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Assessment of *In Vitro* Anti-Diabetic and Anti-Oxidant Potential of *Syzygium nervosum* Leaves Extracts

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Abstract: *Syzygium nervosum* (also recognized as Cleistocalyx operculatus and Eugenia operculata), is widely distributed in tropical areas of South East Asian countries. Hot water brewing of *S. nervosum* leaves or flower buds is well known for treating influenza and some digestive conditions according to traditional medicine. Besides that, *S. nervosum* leaves and flower buds were also used externally for inflammatory conditions, including bruises, acnes, and skin ulcers. This study evaluated *in vitro* antioxidant and anti-diabetic activities of *Syzygium nervosum* leaves extract. The antioxidant activity was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl and superoxide anion radical scavenging abilities of various polarity extract (Ethyl acetate, Ethanol and aqueous extract) of *Syzygium nervosum*. *In vitro* anti-diabetic potential was assessed by evaluating the inhibitory effects of the extracts on the activities of α-amylase, α-glucosidase, maltase and sucrase. Ethanol extract of *Syzygium nervosum* leaves exhibited significantly higher ($p < 0.05$) antioxidant activity in DPPH, superoxide radical scavenging and hydroxyl radical scavenging models. The ethanol extract also exhibited significantly higher ($p < 0.05$) inhibition of α-glucosidase as well as strongest inhibition of α amylase. In conclusion, the result of the present study has demonstrated evidently that extracts of *Syzygium nervosum* leaves ameliorates hyperglycemia through the inhibition of diabetes-related enzymes and the associated oxidative stress.

Keywords: *Syzygium nervosum*, *Cleistocalyx operculatus*, Diabetes mellitus, Oxidative stress, Anti-oxidant activity

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by hyperglycemia, hypertriglyceridemia and hypercholesterolemia, which results from defects in insulin secretion, its actions or both (Lodhi and Kori, 2021). Diabetes mellitus is a major public health issue affecting more than 400 million people worldwide. Type 2 diabetes is portrayed by insulin obstruction and having relative absence of insulin discharge. The greater part of the type 2 diabetic patients are fat

and corpulence itself causes insulin obstruction. There are various reasons for this type of diabetes where the particular etiology is not known and no proof of immune system interruption in the β cells. The disorder is associated with an imbalance of the glycemic index and glucose intolerance, which increases the risk of individuals progressing to type 2 diabetes mellitus. Complications from diabetes, such as coronary artery and peripheral vascular diseases, stroke, diabetic neuropathy, risk of ulcers and amputations, renal failure, sexual dysfunction, and blindness accounts for the increasing disability, reduced life expectancy, and enormous health costs associated with this condition for virtually every society. This metabolic disorder progressively leads to chronic microvascular, macrovascular and neuropathic life threatening complications. DM is caused either by deficiency of insulin secretion, damage of pancreatic β cell or insulin resistance related to non-use of insulin. The homeostasis of glucose in the body is kept up with by various chemicals. Anyway two chemicals to be specific, insulin and glucagon assume a prevailing part in the guideline of glucose homeostasis (Patel and Kori, 2018a, b). Insulin is discharged by β cells when the grouping of glucose rises. Insulin diminishes the degree of blood glucose by the same token. Therefore, it is important to develop antidiabetic agents targeting diabetes and its associated complications. The major conventional classes of drugs for the treatment of hyperglycemia includes sulfonylureas (enhance release of insulin from pancreatic islets); biguanides (reduces hepatic glucose production); peroxisome proliferator-activated receptor-γ (PPARγ) agonists (boosts the action of insulin); α-glucosidase inhibitors (interferes with absorption of glucose in the gut). These classes of drugs are either administered as monotherapy or given in combination with other hypoglycaemics. Severe hypoglycemia, weight gain, lower therapeutic efficacy owing to improper or ineffective dosage regimen, low potency and altered side effects due to drug metabolism and lack of target specificity, solubility and permeability problems are the major drawbacks associated with the use of the above mentioned conventional drugs (Gupta and Kori, 2023). The oxidative stress of hyperglycemia has extensively been implicated to be a central mechanism for development, progression and complications of the condition, thus this necessitates the development of anti-diabetic drugs with multimodal mechanism of action. Most of the antidiabetic drugs currently available act via one known mechanism and may also present with some undesirable effect when used. As alternatives, herbal remedy may provide a comparative advantage by reason of the presence or possession of diverse bioactive secondary metabolites which could exert their effect via different mechanisms (Mayorov, 2011; Adlak *et al.*, 2023). *Syzygium nervosum* (also known as *Cleistocalyx operculatus*, and *Eugenia operculata*) is well known for treating influenza and some digestive conditions according to traditional medicine. Besides that, *S. nervosum* leaves and flower buds were also used externally for inflammatory conditions, including bruises, acnes, and skin ulcers. Phytochemical studies have identified a total of 86 natural compounds from leaves and flower buds of *S. nervosum*. To a certain extent, some pharmacological effects explained the medicinal uses of this plant in folklore medicine, such as antiviral, anti-inflammatory and antioxidant activities (Wang *et al.*, 2016; Chailungka *et al.*, 2017). The anti-diabetic potentials of S. nervosum leaves have not been established. The present study intends to screen the various polarity extract of the leaves of *S. nervosum* for antioxidant and hypoglycemic activity using in vitro model.

