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ROLE OF THE INCRETINS IN HYPOGLYCEMIC EFFECT OF *PHLOGACANTHUS THYRSIFLORUS* NEES IN CHEMICALLY INDUCED DIABETIC MICE

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Keywords:

Phlogacanthus thyrsiflorus Nees, Glucagon like Peptide-1 (GLP-1), Gastric Inhibitory Polypeptide (GIP), High fat Diet, Streptozotocin, Diabetes.

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ABSTRACT: Objective: This study was aimed to evaluate the role of GLP-1, GIP in the hypoglycemic activity of *Phlogacanthus thyrsiflorus* Nees. **Materials and Methods**: We have evaluated the influence of *Phlogacanthus thyrsiflorus* Nees, a traditionally used antidiabetic medicinal plant on some metabolic parameters, glucose homeostasis, insulin production, glucagon like peptide 1 (GLP1) and gastric Inhibitory Polypeptide (GIP) in high fat diet fed (HFD) obese mice and High fat diet + streptozotocin (HFD + STZ) induced diabetic mice. Oral glucose tolerance test (OGTT) was done to study the glucose homeostasis in both HFD fed obese mice and HFD + STZ induced diabetic mice. Effect of *P. thyrsiflorus* on pancreatic beta cells was assessed using histological studies. **Results**: *Phlogacanthus thyrsiflorus* aqueous extract (PTAE) treatment lowered the blood glucose levels in HFD fed obese mice and HFD + STZ induced diabetic mice significantly. PTAE treatment reduced the weight gain in HFD fed obese mice and prevented the constant weight loss in HFD+STZ induced diabetic mice. PTAE treatment decreased the food intake significantly in HFD fed obese mice. PTAE treatment improved the glucose tolerance and the lipid profile in HFD fed obese mice. PTAE treatment decreased the insulin level in HFD fed obese mice and increased the insulin level in HFD + STZ induced diabetic mice. PTAE treatment increased the GLP –1 level and decreased the GIP level significantly in both HFD fed obese mice and HFD + STZ induced diabetic mice. Histological studies of pancreas showed that *P.thyrsiflorus* helps normalizing the pancreatic histoarchitecture. **Conclusion:** This study states that the hyperglycaemic activity of the *P.thyrsiflorus* is due to the modulation of the entero-insular axis in the Hypoglycemic mice. However the study also revealed the doubtful role of GIP as incretins and its role in diabetes treatment.

INTRODUCTION: In response to food ingestion, enteroendocrine cells in the intestinal mucosa release hormones that can stimulate insulin secretion from endocrine pancreas, and thereby reduce blood glucose [1, 2]. This is known as incretin effect and two such hormones, that is Glucagon like peptide 1 (GLP-1) and Gastric Inhibitory Polypeptide (GIP), have been identified as the incretins [1, 2].

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GLP-1 and GIP are rapidly inactivated to GLP-1 (9-37) or GLP-1 (9-36) NH_2 and GIP (3-42) by dipeptidyl peptidase 4 (DPP-4), a ubiquitous proteolytic enzyme. As a result more than half of the GLP-1 that enters the portal circulation is inactivated by DPP-4 before entry into the systemic circulation [2].

The insulin secretory response to incretins accounts for at least 50% of the total insulin secreted after oral glucose. Despite that lacking secretion or faster clearance of incretin are not pathogenic factors in diabetes, GLP-1 has become a molecular target for therapeutics of type 2 diabetes mellitus since its insulinotrotic activity is still active in these patients, but not GIP [1, 2]. Two such strategies have

already been in clinical practice to treat type 2 diabetes, namely GLP-1 analogs and DPP IV inhibitors that degrades both GLP-1 and GIP [1].

The GLP-1 biology has been extensively reviewed [1-4]. This endocrine hormone is secreted from enteroendocrine L cells, found in high density in distal ileum but also throughout the small and large intestine, is stimulated by glucose, amino acids and fats. Its main physiological roles include: (1) the stimulation of glucose dependent insulin secretion from pancreatic β cells (2) stimulation of insulin biosynthesis and insulin sensitivity (3) enhancement of pancreatic β cell proliferation and protection against apoptosis (4) inhibition of glucagon secretion and gastric emptying (5) inhibition of food intake [1-4]. GIP in contrast is produced by the K cells in the proximal duodenum; its secretion is also stimulated by glucose, but is particularly enhanced by fats [5]. GIP upregulates beta cell insulin gene transcription and biosynthesis, as well as the expression of components of beta cell glucose sensor [6]. The physiologic importance of GIP as incretin hormone is illustrated by disruption of GIP action *in vivo*. Elimination of GIPR signaling using GIPR antagonist, receptor specific antisera or by targeted inactivation of murine GIPR gene (GIPR -/-) is associated with impaired oral glucose tolerance and defective glucose stimulated insulin secretion in rodents [7, 8].

There are many publications on the secretion of GIP in type 2 diabetes, both increased, normal and decreased secretion was reported [9]. GIP receptor agonists also potentiate glucose stimulated insulin secretion but unlike GLP-1, GIP does not appear to have significant effects on glucagon secretion, gastric emptying or body weight [2]. Unexpectedly, either GIP receptor activation or ablation of GIP receptor signaling exerts beneficial metabolic action in preclinical models of experimental diabetes [10, 11]. Hence there is considerable ongoing debate as to the extent to which modulation of GIP receptor signaling will be useful therapeutic option in type 2 diabetes.

Phlogacanthus thyrsiflorus Nees is found in the sub tropical Himalayas, upper Gangetic plain, Bihar, North Bengal and Assam [12]. *Phlogacanthus thyrsiflorus* Nees is a medicinal herb which belongs to Acanthaceae family. Whole plant is used in Whooping cough and Menorrhagia. Fruits and leaves are burnt and it is prescribed for fever. The leaves are reported to contain diterpene lactone, Phlogantholide A. A decoction of leaves is also beneficial in liver and spleen diseases [12]. Jaintia tribe of Meghalaya uses fruit and leaf ash of *Phlogacanthus thyrsiflorus* Nees and use it to treat fever [13]. Ethanolic extract of *Phlogacanthus thyrsiflorus* Nees has analgesic activity on experimental mice [14]. *Phlogacanthus thyrsiflorus* Nees has antimicrobial activity also [15].

The generation of free radicals has been implicated in the causation of several diseases of known and unknown etiologies such as Rheumatoid Arthritis, Cancer etc., and compounds that can scavenge free radicals have great potential in ameliorating these disease processes. *Phlogacanthus thyrsiflora* Nees has prominent free radical scavenging property so it may prove as a very good medicinal herb[16]. We previously reported the antihyperglycemic activity of *Phlogacanthus thyrsiflorus* Nees [17].

