



Inhibitory activity of Curcuminoids and *Curcuma longa* on *in vitro* albumin glycation reaction

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ABSTRACT

Hyperglycaemia causes increased protein glycation and the formation of advanced glycation end products which underlie the complications of diabetes. *Curcuma longa* and its active compounds, curcuminoids are known to have antidiabetic effects. However, their role in the diabetic complication prevention is still unknown. The aim of this study was to determine the effects of curcuminoids and *C. longa* on *in vitro* glycation of albumin. With various concentrations of curcuminoids and *C. longa*, albumin glycation was observed and evaluated using the thiobarbituric acid (TBA) method. Inhibition modes of *C. longa* and curcuminoids against albumin glycation activities were measured with increasing concentrations of glucose as a substrate in the absence or presence of *C. longa* and curcuminoids. This type of inhibition was investigated by Eddie Hofstee plot analysis. *C. longa* and curcuminoids were both found to inhibit the glycation reaction of albumin. *C. longa* exhibited competitive and non-competitive inhibition of albumin glycation, while curcuminoids showed only competitive inhibition. The results obtained have shown that *C. longa* and curcuminoids can inhibit the reaction of glycation and this may explain their ability to decrease complication occurrences in diabetes.

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INTRODUCTION

Diabetes is a metabolic disturbance characterized by abnormal glucose metabolism and a risk of developing vascular complications. These vascular complications include microvascular (retinopathy, neuropathy, nephropathy) and macrovascular complications (coronary heart disease, peripheral arterial disease, cerebrovascular disease). The risk of complications increases as a function of the duration and degree of hyperglycemia (Johnsen and Paavonen, 2007; Powers, 2012).

The worldwide prevalence of diabetes has risen dramatically over the past two decades, from estimated 30 million cases in 1985 to 285 million in 2010. Based on current trends, the International Diabetes Federation project that 438 million individuals will have diabetes by

the year 2030 (Powers, 2012).

The chronic hyperglycemic state of an organism predetermines the intensification of glycation (a process that involves addition of glucose to the amine NH₂ group of lysine residues of blood proteins and lipids). Hyperglycemia results in increased reactive oxygen species (by increased activation of mitochondrial electron transport) and advanced glycation end products (AGEs). AGEs have been implicated in the pathogenesis of diabetic complications (Sam and Meeran, 2009; Peppas et al., 2003).

Formation of AGEs correlates with glycaemic control and the most effective way to reduce the risk of diabetic complications is intensive insulin treatment. However, excellent control of hyperglycaemia is achieved in less than 25% of type 1 diabetic patients. A variety of pharmacological intervention have been used to reduce advanced glycation and thereby AGE accumulation, via

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either attenuation of total AGE load or via chemical modification of existing AGEs to inactive form. Aminoguanidine, pyridoxamine, benfotiamine and metformin reduce AGE accumulation by inhibiting AGE formation (Johnsen and Paavonen, 2007).

Some medicinal plants which have been proven to be antidiabetic include *Curcuma longa*, *Allium sativum*, *Eugenia jambolana*, *Momordica charantia*, *Ocimum sanctum*, *Phyllanthus amarus*, *Pterocarpus marsupium*, *Tinospora cordifolia*, *Trigonella foenum graecum* and *Withania somnifera* (Modak et al., 2007). *C. longa* plant, a perennial herb belonging to the Ginger family, is cultivated extensively in South and South-east tropical Asia. The rhizome of this plant is also referred to as the "root" and is the most useful part of the plant for culinary and medicinal purposes. The most active component of *C. longa* is curcumin, which makes up 2 to 5% of the spice (Chattopadhyay et al., 2004).

Curcumin has been shown to have a wide spectrum, including anti-inflammatory, antioxidant, anti-carcinogenic, anti-mutagenic, anti-coagulant, anti-fertility, anti-diabetic, anti-fungal, anti-protozoal, anti-viral, anti-fibrotic, anti-venom, anti-ulcer, hypotensive, and anti-hypercholesterolemia (Chattopadhyay et al., 2004).

Both *C. longa* and curcuminoids have been reported to have antidiabetic effects and also reduced glycosylated hemoglobin significantly (Hussain, 2002; Seo et al., 2008; Arun and Nalini, 2002).

