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USE OF HYALURONIC ACID PRODUCED FROM A LOCAL ISOLATE OF *STREPTOCOCCUS THERMOPHILUS* AS AN ANTIOXIDANT IN CRUDE SUNFLOWER OIL

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Abstract: Hyaluronic acid is a natural antioxidant derived from various sources, including microbial ones, and is utilised in multiple fields, including medicine, food, and cosmetics. A local isolate of *Streptococcus thermophilus*, cultured on whey medium, was used to produce hyaluronic acid, which was identified by the carbazole reaction, high-performance liquid chromatography (HPLC), and Fourier transform infrared (FTIR) spectroscopy. The antioxidant activity of hyaluronic acid was then evaluated by measuring the reducing power of 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide removal, and ferrous ion chelation, in comparison with the synthetic antioxidant BHT. It was then used as an antioxidant to preserve sunflower oil for a 90-day storage period. The results showed that the hyaluronic acid production rate was 0.598 g/L, and that a concentration of 500 µg/ml of the acid (dissolved in saline solution) was most effective in scavenging DPPH free radicals and hydrogen peroxide, with scavenging rates of 68.14% and 72.08%, respectively, while the iron ion binding rate was 81.98%. The addition of hyaluronic acid extract to crude sunflower oil resulted in lower levels of oxidation, acidity, and thiobarbituric acid during storage periods of 30, 60, and 90 days. The study also showed that sunflower oil stored in transparent containers was more susceptible to oxidation than oil stored in opaque containers. Therefore, hyaluronic acid can be used as a natural antioxidant and an alternative to synthetic preservatives to preserve oils and extend their shelf life.

Key words: *natural antioxidant, alternative antioxidant, shelf life, antioxidant activity, sunflower oil, oxidation stability*

INTRODUCTION

Hyaluronic acid (HA) is a linear heteropolysaccharide that belongs to the group of heterogeneous polysaccharides known as glycosaminoglycans (Buckley, Murphy, Montgomery & Major, 2022). Other examples of glycosaminoglycans include heparin sulphate, keratin sulphate, dermatan sulphate, and chondroitin sulphate. While these compounds share a similar structural composition with HA, they lack a sulphur group and are not synthesized by Golgi enzymes with the assistance of proteins (Abatangelo, Vindigni, Avruscio, Pandis & Brun, 2020). Additionally, hyaluronic acid has a higher molecular weight compared to other gly-

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colsaminoglycans. The molecular weight of HA ranges from 2×105 to 1×107 Daltons, and it can significantly influence many of the physical and chemical properties of HA (Maharjan, Pilling & Gomer, 2011).

HA is found naturally in the tissues of all living organisms, in cartilage, in the synovial fluid surrounding joints, in the skin, in the aqueous humour of the eye, and in fruits and vegetables. Several species of the genus *Streptococcus* are capable of producing hyaluronic acid, such as *S. zooepidemicus* (Di et al., 2025), *S. thermophilus* (Mohammed & Niamah, 2022a), *S. pyogenes* (Hurst et al., 2022), *S. equi* (Gedikli et al., 2018), and *S. suis* (Allen et al., 2004).

Antioxidants in food are substances that, in insignificant amounts, can effectively inhibit or significantly delay the oxidation process of easily oxidizable materials such as fats. Consequently, antioxidants in food science are commonly identified as agents that interrupt the chain reaction of lipid peroxidation (Parcheta et al., 2021). Synthetic antioxidants, not found in nature, are incorporated into food products as preservatives to inhibit lipid oxidation (Viana da Silva et al., 2022). Considering the intrinsic instability of natural antioxidants, various synthetic antioxidants, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), have been utilised to enhance the stability of fats and oils. Initially formulated to protect petroleum from oxidative gumming, these compounds serve a similar purpose in the food industry (Dassarma, Mahapatra, Nandi, Gangopadhyay & Samanta, 2025). The antioxidant activity of carbohydrates (monosaccharides, oligosaccharides, and especially complex carbohydrates) is primarily due to their ability to neutralise reactive oxygen free radicals, such as hydroxyl radicals (OH•) and superoxide radicals (O2•). This process occurs through several chemical mechanisms, including hydrogen atom transfer, electron transfer, and complexation with metal ions (Molaei, Tehrani & Shamlouei, 2023).

Synthetic antioxidant additives used in food, such as BHA and BHT, are facing challenges due to a negative perception among consumers. As a result, there is increasing pressure on food producers to explore safer alternatives from nature, such as phytochemicals (e.g., polyphenols, flavonoids, and terpenoids-rich essential oils). These active compounds found in plants have proved antioxidant properties in laboratory experiments and various food items, including meat, fish, oil, and vegetables (Hussein, Niamah & Majeed, 2024). Given the significant annual food wastage caused by lipid oxidation and premature spoilage from inadequate packaging, there is an urgent need for natural antioxidants to replace artificial ones and meet consumer demands (Uhlig, Bucher, Strenger, Kloß & Schmid, 2024).

Many natural compounds have been used to preserve oils and fats and as antioxidants instead of synthetic antioxidants. A previous study demonstrated the antioxidant bioactivities associated with terpenoids and polyphenols in protecting against lipid oxidation in high-fat foods, such as red meat, fatty fish, oils, and vegetable products, while avoiding the formation of rancid tastes, odours, and colour changes due to oxidised lipids (Gutiérrez-del-Río et al., 2021). Antioxidants that function as chain-breaking agents and promote termination (a more common term than "termination enhancer") or exhibit "preventive/scavenging" effects are characteristics of various classes of natural products. The two main mechanisms are: *i)* chain-breaking antioxidants (phenolic properties). This mechanism characterises molecules with phenolic structures (hydroxyl groups attached to an aromatic ring), such as flavonoids, polyphenols, and tocopherol (vitamin E); *ii)* Termination-promoting/preventive antioxidants (non-phenolic properties). This mechanism characterizes non-phenolic compounds, which often lack the structural features necessary for effective direct Hydrogen Atom Transfer (HAT) activity, such as simple sugars, organic acids (like ascorbic acid in specific contexts), and some sulphur-containing compounds. The common mechanism of action for these compounds is metal chelation (López, Enemark, Grosso & Olmedo, 2023). The aim of this study was to extend the shelf life of sunflower oil by adding HA produced by a local bacterial strain, after first investigating its antioxidant properties.

MATERIALS AND METHODS

Bacterial isolate

Streptococcus thermophilus MZ841806 was obtained from the College of Agriculture at the University of Basrah. It was used as the HA-producing strain throughout this study. The isolate was cultured at 37 °C for 24 hours on

deMan, Rogosa and Sharpe (MRS) agar (Hi-Media, India). The process was repeated 3 times to obtain an isolated active substance used in the production of hyaluronic acid.

