



***IN-VITRO* ALDOSE REDUCTASE INHIBITION AND ANTIGLYCATION ACTIVITIES OF *NYAGRODHADI CHURNA*, AN AYURVEDIC FORMULATION**

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Abstract:

Nyagrodhadi churna (NC) is a compound Ayurvedic formulation used in the treatment of diabetes and its associated complications. Aldose reductase (ALR), the key enzyme of the polyol pathway, plays important role in hyperglycemia-induced diabetic complications. Polyol pathway is involved in formation of advanced glycation end-product (AGE) and increased oxidative stress. The accumulation of tissue AGEs together with increased oxidative stress has an important role in the pathogenesis of diabetic complications. Therefore, the present study demonstrates inhibitory effect of NC on ALR and AGEs activity. In vitro ALR inhibition was studied against kidney ALR and antiglycation activity against glycation of bovine serum albumin. NC showed inhibitory effect against kidney ALR and glycation of bovine serum albumin. Therefore, NC exhibited good potential as antioxidant and ALR and AGE formation inhibitor due to these compounds which was further substantiated by experimental study of ALR inhibition and antiglycation effect by NC. This study validates its use in amelioration of diabetic complications.

Keywords:- Ayurvedic formulation, *Nyagrodhadi churna*, Diabetes mellitus, phytoconstituents

1. INTRODUCTION

Diabetes mellitus is a metabolic disorder which develops when the pancreas fails to produce enough insulin (Type 1 diabetes) or the insulin produced is defective and cannot transport glucose into the cells (Type 2 diabetes). Diabetes, if uncontrolled, leads to development of diabetic complications and considered to be major cause of mortality and morbidity. Diabetes control and complication trial (DCCT) demonstrated that the poor control of blood glucose in diabetic patients is likely to cause diabetic complications (1). In Ayurveda, diabetes is called Madhumeha, a type of Prameha, which literally means sweet urine. Prameha correlates in many ways with obesity, metabolic syndrome, and diabetes mellitus (2). The direct nomenclature of diabetic complications is not found in Ayurvedic text; however, it is mentioned under broad term as Madhumehajanya upadrava (3).

The precise mechanism underlying pathogenesis of diabetic complications remains poorly understood. Hyperglycaemia plays a key role in development of complications (4, 5). Hyperglycaemia ensues biochemical pathways such as non-enzymatic glycosylation, polyol pathway, hexose monophosphate shunt pathway and protein kinase C pathway resulting in overproduction of reactive oxygen species leading to oxidative stress and organ damage (6, 7). The polyol pathway is a key mechanism involved in the etiology of diabetic complications. Aldose reductase (ALR), the first enzyme of the polyol pathway catalysing the reduction of glucose to sorbitol. In hyperglycaemia, the polyol pathway results in accumulation of sorbitol in tissues where glucose uptake is insulin independent (8, 9, 10, 11). This excessive sorbitol accumulation results in disruption of cellular osmotic homeostasis (12, 13). In addition, the increased flux of glucose through the polyol pathway and consequent depletion of NADPH may inhibit the activity of other NADPH-requiring enzymes, including those of the glutathione redox cycle. In turn, the decreased levels of reduced glutathione induce intracellular oxidative stress (14, 15).

Non enzymatic glycosylation is the major classic pathway leading to advanced glycation end products (AGEs) formation leading to pathway of diabetic complications. Glucose and Amadori products undergo autoxidation in the presence of transition metals to generate free radicals (16), and damage proteins, lipids, and nucleic acids and leading to tissue damage in diabetes (17). Increased glycation and accumulation of tissue AGEs can alter protein conformation and impair function by altering enzyme activity, modifying protein half-life, altering immunogenicity, and cross-linking of structural proteins (18, 19). Many tissues possess receptors for AGEs, and their interaction with AGEs can generate intracellular oxidative stress and activation of nuclear factor-kB (NF-kB). It is generally accepted that accumulation of tissue AGEs together with increased oxidative stress has an important role in the pathogenesis of diabetic complications (19). Excessive activation of the polyol pathway increases concentrations of sorbitol and reactive oxygen species, and enhances formation of AGE leading to cell damage. The above-mentioned processes related to hyperglycaemia are key steps in the development of diabetic complications, including macro- and micro vasculopathies, neuropathy, cataract, retinopathy and nephropathy (8, 9, 11, 12, 13, 20).

