using the method developed by (Türkolu et al., 2010), in which 1 mL of HA in various concentrations (1-5m/gml) was mixed with 0.6 mL of H2O2 solution (2 mM) prepared in a phosphate buffer (0.1 M, pH 7.4) and the absorption was measured at 230 nm after 10 minutes. As a positive control, ascorbic acid was employed. The following equation was used to determine HA's ability to scavenge activity:

Inhibition % = $[A_c - A_s \backslash A_c] \times 100$

A_c= Absorbance of the control, A_s = Absorbance of the sample

Statistical Analysis

The results were statistically analyzed using a complete randomized design with one factor, and the data were analyzed using the SPSS software (version 26). was used for statistical analysis. The data was analyzed using at $p < 0.05$., differences were judged significant.

RESULTS AND DISCUSSION

Yield Hyaluronic Acid Extraction

The results showed in Figure (1) the percentage of the yield of HA extracted from animal sources, and the findings revealed that there were significant differences at ($P \leq 0.05$)if the percentage of HA output was considered extracted from the manes of from rooster comb was 4.75%, while the percentage of the yield in fish eyes was 3.34%. This result was similar to what was obtained by (Kulkarni, Patil, and Chavan 2018) when 5.43% of HAwas obtained from the crests of roosters, while (Alcântara et al. 2023) found that the percentage of HA in the eyes of tilapia was 5%.

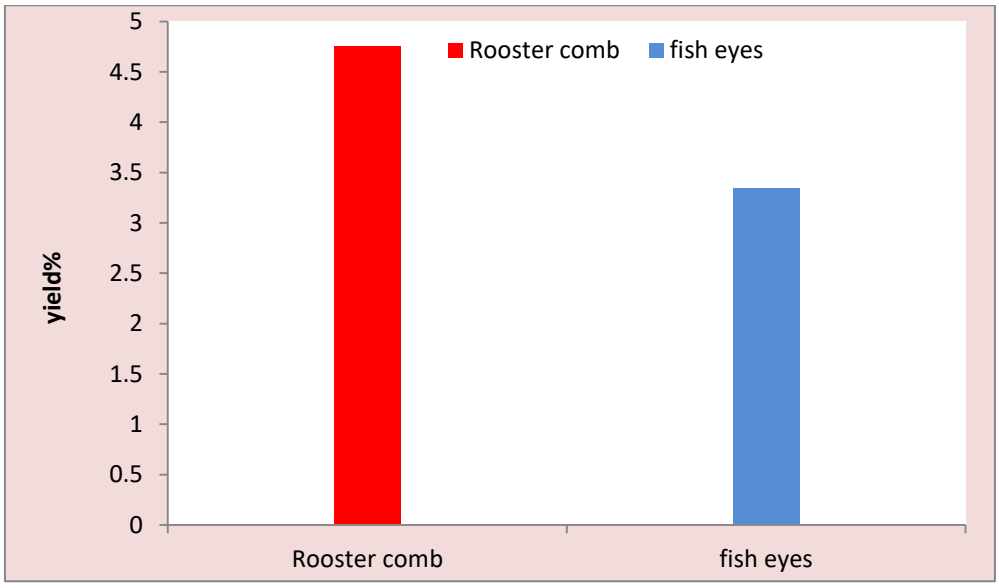

FIGURE 1. A comparison of the proportions of hyaluronic acid extracted from Rooster combs and fish's eyes

L.S.D=0.434

FTIR Analysis

The active and normal totals of HA extracted from rooster comb and fish eyes were diagnosed by using the infrared spectrum and compared with the spectrum of the standard acid as shown in Figure (2). The infrared spectrum can be used as a fingerprint for identification by the comparison of the spectrum from an unknown with previously recorded reference spectra, if the similarity of the peaks of the extracted HA with the active groups of the standard HA is observed, this indicates the presence of the active compounds present in the composition of the HA. Peaks also appeared in the range of 2918-2929 cm^{-1}, which are related to the Methylene group and are caused by the vibration of the C-H bond, while peaks appeared in the range of 1650-1400 cm^{-1}, which due to vibration of the double bond C=O. Which belong to the amid a group of chlorochloronic acid, which is one of the building blocks of hyaluronic acid, and the C-N single bond vibrations of the amine group, as well as peaks appeared in the range of 1064-1040 cm^{-1}, which belong to C-O single bond vibrations belonging to alcohol group

