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Comparison of The Antiglycation Effect of Pyridoxamine and Nettle Hydroalcoholic Extract on Albumin

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ABSTRACT

Background: *Free radicals are highly reactive molecules that cause oxidative damage to fats, proteins, and nucleic acids. Therefore, they play a crucial role in the development of many diseases such as heart disease, cancer, and abnormal brain function. The human body is equipped with antioxidants that neutralize free radicals, preventing their accumulation in tissues. The imbalance between the production of these radicals and the level of antioxidants leads to oxidative stress, where an increase in free radical production reduces antioxidant levels, causing a deficiency in the body. On the other hand, evidence indicates that oxidative stress contributes to the onset and exacerbation of diabetes-related complications and plays a significant role in non-enzymatic glycation of proteins (Riahi et al., 2014). Therefore, any diabetes treatment must directly or indirectly reduce non-enzymatic protein glycation and oxidative stress. Non-enzymatic protein glycation and the formation of AGEs are factors involved in the pathogenesis of chronic diabetes complications. Since glycation reactions are accompanied by oxidation reactions, leading to the improper function of proteins, plant-derived antioxidant compounds can be used as a therapeutic approach to prevent the onset of these chronic complications.*

Keywords: Pyridoxamine, Nettle extract, Antiglycation, Human serum albumin, and Diabetes.

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INTRODUCTION

1.2 Statement of the Problem

Nettle, scientifically known as Urtica dioica, is a herbaceous plant with a warm temperament mostly cultivated in Asia and Northern Europe. It often grows wild in ruins, moist areas, forest roads, gardens, temperate regions, and Mediterranean areas. The parts of this plant used include its flowering stems, Leaves, seeds, and roots. It has four-sided upright stems with branching, fully opposite leaves covered with rough hairs. Its stem can reach up to one meter in height. The stem is covered with conical-shaped hairs and stinging trichomes that stick to the skin upon contact, causing swelling and redness (Bnouham et. al., 2003). Nettle is a useful plant with many therapeutic properties, containing antioxidant Compounds. Antioxidants are compounds that prevent the activity of released free radicals or remove them, thus protecting the body's cells from the harmful effects of these compounds. Its antioxidant property is due to the presence of flavonoids, which include a large group of plant phenols such as flavones, anthocyanins, isoflavonoids, and flavonols. These

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compounds have to rejuvenate power and possess high antifungal and antimicrobial properties (Majd et al., 2003). Flavonoids hydrolyze lipids, eliminate cancer cells, demonstrate cytotoxic properties, and are beneficial in treating cancer-related diseases such as breast and lung cancer. Its root contains lectins that inhibit the abnormal proliferation of prostate cells, prevent epidermal growth factor from binding to its receptor, and are used in the treatment of benign prostatic hyperplasia by inhibiting the enzyme 5α-reductase (Goswami et al., 2022).

Nettle leaves reduce blood sugar levels due to their main flavonoids: rutin, quercetin, and kaempferol. Quercetin reduces glucose levels to normal and protects the beta cells of the pancreas, leading to increased beta cells and insulin secretion. Kaempferol acts as an α-glucosidase inhibitor and improves insulin resistance, and glucose homeostasis. Rutin, similar to quercetin, promotes calcium absorption in the pancreas (Soares et al., 2017). The total content of phenolic and flavonoid compounds plays a significant role in the medicinal properties of nettle. It appears that the compounds present in this plant can also affect the glycation of proteins like albumin.

1.3 Importance and Necessity of Research

Diabetes is one of the most prevalent chronic diseases in the modern world, and its prevalence is significantly increasing. Individuals with diabetes are unable to produce insulin or become resistant to insulin. Insulin is a hormone-responsible for regulating glucose (blood sugar) and allowing body cells to absorb glucose as energy. When cells become resistant to insulin, it means the body can produce insulin, but the body's cells do not respond appropriately to the produced insulin, leading to abnormal increases in blood sugar or hyperglycemia, and also resulting in elevated levels of triglycerides and ketosis similar to tissue catabolism. Diabetes increases the risk of various cardiovascular problems such as heart attacks, chest pain, artery narrowing, high blood pressure, and strokes, and also leads to kidney failure, limb amputation, and blindness. The non-specific reaction of proteins with sugars, called glycation or Maillard reaction is crucial as it plays a significant role in causing diabetes-related complications such as neuropathy, retinopathy, nephropathy, and other diseases. Diabetic patients with high blood sugar undergo a non-enzymatic glycation process, where sugars react with the amino acid lysine's ε-amino group and the α-amino group at the N-terminal end of proteins, known as glycation. The glycation reaction is divided into two stages: in the first stage, reducing sugars like glucose reacts with free amino groups in proteins and then forms Schiff bases. This bond is not stable and is reversible. As a result, rearrangement of intermediate compounds is produced. In the final stages of glycation, these intermediate products slowly and irreversibly create advanced glycation end products (AGEs) through a series of complex reactions (R. Singh et al., 2001).

Glycation products are toxic and have a long lifespan. Serum albumin is one of the proteins present in the plasma that undergo glycation. Human serum albumin is composed of a single polypeptide chain synthesized in the liver and is the most abundant plasma protein in human serum. Albumin has a helical and heart-like structure. This protein has useful functions such as antioxidant activity, regulation of colloid osmotic pressure, and the ability to bind to numerous drugs. Due to its high concentration and low molecular weight, albumin maintains osmotic pressure. Albumin plays a role in separating free radicals, oxygen, and toxic metabolites such as bilirubin, and all these activities are impaired by glycation (Guérin-Dubourg et al., 2012). Glycation in tissues leads to complications in diabetic

patients. This accumulation causes changes in cell immunity, enzyme activity, ligand binding, and protein half-life. Glycated albumin causes oxidative changes, impairs its antioxidant properties, and alters its function (Anguizola et al., 2013).