Curcuminoids have been found to prevent diabetic complications such as delayed streptozotocin induced diabetic cataract formation, enhancing wound healing and providing a protective effect in diabetic nephropathy (Joe et al., 2004; Strimpakos and Sharma, 2008; Nishiyama et al., 2005; Chattopadhyay et al., 2004; Sidhu et al., 1999; Suryanarayana et al., 2005). Inhibition activity of *C. longa* and curcuminoids on albumin glycation is still unknown.

This research therefore investigated the inhibitory activity of *C. longa* and curcuminoids on *in vitro* albumin glycation and their mode of inhibition.

MATERIALS AND METHODS

Curcuminoids contain curcumin, demethoxycurcumin and bisdemethoxycurcumin (Sigma), ethanol, ethyl acetate, hexane, oxalic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA) and phosphate buffered saline (Standefor and Eaton, 1983).

Extraction of *Curcuma longa*

Fresh *C. longa* rhizomes were collected from Manoko Plantation in Lembang, West Java, Indonesia. The plants were identified by taxonomists in the School of Life

Sciences and Technology, Bandung Institute of Technology. *C. longa* rhizomes were dried at 40-60°C for 30-36 h. Hexanic, ethyl acetate and ethanolic extract of *C. longa* were obtained from *C. longa* rhizomes by triplet extraction with three volumes of hexane, ethyl acetate or ethanol, respectively. The filtrate and solvent were then evaporated, using a rotary evaporator (Buchi Rotavapor R-124, Buchi Waterbath R-480) at 60°C for 6 h. An ethyl acetate extract from the residue of the hexane extraction and an ethanol extract from the residue of ethyl acetate extraction were also obtained using the same method. The quantity of extracts obtained from each extraction were 201.2 g of ethanol extract, 35.2 g of ethyl acetate extract and 13.5 g of hexane extract.

Albumin glycation reaction

One ml of 3% glucose solution was added to 1 ml of 5% albumin solution. For the prevention of any environmental contamination, Gentamicin 0.2% was added and incubated for 3 weeks at room temperature in a constant position. After the incubation period, 3 ml of the solution was dialyzed against 10 ml phosphate buffer for 3 h (Sheikh et al., 2004).

Measurement of albumin glycation concentration

Quantitation of albumin glycation was determined using the TBA test: 1 ml of trichloro acetic acid (TCA) 400 g/L was added to 3 ml dialyzed solution (as described above) and then centrifuged for 10 min at 3000 rpm. The supernatant was discarded. This function was done twice to make sure that all of the protein was precipitated. 1 ml phosphate buffer and 0.5 ml oxalic acid were added to the sediment and then transferred into a water bath until it boiled. It was then removed and allowed to cool. After the compound cooled to room temperature, 400 g/L TCA was added to each sample and centrifuged for 10 min at 3000 rpm. The supernatant was then separated and 50 mmol/L TBA was added to 1 ml of each supernatant solution. The mixture was placed in a 40°C water bath for half an hour. The absorbance of each sample was measured at 443 nm by using visible spectrophotometer (Hewlett Packard 8452A) (Sheikh et al., 2004).

Measurement of effects of *C. longa* extract and curcuminoids on albumin glycation

The concentrations that were used in this study were 0.25, 0.5, 1, 10, 15, 20 25 and 30% of *C. longa* and 0.0125, 0.025, 0.05, 0.1 and 0.2% of curcuminoids. 0.1 ml from each of these preparations were added into a solution of 3 ml of 5% albumin and 3% glucose (in

Gentamicin phosphate buffer solution) and incubated at room temperature for 3 weeks. To determine the effect of each concentration on glycation of albumin, the TBA reaction was carried out as explained earlier. All experiment stages and each concentration were done in quintet (Sheikh et al., 2004).