Materials and Methods

Plant material and extract preparation:

The leaves of *Syzygium nervosum* were collected from the Rewa (M.P., India). The plant was identified and authenticated. Fresh leaves were used for Pharmacognostical studies. The leaves were powdered to 60# separately and stored in airtight containers and used for further studies. *Syzygium nervosum* leaves powders were

extracted sequentially with ethyl acetate and ethanol using continuous hot extraction method i.e. soxhlet extraction. The aqueous extracts were obtained by maceration method.

Chemicals and reagents:

Porcine pancreatic a-amylase, rat intestinal α-glucosidase, 1,1- diphenyl-2-picrylhydrazyl, gallic acid, acarbose and *para*-nitrophenyl–gluco-pyranoside were products of Sigma–Adrich Co., St. Louis, USA while starch soluble (extra pure) 1,1-diphenyl-2-picryl hydrazyl (DPPH), DMSO, Ascorbic acid Sodium nitro prusside, Griess reagent (1%w/v sulphanilamide, 2% w/v H_3PO_4 and N-(1-naphthyl) ethylene diamine di hydrochloride), Hydrogen peroxide (H_2O_2), Thiobarbituric acid, Sodium phosphate buffer, 1 mM ferric chloride, Nitro blue tetrazolium Phenazene methosulphate, Nicotinamide adenine phosphate di nucleotide was obtained from J. T. Baker Inc. Other chemicals and reagents were of analytical grade and distilled water was used. Plant extracts were dissolved in dimethysul-phoxide (DMSO) to give stock solutions of 1.0 mg/ml and different concentrations (25, 50, 75, 100 and 200 µg/ml) of the extracts were prepared using a serial dilution method with distilled water. All extracts were stored at 4 °C prior to analysis.

In vitro antioxidant activity:

DPPH free radical scavenging ability: The free radical scavenging ability of the extracts against DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical was evaluated by a modified method of Saha *et al*. (2008). Different concentrations (25 to 200 µg/ml) of the extracts (100 ml) were mixed with 100 ml of 0.4 mmol/l methanolic solution containing DPPH radicals. The mixture was left in the dark for 30 min and the absorbance was measured at 516 nm. The DPPH free radical scavenging ability of each extract was subsequently calculated with respect to the reference (which contains all the reagents without the test sample). Ascorbic acid was used as a positive control.