Fermentation conditions

Twenty percent dried whey (Paras company, India) (1L) with 0.75% yeast extract (Hi-Media, India) was prepared as the fermentation medium was in a 2-litre stirred-tank bioreactor. Internal mixing is achieved using Rushton turbines or inclined-blade fans to ensure oxygen distribution. Adjust the medium to pH 6.8, which was autoclaved (121 °C, 5 min). Cool the culture medium, then inoculate 1% (~0.5×10^8 CFU/ ml) of *S. thermophilus* and incubate at 40 °C for 24 hours at a shaking speed of 150 rpm for HA production.

Isolation and purification of HA

After the fermentation process was complete, HA was extracted and purified according to the method described by Izawa et al. (2009). Briefly, to remove proteins and nucleic acids, 10% trichloroacetic acid (w/v) was added to the solution, which was then incubated for 2 hours at 4°C. The solution was then centrifuged at 15000 × g for 30 minutes at the same temperature. An equal volume of 99% ethanol was then added to the supernatant and incubated overnight at 4 °C. The solution was centrifuged again for 30 minutes under the same conditions. The resulting precipitate was then dialyzed using SpectraPor® regenerated cellulose dialysis membrane with a molecular weight cutoff of 5000 Da. The pH was adjusted to 7.0±0.2 with 0.1N NaOH, and finally, the solution was lyophilized (freeze-dried). The biomass concentration was determined by measuring the dry weight of the cells. The samples were centrifuged at 10,000 rpm for 15 minutes, then washed twice and dried at 100 °C until the weight stabilized.

Determination of glucuronic acid

Glucuronic acid was determined via carbazole reaction (Knutson & Jeanes, 1968). A change from colourless or pale yellow to purple red signifies a positive uronic acid result when 1,2,3, and 4 ml of culture media was combined with 10 ml sulfuric acid with borate [25 ml stock borate solution (24.74 gm borate dissolved in 4M KOH) and made to 1 litre with H_2SO_4. Final solution 0.1 M in H_3BO_3 was heated at 100°C for 10 minutes. The reaction mixture is cooled on ice for 1 hour. Subsequently, 0.4 ml of carbazole solution (0.125 g carbazole in 100 ml 95% ethanol) was added and heated for 12 minutes at 100 °C.

High-Performance Liquid Chromatography (HPLC)

0.20 mg of the produced HA and standard HA (Hi media, India) were resuspended in 10 ml of sodium chloride (1 M), centrifuged and filtered (0.22 μm filter). The resulting solutions were analysed by high-performance liquid chromategraphy (HPLC) (Shimizu Corporation, Japan) using a carbon 18 column and a refractive index detector. The mobile phase contained 0.5 M KH_2PO_4 at pH 2.5 and flowed at a rate of 1 ml/min. The column temperature was continuously maintained at 30 °C (Ruckmani, Shaikh, Khalil, Muneera & Thusleem, 2013).

FT-IR analysis

The functional groups of the produced and standard HA were identified using a Fourier transform infrared (FTIR) spectroscopy instrument, Shimadzu FT-IR 8300, where 4 mg of the produced HA and standard HA was weighed with 196 mg of anhydrous potassium bromide (KBr) and placed inside the instrument, and the spectrum of the functional groups was observed between the wavelength region of 4000-400 cm^{-1} (Polat & Eral, 2022).

Antioxidant activity of HA

The antioxidant activity of the produced HA was estimated at a series of concentrations (100, 200, 300, 400, 500 μg/ml) by dissolving it in 0.15 M NaCl solution and compared with butylhydroxytoluene (BHT) after dissolving it in ethanol using four different methods as follows: *i)* 2,2-diphenyl-1-picrylhydrazyl (DPPH), *ii)* reducing power measurement, *iii)* hydrogen peroxide scavenging, *iv)* ferrous ion chelation (Mohammed & Niamah, 2022b).

Addition of HA to the oil

Different concentrations of hyaluronic acid, produced using whey as a growth medium for the native *S. thermophiles* bacteria, were used (100, 300, and 500 mg/L of oil). The hyaluronic acid was dissolved in a 0.15% sodium chloride solution and then added to the crude sunflower oil obtained from the food processing plant of the General Food Company in Baghdad, Iraq. The mixture was thoroughly blended with lecithin added as an emulsifier. BHT (dissolved in

ethanol at a concentration of 200 mg/L of oil) was also added to the oil as a reference control sample. Another sample of oil free of antioxidants was used as a negative control sample. Lecithin (E322) was added to all oil samples at a concentration of 0.5% as an emulsifying agent. Peroxide value (PV) (Zhang et al., 2021), thiobarbituric acid (TBA) (Piranavatharsan, Jinadasa & Jayasinghe, 2023), total acidity (TA), and iodine value (IV) (Geng *et al*., 2023) were studied for storage periods 0, 30, 60, and 90 days at room temperature (28±2 ºC) using two types of opaque and transparent containers to determine the effect of antioxidants on the shelf life of sunflower oil.

Statistical analysis

Analytical data were obtained from three replicates. The statistical analysis in this study was conducted using a completely randomized design (CRD) using the Statistical Package for Social Sciences (SPSS) version 22. Data were analysed using an analysis of variance (ANOVA) table, and significant differences between the means of the coefficients were determined using the least significant difference (LSD) test at a probability level of 0.01 ($P \leq 0.01$).

RESULTS AND DISCUSSION

Production and identification HA

HA production from the local bacterial isolate (*S. thermophilus* MZ841806) after the end of the fermentation process reached 0.598 g/L and the biomass amount reached 6.08 g/L. After reacting with carbazole solution, the colour changes into purple. The HA was produced and gave the purple colour solution. These results are showed in Fig. 1A. HA produced from whey medium was identified using local isolation and compared to standard hyaluronic acid via HPLC (Fig. 1B and C). The single peak appeared for both whey media and standard HA at retention times of 3.12 and 3.10 minutes, respectively. This close retention time reveals and the lack of other peaks reveals the purity of the produced HA. Colourimetric detection of HA from the local isolate *S. thermophilus* MZ841806 shows that Uronic acid reacts with carbazole solution, producing a purple colour (Cesaretti, Luppi, Maccari & Volpi, 2003). The intensity of this colour depends on the amount of uronic acid produced by bacterial metabolism (Gedikli et al., 2018). The HPLC results showed a single peak for the produced and standard HA, but the residence time of the compound differed by one second. This may be attributed to the purity of the resulting compound and the removal of impurities during the purification process. The results were agreed with Tu and Trang (2013), when they detected HA produced by *S. thermophilus* using rice water as an alternative medium and compared it with standard HA. A single peak appeared at 7.5 minutes, which may be attributed to the purity of the produced HA and the absence of impurities.

