

Antioxidant, antiglycation and inhibitory potential of *Saraca ashoka* flowers against the enzymes linked to type 2 Diabetes and LDL oxidation

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Abstract. – Objective: The present study investigated the antioxidant, antiglycation and inhibitory potential of flavonoid fraction of *Saraca ashoka* flowers (SAF) against α -glucosidase and α -amylase (the enzymes linked to type 2 diabetes) and LDL oxidation.

Materials and Methods: Antioxidant capacity of SAF was evaluated by estimating total antioxidant activity (TAA) and its protective effects against the oxidative stress induced by H_2O_2 on C2C12 cells. Cytotoxicity by MTT assay and markers of oxidative stress: reduced glutathione (GSH), malondialdehyde (MDA) and reactive oxygen species (ROS) were measured.

Results: Pre-treatment of C2C12 cells with SAF prevented the increased formation of MDA and depletion of GSH induced by H_2O_2 . The increased ROS generation induced by H_2O_2 was also reduced by a pretreatment with SAF. Significant inhibitory potential against α -glucosidase and α -amylase enzymes revealed the therapeutic potential of SAF as an antihyperglycemic agent. SAF also demonstrated potent antiglycation property and inhibited LDL oxidation under *in vitro* conditions.

Conclusions: The overall results demonstrate that SAF can be used as an ideal natural remedy for preventing oxidative stress and other complications associated with diabetes.

Key Words:

C2C12 cells, Reactive oxygen species, Antiglycation, α -glucosidase, LDL oxidation.

Introduction

Diabetes mellitus is a chronic disease characterized by hyperglycemia and insufficiency of secretion or action of endogenous insulin. Al-

though both genetic and environmental factors appear to play a role, the cause of diabetes mellitus is still not clear. Increased oxidative stress is a widely accepted participant in the development and progression of diabetes and its complications^{1,2}. Diabetes is usually accompanied by increased production of free radicals³ or impaired antioxidant defenses^{4,5}. Excessively high levels of free radicals cause damage to cellular proteins, membrane lipids and nucleic acids, and eventually cell death. Glucose oxidation in hyperglycemia is believed to be the main source of free radicals in diabetes. Hyperglycemia is also found to promote lipid peroxidation of low density lipoprotein (LDL) by a superoxide-dependent pathway resulting in the generation of free radicals^{6,7}.

Hyperglycemia, a condition characterized by an abnormal postprandial increase in the blood glucose level, has been linked to the onset of type 2 diabetes and associated with oxidative dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels, and has been shown to be also linked to hypertension⁸. A sudden rise in blood glucose levels causing hyperglycemia in type 2 diabetes happens due to hydrolysis of starch by pancreatic alpha-amylase and uptake of glucose by intestinal alpha-glucosidases⁹. An effective strategy for type-2 diabetes management is the inhibition of intestinal alpha glucosidases and pancreatic alpha-amylases¹⁰. Another important source of free radicals in diabetes is the formation of advanced glycation end products (AGEs). Proteins are modified by glucose through the glycation reaction, resulting in the formation of advanced glycation end-products (AGEs). The contribution of AGEs

to diabetes, aging and Alzheimer's disease has received considerable attention in recent years and free radicals have been shown to participate in AGEs formation¹¹. Antioxidants protect against glycation derived free radicals and may have therapeutic potential¹². In addition, recent studies have shown that compounds with combined antioxidant and antiglycation properties with lower inhibitory effect against α -amylase activity and stronger α -glucosidase inhibitory effect can be potentially used as an effective therapy for post-prandial hyperglycemia and diabetes mellitus¹³⁻¹⁵. Thus, the screening and development of natural products with combined antioxidant, antiglycation and antihyperglycemic properties would be beneficial in the treatment of diabetes mellitus.

Saraca ashoka belongs to the family of *caesalpiniaceae* (Synonym: *Saraca indica*) is a small evergreen tree with bright orange flowers. The plant is used in traditional medicine against uterine fibroids, leucorrhoea, piles and dysentery. The flowers have been used widely in the traditional medicine especially due to its wound healing property¹⁶. Flowers of *Saraca ashoka* are edible in nature and possess significant anticancer activities also^{17,18}. *Saraca ashoka* flowers have been widely used against diabetes in Himalayan tribes of India¹⁹. However, there no scientific validations have been done regarding the antidiabetic potential of *Saraca ashoka* flowers.