Recently, there is growing interest in Ayurvedic medicine and many single and compound Ayurvedic formulations are being used by prediabetic and diabetic patients as either an alternative or an adjunctive therapy to prevent the development of diabetic complication. *Nyagrodhadi churna* is a compound Ayurvedic formulation available in classical churna (powder) form. It is official drug in the Ayurvedic Formulary of India (21) and formulated as per Yogaratnakara, a classical Ayurvedic text. It contains 28 ingredients in equal amount (Table 1). *Nyagrodhadi churna* is useful for all three dosha (Vata, pitta and kapha) imbalances. It opens channels and improves mobility of doshas. It is used for mitigation of prameha (Urinary disorder), urinary obstruction, dysuria and diabetic carbuncle (Prameha pidika) (22). It is administered in the dose of 1-3 g with honey and triphala kasaya.

Table 1. The composition of *Nyagrodhadi churna*

S. No.	Ingredients	Scientific name	Parts used	Amount
1.	<i>Nyagrodha</i>	<i>Ficus benghalensis</i> L.	Stem bark	1 Part
2.	<i>Udumbara</i>	<i>Ficus glomerata</i> Roxb.	Stem bark	1 Part
3.	<i>Asvattha</i>	<i>Ficus religiosa</i> L.	Stem bark	1 Part
4.	<i>Syonaka</i>	<i>Oroxylum indicum</i> Vent.	Stem bark	1 Part
5.	<i>Aragvadha-phalamajja</i>	<i>Cassia fistula</i> L.	Fruit pulp	1 Part
6.	<i>Asana</i>	<i>Pterocarpus marsupium</i> Roxb.	Heart wood	1 Part
7.	<i>Amra</i>	<i>Mangifera indica</i> Wall.	Stem bark	1 Part
8.	<i>Kapittha</i>	<i>Kapittha</i>	Stem bark	1 Part
9.	<i>Jambu</i>	<i>Syzygium cumini</i> (L.) Skeels	Stem bark	1 Part
10.	<i>Priyala</i>	<i>Buchanania lanzan</i> Spreng.	Stem bark	1 Part
11.	<i>Kakubha (Arjuna)</i>	<i>Terminalia arjuna</i> (Roxb. ex DC.) Wight & Arn.	Stem bark	1 Part
12.	<i>Dhava</i>	<i>Anogeissus latifolia</i> Wall.	Stem bark	1 Part
13.	<i>Madhuka</i>	<i>Madhuca indica</i> J.F. Gmel.	Stem bark	1 Part

14.	<i>Madhuka (Yasti)</i>	<i>Glycyrrhiza glabra</i> Torr.	Root	1 Part
15.	<i>Lodhra</i>	<i>Symplocos racemosa</i> Roxb.	Stem bark	1 Part
16.	<i>Varuna</i>	<i>Crateva nurvala</i> Buch.-Ham.	Stem bark	1 Part
17.	<i>Paribhadra</i>	<i>Erythrina variegata</i> L.	Stem bark	1 Part
18.	<i>Patola</i>	<i>Trichosanthes dioica</i> Wall. whole plant	Whole plant	1 Part
19.	<i>Mesasringi</i>	<i>Gymnema sylvestre</i> (Retz.) R.Br. ex Sm.	Whole plant	1 Part
20.	<i>Danti</i>	<i>Baliospermum montanum</i> Müll.Arg.	Roots	1 Part
21.	<i>Chitraka</i>	<i>Plumbago zeylanica</i> L.	Roots	1 Part
22.	<i>Patali</i>	<i>Bignonia suaveolens</i> Roxb.	Stem bark	1 Part
23.	<i>Karanja</i>	<i>Pongamia pinnata</i> (L.) Merr.	Seeds	1 Part
24.	<i>Haritaki</i>	<i>Terminalia chebula</i> Retz.	Pericarp	1 Part
25.	<i>Bibhitaka</i>	<i>Terminalia bellirica</i> (Gaertn.) Roxb.	Pericarp	1 Part
26.	<i>Amalaki</i>	<i>Embilica officinalis</i> Gaertn.	Pericarp	1 Part
27.	<i>Sakra (kutaja)</i>	<i>Holarrhena antidysenterica</i> (L.) Wall.	Seeds	1 Part
28.	<i>Suddha Bhallataka</i>	<i>Semecarpus anacardium</i> Buch.-Ham.	Fruits	1 Part