Most modern diabetes treatments have side effects; therefore, finding an effective treatment is essential, and in this regard, the use of compounds found in medicinal plants can be considered. Plant compounds may be beneficial in the prevention and reduction of diabetes complications by inhibiting reactions, breaking down sugar-protein bonds, or anti-glycation. These compounds exhibit beneficial effects by various methods such as trapping and removing active oxygen radicals, blocking carbonyl groups in reducing sugars and breaking down cross-linked structures in final glycation products.

Objective: Considering the biochemical effects of nettle extract and the human inclination towards herbal medicine, we aim to investigate the effect of this plant on serum albumin glycation and compare it with the anti-glycation drug pyridoxamine.

Specific Objectives: Determine the effect of nettle extract at different concentrations on the formation of early and final glycation products and the structure and weight of albumin in the presence of glucose. Investigate the effect of concentration and treatment time on the potential performance of the extract. Evaluate the effect of nettle extract compared to pyridoxamine on albumin

MATERIALS METHODS

The list of materials and equipment utilized in this thesis is presented in Tables (1,2).

Table (1) Equipment Used.

Device model/country of manufacture	Device name
A.N.D-Jenway	scales
Winlab / Germany	pH meter
Memmert /Germany	Incubator
Jenway / England	UV-VIS Spectrophotometer
Sigma / Germany	Centrifuge
IKA / Germany	Vortex
Jenway / England	Spectrofluorometer
IKA / Germany	Vacuum distillation machine
Germany	Sampler
Iranians are the leaders of medicine/Iran	autoclave
Labnet/ China	Centrifuge
Iran	Fraser
L81ST/ Netherlands	heat stirrer

Table (2) Materials used.

Material	Company
Pyridoxamine	Sigma Co
Sodium azide	Sigma Co.
Human Serum Albumin (HSA)	Sigma Co.
Congo red	Sigma Co.
Glucose	Sigma Co.
NaH2PO4	Merck
NaHPO	Merck
Ethanol	Merck
Nitro blue tetrazolium chloride	Sigma Co.

Preparation of Nettle Extract

First, the leaves of this plant are collected, washed, and dried. Then, they are ground and prepared for extraction. Fifty grams of nettle powder is soaked in 70% ethanol (the ratio of plant to solvent is 1:20) and kept for 72 hours in a dark room without light. The temperature should be maintained between 30 and 40 degrees Celsius. After this period, the solution is filtered using filter paper, and then the extract is placed in an oven at a temperature of 45 degrees Celsius to dry. Finally, it is stored in a freezer at a temperature of minus 20 degrees Celsius until use (Sultani et al., 2021).

Preparation of Protein Treatment with Sugar and Nettle Extract

Human serum albumin protein was purchased from Sigma, and a solution with a concentration of 4 mg/mL was prepared in 0.2 molar phosphate buffer. In this study, the albumin was combined with a 50 mM glucose solution in phosphate buffer as a glycation agent. To create glycation conditions, various protein samples of human serum albumin were incubated in the presence and absence of glucose. Samples of serum albumin protein without glucose were considered as negative controls. Furthermore, all stages of sample preparation and sampling were carried out under a laminar hood to prevent fungal and bacterial growth and to maintain sterile conditions. To investigate the anti-glycation properties of nettle extract, a concentration of 50 micromolar human serum albumin was incubated with glucose and 1 millimolar pyridoxamine was used. Pyridoxamine was used as a positive control and also as a known glycation-inhibiting agent.

Preparation of Samples Under Study in Glycation of Human Serum Albumin Protein with Nettle Extract:

- **Group 1**: 50 micromolar human serum albumin that was kept in phosphate buffer with a known pH along with sodium azide and 5 molar glucose for 10 days.
- **Group 2**: Similar to Group 1, but it was kept for 20 days.

- **Groups 3, 4, and 5**: Similar to Group 1, except that 10%, 20%, and 40% weight/volume of nettle extract was added, respectively.
- **Groups 6, 7, and 8**: Similar to Group 2, except that 10%, 20%, and 40% weight/volume of nettle extract was added, respectively, and they were kept for 20 days.
- **Group 9**: This group contains albumin, sodium azide, glucose, and 1 millimolar pyridoxamine, kept for 10 days.
- **Group 10**: This group contains albumin, sodium azide, glucose, and 1 millimolar pyridoxamine, kept for 20 days.

All experiments were conducted with 3 repetitions, and the storage conditions of the solutions were in an incubator shaker at a temperature of 37 degrees Celsius.

Measurement of Fructosamine

When glucose combines with protein, fructosamine measurement is used. After glucose binds to amino groups and forms a Schiff base, e.g., on the amino acid lysine, through reactions known as Amadori, lysine fructosyl or more broadly, fructosamines are formed. These compounds appear on the surface of proteins in the early stages of glycation and play an important role in causing vascular damage. For this test, the NBT reagent is used, added to the samples, and incubated at 37 degrees Celsius for one hour. Then, the absorbance of the samples at a wavelength of 530 nanometers will be measured against a blank. This measurement will be performed for all groups on the 10th and 20th days (Tupe et al., 2015).

Identification of Advanced Glycation End Products

The identification of proteins modified by malondialdehyde as well as pentosidine and argpyrimidine—both derived from arginine—can be conducted, as these compounds exhibit fluorescent properties that allow for their identification. The solutions are diluted, and the emission and excitation wavelengths differ. For the first compound, the emission wavelength is 440 and the excitation wavelength is 370 nanometers; for the second compound, the emission wavelength is 400 and the excitation wavelength is 335 nanometers (Vasarri et al., 2020).