FT-IR analyses

Fig. 2 shows the functional groups of the produced and standard HA using FTIR spectroscopy. The results showed the appearance of a peak at 3411 cm^{-1} for both the product acid and the standard acid, respectively, which is attributed to the vibrations and stretching of the O-H and N-H bonds of the N-acetyl group, characteristic of the HA structure, which contains the D-glucosamine compound. The peak at 2927 cm^{-1}, respectively, is due to the stretching of the C-H bond of the methylene group, which is found in organic compounds. The peaks that appeared at 1637 and 1421 cm^{-1} are due to the vibrations of the double bond C=O of the amide group of glucuronic acid, which is one of the structural units of HA and the vibrations of the single bond C-N of the amine group, respecttively. In addition, the peak that appeared at about 1075 cm^{-1} is due to the vibration of the single bond C-O of the alcohol group (Mirzayeva, Čopíková, Kvasnička, Bleha, & Synytsya, 2021; Yousefi, Kandel & Pleshko, 2018).

The FT-IR spectrum showed similarity in the locations of the peaks belonging to the functional groups of the produced and standard HA, indicating the quality of the purification processes and the purity of the resulting compounds. The peaks and vibrations of the H-N and C-N single bonds, which belong to N-acetyl and amine group, also confirm that the resulting compound belongs to HA (Mirzayeva *et al*., 2021; Yousefi *et al*., 2018).

Antioxidant activity of HA

Fig. 3 shows the antioxidant activity of HA produced from a local isolate of *S. thermophilus* using different concentrations of HA produced from the whey media (100, 200, 300, 400, and 500 µg/ml) in comparison with the synthetic antioxidant butylated hydroxytoluene (BHT).

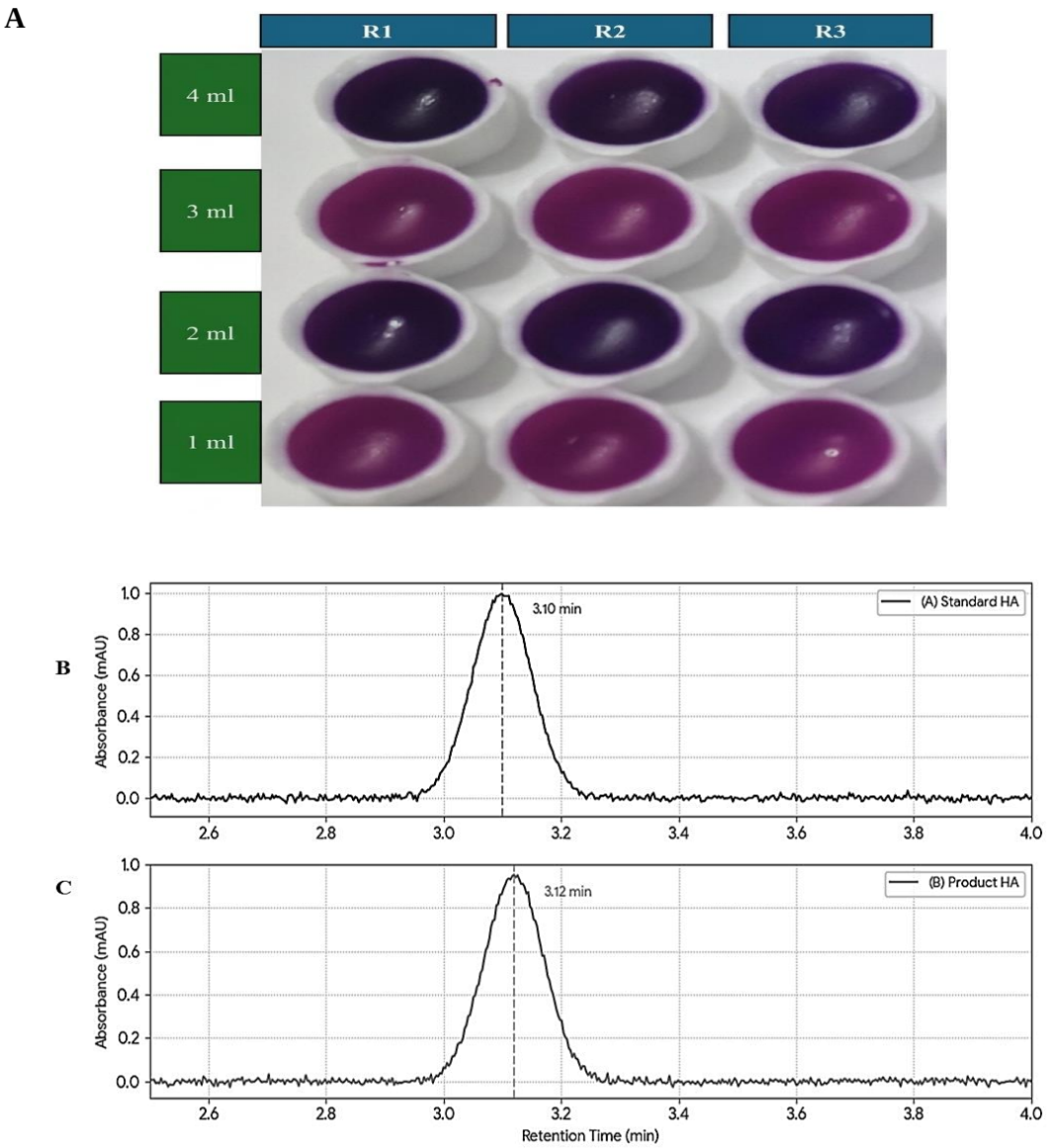
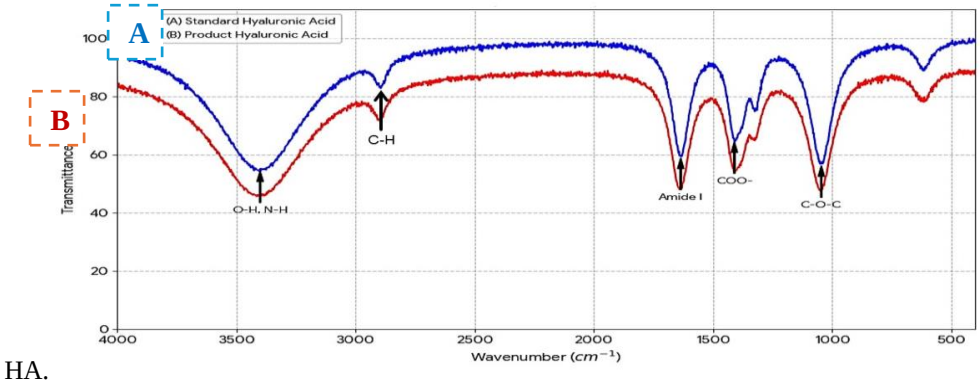


Figure 1. Determination of HA produced from local isolate after growth in whey medium, (A) Colour reaction with carbazole solution, 1-4 ml of produced HA, R1-3: duplicates (B) HPLC spectrum of standard HA, (C) HPLC spectrum of produced



HA.

