Investigation of Effect of *Curcuma longa* L. against Glucose Mediated Glycation of Bovine Serum Albumin

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**Abstract:** The beginning and/or development of an expanding variety of human diseases has been linked to oxidative stress, inflammation, glycation, and advanced glycation endproducts. Large-scale research on complementary therapies with anti-oxidant and anti-inflammatory properties for the treatment or prevention of several diabetes-related diseases has not, however, been advised because there is insufficient data from meta-analyses and clinical trials. However, adverse effects and high cost of allopathic medicines force to investigate plant based therapeutic strategies for management of various diseases. This work aims to determine the impact of *Curcuma longa* extract on AGE production, oxidative stress, and glycation through in vitro experiments. Ascorbic acid's reducing power was comparable to that of *C. longa* extract (at 600 µg/ml). Additionally, *C. longa* suppresses the amount of browning intensity by 60.3%, the percent aggregation index by 57.38%, and the amyloid structure by 61.2%, preventing glycation of BSA's native structure. Consuming *C. longa* can therefore help avoid and treat issues associated with diabetes. To ascertain the processes of *C. longa* components in disease modulations, more thorough chemical and pharmacological investigation is necessary.

**Index Terms** - Diabetes, Glycation, AGEs, *Curcuma longa*.

1. Introduction

Oxidative stress, glycation, and the production of advanced glycation endproducts are associated with most diabetic problems. It has been discovered that employing medicinal plants for the treatment and management of illnesses in a variety of traditional medical systems is a secure and financially sensible substitute for allopathic medicine (Anwar et al., 2021). *Curcuma longa* is a plant used for medicinal purposes that has been shown to be a successful treatment for a number of illnesses. Its antihypertensive, antibacterial, antidiarrheal, digestive, liver tonic, diuretic, analgesic, and skin-protective properties have all been documented in earlier research. Additionally, it is said to provide therapeutic benefits for heart disease, neurological conditions, tumor growth, hyperglycemia, and inflammation (Fuloria et al., 2022; Dosoky and Setzer, 2018).

*Curcuma longa* L., a traditional herbal remedy, has gained popularity recently as a means of preventing complications from diabetic vascular disease. Owing to its anti-inflammatory, anti-cancer, and antioxidant characteristics, it has been extensively researched and has a broad range of therapeutic applications (Sharifi-Rad et al., 2020; Ahmad et al., 2020). Due to its well-documented health advantages and non-toxicity, curcuma has a great deal of potential in preventing a wide range of ailments. According to
Gupta et al. (2012), curcuma therapy is inexpensive. Strong antioxidant and anti-inflammatory properties of curcuma longa have previously been established (Anwar et al., 2022a).

One of the characteristics of diabetes is hyperglycemia, which promotes the production of advanced glycation end products (AGEs) (Rahbar and Figarola, 2003; Miroliaei et al., 2017; Anwar et al., 2021). The emergence of diabetic problems is a direct result of AGEs. The development of microvascular problems unique to diabetes and an acceleration of macrovascular disease are linked to the hyperglycaemic state observed in individuals with diabetes mellitus (Li et al., 2023). Advanced glycation end-products (AGEs) have been shown to be a contributing factor in the development and consequences of these adducts (Anwar et al., 2020a). The Receptor for AGE (RAGE), which AGEs interact with to upregulate receptor expression and initiate a series of cytotoxic pathways, is how AGEs affect the body. According to Hudson et al. (2002), accumulation of AGE/RAGE has been observed at sites of vascular illness in both human and animal models of diabetes.

Proteins generally undergo the spontaneous process of glycation, which significantly affects both their functional and physical characteristics (Shumilina et al., 2019). These modifications to the characteristics of proteins may have a connection to several clinical outcomes, including Alzheimer's disease (Breijyeh and Karaman, 2020), cataracts (Stevens, 1998), and arteriosclerosis (Fishman et al., 2018). Ultimately, the multistage and intricate glycation reaction produces heterogeneous advanced glycation end products. Glycation product formation is enhanced with age and in a number of disease situations, such as diabetes or neurodegenerative disorders (Younus and Anwar, 2018, Brownlee et al., 1986). Because of their comparable folding, well-known fundamental structures, and histories of being linked to the binding of numerous distinct classes of small molecules, bovine serum albumin (BSA) and human serum albumin (HSA) are commonly utilized in biophysical and biochemical research. One notable distinction between human albumin and bovine albumin is that the former has a single tryptophan residue, while the latter has two (Gelamo and Tabak, 2000).

