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Phytochemical Screening and Hypoglycemic Activity of Aqueous, Ethanolic and Methanolic Extracts of *Syzygium Cumini* and *Phyllanthus Amarus* in Streptozotocin Induced Diabetic Rats

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Abstract – Type II diabetes has become an epidemic in the 21st century where India leads the world with largest number of diabetic subjects. Traditional medicines are obtained from medicinal plants and are used by about 40-60% of the world's population. Though there are many approaches to control diabetes and its secondary complications, herbal formulations are preferred due to lesser side effects and low cost. Syzygium cumini and Phyllanthus amarus are medicinal plants used for the potential management of diabetes mellitus. The leaves are used in herbal medicinal preparation. The concerned study reveals the anti-diabetic potential of Syzygium cumini seeds and Phyllanthus amarus leaves in controlling blood glucose level in Streptozotocin induced Diabetic rats. Bioactive compounds like Mycaminose in Syzygium cumini seeds and bioactive compounds like lighans, and galloatnoids, may be responsible for the significant stimulation of β-cells of pancreas in the production of Insulin. In the current work we have studied the anti-diabetic effect of the aqueous, ethanolic and methanolic extracts of Syzygium cumini and Phyllanthus amarus on streptozotocin induced diabetic rats. The ethanolic and methanolic extracts of Syzygium cumini had significantly reduced the blood glucose levels than the aqueous extracts. Similarly the methanolic and aqueous extracts of Phyllanthus amarus, has significantly reduced the blood glucose levels than the ethanolic extract

Keywords- Hypoglycemic, Streptozotocin, Glibenclamide, Postprandial, Diabetes Milletus

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1. INTRODUCTION

Type 2 diabetes is a major public health issue in India, more likely to develop Diabetes at younger ages at lower body mass indices [21,28,35] India has the highest number of diabetes cases worldwide (40 million). Another 30 million Indians have pre-diabetes and are at high risk of developing T2DM.5 T2DM is an economically costly disease [5] and a major cause of mortality and morbidity. Indians with diabetes have worse glycemic control, [13, 27] a higher prevalence of micro-albuminuria, [9] hypertension, retinopathy, and cardiovascular disease, [39] and a higher incidence and faster progression of renal disease than most other diabetic populations. The prevalence of T2DM risk factors, such as insulin resistance, increased fat mass, and central obesity are high in South Asian populations.[31,32,33] T2DM prevention is a priority in Indian populations. Diabetes is a chronic disorder of carbohydrate, fat and protein metabolism characterized by increased fasting and postprandial blood sugar levels. The global prevalence of diabetes is estimated to increase by 5.4% by the year 2025. WHO has predicted that the major burden will occur in developing countries. Studies conducted in India in the last decade have highlighted that not only is the prevalence of diabetes high but also that it is increasing rapidly in the urban population [28]. It is estimated that there are approximately 33 million adults with diabetes in India. This number is likely to increase to 57.2 million by the year 2025. Diabetes mellitus is a complex metabolic disorder resulting from either insulin insufficiency or insulin dysfunction. Type I diabetes (insulin dependent) is caused due to insulin insufficiency because of lack of functional beta cells. Patients suffering from this are therefore totally dependent on exogenous source of insulin while patients suffering from Type II diabetes (insulin independent) are unable to respond to insulin and can be treated with dietary changes, exercise and medication. Type II diabetes is the more common form of diabetes constituting 70-80% of the diabetic population. Symptoms for both diabetic conditions may include: (i) high levels of sugar in the blood; (ii) unusual thirst; (iii) frequent urination; (iv) extreme hunger and loss of weight; (v) blurred vision; (vi) nausea and vomiting; (vii) extreme weakness and tiredness; (viii) irritability,