Figure 2. FT-IR spectrum of HA, (A) standard HA, (B) product HA.

The results imply that the ability of HA and BHT to scavenge the free radical (DPPH) increased with increasing concentration of both HA and BHT.

The highest scavenging capacity of HA was 68.14% at a concentration of 500 µg/ml, while the concentrations of 100, 200, 300, and 400 µg/ml showed the lowest scavenging capacity of the free radical (DPPH), which reached 26.22%, 38.55%, 46.17%, and 57.67%, respectively. This is lower compared to the scavenging capacity of BHT at all concentrations, which reached 66.22%, 88.11%, 95.58%, 98.99%, and 98.99%, respectively. The reducing power of the HA and BHT was estimated at different concentrations ranging from 100 to 500 µg/ml, which were measured at a wavelength of 700 nm, as shown in Figure (3B). The concentration of 500 µg/ml gave an absorbance of 1.3, while the concentrations of 100, 200, 300, and 400 gave an absorbance of 0.3, 0.6, 0.9, and 1.1.

The results of hydrogen peroxide (H2O2) scavenging capacity using different concentrations of HA and BHT (Fig. 3C). The results show that HA has a high hydrogen peroxide scavenging capacity, and this capacity increases with increasing concentration. The HA scavenging percentage reached 20.33%, 29.15%, 41.15%, 66.78%, and 72.08%, respectively, for concentrations of 100, 200, 300, 400, and 500. BHT showed a higher scavenging capacity for all concentrations, reaching 60.12%, 70.01%, 80.88%, 88.15%, and 90.16%, respectively. Fig. 3D shows the ability of HA to bind ferrous ions compared to BHT. The results showed that HA has the ability to bind ferrous ions, but BHT showed a higher ability to bind the ions. The percentage of ferrous ion chelating to HA reached 22.11%, 34.28%, 49.93%, 64.77%, and 81.98%, respectively, while the percentage of BHT reached 41.59%, 55.03%, 69.98%, 95.19% and 96.67%, respectively.

The effectiveness of hyaluronic acid in scavenging free radicals, such as DPPH, is attributed to the functional groups present in its structure, including hydroxyl and carboxyl groups, which bind to free radicals to convert them into more stable compounds, thus halting the free radical chain reaction. The ability of hyaluronic acid to scavenge free radicals or inhibit their formation in the reaction medium increases with increasing concentration, as higher concentration leads to an increase in the number of functional groups (El-Safory, Fazary & Lee, 2010). In a past study, the antioxidant activity of HA produced by *S. thermophilus* was studied at concentrations ranging from 100 to 800 µg/ml. The highest free radical scavenging capacity at 800 µg/ml was achieved at 79.16%, while the lowest value at 100 µg/ml was achieved at 16.88% (Hamad, Taha, Hafez & El Sohaimy, 2017). The difference in the ability of synthetic hyaluronic acid to capture free radicals may be due to its molecular weight, as well as the number of active functional groups within the compound.

The reducing capacity of hyaluronic acid was measured based on the graphical relationship between absorption and concentration (Mohammed & Niamah, 2022b). This capacity was determined by the increase in absorption, which is directly proportional to the reduction of the ferric ion (Fe+3) to the ferrous ion (Fe+2) in the potassium ferrocyanide complex K3Fe(CN)6.

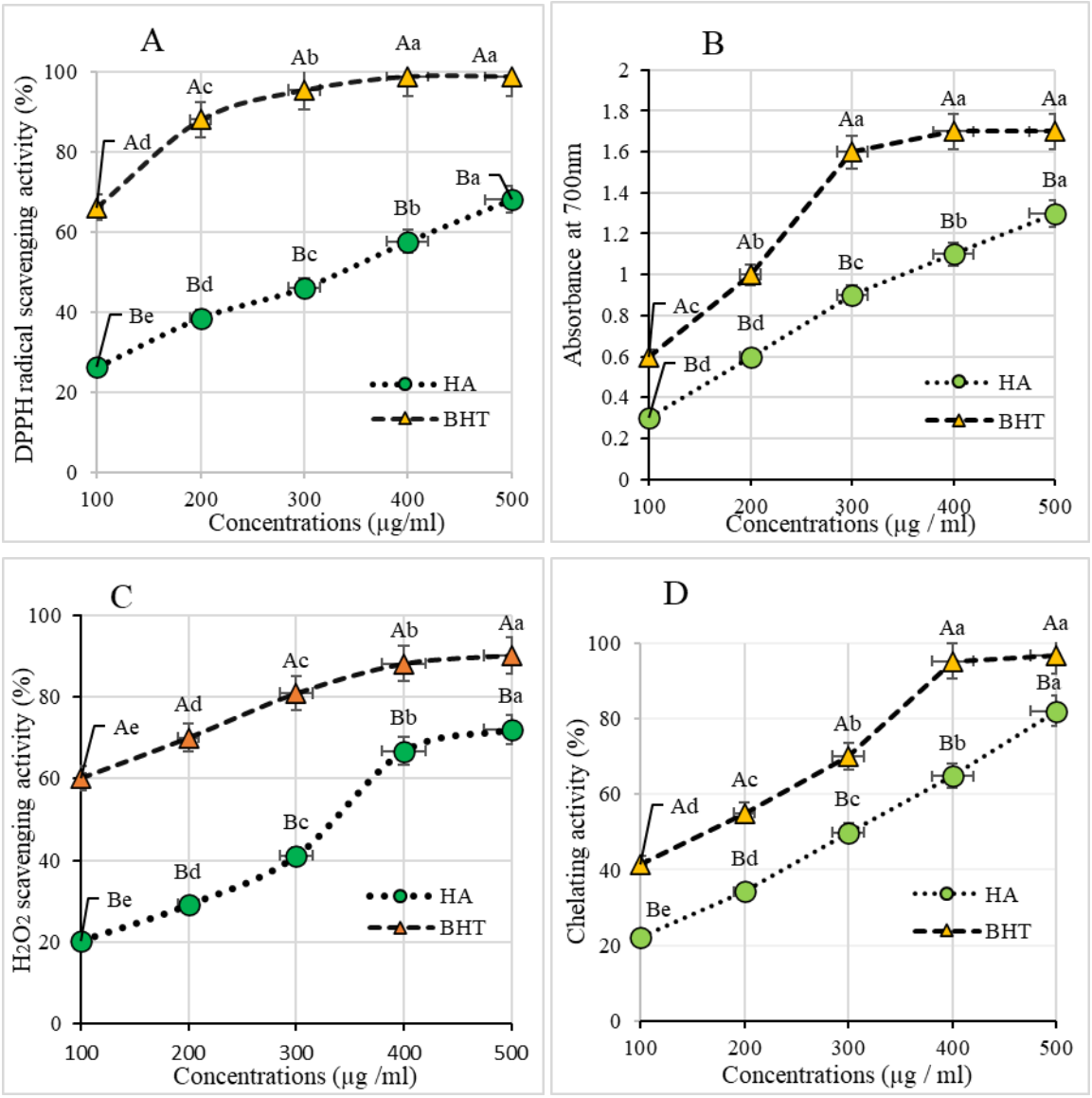
The reducing capacity of hyaluronic acid is attributed to its active functional groups, such as carboxyl and hydroxyl groups, which reduce many metal ions, such as Fe+2 and Cu+2 (Parcheta et al., 2021). The reason for the increased strength with increasing concentration is the possibility of increasing the number of hydrogen-donating functional groups, which contribute to the stability of free radicals and the prevention of chain reactions. The results of the current study were in agreement with what was reported by Kanchana, Arumugam, Giji and Balasubramanian (2013), who observed an increase in the reducing power with increasing concentration of both HA produced from an animal source and ascorbic acid when using different concentrations of 0.2, 0.4, 0.6, 0.8 and 1 mg/ml. It was also found that the absorbance reading of the reducing power of ascorbic acid was higher than that of HA. Hydrogen peroxide (H2O2) is a weak oxidizing agent in its natural form, but it is a source of free radical production, such as hydroxyl radicals and oxygen radicals, and thus their accumulation and potential interaction with metal ions (Abdelshafy et al., 2024). The effectiveness of HA in capturing H2O2 can be attributed to the presence of effective functional groups in its structural composition, such as carboxyl and hydroxyl groups that donate hydrogen atoms, making the free hydroxyl radical (•OH) or free oxygen radical (•O) more stable and preventing the initiation of the oxidative chain reaction (Abbasi et al., 2021). Iron is one of the most powerful oxidising metals. In the Fenton reaction, the ferrous ion (Fe^{+2}) breaks down peroxides and converts (H2O2) into active free radicals (Meyerstein,