Comparison of Amyloid Aggregation Formation from Modified Albumins

For this measurement, Congo red, a specific stain for beta sheets, is used for the identification of amyloids. In this process, the albumin solutions exposed to Congo red will be evaluated at a concentration four times that of albumin and by adding 10% volumetric ethanol to the albumin solution, with light absorption assessed at 530 nanometers (Vasarri et al., 2020).

Visualization of Treated Albumins on Gel

SDS-PAGE electrophoresis will be used. The polyacrylamide gel containing sodium dodecyl sulfate can identify the molecules added to albumin and the change in the weight of these molecules, potentially resulting in the formation of various albumins with different weights on the gel during electrophoresis (Tupe et al., 2015).

Ultraviolet-visible absorbance Spectroscopy (UV-VIS)

Absorbance is a process in which a substance selectively absorbs energy from specific frequencies of electromagnetic radiation, thereby attenuating the original beam of radiation. Ultraviolet and visible spectroscopy involves the absorption of electromagnetic radiation by a substance in the ultraviolet/visible region. Organic molecules, mineral species, and charge-transfer complexes are three important categories of

absorbers in UV and visible spectroscopy. The most significant transitions of organic compounds involve n to π* and π to π* transitions. The electrons responsible for absorption in mineral species are located in d and f orbitals, and charge transfer absorption in complexes is also the result of a type of internal oxidation/reduction process. It is possible that all the energy from a frequency of radiation is not absorbed by the substance. Transmittance is a measure of the quantity of unabsorbed light. Absorbance is also a dimensionless quantity that has a direct relationship with concentration. The Beer-Lambert law states a relationship between absorbance and concentration. One of the factors that affects absorbance spectra is the polarity of the solvent, which, depending on the type of transition, can shift absorption in the red light spectrum toward lower frequencies or in blue light toward higher frequencies.

Method for Determining the Amount of AGE Formed Using UV-Vis Spectroscopy

To examine glycated human serum albumin protein using UV-visible spectroscopy, a quartz glass cell with a thickness of 10 mm is employed in the UV-Vis spectroscopic device. Initially, the device is zeroed with distilled water. For this purpose, a protein sample with a concentration of 20 µM is prepared, and absorbance is measured in the wavelength range of 240-500 nanometers.

Method for Congo Red Binding Assay

Congo Red (CR) is a diazo dye that is widely utilized as a histological marker following the death of amyloid peptide aggregates in the brain tissues of Alzheimer's patients (Khurana et al., 2001). Its utility as an amyloid marker in pathological samples has been demonstrated by its high specificity for binding with amyloid proteins. The conformation of the amyloid fibrils is identified as a significant factor in the binding of CR (Lomakin et al., 1997). The spatial interaction between the dye molecules and amyloid fibrils leads to the formation of hydrogen bonds and hydrophobic and electrostatic interactions between the dye and amyloid fibrils. The absorbance of Congo Red increases at 530 nanometers due to its binding to beta structures. To prepare the Congo Red reagent (100 micromolar), 0.003 grams of Congo Red is dissolved in 5 milliliters of 10% ethanol and then diluted to a final volume of 50 milliliters with PBS solution. This solution is filtered through dark glass and kept in the refrigerator for 15 minutes (Miroliaei et al., 2011). For this test, 107 microliters of the sample is mixed with 40 microliters of the 100 micromolar Congo Red reagent, and after 10 minutes, the absorbance is measured at approximately 530 nanometers.

Measurement of AGE and Pentosidine

Certain types of AGEs, such as pentosidine, possess intrinsic fluorescence and can serve as markers for measuring AGEs. Pentosidine is a crosslink formed by the reaction between lysine and arginine with reducing sugars. Thus, measuring fluorescence intensity is one way to assess the amount of AGE and some of their specific characteristics. The level of AGE is indicated by measuring the fluorescence intensity of samples with maximum excitation and emission wavelengths of 370 and 410 nanometers, respectively. The presence of pentosidine is indicated by fluorescence peaks with excitation and emission wavelengths of 335 and 385 nanometers, respectively. The fluorescence intensity is measured using a spectrofluorometer (Sharma et al., 2002).

Gel Electrophoresis

To observe the electrophoretic mobility changes of both non-glycated and glycated albumin, an 0.8% agarose gel containing ethidium bromide is used as a staining agent.

1-Preparation of Agarose Gel

In the preparation of agarose gel, a buffer and agarose powder are used. The ratio of these components is directly related to the size of the molecules that are to be electrophoresed. For preparing agarose gel, agarose powder is typically dissolved in a suitable buffer. Common buffers used for this purpose include TAE and TBE. Buffer TAE is composed of tris (hydroxymethyl) aminomethane and EDTA, and it is preferred for its acidic environment. The concentration of this buffer in the gel and the electrophoresis tank should be the same to maintain ionic balance during the electric current application, which avoids interference in the movement of albumin samples. In buffer TBE, boric acid is used instead of acetic acid.

2-Steps for Preparing the Gel:

1. **Buffer Preparation:** First, prepare a 5X concentration of TAE buffer. Dilute the buffer with distilled water at a ratio of 1:9 to obtain a 0.5X buffer.
2. **Agarose Gel Preparation:** Dissolve 0.8 grams of agarose powder in 100 milliliters of distilled water and heat until clear. (During heating, the mixture must be stirred to prevent the agarose powder from settling or burning.) Once the mixture reaches boiling and the agarose has dissolved completely, remove it from heat and allow it to cool in a laboratory environment until it reaches about 60°C. (If the gel is poured immediately after being removed from heat, the rapid cooling may result in smaller particle sizes and porosity that are not expected for a gel of this concentration.). Add 4 microliters of ethidium bromide and mix until homogeneous. Place a comb in the gel to create wells.
3. **Transfer the Gel:** Carefully transfer the gel into a gel tray, ensuring that this is done slowly and evenly to prevent air bubbles from forming. If air bubbles do occur, they should be removed using a clean pipette tip or another suitable tool.