Therefore, the present study was designed to investigate the antioxidant and antidiabetic potential via inhibition of key enzymes linked to type 2 diabetes (α -glucosidase and α -amylase) and antiglycation properties of flavonoid fraction of *Saraca ashoka* flowers. This study would help target the use of *Saraca ashoka* flowers as a natural remedy for the management of type 2 diabetes and other related disorders.

Materials and Methods

Chemicals

Plant Material

Saraca ashoka flowers were collected from local areas of Thiruvananthapuram District, Kerala, India, identified and authenticated by Department of Botany, University of Kerala, Thiruvananthapuram, Kerala. A voucher specimen (No. 014/AGP/09) was deposited in our Herbarium for future reference.

Preparation of *Saraca ashoka* Flower (SAF) Extract

The fresh flowers from *Saraca ashoka* (SAF) were air dried and extracted with methanol using Soxhlet extractor. The supernatant was filtered through Whatman No.1 filter paper and concentrated *in vacuo* under reduced pressure at $50 \pm 1^\circ\text{C}$ in a rotavapour (Hiedolph, Schwabach, Germany). The extract was cleared of low polarity contaminants such as fats, terpenes, chlorophyll and xanthophylls by repeated extraction with hexane and then fractionated with ethyl acetate. The ethyl acetate fraction contains the bulk of polyphenols, which also contain flavonoids. This was concentrated *in vacuo* under reduced pressure at $50 \pm 1^\circ\text{C}$ in a rotavapour followed by lyophilization. The lyophilized powder (SAF) was stored at 4°C until analysis.

Determination of Total Flavonoid Content (TFC)

TFC was determined using a colorimetric method with minor modifications²). An aliquot of 10 ml of appropriate dilutions of SAF extract was added to volumetric flask containing 1 ml of 5% (w/v) sodium nitrite and kept for 6 min, followed by reaction with 1 ml of 10% (w/v) aluminium chloride to form a flavonoid-aluminium complex. After the incubation period, 10 ml of 4.3% (w/v) sodium hydroxide was added and the total volume was made up to 25 ml with distilled water. The final solution was mixed well again and the absorbance was measured at 510 nm with UV-VIS spectrophotometer (UV-2450PC, Shimadzu, Japan). The TFC of extract was expressed as mg quercetin equivalents/100 g of extract.

Total Antioxidant Activity

Total antioxidant activity (TAA) of SAF was determined according to the method of Prieto et al(21). Briefly 0.3 ml of sample was mixed with 3ml of reagent solution containing 0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate. Reaction mixture was incubated at 95°C for 90 minutes. Absorbance was taken at 695nm after cooled to room temperature. TAA is expressed as the number of equivalents of ascorbic acid.

Cell Culture and Treatment

C2C12 cell lines were purchased from NCCS, Pune, India were cultured in Dulbecco's modified Eagle's medium (DMEM) medium supple-

mented with 10% fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. Cultures were maintained at 37°C in 5% CO₂ incubator. When the cells were about to cover 80% of the flask area, they were disrupted and seeded on 24 well plates. After attaining ~70-80% confluency, the cells were washed twice with phosphate-buffered saline (PBS) and changed with medium containing SAF extract at different concentrations. After 24 hrs incubation, the cells were treated with 50 µM H₂O₂ and was maintained for 1hr at 37°C.

Cytotoxicity Assay

Cytotoxicity of flavonoid fraction of SAF was evaluated using MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} assay. To carry out this experiment, 1 × 10⁴ cells plated in each well of 96-well plates were placed in the humidified 5% CO₂ incubator at 37°C and allowed to attach to the substrate. Cells grown to 70-85% confluence were exposed to various concentrations (0, 5, 10, 20, 40, 80, 100 and 125 µg/ml) of SAF. The control and treated cells were incubated for 48 h in 37°C, 5% CO₂ incubator. After incubation, the cell viability was evaluated using the MTT assay according to the standard test protocol²².