One of the many roles that albumin, the main serum protein, performs is binding and conveying both endogenous and foreign ligands. Among the various environmental modifiers that affect its molecular structure, glucose is one of the most important ones. Under normal physiological settings, in vivo albumin glycation happens, but in diabetes, it increases. We sought to see whether curcuma extract could stop the glycation of the BSA molecule because it may be used as a model protein in in vitro studies. A possible pharmacological response in a diabetic statin may be found by using BSA to examine binding effects upon glycation, which appears to be a useful model for early studies (Żurawska-Płaksej et al., 2018).

Hyperglycemia exacerbates the COVID-19 sickness because the SARS-CoV-2 virus attaches to the ACE2 receptor more easily when it is glycated (Brufsky, 2020; Anwar et al., 2022b). Significantly greater levels of ACE2 expression are found in diabetic patients. The mechanism of the SARS-CoV-2 virus has been suggested to be directly impacted by ACE2 glycation (Dallalvalasa et al., 2023). Elevated Covid-19 risk factors and AGEs are linked. AGEs attach to their membrane-bound receptor (mRAGE) to cause intracellular signals that express a number of transcription factors involved in inflammation. Through the participation of AGE/RAGE signaling, hyperglycemia-mediated diabetes mellitus has been related to an increase in oxidative stress (Al-Kuraishy et al., 2021). Current research now center on curcuma’s potential utility as an active inhibitor of glycation, which is the non-enzymatic process of joining a free amino group of a protein with a keto or aldehyde group of sugar.

2. Material and methods

DMSO, methanol, aluminum chloride, hydrochloric acid, sodium carbonate, monosodium dihydrogen phosphate, disodium hydrogen phosphate, and sodium hydroxide.

2.1. C. longa rhizome methanolic extract preparation

At room temperature, the rhizome was shed and allowed to dry for three days after a thorough prewashing with water. Following drying, 50 g of the material was roughly crushed to create a homogenous powder, and 500 ml of methanol was used during the five-day extraction process, along with sporadic shaking. The raw extract was filtered, then vacuum-dried, and the dried extract was stored in the refrigerator until it was needed. The filtrate was regenerated in the necessary volume of either dimethyl sulfoxide (DMSO) or water in accordance with the experiment’s specifications. One solvent that is frequently used in drug discovery is DMSO. DMSO may alter the properties of proteins in solution, leading to denaturation, aggregation, or disintegration of the protein.
2.2. Phytochemical screening, polyphenol content evaluation and flavonoid content investigations

Investigations regarding phytochemical screening, polyphenol content evaluation and flavonoid content investigation done according to previous investigations carried in our lab (Anwar et al., 2022).

2.3. Assessment of reduction capacity

The approach outlined in previous publications (Anwar et al., 2020a) was used to conduct this test with a few minor modifications. By using the ferric reducing antioxidant power (FRAP) method, the in vitro antioxidant assay was estimated. 2.5 ml of 0.1 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide were added to 1 ml of aliquots containing varied amounts of ascorbic acid and 50 to 600 µg/ml of C. longa extract. After being incubated for 20 minutes at 50°C, the reaction mixture was quickly cooled with running water. The mixture was then centrifuged for 10 minutes at 3000 rpm after 2.5 ml of 10% trichloroacetic acid was added. Following that, 2.5 ml of the supernatant was gathered and combined with 2.5 ml of deionized water. After briefly vortexing the resulting, 0.5 ml of freshly made 0.1% ferric chloride (FeCl₃) solution was added till the green-colored solution appeared.

Percentage reduction capacity= [(C – S)/S] × 100

On the other hand, S represents the extract-containing solution's absorbance, while C (the control) indicates the absorbance of the extract-free solution.