2022). The antioxidant activity of HA is due to its ability to bind to the ferrous ion Fe^{+2}. Therefore, the functional groups of HA bind the ion, thus reducing the formation of these radicals (Li et al., 2025).

HA as a sunflower oil antioxidant

Peroxide values

The results showed that the initial peroxide value was 5.34 mEq/kg oil, which is in accordance with the Codex Alimentarius Commission specification for vegetable oils CXS 210-1999, which stipulates that the peroxide value should not exceed 15 mEq/kg oil for unrefined oils and 10 mEq/kg oil for refined oils. A continuous increase in peroxide values with advancing storage periods was observed for both crude sunflower oil and oil treated with BHT at a concentration of 200 mg/kg oil and HA, and for all concentrations added to the oil, 100, 300, and 500 mg/kg oil and stored using an opaque container. After 30 days of storage, the peroxide values reached 10.39, 6.93, 9.96, 8.87, and 7.11, mEq/kg oil and at 60 days of storage they were 45.12, 13.01, 30.08, 15.39, and 14.55 mEq/kg



*Different capital letters indicate significant differences ($p < 0.01$) between the standard and product HA concentrations, while different lowercase letters indicate significant differences ($p < 0.01$) between concentrations of the same group.

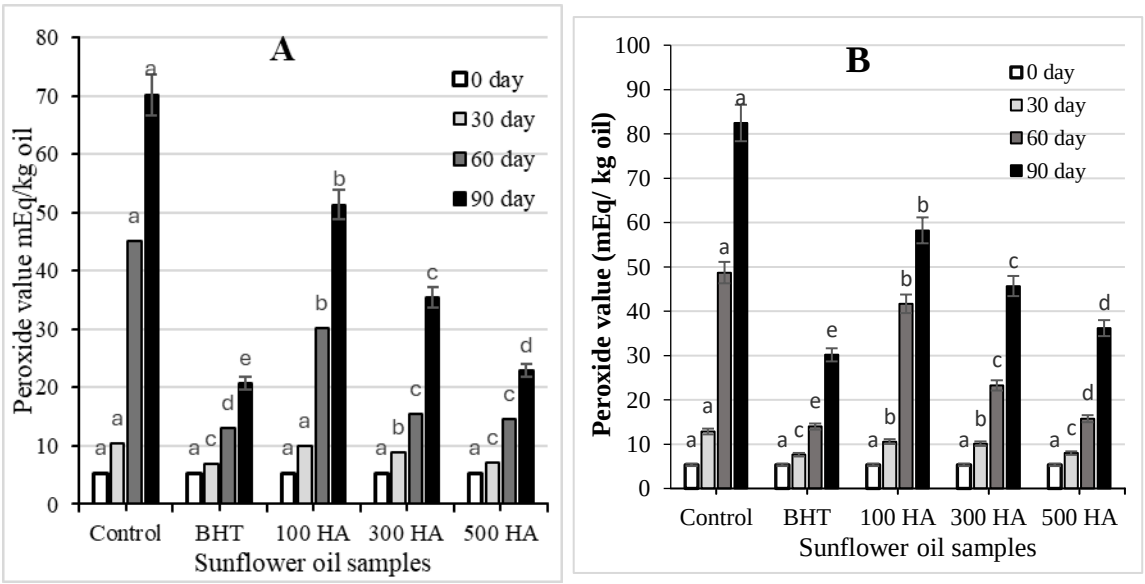
Figure 3. Antioxidant activity of different concentrations of HA, (A) DPPH scavenging activity, (B) reducing power, (C) Hydrogen peroxide (H_2O_2) scavenging capacity, (D) Chelating activity of Iron.

oil while at 90 days they reached 70.2, 20.69, 51.34, 35.41, and 22.92mEq/kg oil, respectively (Fig. 4A). Figure 4B shows that oil stored in transparent containers recorded higher peroxide values compared to oil stored in opaque containers, reaching 12.88, 7.63, 10.56, 10.08, and 8.01mEq/kg oil in 30 days of storage. The highest values were recorded in 60 days, reaching 48.7, 13.98, 41.66, 23.28, and 15.77 mEq/kg oil. The highest values were recorded in 90 days, reaching 82.47, 30.16, 58.25, 45.68, and 36.18 mEq/kg oil, respectively.