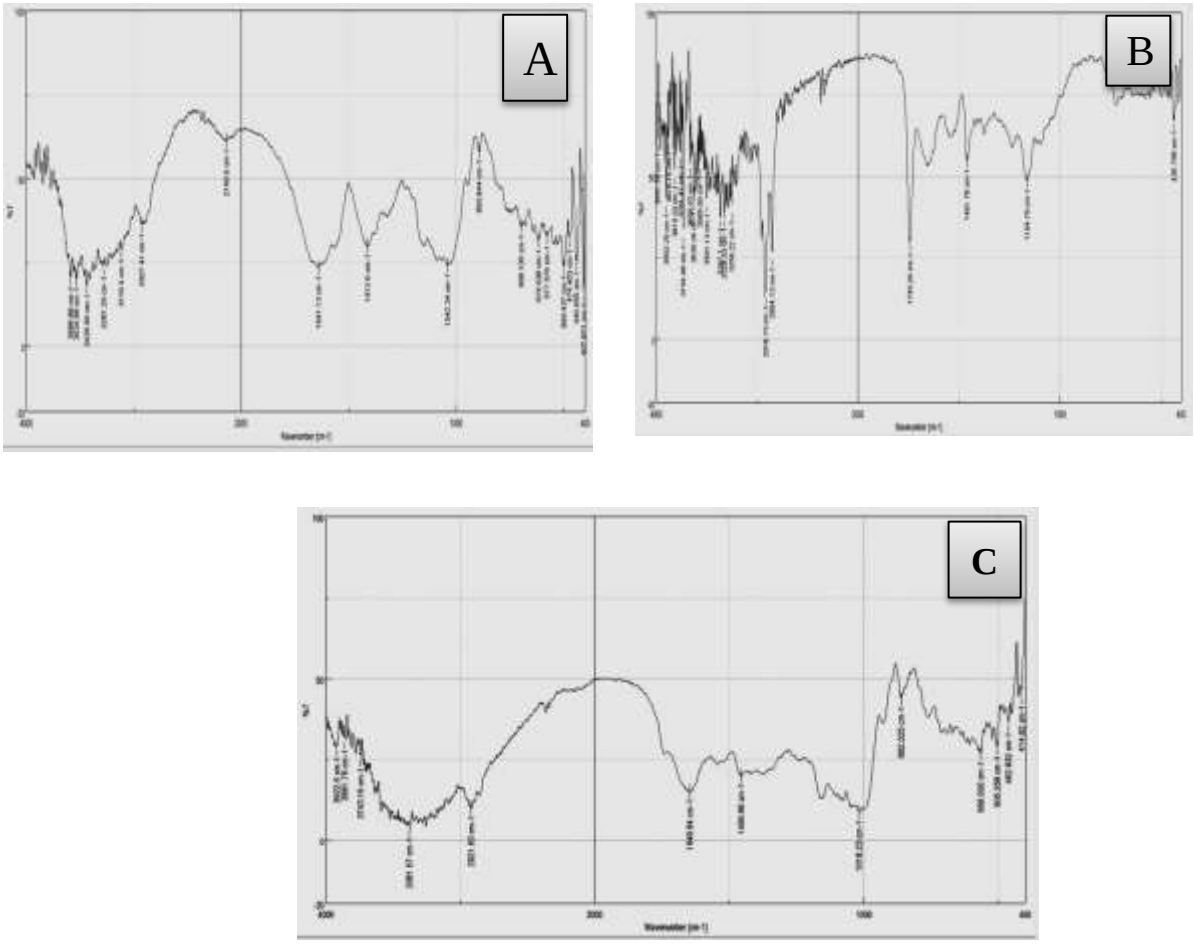

FIGURE 2. FTIR of hyaluronic acid, (A):HA standard, (B): acid extracted from rooster comb, (C): acid extracted from fish eyes