Most of the plant ingredients present in *nyagrodhadi churna* viz. all three *Ficus species* (23), *Terminalia arjuna* (24), *Pongamia pinnata* (25), *Glycyrrhiza glabra* (26), *Feronia limonia* (27), *Buchanania lanzan* (28), *Madhuka indica* (29), *Terminalia belerica* (30), *Piper longum* (31), *Marica (Piper nigrum)* (32), *Pippali (Piper lognum)* (33), *Haritaki (Terminalia chebula)*, and *Vibhtaka (Terminalia belerica)* (34) and *Holarrhena antidysenterica* (35) demonstrated antioxidant activity in various models. Some of the plant ingredients present in *nyagrodhadi churna* viz. *Embilica officinalis* (36), *Terminalia bellerica* (37), *Cassia fistula* (38), *Pterocarpus marsupium* (39, 40) and flavonoids (41) showed aldose reductase inhibition in *in-vitro* and *in-vivo* experimental studies. Moreover, some of the plant constituent ingredients viz. *Oroxylum indicum* (42), *Cassia fistula* (43), *Pterocarpus marsupium* (44), *Mangifera indica* (45) and polyphenolic compounds present in NC (46-51) exhibited antiglycation activity in experimental models and in humans (52).

Pharmacological use of aldose reductase inhibitors (ARIs) as well as inhibition of AGEs formation has been recognized as an important strategy in the prevention and attenuation of long-term diabetic complications (53-58). Involvement of the polyol pathway and oxidative stress in the aetiology of diabetic complications requires inhibition of both processes. Hence, it was thought worthwhile that NC can be effective in prevention of diabetic complications through its effect on ALR inhibition and AGE formation inhibition. The present study demonstrates ALR inhibitory and antiglycation effect using *in vitro* models.

2. MATERIAL AND METHODS

2.1. Drugs and Chemicals: *Nyagrodhadi churna* (Batch No. VHCANC046) was procured from M/s VHCA Ayurveda, New Delhi, India. Folin-Ciocalteu's reagent, aluminium chloride, sodium carbonate (Sisco Research Laboratories, Mumbai), tannic acid (Merck, Mumbai), quercetin and DL-glyceraldehyde, NADPH of SRL make, and aminoguanidine (TCI, USA) were procured from local scientific supplier. All other chemicals used in the experiment were of the highest grade commercially available.

2.2. Quantitative estimation of phytoconstituents: The total phenolic, flavonoid and saponin contents were measured by previously described methods.

2.2.1. Total phenolic content: Total phenolic content was determined spectrophotometrically (59). One ml of Folin-Ciocalteu's reagent, previously diluted (1:20), was added to 1 ml of aqueous solution of NC or tannic acid and mixed thoroughly. To the mixture, 4 ml of sodium carbonate (75 g/L) and 10 ml of distilled water were added and mixed well. The mixture was allowed to stand for 2 h at room temperature. Contents were then centrifuged at 2000×g for 5 min and the absorbance of the supernatant was taken at 760 nm. A standard curve was obtained using various concentrations of tannic acid. Results were expressed as mg of tannic acid equivalents (TAE) per g of formulation.

2.2.2. Total flavonoid content: Total flavonoid content was measured by aluminium chloride colorimetric assay (60). One ml of aqueous solution of NC or standard solution of quercetin was added to 10 ml volumetric flask containing 4 ml of distilled water. To the above mixture, 0.3 ml of 5% NaNO₂ was added. After 5 min, 0.3 ml of 10% AlCl₃ was added. At 6th min, 2 ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. The solution was mixed well and the absorbance was measured against prepared reagent blank at 510 nm. Total flavonoid content of the extracts was expressed as mg of quercetin equivalents per g of formulation.