Peroxide formation is the first stage of oil rancidity, a precise chemical indicator that measures the degree of oil oxidation and quality deterioration during storage (Machado et al., 2023). Several factors influence this process, including oxygen, light, the presence of metals, storage temperature, and the amount of double bonds present in the fatty acids that make up the oil (Choe & Min, 2006). The main reason for the high peroxide values in clear bottles and their low values in opaque bottles is photo-oxidation (oxidation caused by light). When photons of light strike the oil molecules in clear containers, they initiate a chain reaction that ends with the formation of peroxides. The results were consistent with those observed in the study by Gizachew (2020). The effect of storing sunflower oil containing the natural antioxidant (vitamin E) for 5 weeks in the presence of light and darkness showed that peroxide values gradually increased with increasing storage time and also increased in the presence of light compared to samples stored in the dark.

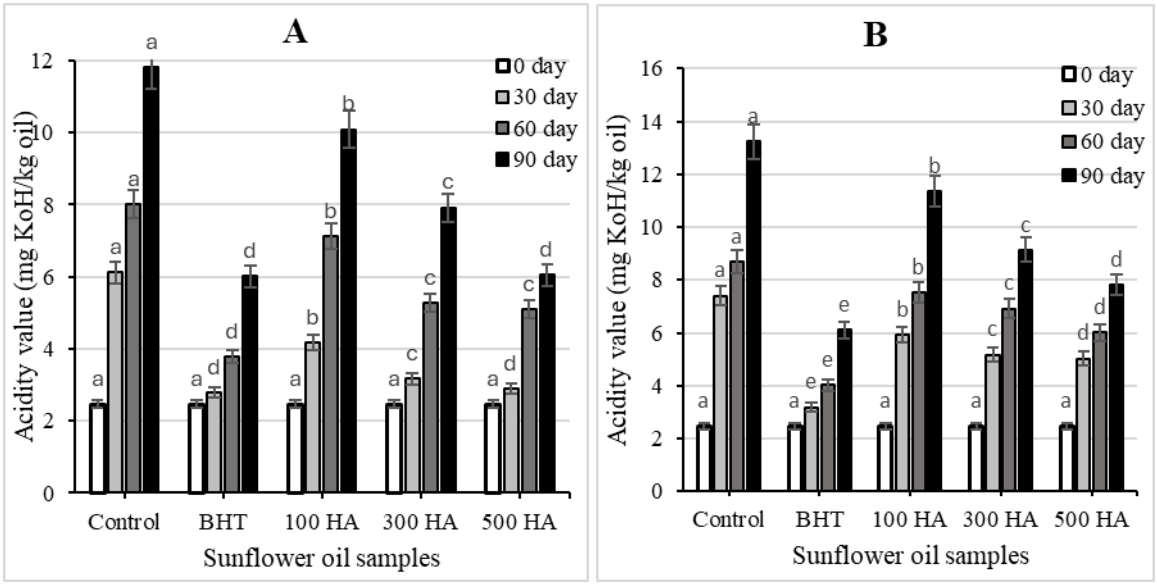
Acidity values

Figure 5 shows the acidity values of sunflower oil samples with different concentrations of HA added compared to the oil sample with BHT added during the storage period (90 days) for opaque and transparent containers. Acidity values decrease with increasing concentration of added HA, while these values increase with increasing storage period. The acidity values in the oil sample, which was supplemented with 100 mg of HA/ kg oil, were 2.47, 4.18, 7.13, and 10.09 mg KOH/ kg oil, respectively, during storage periods of 0, 30, 60, and 90 days. While the acidity values of the oil sample to which 500 mg of HA was added during the storage periods were 2.47, 2.9, 5.11, and 6.05 mg KOH/ kg oil, respectively, and during the storage periods in the opaque containers. The use of transparent containers to store the oil led to higher acidity values. The results showed that the sunflower oil sample with 500 mg of hyaluronic acid added to it reached 2.47, 5.03, 6.01, and 7.83 mg KOH/ kg oil, respectively, during the storage period of 0, 30, 60, and 90 days, while the results of the oil sample with BHT added to it were 2.47, 3.18, 4.02, and 6.11mg KOH/ kg oil, respectively.



[a,b,...] Different lowercase letters indicate significant differences ($p < 0.01$) between oil samples during storage times

Figure 4. Peroxide values (mEq/kg oil) of sunflower oil samples added to HA at different concentrations during 90 days of storage, (A) opaque containers, (B) transparent containers



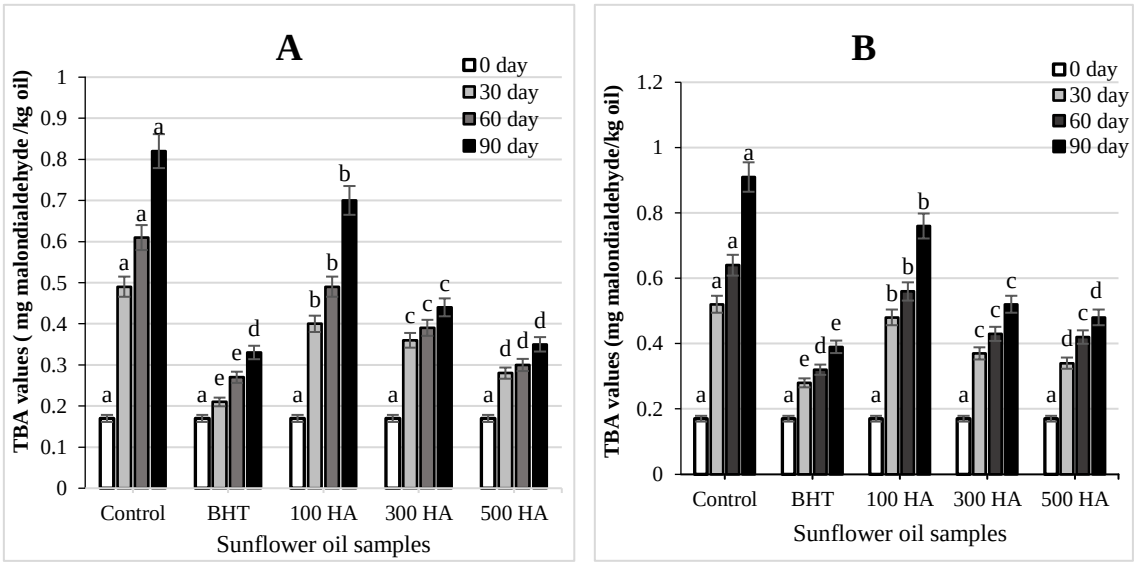
[a,b,…] Different lowercase letters indicate significant differences (p < 0.01) between oil samples during storage times

Figure 5. Acidity values (mg KOH/ kg oil) of sunflower oil samples added to HA at different concentrations during 90 days of storage, (A) opaque containers, (B) transparent containers. *Different lowercase letters indicate significant differences (p < 0.01) between oil samples during storage times