Determination of Reactive Oxygen Species (ROS)

Cytoprotective effect of flavonoid fraction of *Saraca ashoka* flowers against the oxidative stress induced by H₂O₂ was measured by determining intracellular content of ROS. Intracellular ROS levels were measured employing 2',7'-dichlorofluorescein-diacetate (DCFH-DA) as probe using a micro plate reader (Biotek, Winooski, VT, USA)²³. DCFH-DA is cleaved intracellularly by non specific esterase and turn to high fluorescent 2,7-dichlorofluorescein (DCF) upon oxidation by ROS. In brief, cells cultured in multiwell plates were treated with SAF for 24 hrs, the DCFH probe was added for 30 min, and then they were washed twice with PBS before being treated with 50 µM H₂O₂ for 60 minutes.

Evaluation of MDA and GSH in C2C12 Cell lines

After treatment with SAF and H₂O₂, cells were collected and centrifuged and were resuspended in 0.5 mL of Tris-HCl, pH 7.4, and lysed using a sonicator. All the samples were centrifuged at 15,000 rpm for 10 min and 200 µL aliquot of the sample was assayed for malondialdehyde

(MDA). 0.375 ml of 40% (w/v) trichloroacetic acid (TCA) and 0.200 ml of 0.1M thiobarbituric acid (TBA) was added to the samples and was incubated at 90°C for 30 minutes and then, 0.625 ml of distilled water was added. The samples were centrifuged for 10 minutes at 5000 rpm. The absorbance of the sample was monitored at 586 nm, and the concentration of MDA was determined from a standard curve using 1,1,3,3-tetramethoxy propane, as a standard²⁴.

The concentration of GSH in H₂O₂ and extract treated cell lines was evaluated by a fluorometric assay previously described²³. The method takes advantage of the reaction of GSH with o-phthaldehyde (OPT) at pH 8.0 and fluorescence (Biotek, Winooski, VT, USA) was measured at an emission wavelength of 460 nm and an excitation wavelength of 340 nm.

α-Glucosidase Inhibition Assay

α-Glucosidase was assayed²⁵ by using 50 µl of homogenized extract and 100 µl of 0.1 M phosphate buffer (pH 6.9) containing α-glucosidase solution (1.0 U/ml) and incubated in 96 well plates at 25°C for 10 minutes. After pre-incubation, 50 µl of 5 mM p-nitrophenyl-α-D-glucopyranoside solution in 0.1 M phosphate buffer (pH 6.9) was added to each well at timed intervals. The reaction mixtures were incubated at 25°C for 5 minutes. Before and after incubation, absorbance readings were recorded at 405 nm by micro plate reader and compared to a control which had 50 µl of buffer solution in place of the extract. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

$$\% \text{ inhibition} = \frac{\Delta A \text{ control} - \Delta A \text{ extract}}{A \text{ control}} \times 100$$

α-amylase Inhibition Assay

Five hundred microliters of extract and 500 µl of 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) containing α-amylase solution (0.5 mg/ml) were incubated at 25°C for 10 min²⁵. After pre-incubation, 500 µl of a 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9 with 0.006 M sodium chloride) was added to each tube at timed intervals. The reaction mixtures were then incubated at 25°C for 10 min. The reaction was stopped with 1,0 ml of dinitrosalicylic acid color reagent. The test tubes were then incubated in a boiling water bath

for 5 min and cooled to room temperature. The reaction mixture was then diluted after adding 10 ml distilled water and absorbance was measured at 540 nm using multimode- reader (Biotek, Winooski, VT, USA). The results were expressed as % inhibition of enzyme activity and calculated according to the following equation.

$$\% \text{ inhibition} = \frac{\text{Absorbance of control} - \text{Absorbance of extract}}{\text{Absorbance of control}} \times 100$$

Evaluation of Antiglycation Property

Antiglycation property of SAF was determined as per the method described earlier²⁶ with slight modifications. About 500 μ l of albumin (1 mg/ml final concentration) was incubated with 400 μ l of glucose (500 mM) in the presence of 100 μ l of extract at different concentrations, The reaction was allowed to proceed at 60°C for 24 hrs and thereafter reaction was stopped by adding 10 μ l of 100% TCA. Then the mixture was kept at 4°C for 10 min before subjected to centrifugation (Kubota, Fujioka, Japan) at 10,000 g. The precipitate was redissolved in 500 μ l alkaline PBS (pH10) and immediately quantified for relative amount of glycated BSA based on fluorescence intensity at 370 nm (excitation) and 440 nm (emission).