mood changes etc. Though patho physiology of diabetes remains to be fully understood, experimental evidences suggest the involvement of free radicals in the pathogenesis of diabetes [30] and more importantly in the development of diabetic complications [3, 26]. Free radicals are capable of damaging cellular molecules, DNA, proteins and lipids leading to altered cellular functions. Many recent studies reveal that antioxidants capable of neutralizing free radicals are effective in preventing experimentally induced diabetes in animal models [24, 29] as well as reducing the severity of diabetic complications [17]. For the development of diabetic complications, the abnormalities produced in lipids and proteins are the major etiologic factors. In diabetic patients, extra-cellular and long lived proteins, such as elastin, laminin, collagen are the major targets of free radicals. These proteins are modified to form glycolproteins due to hyperglycemia. The modification of these proteins present in tissues such as lens, vascular wall and basement membranes are associated with the development of complications of diabetes such as cataracts, micro-angiopathy, therosclerosis and nephropathy [7]. During diabetes, lipoproteins are oxidized by free radicals. There are also multiple abnormalities of lipoprotein metabolism in very low density lipoprotein (VLDL), low density lipoprotein (LDL), and high density lipoprotein (HDL) in diabetes. Lipid peroxidation is enhanced due to increased oxidative stress in diabetic condition. Apart from this, advanced glycation end products (AGEs) are formed by non- enzymatic glycosylation of proteins. AGEs tend to accumulate on long-lived molecules in tissues and generate abnormalities in cell and tissue functions [12,15]. Although several therapies are in use for treatment, there are certain limitations due to high cost and side effects such as development of hypoglycemia, weight gain, gastrointestinal disturbances, liver toxicity etc [37]. Based on recent advances and involvement of oxidative stress in complicating diabetes mellitus, efforts are on to find suitable antidiabetic and antioxidant therapy. Medicinal plants are being looked up once again for the treatment of diabetes. Many conventional drugs have been derived from prototypic molecules in medicinal plants. To date, over 400 traditional plant treatments for diabetes have been reported, although only a small number of these have received scientific and medical evaluation to assess their efficacy. The hypoglycemic effect of some herbal extracts has been confirmed in human and animal models of type 2 diabetes. The treatment of DM is based on oral hypoglycemic agents and insulin. However, DM is also treated in Indian traditional medicine using anti-diabetic medicinal plants [10, 36]. The oral hypoglycemic agents currently used in clinical practice have characteristic profiles of serious side effects [20]. Hence, there is a need to search for newer anti-diabetic agents that retain therapeutic efficacy and are devoid of side effects that could be important sources of such agents. The Syzygium *cumini* (or Eugenia jambolana) tree belongs to the Myrtaceae family, also called as Jamun, Jambul and Jambol in India. The barks, leaves and seeds extracts of Syzygium *cumini* have been reported to possess anti-inflammatory, antibacterial [4], and antidiarrheal effects [23]. *Phyllanthus amarus* is a monoecious, occasionally dioecious, upright or ascending herb, which grows up to 60 cm high, or occasionally higher, belonging to Phyllanthaceae family, locally known as “Kiru-Nelli”**.** All parts of the plants are used in Ayurvedic medicines because of their medicinal properties. Leaves of the plant are reported to contain lignin, alkaloids, flavonoids and glycosides [11]. *P. amarus* is an important plant of Indian Ayurvedic system of medicine used in the treatment of problems related to stomach, urinogenital tract, liver, kidney and spleen. The whole plant is used in gastropathy, diarrhoea, dysentery, intermittent fevers, ophthalmopathy, scabies, ulcers and wounds. *P. amarus* shows a wide spectrum of pharmacological activities including antiviral, antibacterial, antiplasmodial, anti-inflammatory, antimalarial, antimicrobial, anticancer, antidiabetic, hypolipidemic, antioxidant, and diuretic properties [14]. As the synthetic drugs used for the treatment of diabetes result in many complications. Hence the use of natural source *Syzygium cumini* and *Phyllanthus amarus* for the treatment of diabetes is safe and non-carcinogenic [6, 25]. Hence the present study was designed to evaluate the anti-diabetic activity of aqueous, ethanolic and methanolic extracts of the *Syzygium cumini* seeds and *Phyllanthus amarus* leaves against STZ-induced diabetic rats. The effect of the extracts of the *Syzygium cumini* seeds and *Phyllanthus amarus* leaf was compared to Glibenclamide, used as a standard drug against Diabetes Mellitus.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

All the chemicals procured for this work were of analytical grade, chloroform, ethanol, methanol, Hydrochloric acid (HCL), Sulphuric acid (H_2SO_4) and α- naphthol were procured from S.D. Fine Chem. Ltd, Mumbai, India.

2.2. Collection of plant materials:

Matured seeds of *Syzygium cumini* were collected from Ayanur Kote village and leaves of *Phyllanthus amarus* were collected from Hosahallli village in Shivamogga District, tentatively identified by Dr. T. Parameshwara Naik a renowned botanist in Shivamogga.