2.4. Incubation of C. longa extracts with in vitro glycation system

The fascinating correlation between the inclination of proteins to form amyloid structures and in vitro glycation assays warrants further investigation to ascertain the significance of this relationship in hyperglycemic settings (Arasteh et al., 2014; Anwar et al., 2014). BSA was glycated in vitro using Brownlee's (Brownlee et al., 1968) method. 0.1 M phosphate buffer (pH 7.4) in sterile tubes was mixed with 500 mM glucose and 10 mg/ml BSA, either with or without varying concentrations of extract (50-600 µg/ml). For fifteen days, the tubes were stored in the dark at 37°C. Sterility was ensured by using glass vials with closed caps that were autoclaved. The samples were dialyzed against 50 mM phosphate buffer for a whole night at room temperature after incubation.

2.5. Browning intensity of glycated samples

According to Anwar et al. (2020a) and Kumar and Ali, 2019, there is a claim that the degree of browning in glycated samples can often be used as an accurate diagnostic of glycation. After diluting the various glycated samples with distilled water, the absorbance of each at 420 nm was used to evaluate the browning intensity using a 1-cm path length cell (Brownlee et al., 1986). For each test, there were three tests conducted. The following formula (Rahmani et al., 2022) was used to calculate the relative percentage of browning intensity.

Percentage browning intensity = [(C–S)/C] × 100

In the absence of extract, the absorbance of glycated BSA is expressed as C, however, S represents absorbance of glycated BSA (in the presence of extract).

2.6. Protein aggregation index

Relative aggregation is thought to be indicated by the aggregation index (Schrodel and de Marco, 2005). To ascertain the protective ability of C. longa extract against aggregation, the absorbance of several glycated samples containing (50-600 µg/ml) and without extract was evaluated at 280 and 340 nm. This allowed for the quantification of the percentage of protein aggregation index.

Percentage of the protein aggregation index= [(I340 / (I280 – I340))] × 100

Where, I280 and I340 represent the glycated sample's absorbance at 280 and 340 nm, respectively.

2.7. Determination of fibrillar state by Congo red assay

According to Kumar and Ali (2019) and Rahmani et al., 2022, an examination of the development of amyloid fibrils can be conducted by utilizing the Congo red (CR) dye in conjunction with glycated materials. Previous studies indicated that Congo red was ready. Absorbance was measured independently for native BSA, several glycated samples (including extract or not), and the Congo red backdrop. Briefly stated, 500 µl of AGEs-BSA/native BSA (100 µM) and 100 µl of Congo red (100 µM) were added, and the mixture was allowed to sit at 37°C for ten minutes. Every sample's absorbance was computed at 530 nm.

Percent prevention of amyloid formation= [(C–S)/C] × 100
Where, C denotes to absorbance of glycated sample (not incubated with extract), and S to absorbance of glycated samples having extract.

3. RESULTS

3.1. Preliminary observations, flavonoid, and phenolic content

This study is the second part of our project. We have already published the results of our first part of investigations with phytochemical analysis of the extract, polyphenol content evaluation and flavonoid content investigation in previous article of same extract that we used in this research (Anwar et al., 2022).

3.2. Antioxidant activity

In vitro antioxidant activity of methanolic extracts was determined by ferric reducing antioxidant power method (FRAP) using ascorbic acid as standard. This method is a simple, rapid and reproducible that provides an estimate of antioxidant capacity and is based on the ability of the extract to reduce Fe$^{3+}$ to Fe$^{2+}$. The ascorbic acid solution (100 and 200 µg/ml) showed significant % reducing ability. Further, reducing power ability of extract was found to be comparable to standard (Ascorbic acid).

![Graph showing percentage lowering action of methanolic extracts](image_url)

**Fig. 1.** Methanolic extract's percentage lowering action is shown in Figure 1. Samples 1 through 8 correspond to different amounts of ginger extract (50–600 µg/ml). Samples 9 and 10 included 100 and 200 µg/ml of the standard antioxidant "ascorbic acid" respectively.
3.3. Effect of *C. longa* extract on browning intensity

Browning intensity was substantially lower in samples containing BSA and glucose with varying doses of *C. longa* extract than in samples containing BSA and glucose alone. When comparing the methanolic extract to BSA that was kept glucose-free (without extract), the extract showed 60.3% browning at 600 µg/ml (p<0.05) in Figure 1. The information supports the extract's ability to prevent glycation.