Inhibition of Human LDL Oxidation in vitro

Oxidation of LDL leads to the production of malondialdehyde (MDA) which was measured by reaction with TBA²⁷ with slight modification. LDL (50 μ g/ml) was incubated with different concentrations of extract and the oxidation of LDL was initiated by the addition of 50 μ l copper sulphate (2 mM) at 37°C for two hrs. Final volume of the reaction mixture was made up to 1.5 ml with phosphate buffer (pH 7.4). After incubation, 500 μ l of reaction mixture was mixed with 250 μ l of TBA (1% in 50 mM of NaOH) and TCA (0.28%). Samples were again incubated at 95°C for 45 min. After cooling and centrifugation at 2000 rpm (10 min) fluorescence was taken at 515 nm excitation and 553 nm emission. The result was expressed as % of inhibition of LDL oxidation. Using the amount of MDA formed, % of inhibition can be calculated using the formula.

$$\% \text{ inhibition} = \frac{\text{Oxidation in control} - \text{Oxidation in sample}}{\text{Oxidation in control}} \times 100$$

Statistical Analysis

The experimental results were expressed as mean \pm SD (standard deviation) of triplicate measurements. The data were subjected to one way analysis of variance (ANOVA) and the significance of differences between means were calculated by Duncan's multiple range test using SPSS for windows, standard version 7.5.1, SPSS (SPSS Inc., Chicago, IL, USA) and the significance accepted at $p \leq 0.05$.

Results

Total Flavonoid Content (TFC) and Total Antioxidant Activity (TAA) of SAF

Total flavonoid content of *Saraca ashoka* flowers was estimated to be 45.5 \pm 1.27 mg catechin equivalents/100 g of extract from triplicate measurements. Total antioxidant capacity of flavonoid fraction of SAF is expressed as the number of equivalents of ascorbic acid. The phosphomolybdenum method was based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex. SAF exhibited significant Total antioxidant activity (TAA) and was estimated to be 369.14 mg of ascorbic acid/g of extract from triplicate measurements.

Evaluation of Oxidative Stress in C2C12 cell Lines

With respect to the cytotoxicity of the SAF, MTT assay results suggested that flavonoid fraction of SAF was safe to C2C12 cell lines up to 100 μ g/ml and not cytotoxic. Protective effects of flavonoid fraction of *Saraca ashoka* flowers against H₂O₂ induced oxidative stress in C2C12 cell lines was evaluated by determining intracellular content of ROS, MDA and reduced glutathione (GSH). H₂O₂ treated cells showed increased production of ROS which was significantly reduced ($p \leq 0.05$) by the treatment with SAF (75 μ g/ml) as shown in Figure 1. Treatment with H₂O₂ increased the production of MDA and decreased the concentration of non enzymatic antioxidant, reduced glutathione (GSH) (Figures 2 and 3). On the other hand, SAF treatment (75 μ g/ml) significantly ($p \leq 0.05$) decreased the concentration of MDA and increased the concentration of GSH in H₂O₂ treated cell lines. These data confirm the antioxidant activities of the SAF.

Figure 1. Effect of SAF on intracellular ROS generation on C2C12 cell lines. Each value represents mean±SD (Standard deviation) from three independent experiments and the significance accepted at $p < 0.05$.