Hydroxyl radical scavenging ability: The ability of the plant extracts to prevent Fe^{2+}/H_2O_2 induced decomposition of deoxyribose was carried out using the modified method described by Oboh and Rocha (Oboh *et al.*, 2007). Briefly, 50 ml freshly prepared different concentrations (25 to 200 µg/ml) of the extracts was added to a reaction mixture containing 25 ml 20 mM deoxyribose, 100 ml 0.1 M phosphate buffer, 10 ml 20 mM hydrogen peroxide and 10 ml 500 mM $FeSO_4$, and the volume was made up to 200 ml with distilled water. The reaction mixture was incubated at 37 °C for 30 min, and the reaction was stopped by the addition of 50 ml of 2.8% TCA (trichloroacetic acid), this was followed by the addition of 50 ml of 0.6% TBA solution. The mixtures were subsequently incubated for 20 min and the absorbance was measured at 532 nm by UV-Visible spectrophotometer (Shimadzu UV-Vis1800). Ascorbic acid was used as a positive control.

Nitric Oxide (*NO*) *radical scavenging activity*: Nitric oxide (NO) was generated from sodium nitro prusside (SNP) and was measured by the griess reagent (1% w/v sulfanilamide, 2%w/v H_3PO_3 and 0.1% w/v N-(1-Naphthyl) ethylene diamine dihydrochloride). SNP in aqueous solution at physiological pH spontaneously generates NO, which interacts with oxygen to produce nitrite ions that can be estimated by the use of griess reagent. Scavengers of NO compete with oxygen leading to reduced production of NO. SNP (1 ml of mM) was mixed with 1 ml of selected plants extracts in different concentrations in DMSO, incubated at 25 °C for 180 min. To 1 ml of the incubated solution, 1 ml of griess reagent was added. The absorbance of the chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with N-(1-naphtyl) ethylene diamine dihydrochloride was read at 546 nm by UV-Visible spectrophotometer (Shimadzu UV-Vis1800). All the test analysis were run in triplicate and averaged. Lower absorbance of reaction mixture indicates higher free radical scavenging activity. Ascorbic acid was used as a positive control.

Superoxide radical scavenging ability: Superoxide radical scavenging activity of the extracts was based on the method described by Liu *et al*. (1997). Superoxide radicals were generated in 25 ml of Tris-HCl buffer (16 mM, pH 8.0) containing 25 ml of NBT (50 mM) solution, 25 ml NADH (78 mM) solution and different concentrations (25, 50, 75, 100 and 200 µg/ml) of extracts (50 ml). The reaction started by adding 1 ml of phenazine methosulphate (PMS) solution (10 mM) to the mixture then incubated at 25 °C for 5 min. The absorbance was measured at 560 nm. Ascorbic acid was used as a positive control.

In vitro antidiabetic activity:

α-Amylase inhibitory assay:

α-Amylase inhibitory assay was carried out using a modified method of McCue and Shetty (2004) (McCue and Shetty, 2004). 100 ml *Syzygium nervosum* extract (25 to 200 µg/ml) was taken in a conical flask and added 100 ml of 0.02 M sodium phosphate buffer (pH 6.9) containing α-amylase solution. This solution was pre-incubated at 25 °C for 10 min, after which 100 ml of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at timed intervals and then kept at 25 °C for 10 min. The reaction was terminated by adding 200 ml of dinitrosalicylic acid (DNS) reagent. The flask were then kept in boiling water for 5 min and cooled to room temperature. The reaction mixture was diluted with 5 ml distilled water and the absorbance was measured at 540 nm using a UV spectrophoto-meter. The control was prepared using the same procedure replacing the extract with distilled water while activity of the standard was tested by replacing the extract with acarbose. The α-amylase inhibitory activity was calculated as percentage inhibition, and 50% inhibition of enzyme activity (IC_{50}) was determined.

$$\% \text{ Inhibition} = \frac{\text{Acontrol -- Aextract}}{\text{Acontrol}} \times 100$$

α-Glucosidase inhibitory assay: *α*-Glucosidase inhibitory assay was carried out using a modified method by Adisakwattana *et al*. (2009). Rat intestinal acetone powder (100 mg) was homogenized in 3 ml of 0.9% NaCl solution. After centrifugation (30 min), the crude enzyme (100 ml) was incubated with 5 mM *p*-nitrophenyl glucopyranoside (pNPG), 25 mM maltose or 50 mM sucrose in 0.1 M phosphate buffer (pH 6.9) and 50 ml of the different concentrations (25 to 200 µg/ml) of extracts at 37 °C for 30 min. The reaction mixture was stopped by adding 50 ml of 0.1 M Na_2CO_3. The enzyme activities were determined by measuring the absorbance at 405 nm (α-glucosidase). The control was prepared using the same procedure replacing the extract with distilled water while activity of the standard was tested by replacing the extract with acarbose. Percentage inhibition 50% inhibition of enzyme activity (IC_{50}) was determined.