3-Sample Loading and Equipment Setup:

1. **Sample Preparation:** Mix 15 milliliters of the homogenized sample with 3 milliliters of 6X loading dye and load it into the wells of the gel.
2. **Electrophoresis Setup:** Place the gel along with its tray in the electrophoresis tank, which contains 0.5X TAE buffer, ensuring that the solution completely covers the gel surface. Connect the power supply to the electrophoresis tank. It is important to ensure that the direction of movement is from the negative to the positive electrode. Turn on the device, set it to 30 mA, and wait for 1 hour.
3. **Gel Visualization:** After the electrophoresis is complete, remove the gel from the tank and examine it under UV light.

RESULTS

Results of Fructosamine Measurement

The formation of Amadori products was examined through the fructosamine measurement test. After incubating various serum protein samples of human albumin in the presence and absence of glucose, the formation of Amadori products gradually increased over 10 days and 20 days. The effect of the alcoholic extract of nettle on the formation of Amadori products is illustrated in Figure (1) . These extracts had a delayed inhibitory effect on the formation of these compounds over 20 days, although minimal, and this inhibitory effect increased from the 10-day mark to the 20-day mark. Pyridoxamine, as an inhibitor, led to an increase in the formation of fructosamine. The negative control (lacking glucose) remained constant over 20 days, indicating that the Amadori compound was not formed and demonstrating the validity of the test.

The amount of light absorption resulting from the treatment of different concentrations of nettle extract and pyridoxamine with albumin-glucose solution the average results of the conducted tests are presented in Figure (1 , and the results indicate that over time, in all treatment groups, the level of absorption decreased with increasing concentrations of the extract. The results of the ANOVA statistical analysis show that there is a significant difference between the 10-day and 20-day measurements ($p < 0.05$). Furthermore, comparisons between different treatment groups indicate that the 20-day treatment had the most significant effect, with the treatment containing 40 µg/ml of nettle extract showing the greatest reduction in absorption, nearly equaling the treatment without extract that contains pyridoxamine.

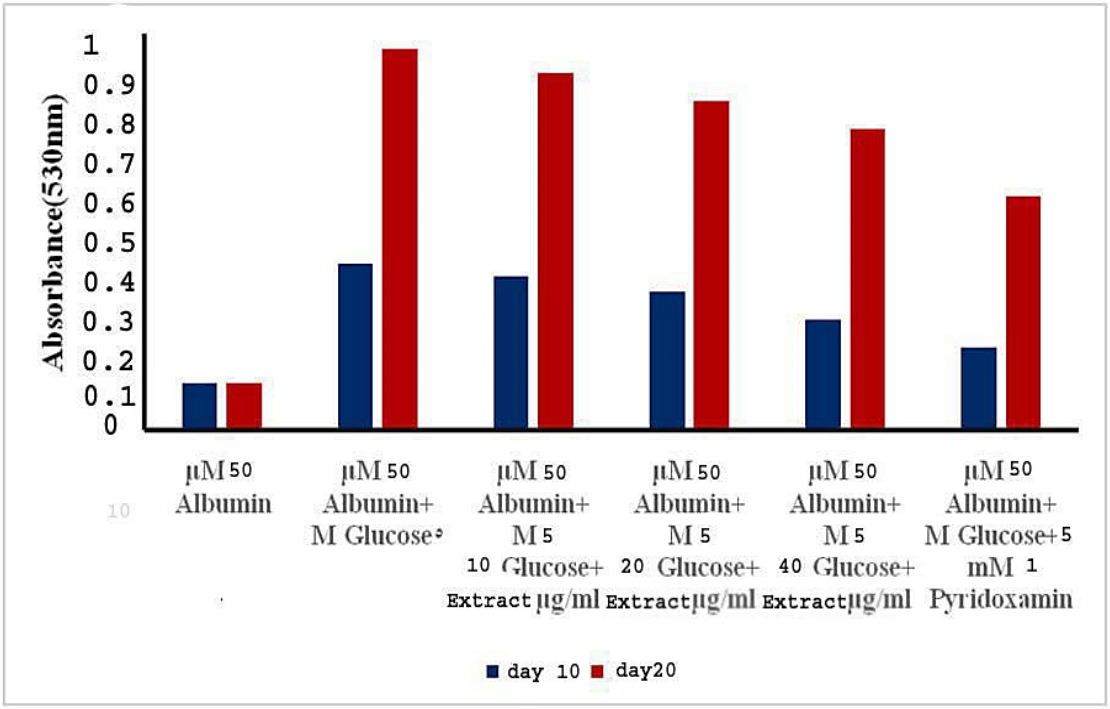

Figure 1: Optical Absorbance Levels Resulting from Treatment of Various Concentrations of Nettle Extract and Pyridoxamine with Albumin-Glucose Solution.

Table 3: Analysis of Variance for Fructosamine Assay.