Higher than normal acidity in oil samples indicates the hydrolysis of glycerides by lipase enzymes, releasing fatty acids, as well as the formation of carboxyl groups as a result of autolysis and an increase in the proportion of di- and triglycerides, the amount of free fatty acids, and phospholipids (Sayyari & Farahmandfar, 2017) and this level decreases with increasing concentration of added hyaluronic acid. Hyaluronic acid may act as a natural inhibitor of lipase enzymes, either by binding to the enzyme or altering its chemical environment, thus preventing it from accessing fat molecules (Stępień et al., 2025). Hyaluronic acid also increases the viscosity of the medium, hindering enzyme movement and diffusion, thereby slowing the rate of lipolysis. Furthermore, hyaluronic acid acts as a protective coating for fats after binding to phospholipids in the cell walls of fat globules (Gupta, Lall, Srivastava & Sinha, 2019). Factors that increase the acidity of oils include storage time, temperature, light, and the presence of metals such as copper. Therefore, the results show a higher acidity level in transparent containers compared to opaque containers (Turek & Stintzing, 2013).*Thiobarbituric acid (TBA) values*

The results showed that the initial TBA value was 0.17 mg malondialdehyde/kg oil for all treatments. The crude oil sample (oil without any additives) showed a continuous increase during storage periods (30, 60, and 90 days) for opaque and transparent containers, reaching 0.49, 0.61, and 0.82 mg malondialdehyde/kg oil, and 0.52, 0.64, and 0.91 mg malondialdehyde/kg oil, respectively. While the BHT treatment was the most inhibitory to the formation of malondialdehyde for all storage periods and for both types of opaque and transparent containers, as it reached 0.21, 0.27, and 0.33 mg malondialdehyde/kg oil and 0.28, 0.32, and 0.39 mg malondialdehyde/kg oil, respectively. The addition of HA to sunflower oil reduced malondialdehyde formation with increasing concentration for all storage periods and for both opaque and transparent containers.

At a concentration of 100 mg of HA, the values reached 0.40, 0.49, and 0.70 mg malondialdehyde/kg oil and 0.48, 0.56, and 0.76 mg malondialdehyde/kg oil, respectively. At a concentration of 500 mg of HA, the values were 0.28, 0.30, and 0.35 mg malondialdehyde/kg oil and 0.34, 0.42, and 0.48 mg malondialdehyde/kg oil, respectively (Fig. 6). The decrease in TBA values with increasing hyaluronic acid concentration is attributed to the increased number of hydroxyl and carboxyl groups. These groups contribute hydrogen atoms to the free radicals produced by the auto-oxidation of oils, and they also have the ability to bind to metals, thus slowing down the oxidation process (Ke, Sun, Qiao, Wang & Zeng, 2011). This suggests the inhibitory effect of hyaluronic acid in inhibiting oxidation and



[a,b,…] Different lowercase letters indicate significant differences ($p < 0.01$) between oil samples during storage times.

Figure 6. TBA values (mg malondialdehyde/kg oil) of sunflower oil samples added to HA at different concentrations during 90 days of storage, (A) opaque containers, (B) transparent containers

the formation of malondialdehyde, a byproduct of peroxide decomposition. Another reason for low TBA values is the ability of HA to bind to metal ions (such as iron and copper) that catalyse oxidation reactions (Balogh, Illés, Székely, Forrai, & Gere, 2003).

Iodine number

Figure 7 shows the inhibitory effect of HA on the iodine value of sunflower oil at different concentrations of 100, 300, and 500 mg/kg oil and for storage periods of 0, 30, 60, and 90 days using two types of opaque and transparent containers and in comparison with 200 mg BHT/kg oil and raw sunflower oil. The initial values of the iodine number of oil samples were 146 g I_2/100 g oil. The results showed a decrease in iodine number during storage periods of 0, 30, 60, and 90 days for all treatments, regardless of the type of container used, which included both opaque and transparent containers.

This decrease varied for sunflower oil with HA concentrations, where the highest HA concentration (500 mg/kg oil) recorded low decrease in iodine number, reaching 146.4, 139.2, 121.3, and 119.8 g I_2/100 g oil for opaque containers and 146.4, 136.5, 121.3, and 119.8 g I_2/100 g oil for transparent containers, respectively, compared to a concentration of 100 mg HA/kg oil, where iodine number reached 146.4, 131.2, 99.3, and 95.2 g I_2/100 g oil and 146.4, 127.9, 97.3, and 92.5 mg iodine/100 g oil, respectively. The 200 mg BHT/kg oil treatment recorded the highest iodine number, reaching 146.4, 141.5, 127.6, and 121.0 g I_2/100 g of oil, and 146.4, 139.5, 124.1, and 120.4 g I_2/100 g of oil in opaque and transparent containers, respectively. The crude oil sample recorded the lowest iodine value compared to all treatments and storage conditions, reaching 146.4, 120.8, 98.0, and 91.0 g I_2/100 g of oil in opaque containers, and 146.4, 119.7, 92.9, and 88.4 g I_2/100 g of oil in transparent containers. A low iodine number is associated with the oil's composition of unsaturated fatty acids, specifically oleic and linoleic acids. A low iodine number indicates a decrease in the number of double bonds in these unsaturated fatty acids (Knothe, 2002). This phenomenon arises from the oxidative degradation of the oil and the resulting decrease in antioxidant activity as their concentrations decline during prolonged storage (Huang, Li, Bao, Li & Wang, 2022). The results of this study indicate that the iodine number increases with increasing HA concentration compared to the crude oil sample (Sudha & Rose, 2014).

The iodine number is a measure of the degree to which oil is not saturated with iodine. During storage, oil undergoes a process called oxidative rancidity, where oxygen reacts with the double bonds in the oil. As these double bonds break down, the oil's ability to absorb iodine decreases, and therefore the iodine number drops (Dymińska et al., 2017).