2.3. Preparation of plant extract:

The air-dried plant material, seeds of *Syzygium cumini* (50-100 g) and leaves of *Phyllanthus amarus* was washed thoroughly to remove the

pulp from seeds and dirt as well impurities and air dried under shade at room temperature and finely grinded with the help of mixer grinder. Extraction of the bioactive compounds was carried out in water, methanol and ethanol. Distilled water was added to the crushed material in a ratio of 1:4 (w/v) and kept at 30^0C for 24 hrs. The organic solvents were added in a ratio of 1:45 (w/v) shaken properly with the residue for 3hrs at their respective boiling temperatures. The extracts were filtered through whatman filter paper and stored at -20^0C for further use.

2.4. Phytochemical screening

The seed extracts of *Syzygium cumini* and leaves of *Phyllanthus amarus* were analyzed for the presence of alkaloids, glycosides, triterpenoids, steroids, saponins, flavonoids, tannins and carbohydrates according to standard methods [19].

2.4.1. Tests for alkaloid: Test solution was prepared by tritrating 40 to 50 mg extract with dilute acid (10 % acetic acid or 1 to 5 % hydrochloric acid). After filtration, 0.5 to 1 mL filtrate was added with 1 to 2 mL of following reagents.

Mayer's test

Mayer's reagent (Solution I: Dissolve 1.36 g $HgCl_2$ in 60 mL water; Solution II: Dissolve 5 g potassium iodide in 10 mL water. Combine these two solutions and add water up to 100 mL). Test solution (0.5 to 1 mL) was added with 1 to 2 mL of Mayer's reagent and development of white or buff colour precipitates indicates the presence of alkaloid.

2.4.2. Test for flavonoid

Test solution was prepared by dissolving 50 to 100 mg extract in 10 mL methanol/water.

Shinoda test: Test solution (1 to 2 mL) was added with a pinch of magnesium metal powder and a few drops of concentrated hydrochloric acid. Development of orange, pink, red to purple colours indicated the presence of flavones, flavonols or xanthones.

Sulfuric acid test: Test solution (1 to 2 mL) was added with few drops of concentrated sulfuric acid from the side wall of test tubes. Flavones and flavonols dissolve into concentrated H_2SO_4, producing a deep yellow coloured solution. Flavanones give orange to red colour.

2.4.3. Test for Triterpenoids: About 5 ml of extract was mixed in 2 ml of chloroform; 2 ml of acetic anhydride and a few drops of concentrated H2SO4 was added. Reddish violet colour indicated the presence of triterpenoids.

2.4.4. Test for Steroids: 10ml of chloroform was mixed with 2ml of extracts and conc. H2SO4 was added to form lower layer. A reddish yellow colour at the interface was an indicative of the presence of steroidal ring.

2.4.5. Test for saponin: Take 0.1 to 0.2 g of extract and add 10 mL distilled water, and shake vigorously. The appearance of froth that stabilizes for 10 to 15 minutes indicates the presence of saponin.

2.4.6. Test for glycoside

Salkowaski's test: To the crude extract (about 50 to 100 mg) taken add 2 mL of chloroform, shake well and then add 2 mL of concentrated H_2SO_4 along the side of the test tube. The development of reddish brown colour at the interface indicates the presence of sterol.

2.4.7. Test for carbohydrates

Molisch's Test: Molisch's reagent (Dissolve 1 g of α- naphthol in 10 mL of methanol or isopropyl alcohol). Test solution (1 to 2 mL) was mixed in a test tube containing 0.5 mL of water, and added with two drops of Molisch's reagent followed by 1 mL of concentrated sulphuric acid from the side of the inclined test tube. Appearance of red brown/violet ring at the interface of acid and aqueous solution indicates the presence of sugars.

2.4.8. Test for tannins

Ferric Chloride Test: Prepare 5 % solution of ferric chloride in 90 % methanol. Test solution (1 to 2 mL) was added with few drops of ferric chloride solution and development of dark green or deep blue colour indicates the presence of tannins.