Source of Variation	Degree of Freedom	Sum of Square	Mean Square	Ratio F	Value p
Rows	1	0/476008	0/476008	23/23314	0/004798
Columns	5	0/439642	0/087928	4/291629	0/067907
Residual error	5	0/102442			0/020488
Total	11				1/018092

Results of the Effect of Pomegranate Extract on Advanced Glycation EndProducts (AGE) Identification:

The formation of Total AGE compounds was evaluated by measuring the fluorescence of these compounds (440 Em, 370 Ex). The fluorescence intensity of these compounds decreased over the incubation period (20 days) and (10 days) in the presence of glucose. Assessment of AGE formation for pomegranate alcoholic extract at concentrations of µg/ml 10, 20, and 40 after 10 and 20 days of incubation was performed. As observed in Figure 2-4, pomegranate extract caused a reduction in the fluorescence intensity of AGE compounds in a concentration-dependent manner. Furthermore, the effect of pomegranate alcoholic extract on the formation process of pentosidine as one of the indicator compounds of AGE was measured using its typical fluorescence (385 Em, 335 Ex). According to Figure 3-4, the fluorescence intensity of pentosidine increased during the incubation period, and pomegranate plant alcoholic extract at various concentrations and in a concentration-dependent manner led to an increase in pentosidine-related fluorescence. The results in these two cases indicate a significant inhibitory effect of pomegranate extract on the formation process of AGE compounds.

The average results of the experiments conducted in Figure 4-2 are presented, indicating that over time, the fluorescence level decreased with increasing extract concentration in all treatments. Statistical analysis using ANOVA shows a significant difference between 10 days and 20 days ($p<0.05$). Furthermore, comparisons between different treatments indicate that the 20-day treatment had the most significant effect, and the treatment containing 40 µg/ml of pomegranate extract had the highest reduction in fluorescence.

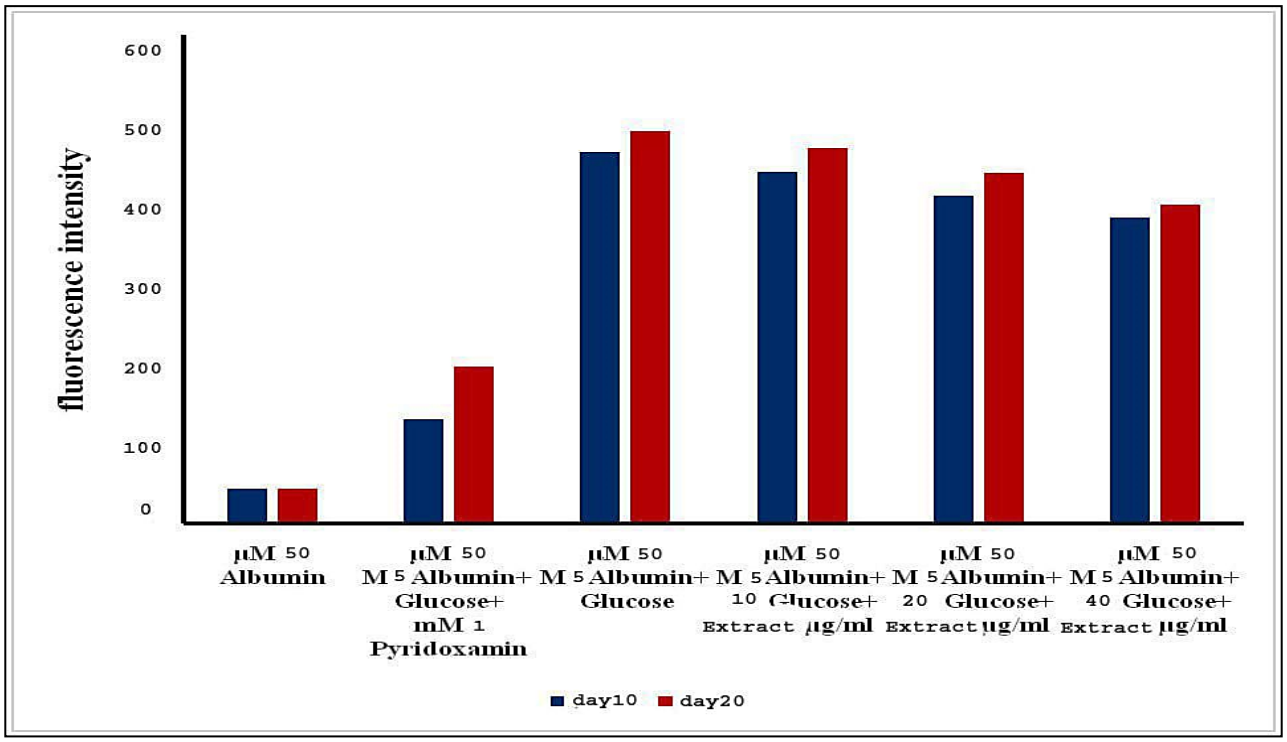

Figure 2: The inhibitory effect of nettle extracts on the formation of AGE compounds in the presence of glucose.

Table 4: Variance analysis of total composition formation (Total AGE).

Source of Variation	Degree of Freedom	Sum of Square	Mean Square	Ratio F	Value p
Rows	1	2214/083	2214/083	9/531822	0/027241
Columns	5	307240/8	61448/15	264/5396	E-06 71/4
Residual error	5	1161/417			232/2833
Total	11				310616/3

The figure presents the average results of the experiments conducted in Figure 3, indicating that over time, the formation of pentos idine increased with increasing extract concentration in all treatments. Statistical analysis using ANOVA shows a significant difference between 10 days and 20 days ($p<0.05$). Furthermore, comparisons between different treatments indicate that the 20-day treatment had the most significant effect, and the treatment con taining 40 µg/ml of pomegranate extract had the highest level of pentosidine formation.