Methodology:

Plant material:

The flowers of *Phlogacanthus thyrsiflorus* Nees were collected from local market in April 2011 and herbarium was prepared. The herbarium was identified for authenticity by the experts of Dept of Botany, Gauhati University, Assam and voucher specimen bearing accession number 177797 was deposited for future reference. The flowers were thoroughly washed and shade dried.

Preparation of Plant extract:

After shade drying the dried flowers were powdered in mixture grinder. The powdered flower was macerated with distilled water for 72 hrs at room temperature with occasional stirring. It was then filtered through Whatman filter paper. The filtrate was air dried and stored in refrigerator for further use as PTAE (*Phlogacanthus thyrsiflorus* aqueous extract). The yield of the extract was 10% (w/w). During experiment the crude extract was diluted with distilled water just before administration to animals.

Chemicals:
Streptozotocin, Sitagliptin and Glibenclamide was purchased from Sigma Chemical Co, St Louis, MO, USA. All other chemicals and reagents used were of analytical grade. All the diagnostic kits were purchased from Crest Biosystems. Mouse Insulin, GIP, GLP-1 ELISA Kits were purchased from Zenith India.

Experimental Animals:
Healthy adult albino mice of both sexes (20-25 g) in-housebred at the Animal house of Gauhati University, Assam, India were used for the study. Mice were housed in polypropylene cages lined with husk in standard environmental conditions and 12:12 hr light: dark cycle. The animals were fed on a standard pellet diet *ad libitum* and had free access to water. The experiments were performed after approval of the protocol by the Institutional Animal Ethics Committee (IAEC) (Registration number: 902/ac/05/CPCSEA, Reference number: IAEC/PER/2012-13/193) and were carried out in accordance with the current guidelines for the care of laboratory animals.

Effect of PTAE on Insulin and GLP-1, GIP levels and lipid profile in HFD fed obese mice:
The amount of fat needed in high fat diet must be in the range of 30 – 60% of the total diet because this amount allows the changes of body weight and endocrine secretion [18]. C3H Mice were *ad libitum* fed either with a basal diet or a high fat diet or a high fat diet with 100 mg/kg PTAE for 5 weeks. The composition of the high fat diet (HFD) and normal diet is given in **Table 1**. The animals were divided into four groups with 4 animals in each:

Group I: Control animals fed with normal diet

Group II: The animals were fed normal diet and were treated with 100mg/kg PTAE

Group III: The animals were fed with high fat diet (HFD)

Group IV: The animals were fed with HFD and were treated with 100mg/kg PTAE

Treatment was continued for 5 weeks. Food, water intake, fasting and post prandial blood glucose and body weight was measured weekly. New portions of food were provided to the mice every day and the leftover food was discarded. Food intake was monitored by weighing the leftover food in each cage and was measured on a cage by cage basis. Oral glucose tolerance test (OGTT) was done after 5 weeks. Two days before sacrifice, the oral glucose tolerance test was performed for the evaluation of blood glucose.

After overnight fasting, the plasma glucose concentration was measured at 0,30,60,90,120 minutes after oral glucose loading (2g/kg b.w). Area under curve (AUC) was calculated for OGTT by trapezoidal rule. On the day of sacrifice, PTAE was orally fed to the respective treated group 30 min before glucose administration, glucose at the dose of 1g/kg was then fed orally [19, 20]. Then the animals were sacrificed.

TABLE 1: COMPOSITION OF HIGH FAT DIET (HFD) AND NORMAL DIET

High fat Diet (HFD)		Normal diet	
Ingredients	**Quantity (%)**	**Ingredients**	**Quantity (%)**
Wheat Bran	18	Wheat Bran	25
Maize Bran	23	Maize Bran	35
Oil Cake	19	Oil Cake	30
Butter	30	Vitamin	5
Vitamin	5	Salt	5
Salt	5		

Collection of blood samples from animals:
During sacrifice blood was collected by cardiac puncture. Then whole blood was collected in an eppendorf containing EDTA and with or without DPP4 inhibitor. Then blood samples was centrifuged in 2000 RPM for estimation of GIP and GLP-1 (with DPP4 inhibitor), Cholesterol, Triglycerides, HDL, Insulin (without DPP4 Inhibitor) [21-23].

Plasma Cholesterol was estimated spectrophotometrically (CHOP-PAP method, Crest Biosystems). Triglyceride was estimated using diagnostic kit (GPO-PAP method, Crest Biosystems), HDL Cholesterol was also estimated using diagnostic kit (PEG ppt method, Crest Biosystems). The VLDL cholesterol was calculated using the formula (TG/5). The Plasma LDL cholesterol was calculated by the method of Friedwald et al. (1972) [24].

Effect of PTAE on Insulin and GLP-1, GIP levels in HFD+STZ induced diabetic mice:

Induction of diabetes:

Experimental diabetes was induced by feeding all the mice high fat diet for 4 weeks and then injecting three intraperitoneal injection of 35mg/kg of Streptozotocin (STZ) freshly dissolved in distilled water in three consecutive days [25, 26]. Control animals received only distilled water. After 48 hrs of Streptozotocin injection animals with fasting blood glucose above 200mg/dl were considered as diabetic and included in the study.

Experimental Animals Grouping:

Animals were divided into 5 groups with 4 animals in each group.

1. **Control group:** This group received only distilled water
2. **Diabetic group:** This group received only distilled water
3. Low dose plant extract treated diabetic group: These were diabetic animals and were treated with 100 mg/kg PTAE
4. High dose plant extract treated diabetic group: These were diabetic animals and were treated with 200 mg/kg PTAE
5. Standard drug treated diabetic group: These were diabetic animals and were treated with glibenclamide (10 mg/kg).

These treatments were continued for 4 weeks. Blood glucose and body weight was monitored weekly. Two days before sacrifice, the oral glucose tolerance test was performed. After overnight fasting, the plasma glucose concentration was measured at 0,30,60,90,120 minutes after oral glucose loading (2g/kg body weight). Area under curve was calculated for OGTT by trapezoidal rule. PTAE was fed 30 min before glucose administration; glucose at the dose of 1g/kg was fed orally. Then the animals were sacrificed.

Collection of blood samples from animals:

Blood was collected by cardiac puncture. Whole blood was collected in an eppendorf containing EDTA and with or without DPP4 inhibitor. Then blood samples were centrifuged in 2000 RPM for estimation of GIP and GLP-1 (with DPP4 Inhibitor) and insulin (without DPP4 Inhibitor).

Histology of pancreas:

Pancreas of all animals were collected and preserved in Bouin's fluid for histological studies. Chrome hematoxylene and Phloxine B was used for staining the pancreatic sections.