$$\% \text{ Inhibition} = \frac{\text{Acontrol -- Aextract}}{\text{Acontrol}} \times 100$$

Statistical analysis was performed using GraphPad Software. The data were analyzed by one way analysis of variance (ANOVA). All the results are expressed as mean ±SEM for triplicate determinations.

Results

Syzygium nervosum leaves extracts revealed a concentration dependent free radical scavenging activity resulting from reduction of DPPH, NO, Hydroxyl radical and superoxide radical to non-radical form. The scavenging activity of Ascorbic acid, a known antioxidant used as positive control, was however, higher. The order of reduction potential was found more by ethanol extract. The ethanol extract of *Syzygium nervosum* leaves at varied concentrations showed remarkable inhibitory effect of nitric oxide radical scavenging activity compared to other extract. Results revealed that all the tested extracts showed the percentage of inhibition in a dose dependent manner. Hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity. The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and

Table 1: IC_{50} values for the free radical scavenging potentials of *Syzygium nervosum* leaves extracts

S. No.	Enzymes	IC_{50} (µg/ml)			
		DPPH	Nitric oxide radical	Hydroxyl radical	Super oxide radical
1	Ascorbic acid	10.91	12.36	20.57	56.23
2	EASN (Ethyl acetate extract)	57.12	59.36	73.67	90.28
3	EOSN (Ethanol extract)	27.28	28.27	33.92	69.49
4	AQSN (Aqueous extract)	70.45	73.52	78.16	99.08

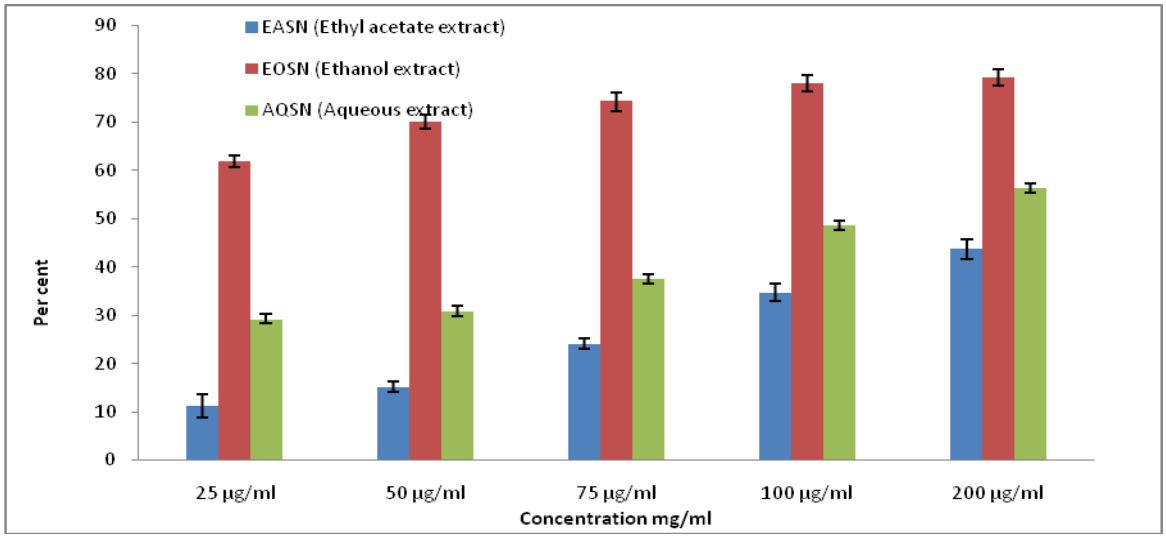

Fig. 1: Effects of *Syzygium nervosum* leaves extracts on DPPH radical scavenging model.