Measurement of kinetics of inhibition of albumin glycation

The mode of inhibition of albumin glycation was measured by increasing concentrations of glucose (3 to 18%) as a substrate in the absence or presence of 1, 10, 15 and 20% of *C. longa* and 0.0125, 0.025, 0.05, and 0.1% of curcuminoids. Inhibition type was determined by Eddie Hofstee plot analysis of the data, which were calculated from the results according to Michaelis-Menten kinetics. Average velocity (V) and velocity/substrate concentration $[S]$ for each concentration was plotted in Eddie hofstee curve. K_m (The substrate concentration that produces a V_i that is one-half of V_{max} is designated as the Michaelis-Menten constant) is slope of the curve. Competitive inhibition means that the molecule bind with the same site as substrate. In the presence of a competitive inhibitor, it takes a higher substrate concentration to achieve the same velocities that were reached in its absence. So while V_{max} can still be reached if sufficient substrate is available, one-half V_{max} requires a higher $[S]$ than before and thus K_m is larger. Meanwhile, non-competitive inhibition means that the molecule bind with a site different from those of substrate, hence V_{max} cannot be reached and K_m is less than without inhibitor.

Statistical analyses

Data were expressed as mean \pm standard deviation. One way analysis of variance (ANOVA) was used to compare the effect of *C. longa* and curcuminoids on inhibiting albumin glycation at different concentrations.

RESULTS

The glycation of albumin *in vitro* was inhibited by the addition of *C. longa*. By using the TBA method, the inhibitory activity on albumin glycation was determined. The results obtained show that 0.25 mg/ml *C. longa* can inhibited (22.06%) albumin glycation. Inhibitory activity of *C. longa* increased significantly ($p=0.001$) with increase in its concentration. Maximum inhibition of 96.9% was observed at a *C. longa* concentration of 30 mg/ml (Table 1). This suggests that inhibitory activity of a *C. longa* depend on its concentration.

The use of pure curcuminoids over a concentration

range of 0.0125 to 0.2 mg/ml also inhibited the glycation of albumin *in vitro* (Table 2). The curcuminoids concentration was lower than that of the *C. longa*. Maximum inhibition of 90.46% was observed at the curcuminoids concentration of 0.2 mg/ml. Inhibitory activity of curcuminoids was also concentration dependent.

Table 3 shows the inhibition concentration (IC_{50}) of *C. longa* and its active compound, curcuminoids compared with Pyridoxamine on the albumin glycation reaction. The inhibitory activity (IC_{50}) of curcuminoids (0.014 mg/ml) was almost the same as Pyridoxamine (0.011 mg/ml), whereas the inhibitory activity of the *C. longa* (0.7 mg/ml) was lower than that of Pyridoxamine.

The inhibition type exhibited by *C. longa* and curcuminoids was determined by increasing glucose concentration as substrate. The use of Eddie-Hofstee plots revealed that the *C. longa* exhibited both competitive and non-competitive inhibition of albumin glycation. Low concentrations of *C. longa* (1 and 10 mg/ml) exhibited competitive inhibition because the K_m ($K_m=-0.56$) was larger than K_m without inhibitor ($K_m=-0.76$). Meanwhile, *C. longa* at high concentration (15 and 20 mg/ml) exhibited non-competitive inhibition because its K_m ($K_m=-0.83$) was less than without inhibitor (Figure 1). Curcuminoids exhibited competitive inhibition type because the K_m ($K_m=-0.88$) was larger than K_m ($K_m=-1.009$) without inhibitor (Figure 2).

DISCUSSION

Much evidences have suggested that AGEs are important pathogenetic mediators of almost all diabetes complications, conventionally grouped into micro- or macro-angiopathies. For instance, AGEs are found in retinal vessels of diabetic patients, and their levels correlate with those of serum as well as with severity of retinopathy. Also, it was known that AGEs accumulate in peripheral nerves of diabetic patients and that the use of anti-AGE agents improves nerve conduction velocities and neuronal blood flow abnormalities (Peppia et al., 2003).

Glucose, acting as the initial substrate, generates early glycation products, termed Schiff bases, which then form more stable Amadori products. These Amadori products (over months to years) undergo further arrangement to yield AGEs. The AGEs-related cross-links are resistant to enzymatic degradation and they are therefore very stable. The rate of AGE formation is dependent on sugar concentration, extent of oxidative stress and time of exposure (Johnsen and Paavonen, 2007).

