WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 8, Issue 11, 24-37. Research Article ISSN 2277– 7105

ANTIGLYCATION AND ANTIOXIDANT ACTIVITY OF A HOMEOPATHIC TINCTURE USED IN SRI LANKA

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Article Received on
01 August 2019,

Revised on 21 August 2019,
Accepted on 11 Sept. 2019,

DOI: 10.20959/wjpr201911-15852

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ABSTRACT

Glycation is the non-enzymatic reaction between a protein and sugars. After a series of complex reactions following glycation, advanced glycation end products (AGEs) are formed. AGEs are responsible for the pathogenesis of diabetic complications. Among the alternative treatments in Sri Lanka, the mother tincture of *Syzygium jambolanum* **(SJ)** is a widely used in homeopathy for treating diabetes mellitus. This study investigates the inhibitory potential on AGE formation by **SJ** and also its antioxidant activity. Nonenzymatic protein glycation inhibitory capacity assessed by Bovine Serum Albumin-Fructose fluorescence spectrometric assay showed that **SJ** at concentrations 4.2-25.2 mg/ml, inhibited AGE formation by 76.7±3.7%-95±0.9%. The positive control aminoguanidine at concentrations of 0.5-5.0 mg/mL, inhibited 65±3.5%-98±1.0%. The total phenolic content of **SJ** was 114.8±1.8 mg GAE/g in the Folin Ciocalteu assay. The percentage radical scavenging activity observed from DPPH assay for **SJ** (1.0-5.0 mg/ml) was 22.4±2.2%-87.7±1.0%, compared to the Ascorbic acid (ASC), positive control (0.5- 2.5 mg/ml) showing 31.9.0±0.4%-95.2±0.3%. Percentage hydroxyl radical scavenging activity observed for **SJ** (1.0- 5.0 mg/ml) was 23.7±0.4%-77.1±0.5% compared to ascorbic acid (0.1-2.5 mg/ml) showing 22.8±0.5% - 97.5±1.1%. Our results indicate the mother tincture of **SJ** can prevent AGE formation and there by prevent the onset of diabetic complications. It also

showed promising radical scavenging activity thus making it a potential remedy for many diseases in which radicals are implicated.

KEYWORDS: Antiglycation potential, antioxidant capacity, homeopathy mother tincture *Syzygium jambolanum.*

INTRODUCTION

Diabetes mellitus is a metabolic disorder which results in serious health problems with high rates of incidence and mortality. South East-Asia (SEA) Region was home to more than 72 million adults with diabetes in 2013 and is expected to exceed 123 million in 2035.[1] Long term effects of diabetes mellitus affect the cardio-vascular & nervous systems, eyes, kidneys etc., contributing to diabetic complications such as retinopathy, nephropathy, neuropathy, cataract among some of them.[2]

It is reported that diabetes is related to the development of several forms of Reactive Oxygen Species (ROS) such as peroxides, superoxide, hydroxyl radical.[3] Over-production of ROS and increasing evidence of oxidative stress play a key role in the pathological process in diabetes and diabetic complications.[4] Several mechanisms have been identified as contributing factors of oxidative stress in hyperglycemia including increased 'polyol pathway flux', increased intracellular formation of Advanced Glycation End Products (AGEs), activation of Protein kinase C and overproduction of superoxide by the mitochondrial electron transport chain. Among them, Advanced Glycation End Product formation may be involved in direct hyperglycemia mediated oxidative stress.[4]

Glycation is the non-enzymatic reaction between a protein and sugars, such as glucose or fructose.[5] These sugars and free amino groups in amino acids or proteins form Schiff base structures. This process is called Maillard reaction or Advanced Glycation. This Schiff base is unstable; hence it rearranges to form Amadori products via chemical transformations. These Amadori products undergo dehydration and rearrangement followed by other reaction such as cyclisation, oxidation, and dehydration and cross linking to form Advanced Glycation End products (AGEs).[6] Formation of AGEs happen in normal physiological conditions, but it is accelerated in hyperglycemia. Accumulations of glycation end products are concomitant with various diseases.[7] AGEs play an important role in the pathogenesis of diabetic complications like retinopathy, nephropathy, neuropathy, cardiomyopathy along with some other diseases such as rheumatoid arthritis, osteoporosis and aging.[8]