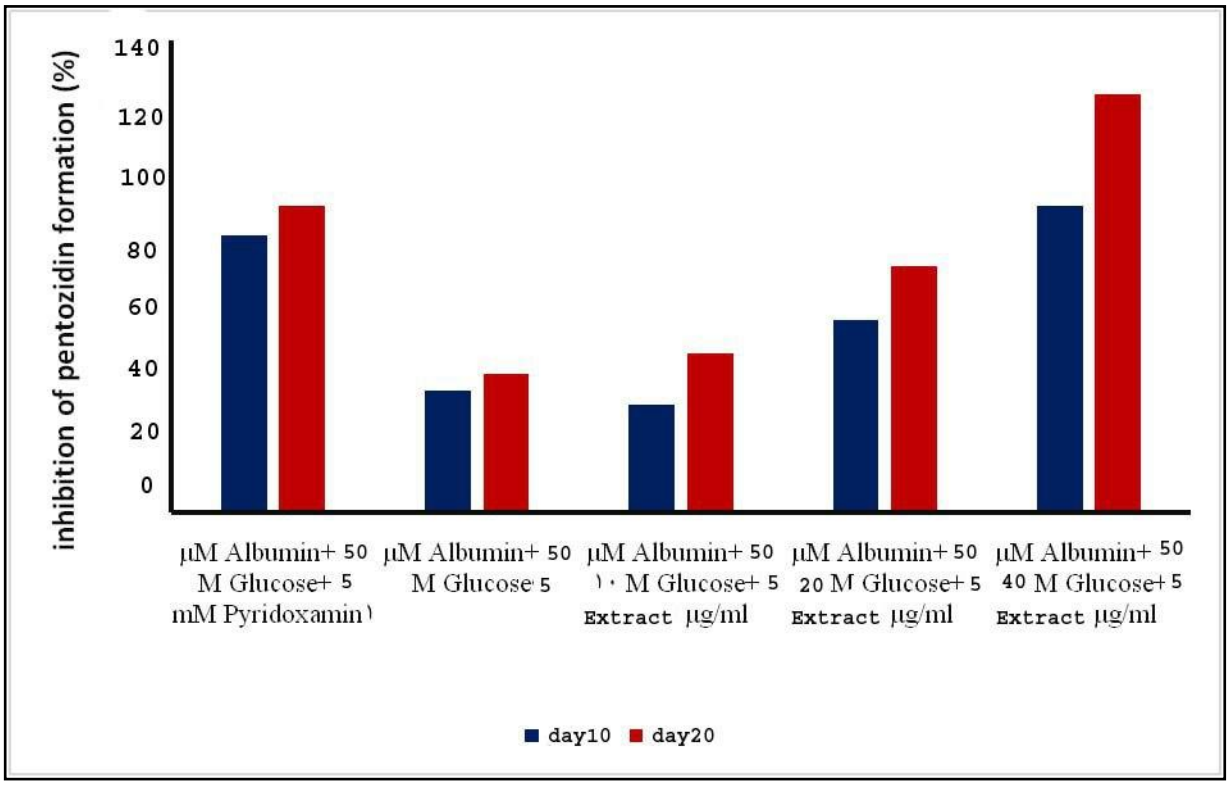

Figure 3: Inhibitory Effect of Pomegranate Plant Extract on Pentosidine Formation as an Indicator Compound of AGE.

Table 5 : Analysis of Variance for Pentosidine Formation.

Source of Variation	Degree of Freedom	Sum of Square	Mean Square	Ratio F	Value p
Rows	1	608/4	608/4	10/5993	0/031207
Columns	5	7184/4	1796/1	31/29094	0/002818
Residual error	5	229/6			57/4
Total	11				8022/4

Examining glycation of albumin using Congo reagent:

Congo red is specific for identifying amyloid fibers caused by glycation and aggregation. The increase in optical absorption of this substance at 530 nm is due to binding to beta structures. According to the increase in absorption in the albumin sample treated with 50 mM glucose compared to albumin, it can be seen that the formation of beta structures in the sugared protein is intensified. While the decrease in absorption in glycated samples is clearly evident in the presence of nettle extract, which indicates a decrease in the binding of Congo Red reagent to the sample, and it can be concluded that nettle extract prevents the change of secondary structures in the protein caused by its glycation.