Several natural products known to have antiglycation activity, including Arbutin (hydroquinone-D-glucopyranoside) which naturally is chemical compound produced by several plant species such as the Ericaceae

Table 1. Effect of different concentrations of *C. longa* extracts on albumin glycation inhibition *in vitro*.

<i>C. longa</i> conc. (mg/mL)	Percentage of albumin glycation inhibition
0.25	22.06±1.62
0.5	40.30± 2.66
1	63.40±2.66
10	75.80±2.60
15	78.60±1.66
20	90.60±1.05
25	95.70±0.75
30	96.90±2.53

Values are given in mean±SD; P=0.001.

Table 2. Effect of different concentrations of curcuminoids on albumin glycation reaction.

Curcuminoids conc. (mg/mL)	Percentage inhibition of albumin glycation
0.0125	20.0±5.14
0.025	52.4±7.02
0.05	67.6±4.39
0.1	72.6±0.53
0.2	90.46±1.61

Values are given in mean±SD; P=0.002.

Table 3. Inhibitory effects of *C. longa* extract and curcuminoids on albumin glycation.

Extract	IC ₅₀ * (mg/mL)
<i>C. longa</i> extract	0.7
Curcuminoids	0.014
Pyridoxamine	0.011

*IC₅₀, Concentration of *C. longa* extract and curcuminoids required to inhibit 50% of albumin glycation reaction.

family (*Arctostaphylos* sp), Betulaceae (*Betula alba*) and Rosaceae (*Pyrus communis* L.). Another plant that appears to possess antiglycation activity is Pomegranate (*Punica granatum* L). Mixtures of some plant extracts such as *Anthemis nobilis* (Roman chamomile), *Crataegus oxyacantha* (hawthorn berry), *Houttuynia cordata* (dokudami) and *Vitis vinifera* (grape leaf) have also been reported to have antiglycation effects (Jedsadayanmata, 2005; Yonei, 2010; Nishigaki et al., 2008).

This study used pyridoxamine to compare its inhibitory activity with a *C. longa* and curcuminoids. Pyridoxamine, a vitamin B6 derivative, inhibits the formation of AGEs. It

scavenges reactive carbonyl intermediates and inhibits the formation of AGEs from Amadori compounds. *In vitro*, pyridoxamine protects renal glomerular cells, including podocytes and mesangial cells, against MGO-induced changes in cell adhesion. The safety and tolerability of the commercial vitamin B6 form, pyridoxine hydrochloride (Pyridon), is currently being evaluated in clinical trials (Johnsen and Paavonen, 2007).

This study has shown that *C. longa* and its active compound group, curcuminoids, inhibit the *in vitro* glycation of albumin. The maximum inhibitory activity of *C. longa* and curcuminoids were 96.9 and 90.46%, respectively. These results indicate high inhibitory activity when compared to previous studies on the inhibitory activity of *Carum petroselinum*, *Psoralea corylifolia*, *Iris tenuifolia*, *Albatrellus dispansus*, *Fumaria parviflora*, *Ficaria siraitia*, *Siraitia grosvenorii*, *Rorippa indica*, *Fagopyrum esculentum*, and *Spiraea ulmaria*, where the maximum inhibition was 76.25, 82.75, 70.4, 80.15, 76.36, 79.5, 70, 65, 82.5 and 70.51%, respectively (Atta-ur-Rahman, 2007).

The calculated IC₅₀ for curcuminoids (0.014 mg/mL) and *C. longa* (0.7 mg/ml) were found to be lower when compared to previous studies on arbutin (IC₅₀=1361 mg/ml). This suggests that the efficacy of *C. longa* and its active compound, in habiting glycation *in vitro* is much greater than arbutin (Jedsadayanmata, 2005).