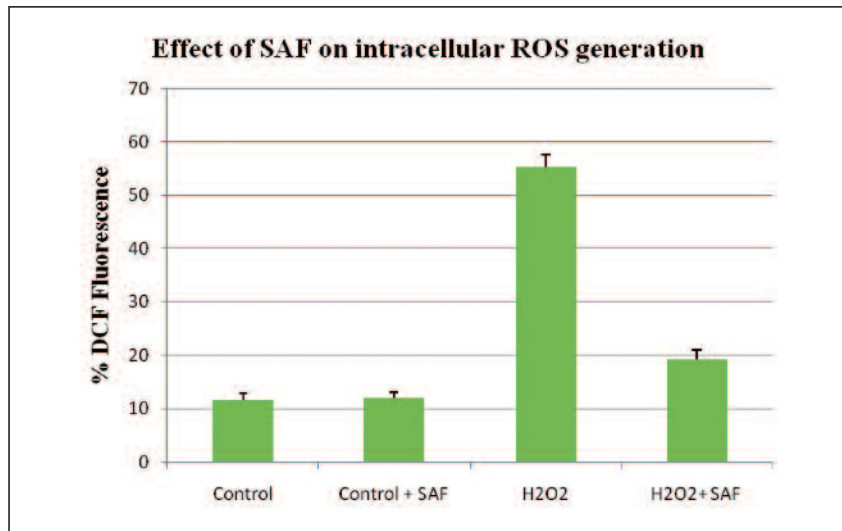


Figure 2. Effect of SAF on MDA concentration. Each value represents mean±SD (Standard deviation) from three independent experiments and the significance accepted at $p < 0.05$.

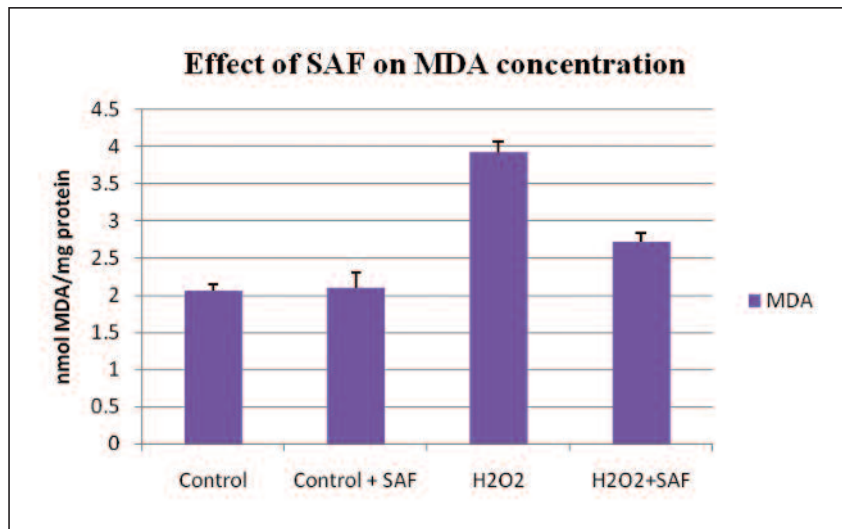
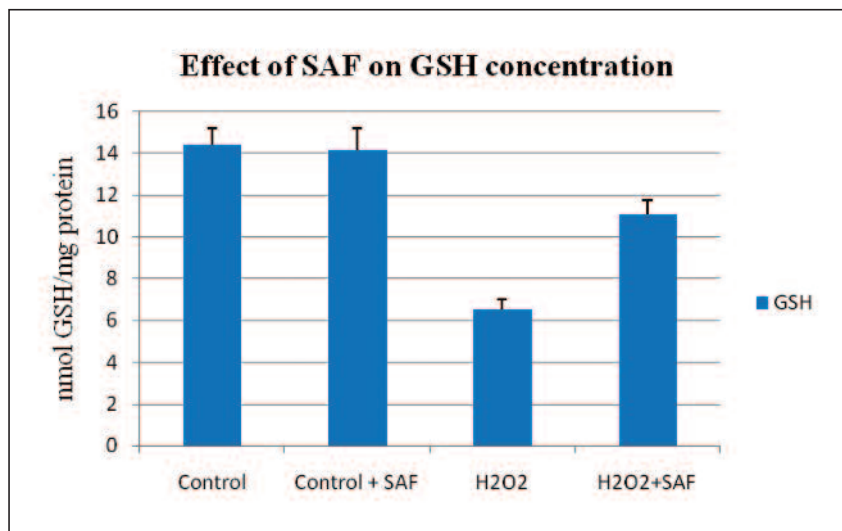


Figure 3. Effect of SAF on GSH concentration. Each value represents mean±SD (Standard deviation) from three independent experiments and the significance accepted at $p < 0.05$.