Scientific literature identifies inhibition of AGE formation as one of the therapeutic approaches to prevent diabetic complications. Aminoguanidine (**AG**) is the first inhibitor of AGEs discovered in 1986.[9] However it may have serious toxicity when administered for diabetic nephropathy.[5]

Therefore, there is considerable interest in the search of plant-based drugs with anti-glycation activity as they may potentially inhibit AGE formation resulting in delaying and preventing the onset of diabetic complications with minimal side effects.[10]

There are various forms of Sri Lankan traditional medicines composed of herbal or herb-mineral ingredients which possess anti-diabetic activity. Among them Homeopathy is a universal method of treatment that uses minor quantities of natural substances originating from plant, minerals or animals.[11] Mother Tincture of the *Syzygium jambolanum* **(SJ)** is used in homeopathy for treatments of type 2 diabetes. Mother tincture is defined as the original tincture prepared with the aid of alcohol, directly from the crude drug.[11] However, the molecular mechanisms responsible for such effects are not investigated.[12] As such this article addresses the investigation of antiglycation potential of the mother Tincture of **SJ** used in homeopathy which in turn could serve as to prevent onset of diabetic complications. Furthermore, determination of total phenolic content and the radical scavenging activity of **SJ** is also investigated in this article.

MATERIALS AND METHODS

Mother Tincture of **SJ** was collected from a general practitioner at Government Homeopathic Hospital, Walisara, Sri Lanka. Density (0.841g/mL) of the mother tincture was taken for calculation of initial concentration (1000mg/ml).

In vitro Protein Glycation inhibition

The inhibitory action of **SJ** on AGE formation was evaluated according to a previously published method with slight modifications.[5],[13] Solutions of Fructose (1000 mM) and Bovine Serum Albumin (BSA 20 mg/mL) were prepared in phosphate buffered saline (200 mM, pH 7.4) containing Sodium Azide (0.02%) to minimize microbial activity during the experiment.[14]

Fructose 4.0 mL was mixed with 5.0 mL of BSA and 1.0 mL of **SJ** (final concentration: 4.2-25.2 mg/mL) to prepare Sample mixtures. A Control was prepared using only BSA and

Fructose in order to induce the formation of the AGEs and to compare the inhibitory activity of the **SJ**. Control blank was prepared using only BSA, whereas Sample blanks were prepared only with the **SJ** with respective concentrations. The total volume of the all tubes was brought up to 20.0 mL with the phosphate buffer (200 mM, pH 7.4).

The formation of fluorescent AGEs was measured by using a Spectrofluorometer (HITACHI, F-2700), at an excitation wave length 355 nm and emission wave length 440 nm.[5] Each tube was kept for a week and the fluorescence emission was measured. Each sample was analyzed in triplicate.

The inhibitory action on AGE formation was determined by the equation given below.[7]

Inhibition of fluorescent AGEs (%) = {(F_C-F_{CB}) - (F_S-F_{SB})/ (F_C-F_{CB})} X100

Where "F" denotes the intensity of fluorescence emission, and subscripts C, CB, S and SB denote control (BSA and fructose), control blank (BSA only), Sample (BSA, Fructose and **SJ**) and Sample blank (**SJ** only) respectively. Aminoguanidine (**AG**) was used as the positive control.[5]

Determination of Total Phenolic Content

Commercially available Folin ciocalteu reagent is used in this study to determine the phenolic content of the tincture based on previously published methods.[15],[16]

A volume of 4 ml of 2% sodium bicarbonate was added to 200 µl of **SJ** and incubated in darkness for 2 minutes. Folin ciocalteu reagent 200 µl was added to the same mixture and incubated in darkness for 30 minutes. The absorbance was measured by the UV/Visible spectrophotometer (Hitachi U 2910) at 750 nm. The blank was prepared by replacing the Folin ciocalteu reagent with 200 µl of ethanol. The same procedure was carried out for standard concentration series using 200 µl of gallic acid instead of **SJ**.