Statistical analysis

All results were expressed as Mean ± SEM. The significance of the difference between the means of test and control studies was established by Student's t-test and the data were expressed as mean ± SEM. P value less than 0.01, 0.001, 0.0001 were considered to be statistically significant.

RESULTS:

Effect of PTAE on blood glucose and body weight in HFD fed obese mice and HFD + STZ diabetic mice:

No significant change in fasting and post prandial blood glucose level was observed in normal mice but in HFD fed obese mice the fasting and post prandial blood glucose increased significantly upto 182.5 mg/dl and 252.5 mg/dl respectively when compared with normal control ($P < 0.0001$) but in the HFD fed mice treated with 100mg/kg PTAE the fasting and post prandial blood glucose level lowered significantly when compared to obese control ($P < 0.0001$) which is shown in **Table 2.** Normal diet fed mice when treated with 100 mg/kg PTAE also decreased both the fasting and post prandial blood glucose when compared to normal control ($P < 0.01$) ($P < 0.001$).

On the other hand, HFD+ STZ diabetic mice had fasting and post prandial blood glucose upto 202 mg/dl and 267 mg/dl respectively in the 4th week of the study which was significantly higher when compared to normal control ($P < 0.0001$) which is shown in **Table 3**. PTAE in both the doses (100mg/kg and 200 mg/kg) reduced the fasting and post prandial blood glucose levels significantly in HFD+STZ induced diabetic mice when compared to diabetic control ($P < 0.0001$) ($P < 0.001$). The body weight of control mice had a slight increase after the experiment onset but the body weight of

HFD fed obese mice increased significantly up to 31 g on the 5th week of the study when compared to normal control (P < 0.0001).

Furthermore the PTAE treatment maintained the body weight in HFD fed animals as the body weight was significantly lower than obese control throughout the period of the study (P < 0.0001) which is shown in Table 2. In the second set of animals, in HFD + STZ diabetic animals, all the animals began to lose weight after STZ injection. The weight loss was maximum in HFD+STZ diabetic control. The mean body weight of HFD+STZ mice was lower than control mice (P < 0.0001). But PTAE in both the doses 100mg/kg and 200 mg/kg controlled the rapid loss of body weight in HFD+STZ mice when compared to diabetic control (P < 0.001) shown in **Table 3.** The result was comparable to standard drug treatment.

Effect of PTAE on food and water intake in HFD fed mice:

There were only slight change in food consumption and water intake in control mice during the whole experimental period. The food consumption increased significantly in the obese control and reached the maximum on the 5th week ie it was 11.6 g/animal in average (P < 0.0001). But in the HFD fed mice treated with100mg/kg PTAE the food consumption remained almost constant throughout the period of study which was significantly lower (P < 0.0001), when compared to obese control which is shown in **Table 2**. In the obese control water intake decreased significantly to 1.65 ml/ animal in average in the 5th week when compared to normal control (P < 0.0001). But in the HFD fed mice treated with 100mg/kg PTAE the water intake remained almost constant throughout the study which was significantly higher than the obese control (P < 0.0001) which is shown in **Table 2**. The food and water intake of normal diet fed mice treated with 100 mg/kg PTAE was almost similar to the normal control.

TABLE 2: EFFECT OF PTAE ON METABOLIC PARAMETERS AND PLASMA BLOOD GLUCOSE LEVELS IN HIGH FAT DIET FED AND NORMAL DIET FED MICE

Parameters	Groups			
	Control	HFD fed obese control	Con + PTAE	HFD + PTAE
Body Weight (g)	25±1.0	31±1.35$^{\neq}$	25±1.15	24±1.08*
Fasting Blood Glucose (mg/dl)	89.5±0.5	182.5±0.5$^{\neq}$	86.5±0.5$^{\#\#}$	125.5±0.5*
Post Prandial Blood glucose (mg/dl)	136.3 ± 0.88	252.5± 2.50$^{\neq}$	122.5± 1.50$^{\#\#}$	154 ± 2.31*
Food intake (g/animal)	6.25±0.05	11.6±0.1$^{\neq}$	6.75±0.05$^{\#\#}$	7.05±0.05*
Water intake (ml/animal)	5.05±0.05	1.65±0.05$^{\neq}$	6.1±0.058$^{\#\#}$	6.25±0.05*

The table represents the metabolic parameters and plasma blood glucose levels of the animals from the four groups; Control , High fat diet fed group (HFD), Control treated with *Phlogacanthus thyrsiflorus* aqueous extract PTAE (Con + PTAE), High fat fed mice treated with *Phlogacanthus thyrsiflorus* aqueous extract PTAE (HFD + PTAE). Values represent Mean ± standard deviation (n=4). Significant difference between control and HFD fed obese control: ≠P < 0.0001, Significant difference between control and Con + PTAE group: ##P< 0.01, ### P < 0.001 Significant difference between HFD fed obese control and HFD + PTAE group: *P< 0.0001.

TABLE 3: EFFECT OF PTAE ON BODY WEIGHT AND BLOOD GLUCOSE LEVEL IN HFD + STZ TREATED DIABETIC MICE

Parameters	Groups				
	Control	HFD + STZ induced diabetic control	HFD + STZ + PTAE (100 mg/kg)	HFD + STZ+ PTAE(200 mg/kg)	HFD + STZ+Glibenclamide
Body Weight (g)	26.2±0.88	21.5 ± 0.50$^{\neq}$	26 ± 1.0$^{\$}$	23 ± 1.0$^{\$}$	24 ± 1.0$^{\$}$
Fasting Blood Glucose (mg/dl)	95 ± 1.0	202 ± 1.0$^{\neq}$	132 ± 2.0*	115.5 ± 0.5*	112.5 ± 2.50*
Post Prandial Blood glucose (mg/dl)	137 ± 1.0	267 ± 1.0$^{\neq}$	164 ± 1.00$^{\$}$	144 ± 1.0$^{\$}$	142 ± 2.0$^{\$}$

The table represents the body weight and plasma blood glucose levels of the animals from the five groups: High fat diet fed + Streptozotocin induced diabetic group (HFD + STZ), High fat diet fed + Streptozotocin induced diabetic group treated with *Phlogacanthus thyrsiflorus* aqueous extract 100 mg /kg PTAE (HFD + STZ+ PTAE), High fat diet fed + Streptozotocin induce diabetic group treated with *Phlogacanthus thyrsiflorus* aqueous extract 200 mg /kg PTAE (HFD + STZ+ PTAE), High fat diet fed + Streptozotocin treated with standard drug glibenclamide 10 mg/kg (HFD + STZ + Std drug. Values represent Mean ± standard deviation (n=4). Significant difference between control and HFD + STZ diabetic control: ≠P < 0.0001. Significant difference between diabetic control and HFD + STZ + PTAE group *P < 0.0001, $^{\$}$P < 0.001.