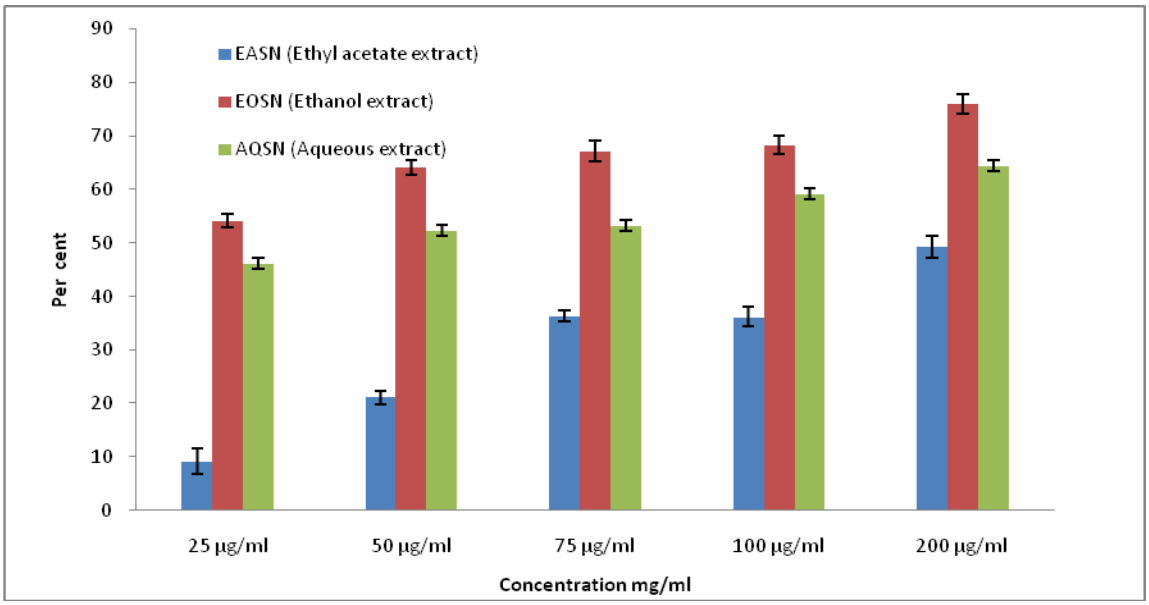

Fig. 2: Effects of *Syzygium nervosum* leaves extracts on Nitric oxide radical scavenging model.

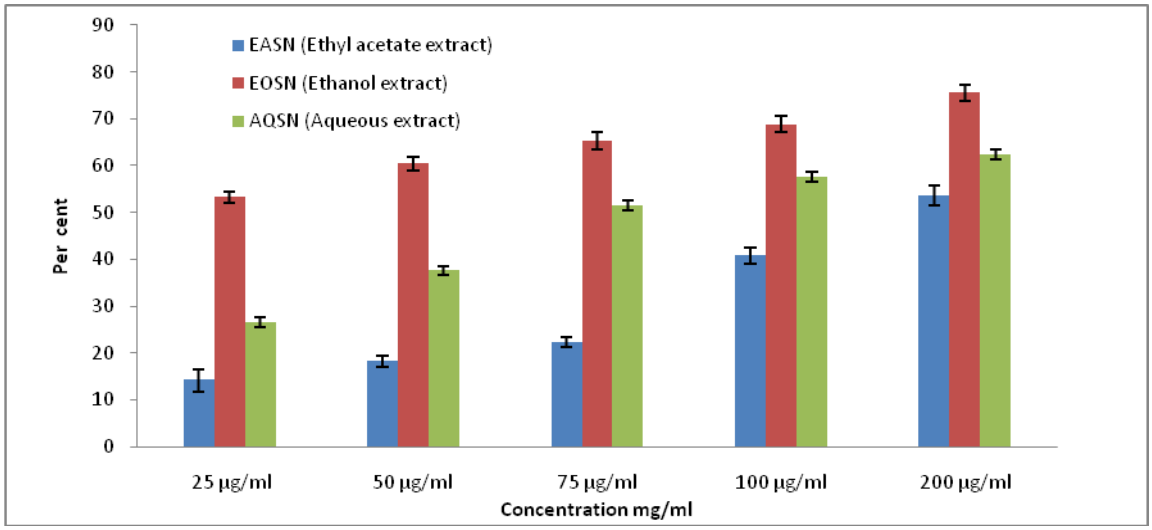

Fig. 3: Effects of *Syzygium nervosum* leaves extracts on Hydroxyl radical scavenging model.