2.2.3. Total alkaloid content: Total alkaloid content was measured by bromo-cresol green colorimetric assay (61). The drug was dissolved in 2N HCl and filtered. One ml of this solution was transferred to a separating funnel and washed 3 times with 10 ml chloroform. The pH of this solution was adjusted neutral with 0.1 ml NaOH. To this, 5 ml of BCG solution and 5 ml of phosphate buffer (pH 4.7) were added. The mixture was shaken and complex formed was extracted with 1, 2, 3 and 4 ml of chloroform by vigorous shaking. The extract was collected in 10 ml volumetric flask and volume was made up to 10 ml with chloroform. The absorbance of the complex in chloroform was measured at 470 nm. A standard curve was obtained using various concentrations of atropine. Total alkaloid content of the formulation was expressed as milligrams of atropine equivalents (AE) per gram of formulation.

2.2.4. Total saponins content: Total saponins were determined according to previously described method of Obadoni and Ochuko (62) with some modifications. NC powder (5g) was put into a conical flask and 50 ml of 20 % ethanol was added. The samples were heated over a hot water bath for 4 h with continuous stirring at about 55°C. The mixture was filtered and the residue re-extracted with another 100 ml of 20% ethanol. This was again reduced to 20 ml on water bath at about 90°C. The concentrate (20 ml) was transferred into a 250 ml separating funnel and 10 ml of diethyl ether was added. The mixture was shaken vigorously and ether layer was discarded. The purification process was repeated. Thirty ml of n-butanol was added. The combined n-butanol extracts were washed twice with 5 ml of 5% aqueous NaCl. The remaining solution was heated in a water bath. After evaporation the samples were dried in the oven to a constant weight. The content was calculated as grams of saponins per 100g of churna.

2.3 Aldose-reductase inhibition assay: Aldose reductase activity was assayed according to the method described by Hayman and Kinoshita (63) with some modifications. The incubation mixture, in a final volume of 250µL, consisted of 67mM potassium phosphate buffer (pH-6.2), 0.4M lithium sulphate, 150µM NADPH, 300µM DL-glyceraldehyde enzyme (50µL) and various concentrations of methanol extracts. Quercetin 0.5 µg/mL was used as standard inhibitor. Appropriate blanks were prepared for quercetin and extracts samples without DL-glyceraldehyde. The reaction was initiated by adding NADPH and absorbance was read at 340 nm for 20 minutes using spectrophotometer in a kinetic mode. The percent inhibition by NC/quercetin was calculated by considering the control value as 100%, using the equation mentioned below. The median inhibitory concentration (IC₅₀) was calculated using regression analysis. The percentage inhibition was calculated with the following formula:

$$\text{Percent inhibition} = 100 - \frac{\Delta A_{340} \text{ Sample} \times 100}{\Delta A_{340} \text{ control}}$$

Where, ΔA_{340} = Change in absorbance at 340 nm

2.4. AGE inhibition assay: Glycation of proteins leads to the formation of early glycation adducts (fructosamine derivatives) and advanced glycation end products (AGEs). AGEs can be quantified by measuring fluorescence (excitation at 370 nm and emission at 440nm). This assay was done as previously described by Rahbar *et al.* (264) with minimal modifications. BSA, NaN₃, and glucose were dissolved in Na phosphate buffer (pH 7.4, 0.2 M), to a final concentration of 50 mg/ml BSA, 0.02% NaN₃, 50 mM glucose. All solutions were filter sterilized and the experiments were done under aseptic conditions to prevent microbial contamination. Reaction mixtures were prepared by adding BSA, NaN₃ and glucose solutions with or without *nyagrodhadi churna* extract (20, 40, 60, 80, 100,

120, 140, 160, 180, 200 mM). The reaction mixtures were incubated in 96-well sterile plates (200 µl/well) at 37°C for 7 days and their fluorescence was examined at 370/440 nm excitation/emission wavelength using a fluorescence spectrophotometer (Synergy, Biotek, USA). The activity of *nyagrodhadi churna* was assessed by calculating the percentage of inhibition versus the negative control (reaction mixture without *nyagrodhadi churna*) on the day 7 according to the formula:

$$\text{Percentage inhibition (\%)} = \left\{ 1 - \frac{\text{fluorescence of sample}}{\text{fluorescence of control}} \right\} \times 100$$

The half-maximal inhibitory concentration (IC₅₀) for *nyagrodhadi churna* was then found using Graph Pad prism, version 4.0. The assay was run in triplicates.