2.5. Animal groups and experimental design:

Seventy five Wistar rats were taken initially for this study, which were weighing 150-180g, were kept in separate cages under standard temperature and fed with standard diet, and water. They were divided into nine groups, 1st and 2nd groups included normal (non-diabetic) and diabetic control rats (3 each) that received only distilled water that was free from dissolved salts and colloidal particles which could interfere with the results of the present research and standard diet throughout the trial. Diabetic rats were fed with *Syzygium cumini* and *Phyllanthus amarus* extracts dissolved at the levels of 100, 200, and 300 mg/kg in distilled water, ethanol, methanol and administered orally as a daily dose for 2 weeks. Out of 75 rats, 7 rats died before grouping and 5 rats were omitted from the study due to mild hyperglycemia (below 150 mg/dL). Remaining 63 rats were divided in to 9 groups, Group-I, Group-II and G-III of 3 Wistar rats

in each group and Group IV to IX had 9 Wistar rats in each group [8].

These groups are as follows:

GROUP-I: Normal (Rats injected with normal saline is considered as vehicle control).

GROUP-II: Diabetic control (was administered with 20% Glucose Soln. for 14 days)

GROUP-III: STZ induced diabetic Wistar rats were administered with standard drug (Glibenclamide) 5 mg/kg for 14 days, served as positive control.

GROUPS-IV to IX: STZ induced diabetic Wistar rats were administered with Aqueous, ethanolic and methanolic extracts of *Syzygium cumin* and *Phyllanthus amarus*, at the dose of 300 mg/kg rat body weight respectively.

On day 14 all animals were deprived of food, and water was given ad libitum. On day 15 blood glucose levels were measured using Glucometer (Accu-chek) by tail pinching method. Data was analyzed using one way ANOVA followed by suitable post-hoc test. All the values are reported as Mean ± SEM. Statistical significance was set at pH 0.05. Gloves, face mask was used in addition to protective clothing's and slippers to ensure adequate personal health and safety and to avoid inhalation and skin contact with the test items.

2.6. Acute toxicity studies

Following the guidelines of OECD (Organization for Economic Co-operation and Development), the acute oral toxicity [32], was performed on the adult Wistar rats. The rats were kept fasting for overnight providing only water, after which the extracts (aqueous, ethanol, and methanol) were administered orally at the dose level of 5 mg/kg. The animals were observed continuously for 3 hours for general behaviour, neurological and autonomic profiles and then every 30 minutes for next 3 hours and finally for mortality after 24 hours till 7 days. If mortality was observed in one animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher dose such as 100, 200, and 300 mg/kg body weight.

2.7. Experimental induction of diabetes

Diabetes was induced in the adult Wistar rats by injecting intra-peritoneally with a single dose of 50 mg/kg streptozotocin after dissolving it in freshly prepared ice-cold citrate buffer (0.1M, pH 4.5) after 18 hrs of fasting. After the injection, they had free access to feed and water and were given 20% glucose solution for 24 hrs to prevent initial drug induced hypoglycemic shock or mortality [26]. The normal control rats received only distilled water and standard diet. Development of diabetes mellitus in the rats were confirmed by testing fasting blood glucose level (FBGL) using (Accu-chek) Glucometer, after 48 hrs of STZ injection. The rats with FBSL higher than 200 mg/dL were considered diabetic and were selected for the study [2] [Table-1].

2.8. Drug Administration:

The group-I control rats received normal saline and fed on normal diet. The group-II was diabetic control. The group-III rats received a standard anti-diabetic drug Glibenclamide of 5 mg/kg orally dissolved in normal saline solution. The *Syzygium cumini* seeds and *Phyllanthus amarus* leaf extract at the dose of 100, 200, and 300 mg/kg body weight was given orally to the groups IV to IX respectively. Then blood glucose levels were estimated on every 1st, 7th, and 15th day.