HPLC Chromatography

Figure (3) shows the diagnosis of HA extracted from rooster comb and fish eyes, compared with standard HA, where one peak appeared for each source with a slight difference in retention time ranging between 1.80-2.052 minutes, where the retention time was for standard solution and the acid extracted from rooster comb and fish eyes is 1.945and 1.790 min, and the appearance of one peak in all sources may indicate the purity of the extracted HA and were recorded for rooster comb and fish eyes peak and found to be within acceptable limits. . The results were agreed with what was found by (Liu et al. 2013), where it was found that the retention time 1.85-1.95 min (de Oliveira, Nakamura, and Auzély-Velty 2020) found the retention time for standard HA to be 2.4 min.

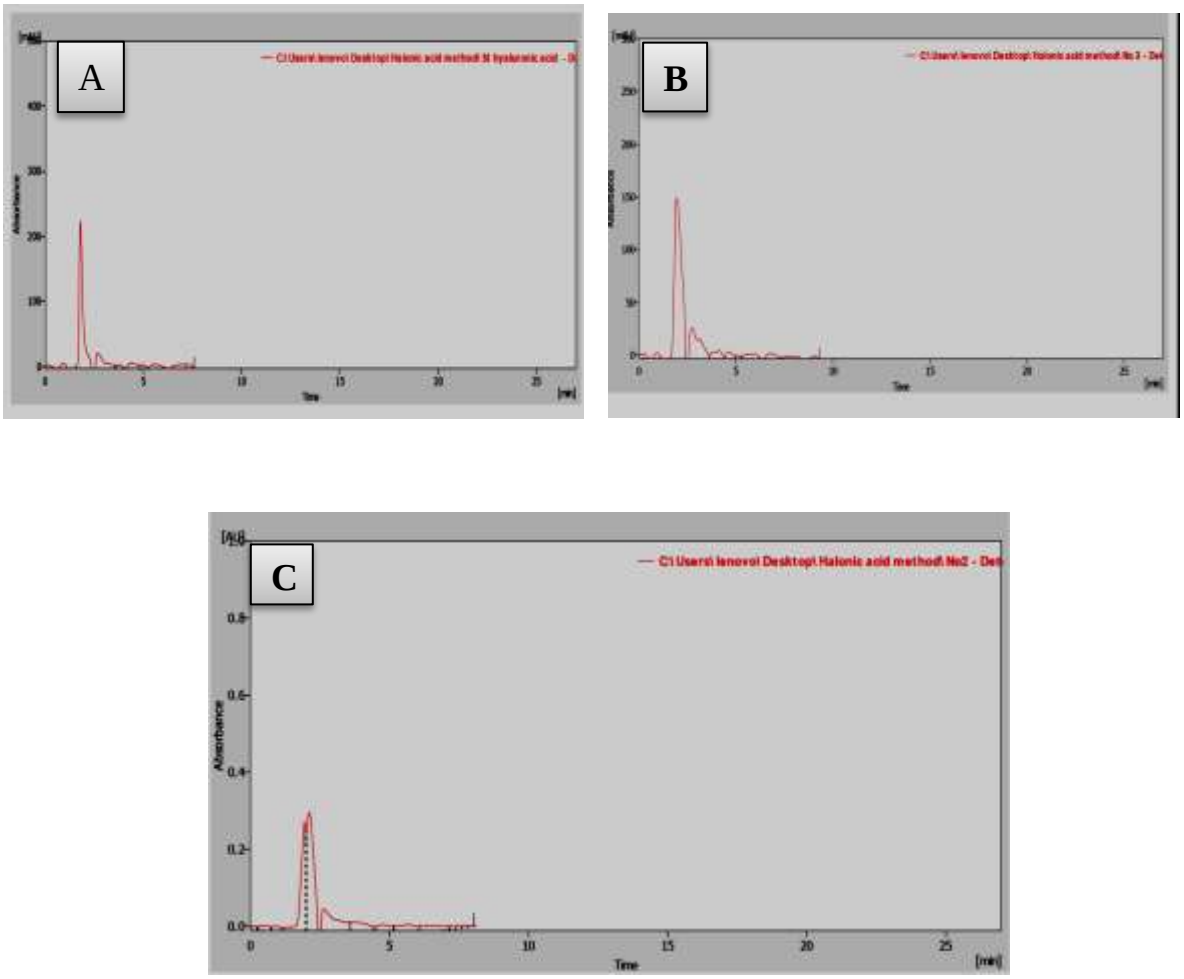

FIGURE 3. HPLC of hyaluronic acid, (A):HA standard, (B): acid extracted from rooster comb, (C): acid extracted from fish eyes

ANTIOXIDANT ACTIVITIES

Radical Scavenging Activity at DPPH

Figure (4) shows the antioxidant effectiveness of HA extracted from animal sources and compared with synthetic antioxidants (BHT) using different concentrations ranging from 25-125 mg ml. It is noted that the extracted HA can scavenging the free radical (DPPH). Where this ability increases with increasing concentration, as the ability to