Different concentration of Gallic acid were prepared and the absorbance measured to obtain a calibration plot.[16],[17]

In vitro Antioxidant activity

DPPH Radical Scavenging Activity

The free radical scavenging activity of the tincture was measured *in vitro* by 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay according to the previously published methods.[18]

A volume of 2 ml of 2,2-diphenyl-1-picrylhydrazyl (DPPH, 0.02mg/mL) working standard was added to 2 ml of each concentration (0.5- 5.0 mg/mL) of the **SJ**. Each sample reaction mixtures were shaken well and incubated in the dark for 15 minutes at room temperature. The absorbance was measured at 517 nm using a UV Visible Spectrophotometer (Hitachi U 2910). The solution mixture of 2 ml DPPH (0.02mg/ml) with 2 ml of Methanol (95%) was used as the control.

The same procedure was carried out for standard concentration series of Ascorbic acid (**ASC**), the positive control instead of the **SJ**.[16],[18]

Percentage of scavenging activity (%RSA) was calculated using the following equation.[16],[18]

RSA (%) = {(Absorbance of Control-Absorbance of Sample) / (Absorbance of Control)} X100

Hydroxyl Radical Scavenging activity

Hydroxyl radical scavenging capacity of **SJ** was measured by the capacity of the different concentration of the tincture samples to scavenge the hydroxyl radicals generated by the Fe^{3+}-ascorbate-EDTA-H_2O_2 system.[18]

A volume of 500 µl of 2-deoxyribose, 200 µl of premixed ferric chloride ($FeCl_3$) and ethylenediaminetetraacetic acid (EDTA 1:1 v/v), 100 µl of hydrogen peroxide (H_2O_2) were added to 100 µl of each concentration of the **SJ**. The reaction was triggered by adding 100 µl of ascorbic acid and the reaction mixtures were incubated for 1 hour at 37 ℃. A volume of 0.5 ml of the reaction mixture was added to 1 ml of trichloroacetic acid and then 1 ml of thiobarbituric acid was added into the same mixture. The mixtures were heated for 15 min on a boiling water bath. After the mixture reached room temperature, the absorbance at 532 nm was measured. The control was prepared using a mixture of 2-deoxyribose, phosphate buffer, premixed ferric chloride, EDTA and H_2O_2. The same procedure was carried out for standard concentration series of Ascorbic acid, positive control instead of the **SJ**.

Percentage Hydroxyl Radical Scavenging activity was calculated as follows.

Hydroxyl Radical Scavenging activity (%) ={(1-Absorbance of Sample)/(Absorbance of Control)}X100

DATA ANALYSIS

All tests were carried out in triplicate and the data shown in the table and the graphs are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

In vitro Protein Glycation inhibitory activity

Glycation is a regular process that occurs in our body at very slow rates and mainly involves non-enzymatic reaction between reducing sugars with amino group of amino acid, peptide or protein.[5] It is known that the levels of hemoglobin A1c (HbA1c), a naturally occurring minor species of hemoglobin in humans is elevated in patients having diabetes mellitus.[6]

HbA1c is a post- translational adduct of glucose where the N-terminal valine of the β-chain of hemoglobin is attached to glucose nonenzymatically to form a Schiff base structure.[5] This Schiff base is unstable; hence it follows Amadori rearrangement via chemical transformations. Furthermore, it undergoes dehydration and rearrangement followed by other reactions such as cyclisation, oxidation, and dehydration and cross linking to form Advanced Glycation End products (AGEs). Increased blood glucose levels will increase protein glycation and leads to the formation of Amadori products and subsequently, AGEs.[6]