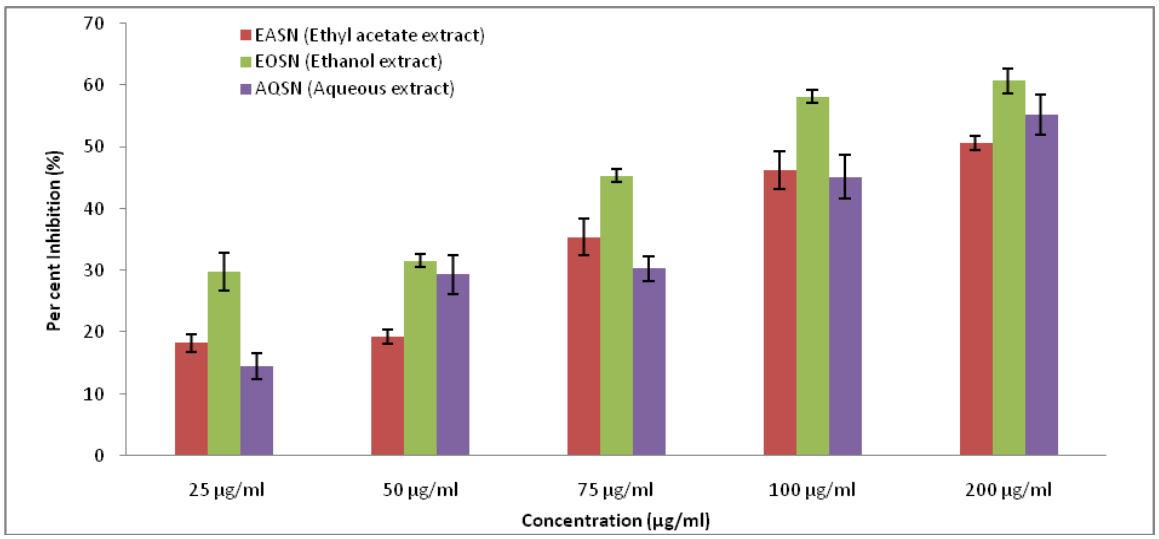

Fig. 4: Effects of *Syzygium nervosum* leaves extracts on Super oxide radical scavenging activity.

proteins. All the results showed hydroxyl radical scavenging activity in a dose dependent manner.

Syzygium nervosum ethanol extract at varied concentrations showed remarkable inhibitory effect of Hydroxyl radical scavenging activity compared to ethyl acetate and aqueous extract. Superoxide is a reactive oxygen species, which can cause damage to the cells and DNA leading to various diseases. The ethanol extract of *Syzygium nervosum* leaves at varied concentrations showed remarkable inhibitory effect of superoxide radical activity scavenging compared to ethyl acetate and aqueous extract. The ethanol extract of *Syzygium nervosum* leaves extract at varied concentrations showed remarkable inhibitory effect of superoxide radical scavenging activity compared to ethyl acetate and aqueous extract. The IC_{50} value for various free radical scavenging abilities of the *Syzygium nervosum* leaves extracts is depicted in Table 1 and Figures 1-4. Ethanol extract had the lowest IC_{50} for DPPH (27.28 mg/ml), which is significantly lower ($p < 0.05$) than other extracts and comparable to standards (ascorbic acid). Ethanol extract possessed the lowest IC_{50} (33.92 mg/ml) for hydroxyl radical scavenging ability. Aqueous extract also displayed the lowest IC_{50} value for the inhibition of superoxide anion radicals, which is significantly different from other extracts and standard (ascorbic acid).