C. longa has been known to have antidiabetic effect in preclinical study with 50 and 100 mg/kg doses (Sukandar et al., 2011). Clinical trial of activity of a combination of *C. longa* and *Allium sativum* with 2.4 g/day dose as antidiabetic against type-2 diabetes patients with dyslipidemia have also been conducted (Sukandar et al., 2010). The concentration of *C. longa* tested in this study was higher compared to the dose that used in preclinical or clinical study. This suggested that *C. longa* might be acting in another way to exert antidiabetic effects. *C. longa* stimulated adipocyte differentiation and showed activity to PPAR_γ (Kuroda et al., 2005). Previous study also showed that 25 mg/kg curcuminoids have antidiabetic effect in preclinical study. However, curcumin have poor bioavailability in human body, previous study showed that maximum blood concentration of curcumin was only 149.8 ng/g (Antony et al., 2008). This is almost 10-fold lower than the lowest dose of curcuminoids tested in this study. Hence, curcuminoids also might be acting in another way, other than by inhibition of glycation, to exert their antidiabetic effects. Other study showed that curcumin protected β cell function from oxidative stress to exert its antidiabetic effect (Kanitkar and Bhonde, 2008).

The products of albumin glycation reactions, so-called advanced glycation end products (AGEs) play an important role in microvascular and macrovascular complications in diabetic patients. AGEs are a heterogeneous group of molecules formed from the non-enzymatic reaction of reducing sugars with free

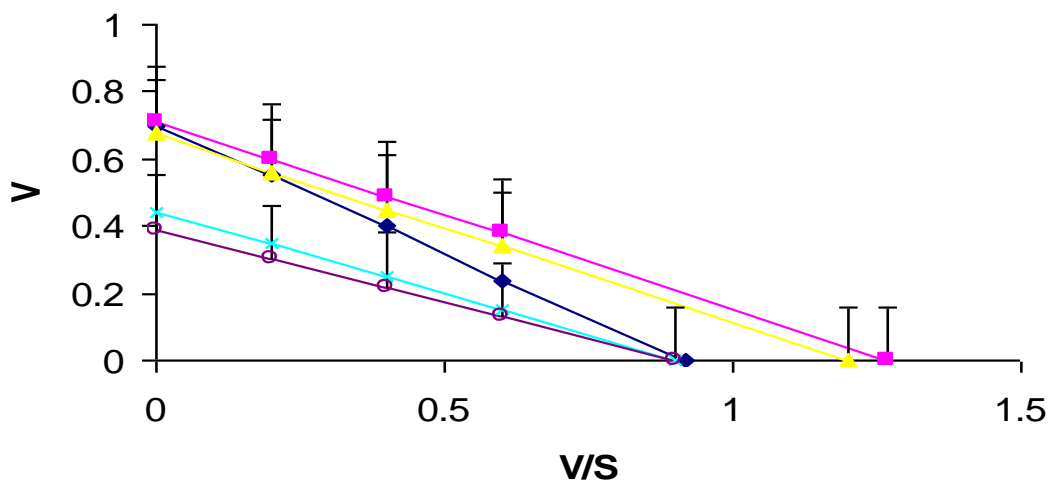


Figure 1. Eddie Hofstee plot of the reaction of albumin glycation in the presence of *C. longa* extracts. Diamonds, no inhibitor; squares, 1 mg/ml; triangles, 10 mg/ml; crosses, 15 mg/ml; circles, 20 mg/ml. V, velocity; V/S, velocity/substrate concentration.