Effect of PTAE on Oral Glucose Tolerance in HFD fed obese mice and HFD+STZ induced diabetic mice

OGTT can be used to study the blood glucose homeostasis in normal as well as diabetic mice. OGTT was done to evaluate the blood glucose homeostasis in both HFD fed obese mice and HFD + STZ induced diabetic mice which is shown in **Table 4** and **5**. Total area under curve (AUC) was calculated for all groups which are shown in **Fig.1** and **2.** HFD fed obese mice and HFD + STZ induced diabetic mice showed impaired glucose tolerance i.e significantly higher blood glucose at all time points as well as greater AUC when compared with normal control (P< 0.0001).

The blood glucose level was highest in 30 minutes after the glucose load in obese control ie it was 200.5 mg/dl which was significantly higher when compared to normal control (P < 0.0001) and PTAE treated HFD fed mice which showed the blood glucose level of 161 mg/dl in 30 minutes which was significantly lower when compared to obese control (P < 0.0001). HFD fed mice cleared the glucose in blood less efficiently when compared to normal control and PTAE treated HFD fed mice. PTAE treated HFD fed mice showed significantly lower blood glucose at all time points and lower AUC when compared with obese control (P < 0.0001). PTAE treated normal mice also showed lower glucose levels at all time points and lower AUC when compared to normal control (P < 0.001). In HFD + STZ diabetic mice postprandial blood glucose level showed a significant change after glucose loading, increasing in all groups of diabetic mice within 30 minutes and remaining high for over the next 120 minutes in diabetic control mice.

In diabetic control the blood glucose was 282.3 mg/dl after 30 minutes of the glucose load which was significantly higher than the normal control (P < 0.0001). But PTAE in both the doses ie 100 mg/kg and 200 mg/kg and glibenclamide reduced the blood glucose level signifiantly at all time points when compared to diabetic control (P < 0.0001), the blood glucose levels normalized after 120 minutes. AUC of PTAE treated HFD+STZ mice was significantly lower when compared to diabetic control (P < 0.0001) which is shown in Fig 2. PTAE treatment improved oral glucose tolerance in HFD fed obese mice and HFD + STZ induced diabetic mice.

TABLE 4: EFFECT OF PTAE ON ORAL GLUCOSE TOLERANCE TEST OF NORMAL DIET FED MICE AND HIGH FAT FED MICE

Animal group	0 min (mg/dl)	30 min (mg/dl)	60 min (mg/dl)	90 min (mg/dl)	120 min (mg/dl)
Normal control	91±0.58	150.5±0.5	139±0.58	129.5±0.5	121±0.58
High fat diet fed group	151±0.58$^{\neq}$	200.5±0.5$^{\neq}$	189±0.58$^{\neq}$	161±1.0$^{\neq}$	151±0.58$^{\neq}$
Normal+100mg/kg PTAE	91±0.58	134±0.58$^{\neq\neq}$	127.5±0.5$^{\neq\neq}$	114±0.58$^{\neq\neq}$	100.5±0.5$^{\neq\neq}$
HFD+100mg/kg PTAE	120±1.15*	161±1.0*	152.3±1.45*	141±1.0*	131.3±0.88*

The table represents the Oral Glucose Tolerance Test and the figure represents the calculation of Area under Curve of OGTT of the animals from the four groups; Control; High fat diet fed group (HFD), Control treated with *Phlogacanthus thyrsiflorus* aqueous extract PTAE (Con + PTAE), High fat fed mice treated with *Phlogacanthus thyrsiflorus* aqueous extract PTAE (HFD + PTAE). Values represent Mean ± standard deviation (n=4). Significant difference between control and HFD fed obese control: ≠P < 0.0001. Significant difference between control and Con + PTAE group:

≠≠P < 0.001 Significant difference between HFD fed obese control and HFD + PTAE group: *P< 0.0001

TABLE 5: EFFECT OF PTAE ON ORAL GLUCOSE TOLERANCE TEST IN HFD + STZ DIABETIC MICE

Animal group	0 min (mg/dl)	30 min (mg/dl)	60 min (mg/dl)	90 min (mg/dl)	120 min (mg/dl)
Normal control	93.6 ± 0.88	151.5 ± 1.5	132 ± 1.15	112 ± 2.0	96.3 ± 0.88
HFD + STZ diabetic control	201.5 ± 1.5$^{\neq}$	282.3 ± 1.2$^{\neq}$	262 ± 2.0$^{\neq}$	236 ± 0.58$^{\neq}$	208 ± 2.0$^{\neq}$
HFD+ STZ + PTAE100 mg/kg	133 ± 1.53*	222 ± 2.0*	205.6± 0.67*	183.5 ± 3.5*	137 ± 1.15*
HFD+STZ+200 mg/kgPTAE	125.5 ± 0.50*	202 ± 1.15*	182 ± 2.0*	163 ± 0.58*	131 ± 1.0*
HFD+STZ+10 mg/kg glibenclamide	134 ± 1.15*	203.5 ± 1.5*	175.6 ± 1.2*	156.3 ± 0.8*	136 ± 2.31*

The table represents the Oral Glucose Tolerance Test and the figure represents the calculation of Area under Curve of OGTT of the animals from the five groups: Control, High fat diet fed + Streptozotocin induced diabetic control (HFD + STZ), High fat diet fed + Streptozotocin induced diabetic group treated with *Phlogacanthus thyrsiflorus* aqueous extract 100 mg /kg PTAE (HFD + STZ+ PTAE), High fat diet fed + Streptozotocin induce diabetic group treated with *Phlogacanthus thyrsiflorus* aqueous extract 200 mg /kg PTAE (HFD + STZ+ PTAE), High fat diet fed + Streptozotocin treated with standard drug glibenclamide 10 mg/kg (HFD + STZ + Std drug. Values represent Mean ± standard deviation (n=4). Significant difference between control and HFD + STZ diabetic control: ≠P < 0.0001. Significant difference between diabetic control and HFD + STZ + PTAE group *P < 0.0001.