Inhibitory effects of different extracts of

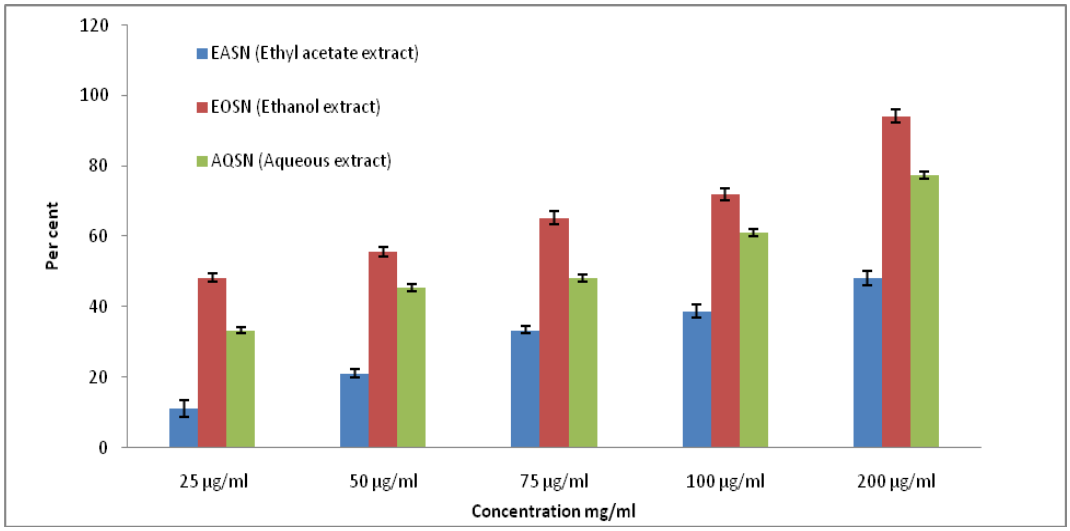

Fig. 5: *In vitro* alpha amylase inhibitory activity of *Syzygium nervosum* leaves extracts.

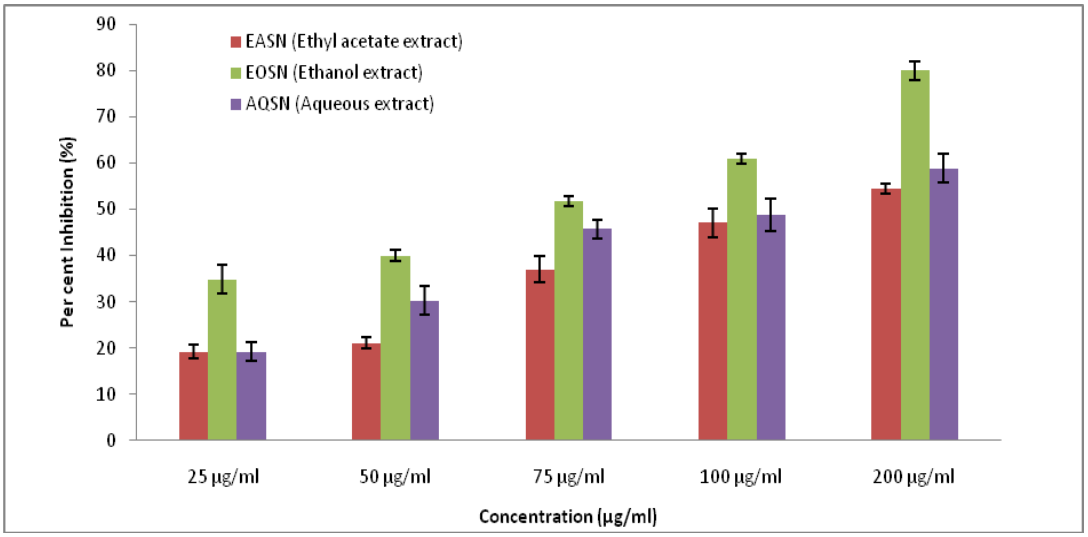

Fig. 6: *In vitro* Alpha Glucosidase inhibitory activity of *Syzygium nervosum* leaves extracts.