3. RESULTS

3.1. Phytochemical Screening of *S. cumini* seeds and *Phyllanthus amarus* leaf extracts:

The phytochemical analysis of aqueous, methanolic, and ethanolic extracts of *S. cumini* seed powder revealed the presence of alkaloids, flavonoids, triterpenoids, steroids, saponins, glycosides, carbohydrates and tannins. Tannins were present in abundance in all the three extracts, saponins and flavonoids were moderately present except methanolic extract which showed abundance of flavonoids. Alkaloids, steroids and glycosides were present in all the three extracts used. Triterpenoids and carbohydrates were present only in the methanolic and ethanolic extract. The aqueous extracts did not show the presence of triterpenoids and carbohydrates. The phytochemical analysis of aqueous, methanolic, and ethanolic leaf extracts of *Phyllanthus amarus* powder revealed the presence of alkaloids, flavonoids, triterpenoids, steroids, saponins, glycosides, carbohydrates and tannins. Alkaloids were present in all, but abundantly in aqueous extract. Flavanoids were present in all but moderately in methanolic extracts. Triterpenoids were moderately present only in the aqueous and present in ethanolic extracts. Steroids were moderately present methanolic, and just present in other extracts. Saponins were present abundantly in aqueous, moderately in methanolic and just present in ethanolic extracts. Glycosides were moderately present in aqueous and methanolic extracts. Carbohydrates were present only in the aqueous and methanolic extracts. Tannins were moderately present in aqueous and methanolic extracts than ethanolic extracts (Table-1).

Table-1. Phyto-chemical screening of *Syzygium cumini* Seed and *Phyllanthus amarus* leaf extract

PLANT SPECIES	FAMILY	PART USED	EXTRACT	PHYTOCHEMICALS PRESENT							
				Alkaloids	Flavanoids	Triterpenoids	Steroids	Saponins	Glycoside	Carbohydrates	Tannins
Syzygium cumini	Myrtaceae	Seeds	Water	+	++	-	+	++	+	-	+++
			Methanol	+	+++	+	+	++	+	+	+++
			Ethanol	+	++	+	+	++	+	+	+++
Phyllanthus amarus	Phyllanthaceae	Leaves	Water	+++	+	++	+	+++	++	+	++
			Methanol	+	++	-	++	++	++	-	++
			Ethanol	+	+	+	+	+	+	+	+

'+++' = present in high quantity, '++' = present in moderate quantity, '+' = present, '-' = absent

3.2. Acute toxicity studies

This study showed no mortality up to the dose of 500 mg/kg body weight. So, the extracts were safe for long term administration.

3.3. Anti-diabetic activity

The blood glucose levels measured in normal and experimental rats in initial and at the 1st 7th and 15th days of treatment are given in Table-2. Streptozotocin-induced diabetic rats showed significant increase in the levels on blood glucose as compared to normal rats. Oral administration of aqueous, ethanolic and methanolic extracts (300 mg/kg) showed significant decrease ($p<0.05$) in blood glucose level. The ethanolic and methanolic extract of *Syzygium cumini* significantly showed reduction ($p<0.05$) in blood glucose level than the aqueous extract. Whereas the oral administration of methanolic and aqueous extracts (300 mg/kg) showed significant decrease ($p<0.05$) in blood glucose level than the ethanolic extract in *Phyllanthus amarus*. The standard drug, Glibenclamide decreased blood glucose level in 15 days treatment.

Table: 2. Effect of aqueous, ethanolic and methanolic extracts of *Syzygium cumini* and *Phyllanthus amarus* on blood glucose levels in Streptozotocin induced diabetic Wistar rats.

Group & Treatment	Dose (mg/kg rat b.wt.)	Blood Glucose (mg/dL)			
		Pre-treatment 0 Day	Pre-treatment 0 Day	Pre-treatment 0 Day	Pre-treatment 0 Day
G1 Vehicle Control (28% Glucose sols.)	10 mL/kg	109.50 ± 3.64	107.66 ± 2.44	103.55 ± 3.48	90.33 ± 2.66
G2 Diabetic Control (28% Glucose sols.)	10 mL/kg	338.66 ± 24.61*	342.66 ± 21.40*	353.60 ± 24.60*	367.5 ± 21.92*
G3 Glibenclamide	5	341.60 ± 19.97*	339.66 ± 16.17*	266.66 ± 18.17*	132.66 ± 6.38
G4 Aqueous Extract of *Syzygium cumini*	300	355.33 ± 19.15*	354.33 ± 17.38*	294.66 ± 27.18*	238.66 ± 20.00*
G5 Ethanolic Extract of *Syzygium cumini*	300	361.66 ± 15.57*	359.5 ± 14.37*	280.33 ± 12.34*	212.66 ± 21.26*
G6 Methanolic Extract of *Syzygium cumini*	300	371.66 ± 14.44*	373.33 ± 14.23*	289.83 ± 28.26*	217.55 ± 24.15*
G-7 Aqueous Extract of *Phyllanthus amarus*	300	365.30 ± 12.17*	362.18 ± 15.17*	277.40 ± 29.10*	190.22 ± 8.28*
G-8 Ethanolic Extract of *Phyllanthus amarus*	300	371.66 ± 14.56*	370.08 ± 12.84*	329.30 ± 12.34*	267.83 ± 19.28*
G-9 Methanolic Extract of *Phyllanthus amarus*	300	374.80 ± 8.64*	346.30 ± 19.22*	254.53 ± 28.36*	160.15 ± 8.45*

Values are expressed as mean ± SEM; n=9 *p= 0.05 statistically significant Vehicle control versus Diabetic control/ Treatment groups (G4-G9) #p 0.05= statistically significant Vehicle control versus Diabetic control/ Treated groups (G4-G9).