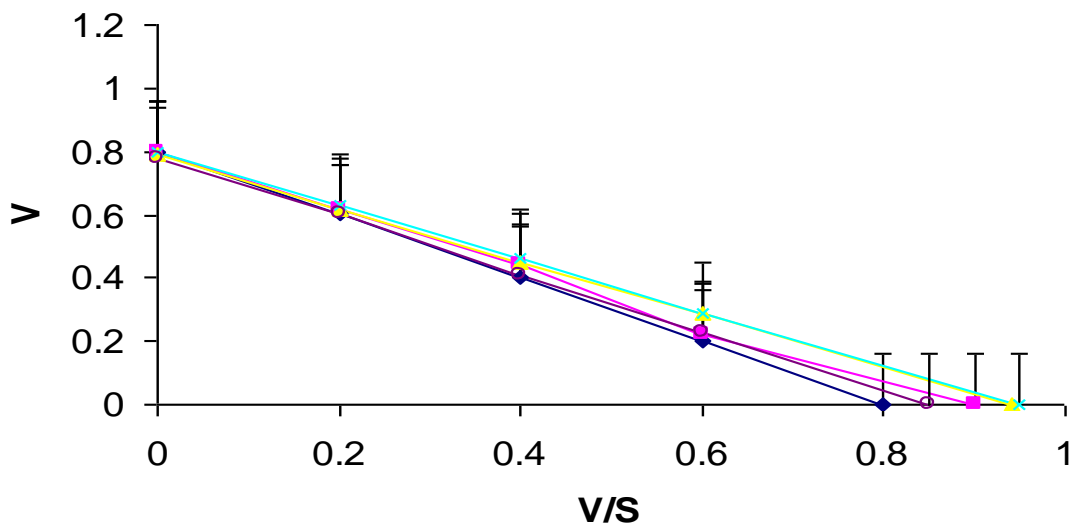


Figure 2. Eddie Hofstee plot of the reaction of albumin glycation in the presence of Curcuminoids. Diamonds, no inhibitor; squares, 0.0125 mg/ml; triangles, 0.025 mg/ml; crosses, 0.05 mg/ml; circles, 0.1 mg/ml. V, velocity; V/S, velocity/substrate concentration.

amino groups of proteins, lipids, and nucleic acids. The initial product of this reaction is called a Schiff base, which spontaneously rearranges itself into an Amadori product, as is the case of the well-known hemoglobin A1c. These initial reactions are reversible depending on the concentration of the reactants. A lowered glucose concentration will unhook the sugars from the amino groups to which they are attached. Conversely, high glucose concentrations will have the opposite effect, if persistent. The rate of AGE formation is dependent on sugar concentration, extent of oxidative stress and time

of exposure (Johnsen and Paavonen, 2007; Peppas et al., 2003).

C. longa and curcuminoids inhibited the albumin glycation reaction. Inhibition type was observed with increasing glucose concentration. The *C. longa* exhibited both competitive and non-competitive inhibition of the glycation reaction. At low concentrations, they compete with albumin to bind with glucose at the same site. However, at higher concentration, they have non-competitive inhibition (Figure 1). The possible explanation for this may be due to the fact that extract had more than

one antiglycation inhibitor.

Curcuminoids exhibited competitive inhibition of the glycation reaction. Eddie hofstee plot (Figure 2) showed that in the present of curcuminoids as inhibitor, Km was larger than without curcuminoids hence when blood glucose level was raised, AGEs formation could still proceed.

AGEs formation is correlated with blood glucose control. *C. longa* and its active compound, curcuminoids, have been reported to have antidiabetic effects (Hussain, 2002; Honda et al., 2006; Kuroda et al., 2005; Seo et al., 2008). Administration of *C. longa* and curcuminoids to diabetic mice decreased blood glucose levels and also reduced glycosylated haemoglobin (Arun and Nalini, 2002). The result of the present study support previous studies that show that both *C. longa* and curcuminoids prevent diabetes complications such as cataract, diabetic nephropathy and enhanced wound healing. Diabetes complications are correlated with increasing HbA1c levels that result from haemoglobin glycation (Joe et al., 2004; Strimpakos and Sharma, 2008; Nishiyama et al., 2005; Chattopadhyay, 2004; Sidhu, 1999; Suryanarayana, 2005).

Conclusion

C. longa and its active compound group, curcuminoids, can inhibit albumin glycation. The activity of *C. longa* and curcuminoids on albumin glycation *in vitro* among several concentrations used in this study; *C. longa* with a concentration of 30 mg/ml had the highest inhibitory activity, while the maximum inhibitory activity of curcuminoids occurred at a concentration of 0.2 mg/ml. The inhibitory activity on albumin glycation reaction of curcuminoids was greater than the *C. longa*. Inhibitory activity of curcuminoids was also better than pyridoxamine. *C. longa* exhibited both a combination of competitive and non-competitive inhibition, while curcuminoids exhibited only competitive type inhibition. The inhibitory activity of *C. longa* and curcuminoids on glycation may explain why they are considered to have an important role in preventing complications associated with diabetes.

The findings suggest that *C. longa* and curcuminoids might inhibit AGEs formation and explain why they may be useful in decreasing the microvascular and macrovascular complication associated with diabetes.

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