α -Glucosidase and α -Amylase Inhibitory Potential of SAF

Flavonoid fraction of SAF exhibited significant α -glucosidase and α -amylase inhibitory potential in a dose dependant manner (Figures 4 and 5) and can be compared with that of standard compound acarbose. The estimated EC₅₀ values of α -glucosidase and α -amylase inhibition by SAF is 63.62 μ g/ml and 411. 83 μ g/ml respectively while that of acarbose is 45.20 and 360.35 μ g/ml respectively.

Antiglycation Potential of SAF

Inhibitory potential of SAF against the production of advanced glycation end products (AGEs) was studied with different concentration of SAF. The results revealed that SAF was able to inhibit the production AGEs by 72.89% in 50 μ g/ml concentration as against the same concentrations of vitamin C, which resulted in 77.34% inhibition (Figure 6).

Inhibition of LDL Oxidation

SAF significantly inhibited LDL oxidation in a dose dependent manner (Figure 7) under *in vitro* condition with an EC₅₀ value of 40.11 μ g/ml while that of standard compound ascorbic acid was 24.5 μ g/ml. The result shows that flavonoid fraction of SAF can effectively inhibit the oxidation of LDL and its activity is comparable to that of ascorbic acid.

Discussion

It is well established that oxidative stress and non enzymatic protein glycation are key process in the development of diabetes¹⁴. Hyperglycemia engenders free radicals and it also impairs the endogenous antioxidant defence system in many ways during diabetes. In our study, flavonoid fraction of *Saraca ashoka* flowers exhibited significant total antioxidant activity (TAA). In C2C12 cell lines, SAF reduced the concentration of MDA and increased the concentration of reduced glutathione (GSH) confirms the antioxidant efficacy of SAF. MDA is one of the major products of lipid peroxidation and has been found elevated in various diseases thought to be related to free radical damage. It has been widely used as an index of lipid peroxidation in biological and medical sciences²⁸. High concentration of MDA in H₂O₂ treated cells shows the oxidative damage of cell. GSH is one of major non enzymatic antioxidant defence within the cell and plays an important role in protection against oxidative stress. Depletion of cellular GSH increases cell vulnerability to oxidative stress²⁹. H₂O₂ treatment decreased the concentration of GSH represents increased utilization due to the stress produced by H₂O₂. On the other hand, SAF treatment increased the concentration of GSH and prevented the elevation of MDA shows that SAF can prevent the oxidative damage induced by H₂O₂.

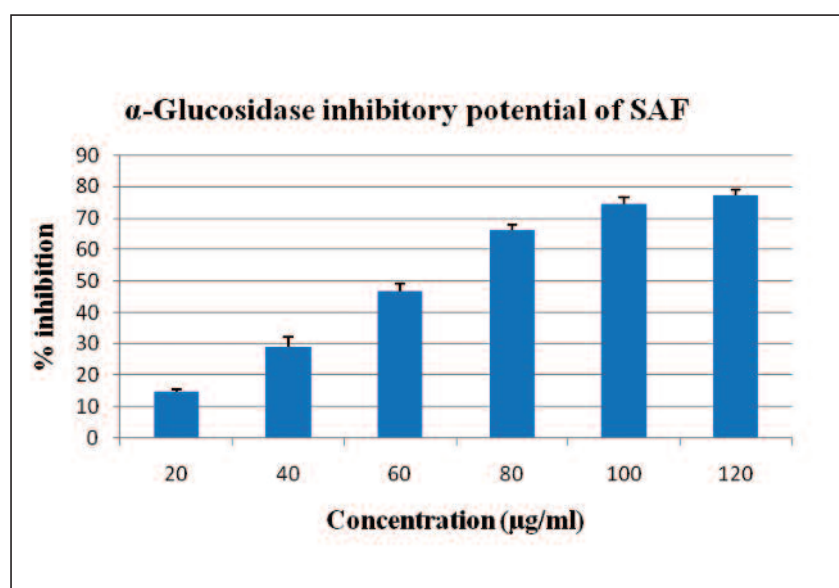


Figure 4. α -Glucosidase inhibitory potential of flavonoid fraction of SAF. Each value represents mean \pm SD (Standard deviation) from three independent experiments and the significance accepted at $p < 0.05$.