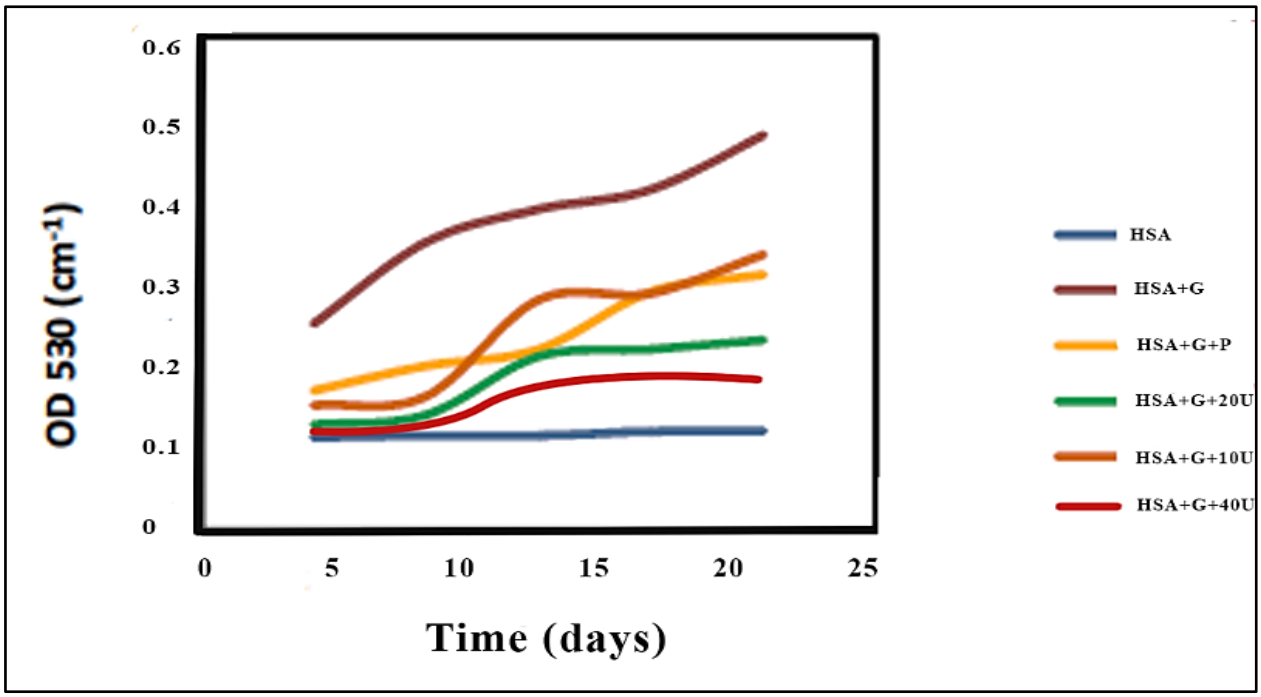

Figure-4: Spectroscopy chart at 530nm wavelength in the presence of Congo Red reagent in samples of control albumin (HAS), glycated albumin (HAS+G) + glycated albumin with extract with concentrations of 10, 20, 40 and glycated albumin with pyridoxamine.

Examination of glycation of treated albumins on the gel:

Various samples of human serum albumin protein in the presence and absence of glucose in phosphate buffer with a concentration of 1/0 M and a pH of 4/7 were incubated for 10 days and 20 days at a temperature of 37°C. They were then electrophoresed on a 5/1% agarose gel at a voltage of 70 mA for 55 minutes. When albumin was uniformly run in the electrophoresis gel, it moved briefly, and a single band was observed at the top of the gel due to its linear relationship in gel migration with the size algorithm of the fragment. As seen in Figure (5), albumin samples in the presence of glucose, pomegranate extract, and pyridoxamine on SDS-PAGE gel show different bands at 10 days and 20 days. Fragmentation of glycated albumin molecules with thick multiple bands can be seen due to pomegranate extract preventing protein polymerization. The results obtained in this study indicate structural changes during the 10 and 20 days of treatment, with longer treatment times with glucose resulting in a higher percentage of albumin degradation. This study demonstrates the protective role of pomegranate extract against molecular structure degradation of albumin.

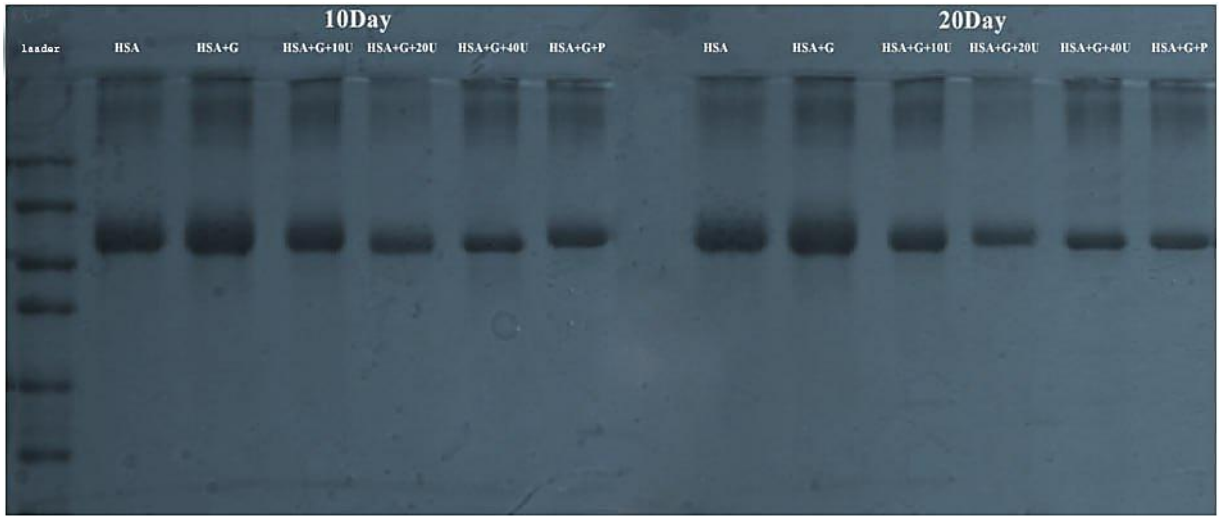

Figure 5-: SDS-PAGE related to samples of glycated albumin with extract with concentrations of 10, 20, and 40 and glycated albumin with pyridoxamine.

DISCUSSION AND CONCLUSION

In diabetic patients, poor control of glucose levels over time leads to hyperglycemia, which causes damage to tissues and creates dangerous complications such as infertility (Kovac et al., 2013). Studies indicate that the irreversible complications of diabetes arise from glucose metabolites, which result in changes in the composition of cholesterol, albumin, collagen, and hemoglobin, thereby facilitating the emergence of diabetic complications in diabetic individuals (Shamsi et al., 2010). Type 2 diabetes is the most common and serious metabolic disease worldwide, characterized by

impaired function of pancreatic beta cells and insulin resistance. Under diabetic conditions, chronic hyperglycemia leads to an increase in reactive oxygen species (ROS), which damage beta cell function and contribute to heightened insulin resistance, ultimately exacerbating Type 2 diabetes. It has been shown that ROS are produced in various tissues under diabetic conditions. There are several sources of ROS within cells, including non-enzymatic glycosylation reactions, the electron transport chain in mitochondria, and NADPH oxidase associated with membranes. Due to the increasing prevalence of diabetes and its late-onset complications, it is crucial to understand that the primary cause of these complications is the glycation of macromolecules. The non-enzymatic reaction of glucose with macromolecules such as proteins has been a significant area of focus for researchers (Li et al., 2007). Elevated blood sugar levels enhance non-enzymatic glycosylation or glycation of intracellular and extracellular proteins. The glycation process leads to the production of various free radical species. Well-known products of this process include Schiff bases, Amadori products, and advanced glycation end products (AGEs), all of which have highly oxidative properties and are generally destructive. Since the function of every protein is dependent on its structure, glycation can alter the structure and function of numerous intracellular and extracellular proteins, leading to many of the secondary complications of diabetes.