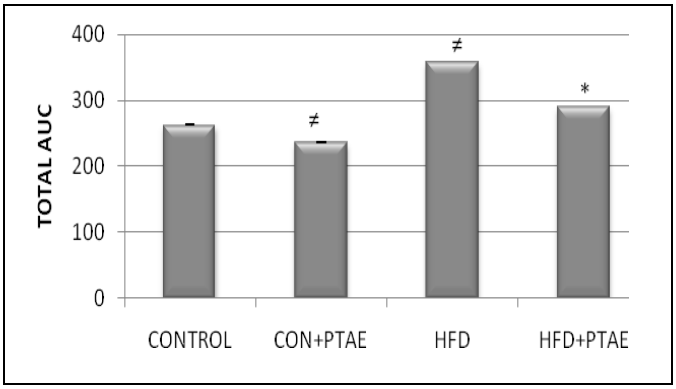


FIG.1: CALCULATION OF TOTAL AREA UNDER CURVE OF OGTT OF HFD FED OBESE MICE

The figure represents the calculation of Area under Curve of OGTT of the animals from the four groups; Control; High fat diet fed group (HFD), Control treated with *Phlogacanthus thyrsiflorus* aqueous extract PTAE (Con + PTAE), High fat fed mice treated with *Phlogacanthus thyrsiflorus* aqueous extract PTAE (HFD + PTAE). Values represent Mean ± standard deviation (n=4). Significant difference between control and HFD fed obese control: ≠P < 0.0001. Significant difference between control and Con + PTAE group; Significant difference between obese control and HFD + PTAE group: *P< 0.0001.

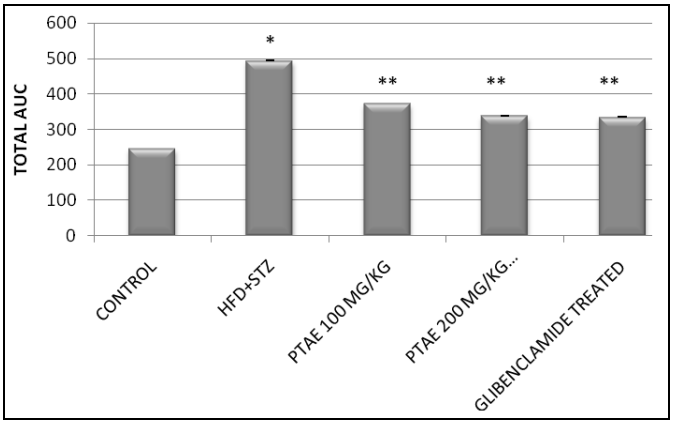


FIG 2: CALCULATION OF TOTAL AREA UNDER CURVE OF OGTT OF HFD+STZ DIABETIC MICE

The figure represents the calculation of Area under Curve of OGTT of the animals from the five groups: Control, High fat diet fed + Streptozotocin induced diabetic control (HFD + STZ), High fat diet fed + Streptozotocin induced diabetic group treated with *Phlogacanthus thyrsiflorus* aqueous extract 100 mg /kg PTAE (HFD + STZ+ PTAE), High fat diet fed + Streptozotocin induce diabetic group treated with *Phlogacanthus thyrsiflorus* aqueous extract 200 mg /kg PTAE (HFD + STZ+ PTAE), High fat diet fed + Streptozotocin treated with standard drug glibenclamide 10 mg/kg (HFD + STZ + Std drug. Values represent Mean ± standard deviation (n=4). Significant difference between control and HFD + STZ diabetic control: *P < 0.0001. Significant difference between diabetic control and HFD + STZ + PTAE group **P < 0.0001.

Effect of PTAE on lipid profiles of high fat fed mice: As shown in **Table 6** the Plasma Cholesterol, Triglycerides, Low Density Lipoprotein (LDL) and Very Low Density Lipoprotein (VLDL) in HFD fed mice was significantly higher but High Density Lipoprotein (HDL) was lower when compared to normal control ($P < 0.0001$) ($P < 0.001$). Treatment of PTAE significantly lowered plasma Cholesterol, Triglycerides, LDL and VLDL and increased HDL when compared to obese control ($P < 0.0001$) ($P < 0.001$).

TABLE 6: EFFECT OF PTAE ON LIPID PROFILES OF NORMAL DIET FED MICE AND HIGH FAT FED MICE

Animal group	Total cholesterol (mg/dl)	Triglyceride (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)
Normal control	94.5 ± 0.5	72.3 ± 1.20	32.3± 1.15	46.8 ± 1.67	14.4 ± 0.24
High fat diet fed group	260.2± 2.89$^{\neq}$	217.3 ± 1.45$^{\neq}$	16.3±0.8$^{\#\#}$	200.2± 4.0$^{\neq}$	43.4 ± 0.29$^{\neq}$
Normal+100 mg/kg PTAE	90.3± 0.88$^{\#\#}$	64.5 ± 0.5$^{\#\#}$	28.5 ± 0.5	49.1±2.1$^{\#\#}$	12.9 ± 0.1
HFD+100 mg/kg PTAE	122.5 ± 2.50*	145.6 ± 1.0*	31.5±1.0**	62.5 ±1.7*	29.9 ± 0.2*

The table represents the lipid profile of the animals from the four groups; Control, High fat diet fed group (HFD), Control treated with *Phlogacanthus thyrsiflorus* aqueous extract PTAE (Con + PTAE), High fat fed mice treated with *Phlogacanthus thyrsiflorus* aqueous extract PTAE (HFD + PTAE). Values represent Mean ± standard deviation (n=4). Significant difference between control and HFD fed obese control: $\neq P < 0.0001$, $\#\# P < 0.001$. Significant difference between control and Con + PTAE group: $\#\#\# P < 0.01$ Significant difference between HFD fed obese control and HFD + PTAE group: $*P< 0.0001$, $** P < 0.001$

Effect of PTAE on plasma insulin, GLP-1 and GIP levels in HFD fed obese mice and HFD+ STZ induced diabetic mice:

The plasma insulin level of HFD fed mice was significantly higher when compared to normal control ($P < 0.0001$). But the treatment of HFD fed mice with PTAE lowered the insulin level significantly when compared to obese control ($P < 0.0001$). The plasma GLP-1 level of HFD fed mice was significantly lower when compared to normal control ($P < 0.0001$). But the treatment of HFD fed mice and normal mice with PTAE increased the GLP-1 level significantly when compared to obese control ($P < 0.001$). The plasma GIP level was significantly higher when compared to normal control ($P< 0.001$). But the treatment of HFD fed mice with PTAE decreased the GIP level significantly when compared to HFD fed mice ($P < 0.001$). The result is shown in **Table 7**.

From the previous study it can be concluded that HFD fed mice developed insulin resistance. When HFD induced insulin resistance was combined with partial islet damage elicited by low dose of STZ, mice exhibits loss of beta cells, decreased circulating insulin level and hyperglycemia. It may be due to this fact that the postprandial insulin level of HFD + STZ diabetic mice was lower than the control. The plasma insulin level of HFD + STZ diabetic mice was significantly lower when compared to normal control ($P < 0.0001$). But the treatment of HFD + STZ diabetic mice with PTAE in both doses i.e. 100 mg/kg and 200 mg/kg increased the insulin level significantly when compared to diabetic control ($P < 0.0001$).