Acceleration of AGE levels in the body is a major cause for micro- and macrovascular complications such as retinopathy, nephropathy, atherosclerosis and other diabetes related complications.[19] Recent studies suggest inhibition of AGE formation as one of the therapeutic approaches to prevent diabetic complications. Aminoguanidine has been identified as an inhibitor of AGE formation by trapping reactive dicarbonyl species formed prior to the Amadori rearrangements.[6]

However, due to the toxic effect of the synthetic amnioguanidine, which was the first anti-AGE drug, plant-based remedies were sought for, which possess fewer side effects.[5]

One of the main focuses in this study was to evaluate the effectiveness of mother tincture of *Syzygium jambolanum* **(SJ)** in the reduction of AGEs formation via its potential antiglycation

activity. This was carried out using a mixture of BSA and fructose which were then treated with different concentrations of the **SJ** samples and aminoguanidine (positive control).

Fructose was chosen as the sugar to induce AGE formation in BSA as it has been shown that fructose can lead to the formation of glycated products faster and these products have high fluorescence intensity compared to the products from glucose.[20],[21] Apart from this reason it has been discovered that when compared to glucose, fructose can produce more dicarbonyl compounds and hydroxyl radicals by auto-oxidation.[22] [23]

The basis of testing for the antiglycation activity was the analysis of the fluorescence intensities following a week of incubation whereas the intensity would vary depending on the concentrations of fluorescent AGEs present in the samples. BSA gives fluorescence due to aromatic amino acid residues present in the protein. Following glycation with fructose (Frc), BSA yields AGEs, some of which are fluorescently active. These fluorescent AGEs show higher fluorescence emission than BSA alone.[5]

The fluorescence intensity increased considerably throughout the period, and the introduction of **SJ** to the reaction mixtures (0.5-33.3 mg/mL) demonstrated drastic reduction in the fluorescent intensity of mixtures as shown in Figure 1(a) compared to BSA with Frc. This indicate the reduction of formation of fluorescent AGEs by **SJ**.

Based on the recorded intensities, percentage inhibition was calculated. At 25mg/mL concentration, **SJ** showed 95% inhibitory potential towards fluorescent AGE formation. At 5mg/mL **SJ** showed 85% inhibition while aminoguanidine (AG), positive control at the same concentration showed 98% as indicated in Figure 1(b).

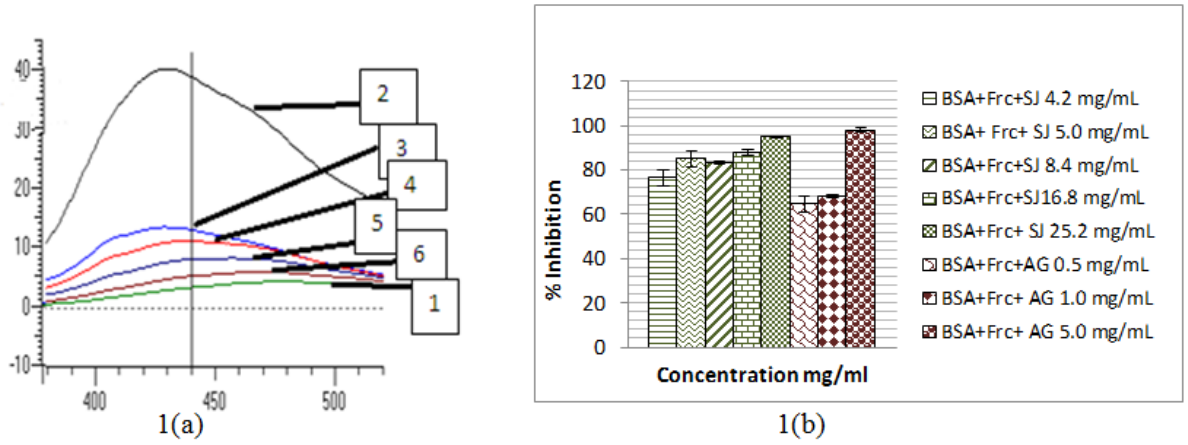

Figure 1(a): The overlay of the fluorescence emission spectra after one week for the samples that contain (1) BSA only, (2) BSA +Frc only, (3) BSA+ Frc+ SJ 4.2 mg/ mL only, (4) BSA+ Frc+ SJ 8.4 mg/mL only, (5) BSA+ Frc+ SJ 16.8 mg/mL only (6) BSA+ Frc+ SJ 25.2 mg/mL only. Figure 1(b): Comparison of percentage inhibition on AGE formation caused by SJ and AG at different concentrations.