Figure 5. α -amylase inhibitory potential of SAF. Each value represents mean \pm SD (Standard deviation) from three independent experiments and the significance accepted at $p < 0.05$.

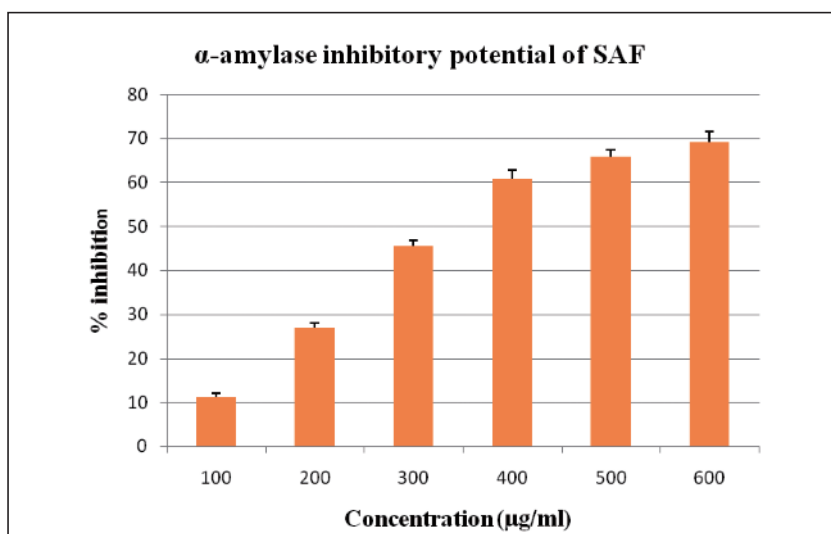


Figure 6. Antiglycation potential of SAF. Each value represents mean \pm SD (Standard deviation) from three independent experiments and the significance accepted at $p < 0.05$.

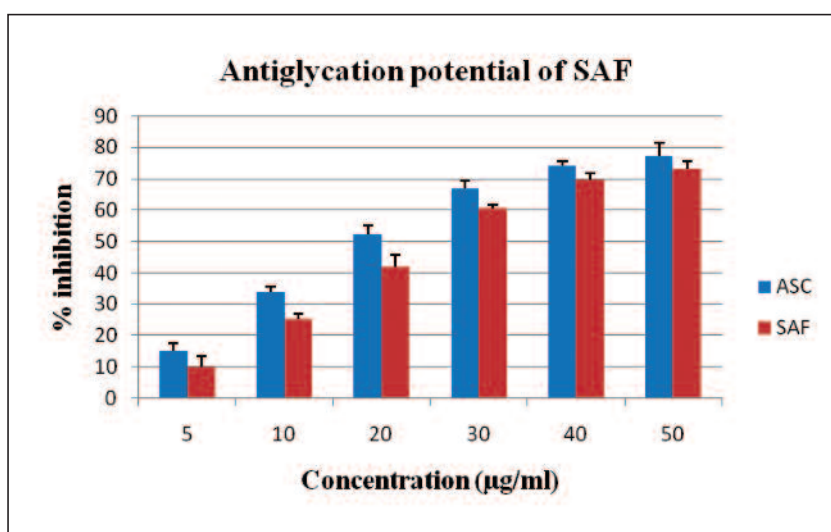
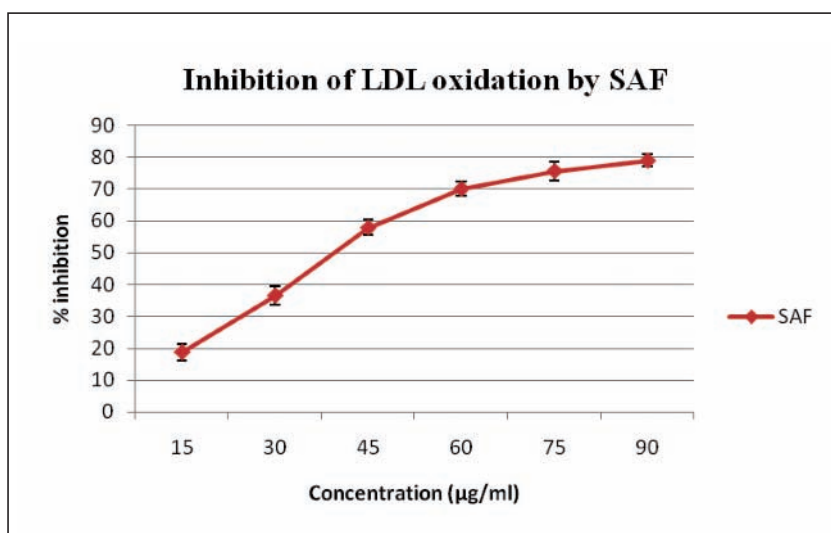