absorb HA extracted from rooster combs reached 30, 38, 42, 52, 61%, respectively, while the ability to absorb HA extracted from fish eyes was 22, 30, 42, 48, 54. These results came close to what was mentioned by (Kanchana et al. 2013), where HA was extracted from animal sources, where concentration 0.2 mg ml gave 19.77%, while it gave the highest concentration of 54.42% at 1 mg ml. While the industrial antioxidant (BHT) showed the ability to scavenging the free radical (DPPH) higher for all concentrations, if it reached 40, 61, 68, 82, 90%, respectively. Antioxidant effect enhanced when the concentration was increase (Al-Ali et al., 2021; Al-Hilifi et al., 2022). The effectiveness of HA in scavenging free radicals (DPPH) is caused by functional groups such as the carboxyl and hydroxyl groups that connect with free radicals and transform them into more stable molecules, so ending the process of free radical reactions. Mohammed and Niamah, 2022; Campo et al., 2004).

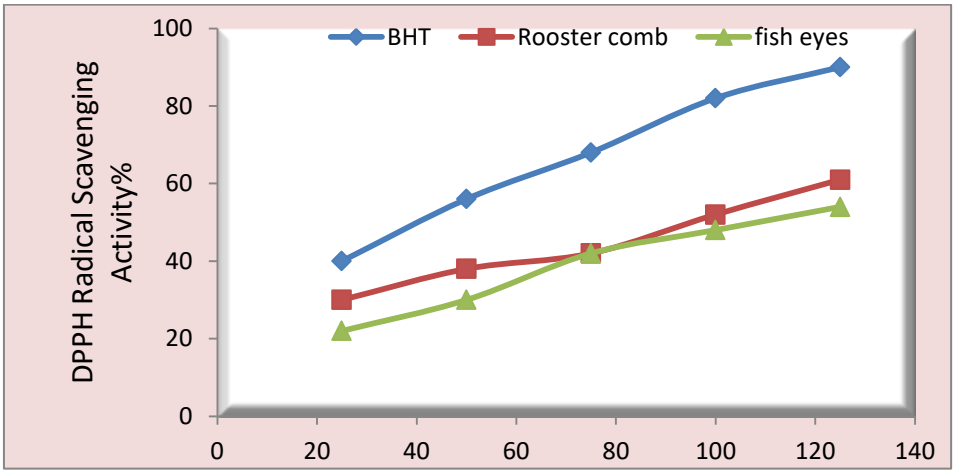

FIGURE 4.. DPPH radical scavenging activity of hyaluronic acid extraction and BHT. L.S.D=0.0712