Based on the results that were obtained, it is evident that **SJ** elicits a significant percentage inhibition of protein glycation relative to the inhibitory activity of the positive control; aminoguanidine.

Antioxidant activity

Enhanced oxidative stress and the changes in antioxidant capacity, which are observed in both clinical and experimental Diabetes Mellitus, are supposed to contribute to the etiology of chronic diabetic complications. Previous studies have reported the relationship between antioxidants and nonenzymatic protein glycation.[24] There have been a growing number of investigations on the potential of antidiabetic medicinal plants as possible antioxidants to prevent or delay chronic diabetic complications.[25] As such, in addition to evaluating effectiveness of **SJ** in antiglycation, the antioxidant properties of **SJ** were also evaluated.

Total phenolic content

The antioxidant activity of plants have been associated with polyphenolic compounds probably due to the ability of phenol compounds to act as electron donors as well as chelate metal ions.

The total phenolic content was assessed using the Folin-Ciocalteau method. Hexavalent phosphor-tungstic complexes are present in the Folin reagent and it has a yellow colour.[27]

Under alkaline conditions phenolic compounds dissociate to an anionic form which reacts with Folin reagent, If the test is positive, the reagent gives a colour change from yellow to dark blue due to the reduction of phosphomolybdate-phospho-tungstate complex forming Mo (+V) species.[28]

The total phenolic content for **SJ** was calculated as 114.8±1.8 mg GAE/g.

DPPH (2,2-diphenylpicrylhydrazyl) assay

The DPPH assay has been conducted to determine the ability of the **SJ** to scavenge the DPPH radicals. DPPH (2,2-diphenylpicrylhydrazyl) is a nitrogen centered free radical that is stable and commonly used for testing the radical scavenging activity of plant extracts.[29] The decrease in the DPPH radical concentration as determined by the fading of the purple colour is proportional to the increasing radical scavenging activity.[27] This process is carried out by the donation of electrons by the antioxidants to the free radicals and thus turning it into a yellow colored di-phenyl-picryl-hydrazine radical.[30] As a result of this activity, compounds that allow this conversion can be identified as antioxidants.

The calculated percentage of DPPH radical scavenging activity (RSA) of **SJ**, and calculated %RSA for different concentrations of the Ascorbic acid (positive control) are shown in figure 2.

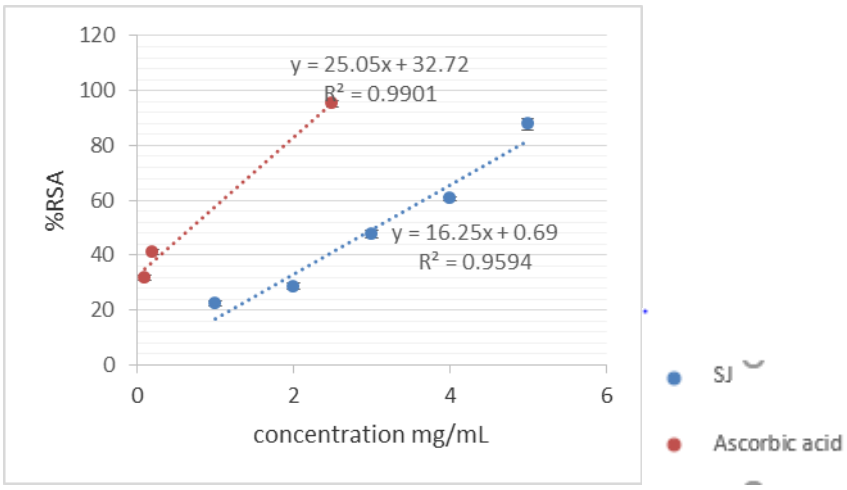

Figure 2: Comparison of radical scavenging activity of SJ versus ascorbic acid in DPPH radical scavenging activity assay. Results are presented as mean ± standard deviation of three independent experiments.