The plasma GLP-1 level of HFD + STZ diabetic mice was significantly lower when compared to normal control ($P < 0.0001$). But the treatment of HFD + STZ diabetic mice with PTAE in both doses i.e. 100 mg/kg and 200 mg/kg increased the GLP-1 level significantly when compared to diabetic control ($P < 0.0001$). The plasma GIP level of HFD + STZ diabetic mice was significantly higher when compared to normal control ($P < 0.001$). But the treatment of HFD + STZ diabetic mice with PTAE in both doses i.e. 100 mg/kg and 200 mg/kg reduced the GIP level significantly when compared to diabetic control ($P < 0.001$). The results are comparable with the standard drug treatment. The result is shown in **Table 8.**

TABLE 7: EFFECT OF PTAE ON INSULIN, GLP-1 AND GIP LEVELS IN HIGH FAT DIET FED MICE

Animal group	Insulin (pmol/l)	GLP 1 (pmol/l)	GIP (pmol/l)
Normal control	282.5 ± 1.6	162.8 ± 1.1	11.6 ± 0.88
High fat diet fed obese control	468.6 ± 1.5$^{\neq}$	87.1 ± 2.0$^{\neq}$	38.8 ± 2.0$^{\#\#}$
Normal+100mg/kg PTAE	279.5 ± 0.5	182.2 ±1.0$^{\#\#}$	12.3 ± 1.0
HFD+100mg/kg PTAE	343 ± 3.0*	123.5 ± 1.0**	21.5± 1.5**

The table represents the insulin and incretins level of the animals from the four groups; Control, High fat diet fed group (HFD), Control treated with *Phlogacanthus thyrsiflorus* aqueous extract PTAE (Con + PTAE), High fat fed mice treated with *Phlogacanthus thyrsiflorus* aqueous extract PTAE (HFD + PTAE). Values represent Mean ± standard deviation (n=4). Significant difference between control and HFD fed obese control: ≠P < 0.0001, ## P < 0.001. Significant difference between control and Con + PTAE group: ###P < 0.001 Significant difference between obese control and HFD + PTAE group: *P< 0.0001, ** P < 0.001.

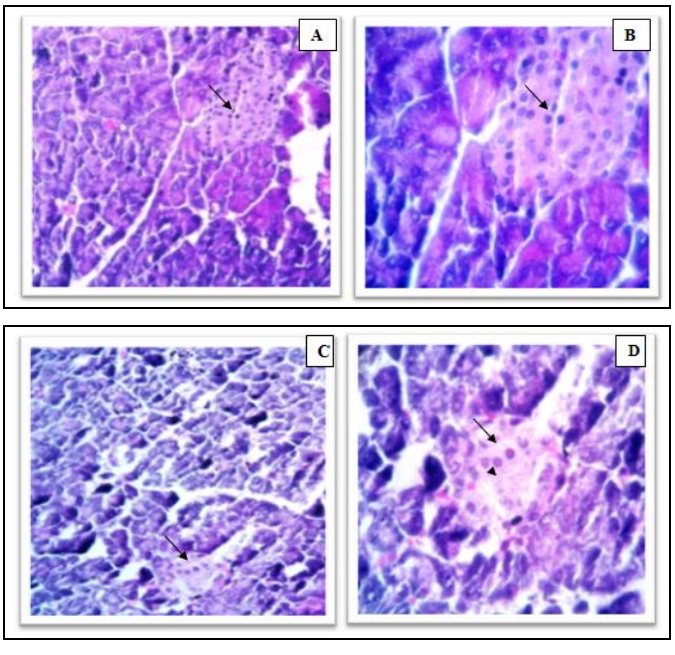
TABLE 8: EFFECT OF PTAE ON INSULIN, GLP-1 AND GIP LEVELS IN HFD + STZ INDUCED DIABETIC MICE

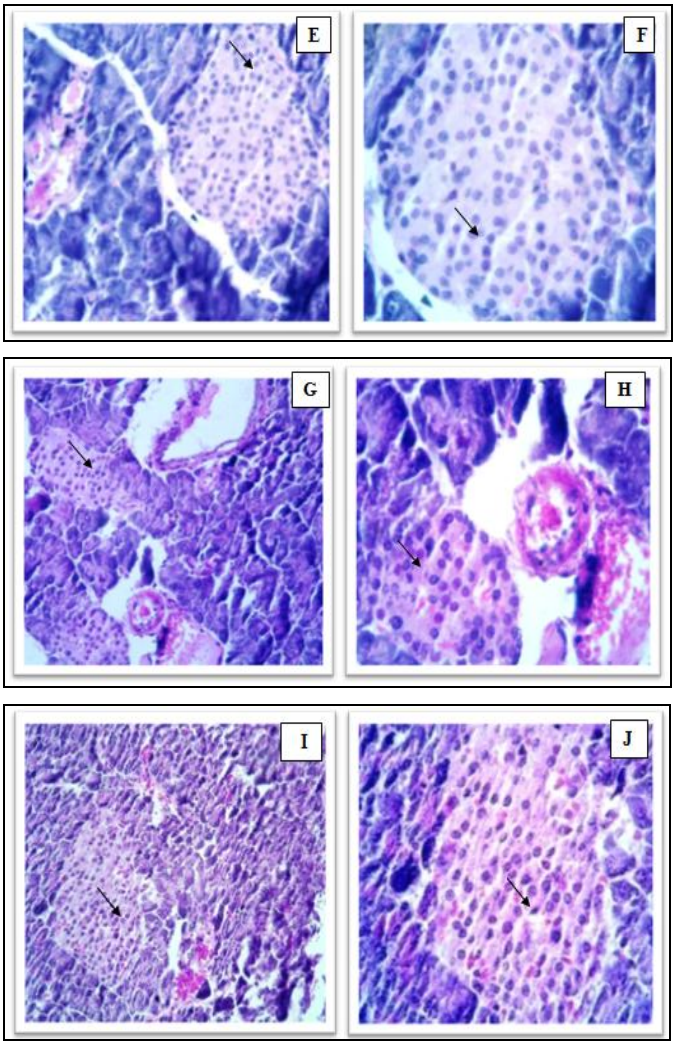
Animal group	Insulin (pmol/l)	GLP 1 (pmol/l)	GIP (pmol/l)
Normal control	295.5 ± 1.4	172.7 ± 1.2	12.6 ± 1.4
High fat diet fed+ STZ diabetic control	219 ± 1.0$^{\neq}$	80.6 ± 2.0$^{\neq}$	42.8 ± 2.5$^{\#\#}$
HFD+ STZ + PTAE100 mg/kg	257.2 ± 1.0*	132.6 ± 2.0*	25.5 ± 1.0**
HFD+STZ+200 mg/kgPTAE	272.2 ± 2.5*	153.8 ± 1.5*	18.4 ± 1.0**
HFD+STZ+10 mg/kg glibenclamide	276.4 ± 1.5*	161.2 ± 1.5*	20.2 ± 1.5**