2.5. Statistical Analysis: The data obtained were analysed with linear or nonlinear regression analysis wherever required for calculation of the half-maximal inhibitory concentration (IC₅₀) and one-way ANOVA followed by Tukey's multiple comparison post hoc tests, respectively wherever necessary using Graph Pad prism, version 4.0. A value of P<0.05 was considered significant in all the cases.

3. RESULTS

3.1. Quantitative estimation of phytoconstituents: The total flavonoid and phenolic content contents of *nyagrodhadi churna* were 49.81±0.971 mg quercetin equivalents/g of *nyagrodhadi churna* and 75.75±0.465 mg tannic acid equivalents/g of *nyagrodhadi churna*, respectively. The total alkaloid content was 29.62±1.196 mg atropine equivalents/g of *nyagrodhadi churna* while total saponin content present was 0.794±0.009 per 100 g dry powder of *nyagrodhadi churna*.

3.2. Aldose-reductase inhibition assay: *Nyagrodhadi churna* (2.5-20 µg/ml) and standard, quercetin (0.25-2 µg/ml) showed inhibition of aldose reductase as indicated by concentration-dependent decrease in the absorbance. Non-linear regression analysis of concentration versus percent inhibition was carried out. The regression coefficient of *nyagrodhadi churna* and quercetin were r²=0.9823 and r²=0.9776, respectively. The IC₅₀ value of *nyagrodhadi churna* and quercetin were 8.040 and 0.620 µg/mL, respectively. The results are expressed in Table 2.

Table 2. Effect of *Nyagrodhadi churna* (NC) on aldose-reductase activity

Concentration (µg/ml)	% Aldose reductase inhibition	IC ₅₀ Value
NC	2.5	8.040 µg/ml
	5.0	
	10	
	20	
	50	
Quercetin	0.25	0.620 µg/ml
	0.5	
	1.0	
	1.5	
	2.0	

Values are mean± SEM; n=3; IC₅₀= 50% Inhibitory concentration

3.3. Assessment of the Antiglycation effect of *Nyagrodhadi churna*: Two-way ANOVA showed that *nyagrodhadi churna* exhibited a significant effect on the glycation of bovine serum albumin (BSA) in terms of relative fluorescence unit (RFU). Post hoc test showed that *nyagrodhadi churna* caused significant (p<0.001) decrease in glycation of bovine serum albumin in concentration and time-dependent manner as indicated by a decrease in relative fluorescence unit and area under the curve as compared to glycosylated BSA (Figures 1 and 2). The results were comparable to standard drug, aminoguanidine (Figures 3 and 4).

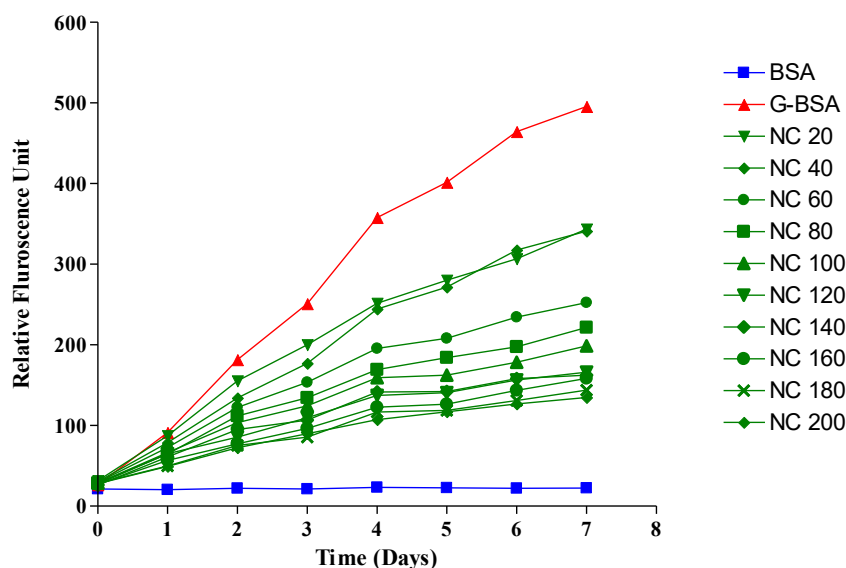


Figure 1: Effect of *Nyagrodhadi churna* on glycation of BSA in RFU. Results are expressed as mean $\pm$ SEM (n=3). *P<0.001 when compared to BSA; \$P<0.001 when compared to G-BSA