As per the Figure 2 it has been shown that the radical scavenging activity has been increased with increasing concentration of **SJ** samples. Percentage RSA of 87% was observed at 5 mg/mL concentration of **SJ** whereas Ascorbic acid showed similar activity around 2.5mg/mL. Bearing in mind that **SJ** contains crude plant extract this is quite significant.

Hydroxyl radical scavenging activity

Among other radicals, hydroxyl radicals play a major role in biological systems since it is a highly potent reactive oxygen Hydroxyl radicals react with polyunsaturated fatty acid moieties in phospholipids and damage the cell membranes and as a result, these radicals contribute to carcinogenesis, Mutagenesis and cytotoxicity.[18]

Hydroxyl radical scavenging activity was measured by the ability of the different concentrations of **SJ** in order to scavenge the hydroxyl radicals generated by the "Fe^{3+} - ascorbate-EDTA-H_2O_2" System.[17]

Absorbance values collected for Hydroxyl assay using different concentrations of **SJ** samples are used to calculate the percentage radical scavenging activity (% RSA) according to the Equation (3). The calculated percentage RSAs for different concentrations of **SJ** are shown in the Figure 3.

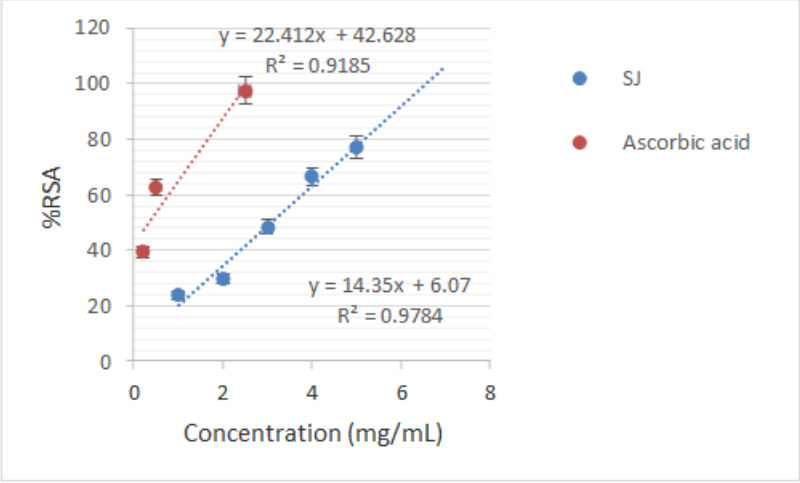

Figure 3: Comparison of hydroxyl radical scavenging activity of SJ versus ascorbic acid. Results are presented as mean ± standard deviation of three independent experiments.

Results from this assay indicate that the antioxidant activity of **SJ** behaves in a dose dependent manner.

This study reveals that **SJ** has high glycation inhibitory potential. It also exhibits a high TPC value and promising radical scavenging properties (according to the DPPH and hydroxyl radical scavenging assays).

CONCLUSION

The total phenolic content and the DPPH radical scavenging activity of **SJ** demonstrates its potential to relieve oxidative stress. The hydroxyl radical scavenging activity of **SJ** highlights its ability to combat carcinogenesis, Mutagenesis and cytotoxicity (*vide supra*). Due to its inhibitory potential on Advanced Glycation end product formation, **SJ** is capable of reducing onset of diabetic complications during chronic hyperglycaemia.

In conclusion this study shows that the Homeopathic mother tincture of *Syzygium jambolanum* has a potential to inhibit AGE formation and counteract diabetic complications. The radical scavenging activity is also significant as radicals are implied in many diseases as well as aging.

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