The table represents the insulin and incretins level of the animals from the five groups: Control, High fat diet fed + Streptozotocin induced diabetic group (HFD + STZ), High fat diet fed + Streptozotocin induced diabetic group treated with *Phlogacanthus thyrsiflorus* aqueous extract 100 mg /kg PTAE (HFD + STZ+ PTAE), High fat diet fed + Streptozotocin induce diabetic group treated with *Phlogacanthus thyrsiflorus* aqueous extract 200 mg /kg PTAE (HFD + STZ+ PTAE), High fat diet fed + Streptozotocin treated with standard drug glibenclamide 10 mg/kg (HFD + STZ + Std drug. Values represent Mean ± standard deviation (n=4). Significant difference between control and HFD + STZ diabetic control: ≠P < 0.0001, ## P < 0.001. Significant difference between diabetic control and HFD + STZ + PTAE group *P < 0.0001, ** P < 0.001.

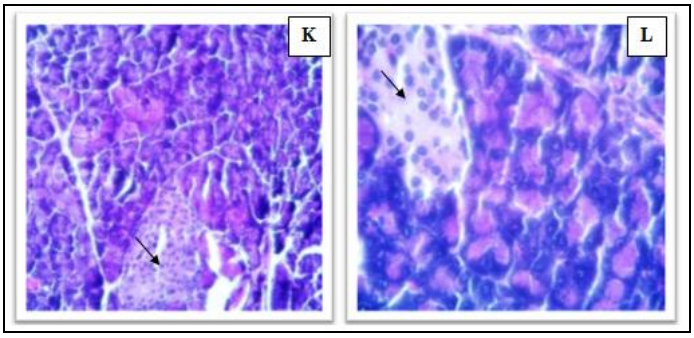
Histology of Pancreas:

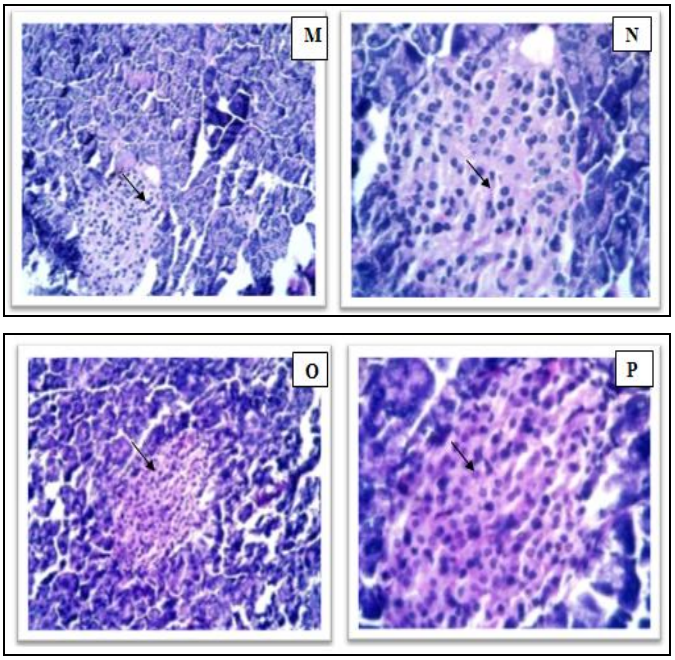
Pancreatic islets of diabetic control mice revealed significant reduction of size and number of acinar cells around the islets seems to be extensively damaged. This showed that islets were damaged, and shrunken in size. But in HFD fed obese mice beta cell proliferation was more when compared to normal control, HFD increases beta cell proliferation it may be the reason for insulin resistance and abnormal increase in insulin level in HFD fed obese mice. The presence of necrosed area within islets indicated the damage caused by STZ injection. In the PTAE treated group (100mg/kg b.w.) the beta cells regeneration in islets was seen. The islet cells of 200 mg/kg b.w. PTAE treated group were seen to be in normal position and it showed rapid proliferation of beta cells when compared with diabetic control. The acinar cells also seen to be normal in the PTAE treated groups. The islet cells of glibenclamide treated groups seem to be in normal position. The size of the cells was back in normal position, the islet cells were compactly arranged with negligible intercellular space. But in HFD fed mice treated with 100 mg/kg b.w. PTAE, beta cells number as well as islet cell size decreased as PTAE seems to control the abnormal increase of beta cells and islet size in HFD fed obese mice. This may be the reason of decreased insulin level in PTAE treated HFD fed obese mice.





PHOTOGRAPHS OF HISTOLOGICAL SLIDES OF PANCREAS: A: NORMAL CONTROL (10 X MAGNIFICATION); B: NORMAL CONTROL (40 X MAGNIFICATION); C: DIABETIC CONTROL (10X MAGNIFICATION); D: DIABETIC CONTROL (40 X MAGNIFICATION); E: PTAE AT THE DOSE OF 200 MG/KG B.W TREATED GROUP (10X MAGNIFICATION); F: PTAE TREATED AT THE DOSE OF 200 MG/KG B.W GROUP (40X MAGNIFICATION) G: PTAE TREATED AT THE DOSE OF 100 MG/KG B.W (10X MAGNIFICATION); H: PTAE TREATED AT THE DOSE OF 100 MG/KG B.W (40X MAGNIFICATION);I: GLIBENCLAMIDE TREATED GROUP; J: GLIBENCLMIDE TREATED GROUP. [ARROWS INDICATES THE PRESENCE OF BETA CELLS, ARROW HEADS INDICATES THE NECROSED AREA IN DIABETIC ANIMAL





PHOTOGRAPHS OF HISTOLOGICAL SLIDES OF PANCREAS: K: NORMAL CONTROL (10 X MAGNIFICATION); L: NORMAL CONTROL (40 X MAGNIFICATION); M: HFD FED OBESE CONTROL (10 X MAGNIFICATION) N: HFD FED OBESE CONTROL (40 X MAGNIFICATION) O: PTAE (100 mg/kg B.W) TREATED OBESE MICE (10 X MAGNIFICATION); P: PTAE (100 mg/kg B.W) TREATED OBESE MICE (40 X MAGNIFICATION) [ARROWS INDICATES THE PRESENCE OF BETA CELLS]

DISCUSSION: In the present work, we have evaluated for the first time the effect of PTAE on incretins, glucose and lipid metabolism in chemically induced diabetic mice. An attempt has been made in the present study to examine if PTAE helps in the modulation of enteroinsular axis or incretins in diabetic mice. In the present study, the HFD fed obese mice and HFD + STZ induced diabetic mice manifests hyperglycemia associated with insulin resistance and impaired insulin secretion, so it was chosen as a model of type 2 diabetes to study the effects of PTAE on the incretins which in turn is related to insulin secretion.