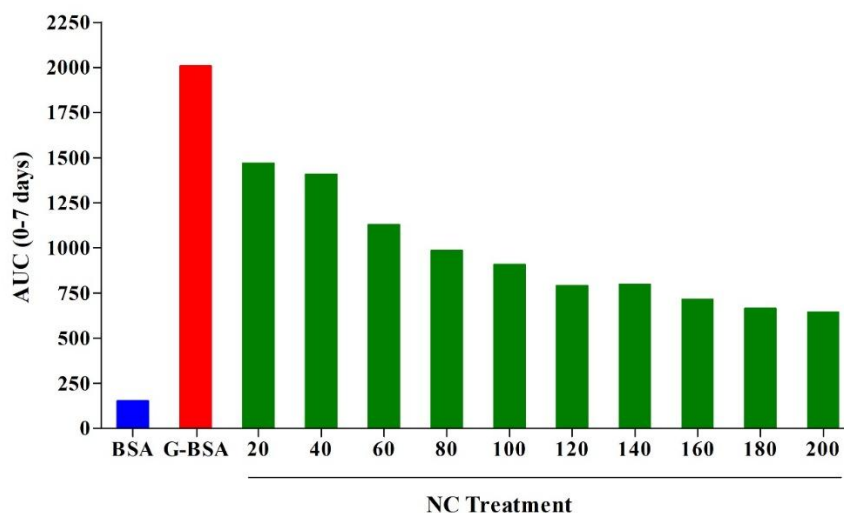


Figure 2: Effect of *Nyagrodhadi churna* on AUC of glycation of BSA. Results are expressed as mean are under the curve (AUC)

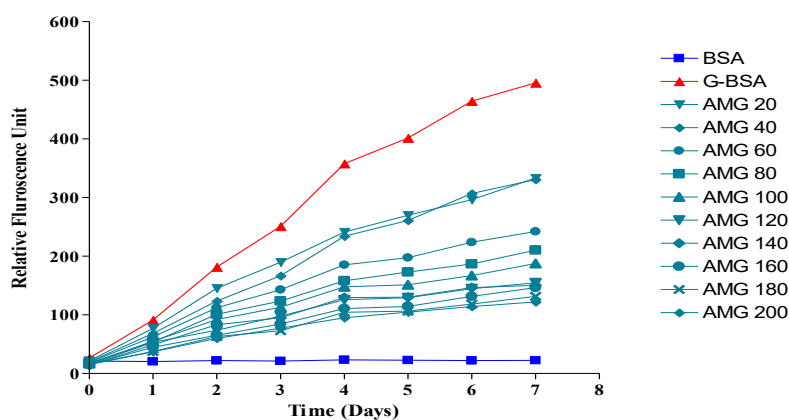


Figure 3: Effect of aminoguanidine on glycation of BSA in RFU. Results are expressed as mean $\pm$ SEM (n=3). *P<0.001 when compared to BSA; \$P<0.001 when compared to G-BSA