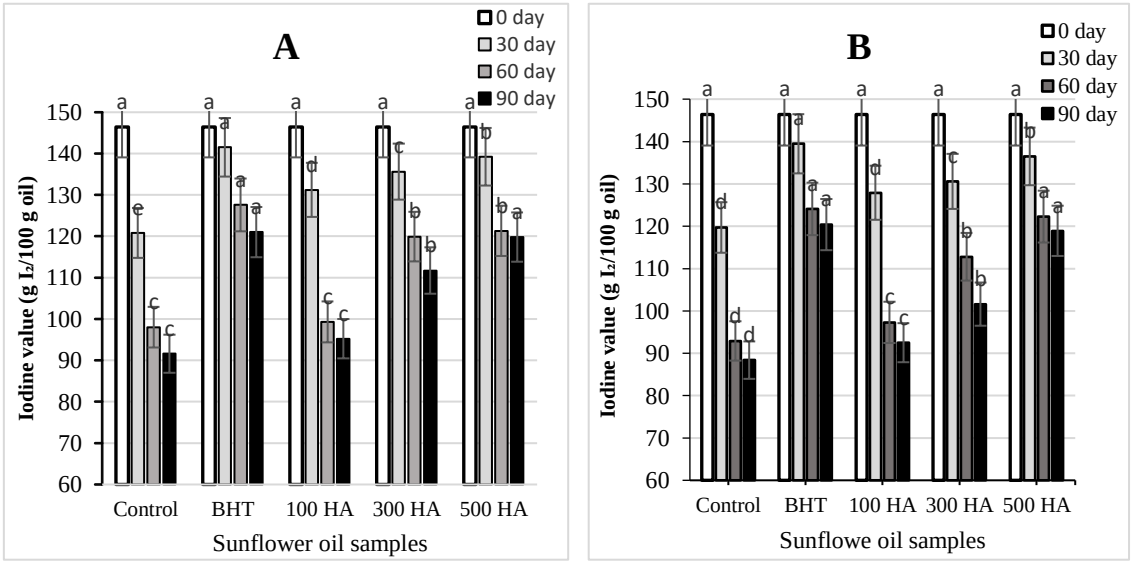


Figure 7. Iodine number values (g I_2/ 100 g oil) of sunflower oil samples added to HA at different concentrations during 90 days of storage, (A) opaque containers, (B) transparent containers. *Different lowercase letters indicate significant differences ($p < 0.01$) between oil samples during storage times

Added acids (such as hyaluronic acid or similar organic acids) act as a protective agent through two mechanisms: *i)* the added acid absorbs reactive oxygen atoms before they can attack the C=C double bonds in the oil, thus maintaining a high and stable iodine number; *ii)* molecular protective layer formation. In some cases, the acid forms a microscopic protective layer around the oil molecules, preventing penetration by external oxidising agents (Mohammed & Niamah, 2022b).

The oil used in this study is crude oil, which may contain enzymes (such as lipoxygenase) or impurities that catalyse the breakdown of double bonds in fatty acids. The long storage period of three months (90 days) is sufficient for significant oxidation to occur, especially if the sample is stored in containers that allow light or air to pass through. Furthermore, atmospheric oxygen reacts with the double bonds in the oil even in the absence of high heat, leading to the formation of hydroperoxides and the breakdown of the double bonds, thus reducing the iodine value. This explains the significant decrease in iodine number. Crude sunflower oil, with its high oleic acid (18.72-79.30%) and linoleic acid (10.11-51.72%) content (Li et al., 2024), is a very sensitive substance and more prone to oxidation, which negatively affects the iodine value of the oil.

The initial values of peroxide, acidity, TBA, and iodine number of sunflower oil were in accordance with the Codex Alimentarius standard for vegetable oils CXS 210-1999. Peroxide value, acidity value, and TBA value increased. In contrast, iodine number decreased throughout the storage periods of all sunflower oil samples, particularly in transparent containers compared to opaque containers (Randhawa & Mukherjee, 2023). This is attributed to the synergistic effect of light and oxygen in facilitating the oxidation process, accelerating the formation of peroxides (Machado et al., 2023).

CONCLUSIONS

Hyaluronic acid is a naturally occurring bioactive compound, and this study demonstrated its efficient production using probiotic bacterial strains (*S. thermophilus*) and whey as a low-cost culture medium, thus supporting the concept of environmental sustainability. Hyaluronic acid exhibited a superior ability to inhibit the oxidation of oils and fats, making it a safe and effective natural alternative to traditional synthetic antioxidants such as butylhydroxytoluene (BHT). The results confirmed that packaging plays a crucial role in oil stability; opaque packaging outperformed transparent packaging in protecting the product from photo-oxidation caused by UV penetration. Incorporating natural probiotic metabolites into food products enhances their health and functional properties, while demonstrating a high level of safety compared to chemical compounds, thereby reducing

the likelihood of side effects associated with synthetic additives.

AUTHOR CONTRIBUTIONS

Conceptualization, data curation, formal analysis, investigation, methodology, software, visualization, writing−original draft, A.A.M.; Conceptualization, data curation, investigation, methodology, project administration, S.T.G.A-S.; Supervision, validation, writing − original draft, writing − review & editing, A.K.N.

DATA AVAILABILITY STATEMENT

Data contained within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest**.**

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UPOTREBA HIJALURONSKE KISELINE DOBIJENE IZ LOKALNOG IZOLATA *STREPTOCOCCUS THERMOPHILUS* KAO ANTIOKSIDANTA U SIROVOM SUNCOKRETOVOM ULJU

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Sažetak: Hijaluronska kiselina je prirodni antioksidans dobijen iz različitih izvora, uključujući mikrobne, sa širokom primenom u medicini, prehrambenoj industriji i kozmetici. U ovoj studiji lokalni soj *Streptococcus thermophilus*, kultivisan na podlozi od surutke, korišćen je za proizvodnju hijaluronske kiseline, koja je identifikovana karbazolnom reakcijom, HPLC analizom i FTIR spektroskopijom. Antioksidativna aktivnost hijaluronske kiseline je procenjena merenjem sposobnosti neutralisanja DPPH slobodnih radikala, uklanjanja vodonik-peroksida i helacije jona gvožđa, u poređenju sa sintetičkim antioksidansom BHT. Tako dobijena hijaluronska kiselina korišćena je kao antioksidans u očuvanju suncokretovog ulja u to 90 dana skladištenja. Brzina produkcije hijaluronske kiseline je iznosila 0,598 g/L. Najveća efikasnost postignuta je pri koncentraciji od 500 µg/ml (u slanom rastvoru), sa stopama neutralisanja od 68,14% (DPPH) i 72,08% (H_2O_2), dok je stopa vezivanja jona gvožđa iznosila 81,98%. Dodavanje hijaluronske kiseline u nerafinisano suncokretovo ulje tokom skladištenja od 30, 60 i 90 dana rezultiralo je smanjenjem stepena oksidacije, kiselosti i sadržaja tiobarbiturne kiseline. Ulje čuvano u providnim posudama pokazalo je veću podložnost oksidaciji u odnosu na ulje u neprovidnim posudama. Rezultati ukazuju da hijaluronska kiselina može služiti kao prirodni antioksidans i održiva alternativa sintetičkim konzervansima u očuvanju jestivih ulja i produženju njihovog roka trajanja.

Ključne reči: *prirodni antioksidans, alternativni antioksidans, rok trajanja, antioksidaciona aktivnost, suncokretovo ulje, oksidaciona stabilnost*

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