Blood Glucose Control and Glycation

Blood glucose levels are regulated by various factors that affect the digestion and absorption of carbohydrates, glucose control in the liver, storage, production, and insulin efficiency in stimulating glucose uptake. Glycation is a non-enzymatic reaction between proteins and sugars that leads to the formation of advanced glycation end products (AGEs). To prevent the production of AGEs, there are numerous natural and synthetic inhibitors available. Oxidative damage caused by free radicals contributes to the development of many diseases, leading to significant and fundamental damage to cells and tissues. Since albumin protein plays many essential roles, including regulating blood pressure and transporting metabolites, fatty acids, and amino acids, studying the changes induced by glycation of serum albumin is of great importance. Considering the mentioned points, it seems that research on protein glycation, its complications, and strategies to mitigate and combat this process could be beneficial and effective for public health by preventing various diseases in humans. In this regard, various studies have been conducted, and many therapeutic processes often involve synthetic drugs and inhibitors to mitigate glycation, which unfortunately often have various side effects.

Therefore, it is necessary to investigate the natural substances that can prevent protein glycation and, to the extent possible, replace synthetic drugs. This study aims to investigate the effects of nettle extract on the glycation of human serum albumin (HSA). In the Congo red binding method, since the beta structures formed in the protein bind to the Congo indicator, this indicator was utilized to detect cross-β structures. The results obtained from this investigation, in the absence of extract, indicate that amyloid structures in albumin protein increase as a result of glycation, and the reduction in binding in the presence of the extract can be attributed to the inhibiting effect of the extract on glycation and the formation of beta structures. Using the SDS-PAGE method, it was clear that the albumin molecule undergoes structural changes during glycation. SDS plays a role in equalizing protein charge, and under its influence, proteins are separated from each other based on molecular weight. Therefore, in the case of glycated albumin

samples, the increase in Cross-beta structures and the formation of dimers and tetramers during glycation may lead to an increase in the molecular weight of the albumin molecule in the final stages of glycation. The fragmentation of glycated albumin molecules with thick multi-band is observed. In the presence of pomegranate extract, this fragmentation decreases, which is probably due to the inhibitory function of the extract in preventing the formation of dimers, tetramers, and polymerization of proteins. The results of measuring fructosamine, which indicates the level of early glycation compounds, during our research also confirm this issue.

The pomegranate extract had a very slight inhibitory effect on the formation of fructosamine compounds over 3 weeks, and despite its high antioxidant activity, it was able to inhibit these compounds with an increase in concentration, although slightly. Over a period from 10 days to 20 days, this inhibitory effect increased. In contrast, in examining the content of total AGEs using fluorescence intensity measurement of samples at 10 days and 20 days, we observed the effect of pomegranate extract on reducing the level of AGEs. The results indicate the inhibitory effect of pomegranate extract on the formation of AGEs, which this inhibitory effect increased significantly with an increase in concentration. In conclusion, glycation reactions are divided into two stages: reactions leading to the production of early glycation compounds in the first stage, and in the second stage, the compounds produced lead to the production of various AGE compounds (Vlassara, Bucala, and Striker 1994).

It has been suggested that oxidative reactions and free radicals do not play a role in the production of early glycation compounds in the first stage, while they play an important role in the formation of AGE compounds in the second stage (Sakurai and Tsuchiya 1988) and (Fu et al. 1994). In our studies, measuring the level of fructosamine indicates the level of production of early glycation compounds during the glycation process. The results showed that the pomegranate extract has an effect on inhibiting the formation of early glycation compounds, and this inhibitory effect, although small, increased over time, albeit slightly, and increased significantly from ten days to 20 days. Based on this discussion, our results indicate two points: the intervention of oxidative reactions in AGE formation and the possibility of using natural antioxidants as AGE inhibitors, versus the use of specific fluorescence as an accepted method for identifying final AGE compounds in our studies. The results indicate the inhibitory effect of pomegranate extract on the formation of overall AGE and pentosidine compounds, and this inhibitory effect increased significantly with an increase in concentration.

Ethics Committee Approval Statement

This study, titled "Comparison of the Antiglycation Effect of Pyridoxamine and Nettle Hydroalcoholic Extract on Albumin," was conducted by the ethical standards outlined in the Declaration of Helsinki. The Ethics Committee of the College of Medicine, University of Al-Qadisiyah, Iraq reviewed and approved the research protocol. Informed consent was obtained from all participants involved in the study, ensuring their rights and welfare were prioritized throughout the research process. All procedures were conducted in compliance with institutional guidelines for ethical research practices.

Suggestions

- **Investigation of Interactions**: Examine the interactions of other plant compounds and herbal medicines with human serum albumin.
- **Isolation and Identification**: Study the isolation and identification of the

active components and materials in nettle (Urtica dioica).

- **In Vivo Studies**: Conduct studies and experiments using animal models to evaluate the effects of plant compounds in in vivo conditions.
- **Antimicrobial Activity**: Investigate the antimicrobial effects of the compounds and plant extracts.

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