Syzygium nervosum leaves extract against α – amylase and α-glucosidase are presented in Figures 5 and 6. In α -amylase assay, there were significant differences ($p < 0.05$) in the percentage inhibition of the enzyme amongst all the extracts tested except at 25 and 50 µg/ml. At all concentrations tested, there were significant differences ($p < 0.05$) amongst all the extracts in the α-glucosidase assay and α-amylase. However, at higher concentrations, ethanol extract exhibited highest inhibition which is significantly different than ethyl acetate and aqueous extracts. Ethanol extract exhibited the lowest (48.71 µg/ml) IC_{50} for the inhibition of a-amylase and α-glucosidase (74.65 µg/ml) this is significantly comparable with ($p < 0.05$) the standard. The *in vitro* alpha amylase inhibitory studies demonstrate that the ethanol extract having good alpha amylase inhibitory activity. The percentage of inhibition at various concentrations of *Syzygium nervosum* leaves showed a concentration dependent reduction in the activity. The highest concentration (200 µg/ml) showed maximum inhibition of nearly 94.27%.

Discussion

Oxidative stress is a suggested mechanism for the induction and progression of diabetes and diabetic complications, which results from imbalance between radical generating and radical scavenging

system (Hassan *et al*., 2015). Hyperglycemia is strongly associated with increased superoxide generation via the mitochondrial system. Though it is a weak oxidant, it gives rise to the generation of powerful and dangerous hydroxyl radicals as well as singlet oxygen, both of which contribute to oxidative stress (Tiwari *et al*., 2013). The hydroxyl radical is regarded as a detrimental reactive oxygen species in pathophysiological processes and capable of damaging almost every biomolecule. It is also implicated in carcinogenesis, mutagenesis, cytotoxicity and pathogenesis of chronic disease. The antioxidant activity of the extracts *in vitro* was evaluated by DPPH, hydroxyl, nitric oxide, superoxide scavenging and reducing power assays. The result of this study revealed that the ethanolic extract of *Syzygium nervosum* possesses phytochemicals with high capacity to scavenge free radicals and reduce highly damaging oxidative intermediates. These components could be flavonoids and phenolics which have been shown to be highly antioxidative via their reducing power or electron/hydrogen donating/transfer potentials.

The antioxidant activities of plants may act by preventing the production of free radicals or by neutralizing/scavenging free radicals produced in the body or reducing the transition metal composition. The results showed that ethanol extract had the lowest IC_{50} for the DPPH, superoxide anion scavenging, hydroxyl radical and nitric oxide inhibition ability. This radical was also effectively scavenged by ethanol extract of *Syzygium nervosum* leaves compared to the other extracts and had similar results to the standard (ascorbic acid). The control of postprandial plasma glucose levels is important in the treatment of diabetes mellitus and its associated complications. Inhibition of enzymes involved in the metabolism of carbohydrates such as α-amylase and α-glucosidase, is an important therapeutic approach for reducing postprandial hyperglycemia. α - amylase found in the saliva and pancreatic juice, catalyses the hydrolysis of polysaccharides (such as starch) to disaccharides (like maltose and sucrose).

Though ethanol extract of *Syzygium nervosum* leaves displayed the more inhibition of α-amylase (IC_{50} 48.71 µg/ml) and comparable to standard. Enzyme α-glucosidases are located in the mucosal brush border of the small intestine and catalyse the conversion of disaccharides to monosaccharides like glucose. In this study, ethanol extract of *Syzygium nervosum* exhibited the strongest inhibition of α -glucosidase. This is depicted by the lowest IC_{50} value of ethanol extract (74.25 µg/ml) The antioxidant and anti-diabetic activities of different extracts of *Syzygium nervosum* may be due to the presence of various phytochemicals like alkaloids, tannins, saponins and glycosides. The findings of present study exhibited that the ethanolic extract of *Syzygium nervosum* leaves has high potential to improve hyperglycemic condition as well as the associated oxidative stress. The ethanol exhibited the highest activity. This study supports the traditional usage of *Syzygium nervosum* leaves in the management of diabetes. This provides scientific evidence for the ethno medicinal use of this plant and a baseline data for further work to explore the extract.

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