Reducing Power

The results show in Figure (5) a reductive strength of HA extracted from rooster combs and fish eyes and compared with industrial antioxidant (ascorbic acid) and using different concentrations ranged between (25-125) mg / ml. The reducing power of all the HA extracts increased with increase in concentration., as HA extracted from rooster combs showed the highest reducing ability, followed by fish eyes, and when compared with ascorbic acid, all results were lower than ascorbic acid. For HA extracted from rooster combs, the absorbance was 1.197, 1.358, 1.590, 1.705, 1.956, respectively, while the absorbance of HA extracted from fish eyes was 0.989, 1.178, 1.328, 1.402, and 1.636, respectively**.** The reducing ferric ion Fe^{+3} increased rapidly as the concentration increase (Zhang et al., 2022). These results are in agreement with Sadhasivam et al. (2013) and Al-Hilifi et al. (2022), noting the increase in reductive power with increasing concentration used**.**

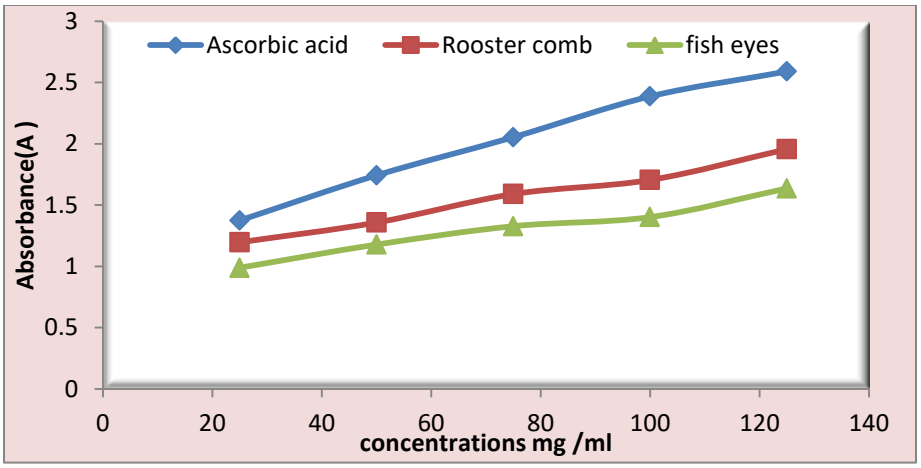

FIGURE.5. Reducing power of hyaluronic acid extraction and ascorbic acid. L.S.D=0.015

Chelating of Ferrous Ion

Figure (6) shows the ability of HA extracted from animal sources to bind the ferrous ion and compare it with the industrial antioxidant EDTA-2Na, using different concentrations that ranged between (1-5) mg ml. If it is noted that the extracted HA can bind the ferrous ion. This ability increases with increasing concentration, as the ability to bind the ferrous ion HA extracted from rooster combs was 59, 61, 63, 68, 73%, respectively, while the ability to bind the ferrous ion to HA extracted from fish eyes was 44, 48, 58, 67.72%, respectively**.** The industrial antioxidant (EDTA-2Na) showed a higher binding capacity for all concentrations, if it reached 33, 42, 58, 78, 96%, respectively, and these results were consistent with what was mentioned by (Mohammed and Niamah 2022),if where was found that with increasing concentration, the ability of HA increases On the binding of iron ions, where the results indicate that the binding ability of HA at 50 µg ml was 7.11%, while it reached 73.74% at concentration of 1300 µg ml. the Bioactive compounds act as hydrogen peroxide scavengers by donating hydrogen atoms to reduce the hydrogen peroxide to H_2O, the breakdown of peroxides and the decomposition of hydrogen peroxide (H_2O_2) into effective free radicals, and this is done using the Fenton reaction (Pisoschi et al., 2021).