3.4. Discussion

The phytochemical analysis of aqueous, methanolic, and ethanolic extracts of *S. cumini* seed and *Phyllanthus amarus* leaf powder revealed the presence of alkaloids, flavonoids, triterpenoids, steroids, saponins, glycosides, carbohydrates and tannins. The aim of the present study was to evaluate the antidiabetic effect of aqueous, ethanolic and methanolic extracts of *Syzygium cumini* and *Phyllanthus amarus* against streptozotocin-induced diabetic rats (Figure-1). The continuous treatment of the extracts of *Syzygium cumini* and *Phyllanthus amarus* for a period of 15 days produced a significant decrease in the blood glucose levels of diabetic rats. The ethanolic and methanolic extracts of *S. cumini* seeds showed significant ($P<0.05$) reduction in blood glucose levels than the aqueous extracts. Similarly the methanolic and aqueous extract in *Phyllanthus amarus* showed a significant reduction ($P<0.05$) in blood glucose levels of diabetic rats. The possible mechanism by which the seeds of *Syzygium cumini* and leaves of *Phyllanthus amarus* brings about a decrease in blood glucose level may be by potentiation of the insulin effect of plasma by increasing the pancreatic secretion of insulin from β-cells of the islets of Langerhans. A number of other plants have been reported to exert hypoglycemic activity through insulin release-stimulatory effects [18]. The anti-Diabetic activity of *Phyllanthus amarus* may be due to the presence of bioactive compounds like lighans, alkaloids, flavonoids, galloatnoids and glycosides. Similarly the anti-diabetic activity of *Syzygium cumini* seeds may be due to the bioactive compound mycaminose. This was almost similar to results obtained with reference drug glibenclamide (341.60- 132.66 mg/dL). Previous studies have showed that lighans, alkaloids, flavonoids, galloatnoids, glycosides and Mycaminose was able to regulate blood glucose levels by enhancing not only insulin secretion and sensitivity but also insulin utilization in insulin deficient rats [16]. According to studies carried out, *Phyllanthus amarus* extract may contain some biomolecules that may sensitize the insulin receptor to insulin or stimulates the β-cells of islets of langerhans to release insulin which may finally lead to improvement of carbohydrate metabolizing enzymes towards the reestablishment of normal blood glucose level [22]. The standard drug, Glibenclamide has been used for many years to treat diabetes, to stimulate insulin secretion from pancreatic β-cells [38]. But, the *Syzygium cumini* seeds collected from Ayanur village and *Phyllanthus amarus* leaves, which was collected from Hosahalli village in Shivamogga district of Karnataka state, India have anti-diabetic properties. These results confirmed the use of *Phyllanthus amarus* leaves and seeds of *Syzygium cumini* in the Indian traditional practice as an anti-diabetic agent [1]. Further comprehensive chemical

and pharmacological investigations are needed to elucidate the exact mechanism of the hypoglycemic effect of seeds of *Syzygium cumini* and leaves of *Phyllanthus amarus*.

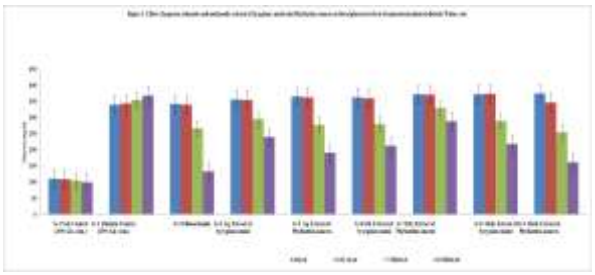

Figure-1. Effect of aqueous, ethanolic and methanolic extracts of *Syzygium cumini* and *Phyllanthus amarus* on blood glucose levels in Streptozotocin induced diabetic Wistar rats.

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