![Browning intensity graph](image)

**Fig. 2.** Browning intensity decreases when *C. longa* is present. Sample 1 shows BSA having glucose only and is considered to be 100% glycated (or browning) after being stored for 15 days. There were 50, 75, 100, 200, 300, 400, 500, and 600 µg/ml of extract in samples 2 to 9. The browning intensity (or maybe glycation) is shown to be decreasing with an increase in extract concentration. Sample 10 only included BSA (without glucose or extract), and because of its low browning intensity, it may have undergone very few structural alterations. The data (n = 3, p<0.05) are displayed as means ± standard error of the means.

3.4. Protein aggregation Index

According to the study's findings, *C. longa* extract greatly decreased protein aggregation. The aggregation index of BSA samples containing both glucose and extract was significantly lower in a concentration-dependent manner (57.38% at 600 µg/ml of extract in sample 9) (Figure 3) (p<0.05) as compared to BSA samples incubated with glucose alone (100% in sample 1).

![Aggregation index graph](image)
Fig. 3. There is a decrease in the aggregation index when extract is present. As a result, Sample 1, which is BSA treated with glucose for 15 days, has the highest glycation aggregation index. Sample 1 also has the highest aggregation index. Samples 2 through 9 included extract at concentrations of 50, 75, 100, 200, 300, 400, 500, and 600 µg/ml. The sample with the lowest aggregation index was sample 10, which had BSA grown without glucose or extract. The means ± standard error of the means are presented for the data (n = 3, p<0.05).

3.5. Congo red test

Figure 4 presents the findings from the CR binding experiment. According to the study's findings, extract dramatically decreased the synthesis of cross amyloid at higher dosages. When BSA samples with both glucose and extract were compared to BSA samples incubated with glucose alone, there was a significant, concentration-dependent decrease in cross amyloid aggregates (p<0.05). The percent amyloid structure in glycated BSA samples containing both glucose and extract was significantly lower in a concentration-dependent manner (61.2% at 600 µg/ml of extract in sample 9) (Figure 4) (p<0.05) as compared to BSA samples incubated with glucose alone (100% in sample 1).

Fig. 4. Cross-amyloid aggregation reduction brought on by the presence of extract. As BSA was treated with glucose for 15 days, Sample 1 is thought to exhibit 100% structural alterations. Cross amyloid aggregates were seen to decrease with increasing extract content in samples 2-9, which contained 50, 75, 100, 200, 300, 400, 500, and 600 µg/ml of extract. Sample 10 (green column) included BSA that had not been treated with extract or glucose.

4. Discussion

With fewer side effects, natural products and the bioactive molecules they contain are important in the management of disease. Previous research has demonstrated that natural compounds or their active ingredients can enhance health by controlling diabetes, reducing oxidative stress and inflammation, and assisting in the control of cell signaling pathways (Alam et al., 2022; Zhou et al., 2022).

Plants contain a wide range of polyphenols and these polyphenols contribute to various health's beneficial activities of these plants (Zagoskina et al., 2023). Due to the presence of molecules or secondary metabolites as antioxidants, plants and their products can combat oxidative stress and reactive oxygen species (ROS) and even avert oxidative damage (Pandey and Rizvi, 2009; Anwar et al., 2023). Undertaking an analysis of plant-derived natural products as a possible supply of antioxidant molecules relevant to medicine that are necessary for therapeutic purposes is highly attractive from this angle (Anwar et al., 2020; Meenatchi et al., 2016).