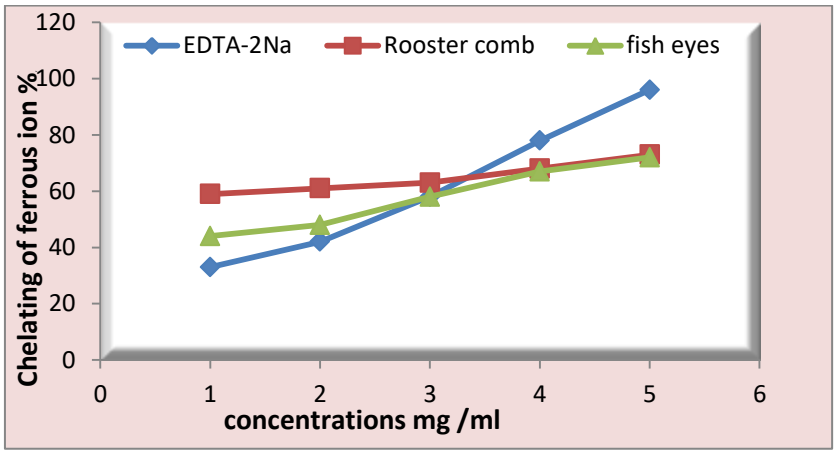

FIGURE 6. Chelating of ferrous ion of hyaluronic acid extraction and EDTA-Na2. L.S.D=0.043

Hydrogen Peroxide Scavenging Activity

Figure (7) demonstrates the potential of HA extracted from animal sources to scavenging the hydrogen peroxide and compared it with the (BHT) using different concentrations that ranged between (1-5) mg / ml. The rustle showed that HA had an effective hydrogen peroxide scavenging activity, , as this ability increases with increasing concentration, as the ability of HA extracted from rooster combs to inhibit hydrogen peroxide was 15%,23%, 38%,51%,63%, respectively**.** While the ability to scavenge HA extracted from fish eyes was 5%,11%, 27%, 32%, 39%, respectively. while, at the same concentration BHT showed 52%, 74. %, 82. %, 93. %, 98. activity respectively Hydrogen peroxide (H_2O_2) is a weak oxidizing agent in its natural form, but it is a source for production of free radicals such as hydroxyl radicals and oxygen radicals, and thus their accumulation and the possibility of their interaction with ions mineral. The ability of HA to scavenging hydrogen peroxide may be due to its possession of active groups in its structural composition, such as the carboxyl and the hydroxyl donor of the hydrogen atom, which make the hydroxyl radical ($OH^{\cdot}$) or the free oxygen radical ($O^{\cdot}$) more stable and prevents the oxidative stress and the proliferation of free radicals (AlMamary and Moussa., 2021; Ofoedu et al., 2021) .

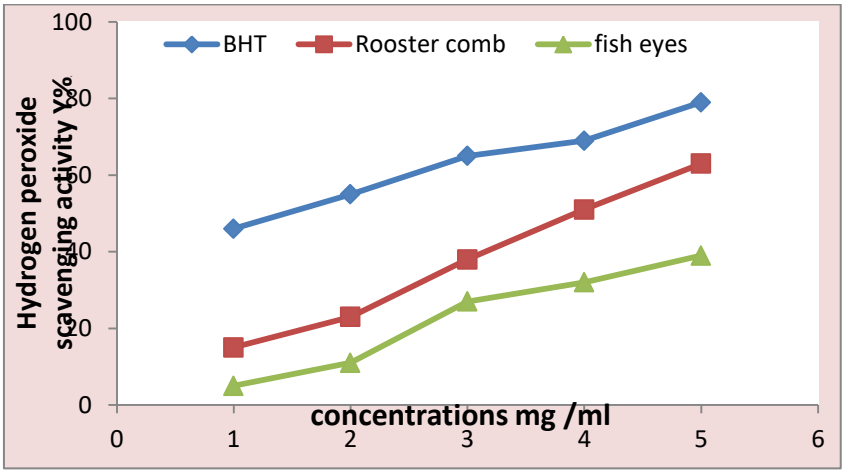

FIGURE 7. Hydrogen peroxide scavenging activity of hyaluronic acid extraction and BHT

CONCLUSION

Hyaluronic acid has been intensively researched for its potential applications in biomedicine. Poultry slaughterhouse waste and fish waste have been employed as alternative low-cost sources of HA production, as well as to lessen the environmental pollution caused by these waste. These hangovers are ideal for HA, in our opinion. In vitro antioxidant studies revealed that HA has substantial antioxidant activity. As a result, this chemical has the potential to be effective in biomedical, aesthetic, and nutritional applications

. ACKNOWLEDGMENTS

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