Figure 7. Inhibitory potential of SAF on LDL oxidation. Each value represents mean \pm SD (Standard deviation) from three independent experiments and the significance accepted at $p < 0.05$.



Accumulation of ROS gives an indication of the oxidative damage to living cells²³. Prooxidants can directly oxidize DCFH to fluorescent DCF, and it can also decompose to peroxy radicals and generate lipid peroxides and ROS, thus increasing fluorescence¹⁴. H₂O₂ treated cells showed significant increase in ROS generation when compared to control cells. SAF treatment efficiently quenched the ROS in cells resulting in a reduced cell damage and lipid peroxidation.

Like antioxidant potential, studies on inhibitory potential of SAF against enzymes involved in the digestion of carbohydrates showed a significant α -glucosidase and α -amylase inhibitory potential. Significant α -glucosidase and α -amylase inhibitory potential indicates that SAF can be act as a potent antihyperglycemic agent. Inhibitors of α -glucosidase and α -amylase delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise³⁰. SAF showed lower inhibitory effect against α -amylase activity and stronger α -glucosidase inhibitory effect and, therefore, can be potentially used as an effective therapy for postprandial hyperglycemia. Excessive production of free radical accelerates nonenzymatic protein glycation reactions. Proteins are modified by glucose through the glycation reaction, leading to the formation of AGEs³¹. In the present study, SAF was demonstrated to possess significant antiglycation property when tested by glucose-BSA assay. Increased rate of glycation and the build-up of tissue AGEs plays an important role in diabetes. Free radicals generated by glycation reaction can cause oxidation of nucleic acids and lipids and protein fragmentation³². Significant antioxidant and antiglycation property of SAF may be due to its protective effects against oxidative stress and glycation reaction.

ROS can degrade LDL to conjugated dienes and further oxidation leads to the formation of aldehydes including MDA. The oxidized LDL is processed by a scavenger receptor of macrophages, leading to cholesterol ester accumulation. These lipid-laden macrophages become foam cells which, in time, create fatty streaks in blood vessel walls. In addition, oxidized LDL has many other atherogenic effects in vascular sub endothelium³³. There were reports that individuals with diabetes have a higher risk for atherosclerotic cardiovascular diseases than

non diabetic patients. Oxidation of low-density lipoprotein (LDL) has been implicated as one of the main reason for human atherosclerosis³⁴. It has been reported that dietary antioxidants and free radical scavengers are able to prevent LDL oxidation and AGEs formation which can reduce the risk of atherogenesis and diabetes^{35,36}. In the present study, SAF significantly inhibited the oxidation of LDL and its effect may be due to its antioxidant and free radical scavenging activity.

From all these observations it can be concluded that flavonoid fraction of *Saraca ashoka* flower can act as an excellent antioxidant and antidiabetic agent. SAF treatment effectively attenuated H₂O₂ induced oxidative stress in C2C12 cell lines and shows significant antihyperglycemic activity by inhibiting α -glucosidase and α -amylase. It also exhibited significant antiglycation property and inhibited LDL oxidation under *in vitro* conditions. Results of the study reveal that as a natural antioxidant, *Saraca ashoka* flowers might be helpful in preventing oxidative stress and other complications associated with diabetes. These effects may explain the extensive use of *Saraca ashoka* flowers in daily life and its possible health benefits and may serve as an ideal candidate for biopharmaceutical industries with fewer side effects.

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