Our study revealed that C. longa exhibited strong antioxidant activity on par with ascorbic acid, which may account for its potential therapeutic benefits against oxidative stress and the generation of AGEs. Numerous clinical disorders associated with diabetes are brought on by oxidative damage to various biological macromolecules (Forcados et al., 2021). Glycation of human serum albumin (HSA) is one of the
proteins that is particularly interesting. When reducing sugars interact with HSA in vitro, structural and functional changes occur. Because of how effective and consistent these modifications are, HSA and BSA may now be used as novel, unique disease marker for diabetes rather than HbA1C. After reviewing the BSA aggregation in terms of the structural and biological effects of glycation on the protein, reporting records that suggest glycated albumin may be employed as a particular diabetes marker were examined (Arasteh et al., 2014).

Glycation is assumed to play a major role in the development of diabetes and its consequences (Younus & Anwar, 2018; Vlassara and Urribarri, 2014). Aggregates may occur as a result of the structural defect. The formation of protein aggregates is also connected to various disorders and associated repercussions (Fassler et al., 2021; Serangelo and Iannuzzi, 2021). It was anticipated that antioxidants will play a novel role in the prevention and treatment of amyloid disorders and diabetes by acting as potential inhibitors of AGEs and glycation-induced protein aggregation. Furthermore, the design and development of potential medications for the treatment and prevention of AGEs and disorders linked to protein aggregation was greatly aided by these inhibitory mechanisms (Liu et al., 2022). As a result, it is critical to explore the protective efficacy of natural products against glycation-induced protein aggregates formation to establish a strategy for treating and managing disorders associated with the aggregate formation or structural alterations caused by glycation (Anwar et al., 2020).

Anything above 320 nm in absorbance signal is caused by aggregate particles scattering light. Its fluorescence intensity increases as it aggregation proceeds from a polar to a non-polar environment. In fact, the ratio of intensities at 280 and 340 nm (I280/I340) is completely independent of concentration and entirely linked to the extent of sample aggregation. The wavelength at 340 nm appears to be very significant for proteins since it robustly relates directly to intrinsic protein fluorescence (Raynal et al., 2014). A complex network of enzymes and antioxidant compounds protect against oxidative stress and oxidative stress cause a number of health problems (Pizzino et al., 2017) such as liver ailments (Yahia and Anwar, 2020). Therefore, antioxidants such as superoxide dismutase (SOD) may function as protective mechanisms against a variety of medical conditions (Younus, 2018). These antioxidant molecules like SOD also become glycated and lose their activity and functions that aid to disease ailments (Anwar et al., 2014).

The capacity of the extract to reduce free radicals revealed that C. longa inhibited free radicals in a concentration-dependent way. Higher levels of polyphenolic compounds may be responsible for the considerable reduction of free radicals (Checkouri et al., 2020). In this investigation, C. longa extract significantly and concentration-dependently reduced the development of cross amyloids, AGEs, as well as browning intensity as seen by different in vitro experiments. Prior research has indicated a potential correlation between polyphenolic content, antioxidant activity, and the suppression of AGE production (Anwar et al., 2020a). Therefore, myrrh extract's capacity to inhibit the AGE-producing process may be due to its high concentration of polyphenolic compounds with strong antioxidant activity. Even though higher concentrations of natural substances can be toxic, they are not always dangerous. They may occasionally be toxic to a particular organ, harming tissue or impairing metabolism. Natural goods should be used carefully because large amounts of them might be hazardous. Our findings provide evidence for the potential therapeutic effects of C. longa against the pathophysiology of several glycation-related health conditions through in vitro studies.

5. Conclusion

Our research concluded that C. longa was successful in preventing the glycation as well as production of AGEs. The antioxidant activity could be a contributing factor to its antiglycation actions. The primary mechanism by which C. longa inhibited protein glycation was its capacity to react with ROS. Moreover, According to these findings, C. longa may help to prevent or treat chronic illnesses linked to AGE. Our previous study confirmed anti-inflammatory potential through various in vitro investigations (Anwar et al., 2022). Combining the results of this study with previous findings of our project linked with exploration of anti-inflammatory, antioxidant and antiglycating potential C. longa, it can be assumed C. longa may be a good choice for use in research examining the role of natural herbal supplements in the prevention of diabetic complications. However, clinical data and in vivo investigations for efficacy, toxicity, safety, and dosage determination are recommended in future.

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