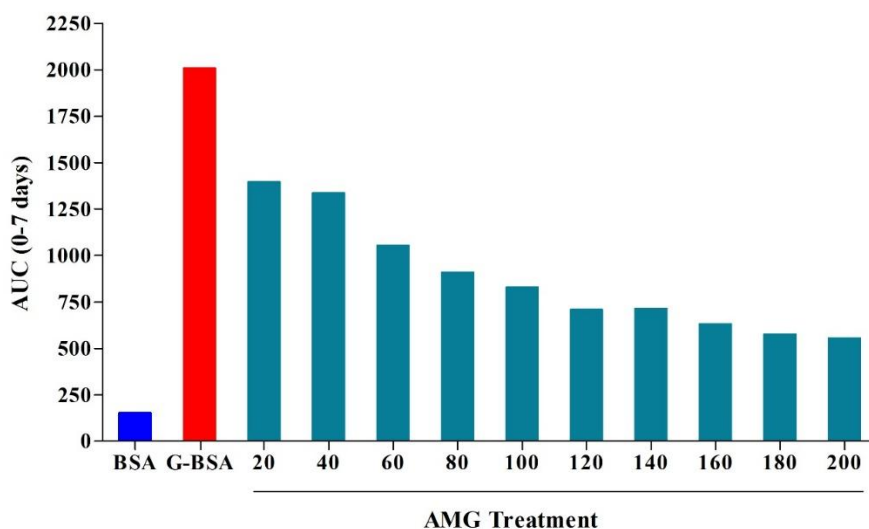


Figure 4: Effect of aminoguanidine on AUC of glycation of BSA. Results are expressed as mean are under the curve (AUC)

2.4 DISCUSSION & CONCLUSION

Nyagrodhadi churna is a compound Ayurvedic formulation used to treat various types of ailments in the traditional system of medicine including the prameha. In view of the use of *nyagrodhadi churna* in the treatment of prameha as well as presence of ALR inhibitory ingredients, it was proposed to investigate its effect on ALR enzyme system and its potential for inhibition of glycation. The preliminary phytochemical screening showed presence of fair amount of phenolic and flavonoid contents. It also showed good amount of alkaloid and saponin contents. In the present study, *nyagrodhadi churna* showed significant concentration dependent ALR inhibitory activity with maximum inhibition of 84 % at 50 $\mu\text{g/ml}$ and IC_{50} value of 8.040 $\mu\text{g/ml}$. The inhibitory effect was comparable to that of standard ALR inhibitor, quercetin with IC_{50} value of 0.620 $\mu\text{g/ml}$. ALR reduces glucose to sorbitol and then metabolized to fructose by sorbitol dehydrogenase. Normally this accounts for less than 3% of glucose consumption. However, in the presence of high glucose, the activity of this pathway is substantially increased and leads up to 30% of total glucose consumption. So, ALR inhibition in the early onset of the secondary complication in the diabetes is beneficial. As *nyagrodhadi churna* showed good inhibitory activity against ALR, so it can be potentially used to treat diabetic complication in the early stage. The phytochemical analysis of *nyagrodhadi churna* revealed that it contains fair amounts of flavonoids and phenolic compounds. It has been earlier reported that the ingredients plants of *nyagrodhadi churna* contain various flavonoids and phenolic (65, 66, 67, 68, 69). The possess aldose reductase inhibition (71, 72, 73), it appears that the observed aldose reductase inhibition effect by *nyagrodhadi churna* may be subsequent to the antioxidant effect shown by the flavonoids or phenolics present in *nyagrodhadi churna*. There are reports of ALR inhibiting activity of plant ingredients present in these formulations such as *Embilica officinalis* (73, 74), *Terminalia bellerica* (75), *Cassia fistula* (76), Shunthi (*Zingiber officinalis*), and *Pterocarpus marsupium* (77, 78).

Glycation reactions involve several species including electrophilic reactive carbonyl species (RCS) and ROS (58). These reactions can take place in both the intra- and the extracellular milieu. In the present study, *nyagrodhadi churna* showed remarkable anti-glycation activity as indicated by concentration dependent reduction in glycation of bovine serum albumin. This can be attributed to several possible mechanisms. The phenolic compounds, due to their acidic nature, are reported to form negatively charged compounds at physiological pH and thus facilitate carbonyl compound trapping. Nucleophilicity is essential for scavenging reactive carbonyl compounds. Further, the phenolic compounds present in the ingredients possess a hydroxyl group that can provide a nucleophilic centre to attack the electrophilic reactive carbonyl compounds to form hemiacetal adducts instead of glycated products (58). It has been shown that several phenolic acids including

ferulic acid, gallic acid, chlorogenic acid, and vanillic acid can remarkably inhibit glucose-induced protein modification and crosslinking (47). The antiglycation mechanisms exerted by such phenolic acids mainly include anti-oxidation, anti-inflammation reactive carbonyl compounds trapping, and the intriguing mechanism of protein wrapping. It is therefore possible that the antiglycation effect of *nyagrodhadi churna* may be attributed to the presence of the various phenolic compounds present in it.

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DECLARATION OF INTEREST

Authors declare no conflict of interest.

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