In this study, it was showed that HFD fed mice developed insulin resistance. When HFD induced insulin resistance was combined with partial islet damage elicited by low dose of STZ, mice exhibits loss of beta cells, reduced insulin level and hyperglycemia [27]. The HFD fed obese mice manifests hyperglycemia associated with insulin resistance. The initial stage involves beta cell hypertrophy and hyperplasia along with increased insulin secretion to counter the development of

insulin resistance resulting from obesity. It may be due to this fact that the insulin level of HFD fed obese mice was higher than the control. In the present study it was found that postprandial insulin level was higher in HFD fed obese mice but lower in HFD+STZ diabetic mice but HFD fed obese mice treated with PTAE showed lowered insulin level when compared with obese control which is shown in **Table 7**. PTAE in 100 and 200 mg/kg treatment increased insulin level in HFD+STZ induced diabetic mice in both the doses significantly when compared to diabetic control which is shown in **Table 8**.

Postprandial GLP-1 levels were significantly lower and GIP level was significantly higher in HFD fed obese mice as well as HFD+ STZ diabetic mice when compared to normal control. But PTAE in both 100 and 200 mg/kg dose increased GLP-1 level and decreased GIP level in HFD fed obese mice and HFD+STZ induced diabetic mice when compared to obese control and diabetic control which is shown in **Table 7** and **8**.

These results are in agreement with the study of Vilsboll *et al*., 2003 [28] where they reported postprandial insulin level was lower in type 2 diabetes mellitus (T2DM) patients when compared to normal. In response to oral glucose load total GLP-1 levels were decreased in T2DM patients when compared to control and total GIP increased in T2DM patients when compared to normal. Another study of Toft Neilson *et al*., 2001[29] reported that postprandial GLP-1 levels and AUC were decreased in type 2 diabetic patients and persons with impaired glucose tolerance (IGT) when compared to persons with normal glucose tolerance (NGT). Insulin level was lower in type 2 diabetic patients and higher in persons with IGT when compared with persons with NGT. GIP level was increased in patients with T2DM and IGT when compared to persons with NGT.

In the present study significantly increased GIP level was observed in obese as well as diabetic mice. Marks *et al*., 1988 [30] hypothesized that GIP might function as obesity promoting hormone, Miyawaki *et al* in 2002 [31] showed that GIP receptor knockout mice gained less weight than normal mice when treated with high fat diet [32]. In addition, GIP has been reported to stimulate fat deposition in adipocytes and has been postulated to link over nutrition to obesity [33]. These observations suggest that GIP may be an important factor for increased triglyceride accumulation in the adipose tissue and nutrient uptake, high GIP may lead to the development of obesity. Continuous treatment of PTAE resulted in significant improvement in glycemic control and metabolic profile. The improved glucose homeostasis may be associated with dose dependent increase in beta cell mass which is evident from the histological studies. These findings highlight the potential utility of PTAE in tackling underlying pathogenic cause of type 2 diabetes, namely the progressive loss of beta cell mass. It is possible that the observed beneficial effect of PTAE on beta cell could simply be a result of improvement in glycemia control.

High fat diet increased body weight in mice significantly which is shown in **Table 2**. But in HFD + STZ diabetic mice after the STZ injection the body weight reduced significantly. One mice died due to excessive weight loss after 5 days of STZ injection during the experimental period. The HFD provides more calories than the normal diet, resulting in high level of fat storage in the periepididymal region. PTAE (100mg/kg) administration prevented the weight gain significantly when compared with obese control. However PTAE treatment in both doses (100mg/kg and 200 mg/kg) prevented the weight loss in HFD+STZ mice when compared to diabetic control which is shown in **Table 3.**

Chatterjee and Shinde (2002) [34] mentioned in their reports that STZ causes reduction in body weight due to loss of tissue protein and increased muscle wasting [35]. The high fat or cafeteria diet has been reported to induce hyperphagia in rats [36] which results in high fat stores resulting in increased body weight [37, 38]. The result of our present study supports the above findings as we have observed increase in body weight and food consumption in the high fat diet fed mice but the water intake was significantly lower in HFD fed mice. Administration of PTAE reduced the consumption of food and calories in HFD fed obese mice and increased the water intake which is shown in **Table 2**. The administration of PTAE decreased both the

fasting and post prandial blood glucose level significantly in the HFD fed obese mice and HFD + STZ diabetic mice as well as normal diet fed mice which is shown in **Table 2** and **3**. Blood glucose levels in HFD+STZ induced diabetic mice increased significantly when compared to control which supported the findings of Poitout and Robertson (2002) [39], where they have mentioned that STZ cause destruction of pancreatic beta cells and it makes the cells less active to be sensitive enough towards insulin for glucose uptake and this will cause high glucose concentration in blood[40]. Insulin mediated glucose uptake causes hyperglycemia in rats [35].

OGTT and AUC results showed that HFD fed mice and HFD + STZ diabetic mice developed severe glucose intolerance after the glucose load. PTAE treated HFD fed as well as HFD + STZ diabetic mice showed significantly lower blood glucose at all time points and lower AUC when compared to obese control and diabetic control which is shown in Table 4, 5 and Figure 1, 2. PTAE treated normal mice also showed lower glucose levels at all time points and lower AUC when compared to normal control. The results are in agreement with the findings of Winzell and Ahren, 2004; Paul *et al*., 2011[41,42]. It may be due to the presence of hypoglycemic effect of flavonoids, phenols and saponins present in PTAE which we have reported in our previous study. It was proved from the results that PTAE can improve glucose homeostasis.

The abnormality in lipid metabolism in type 2 diabetes mellitus has caused hyperlipidemia in diabetic patient. HFD fed mice also shows the similar situation, hyperlipidemia. This finding further supports the idea of Lombardo and Chicco (2006)[44] where it was shown that those rats administered with HFD cause dyslipidemia and other syndromes in diabetics[44]. HFD also effects the insulin secretion. Insulin plays an important role in stimulating lipogenesis in mammals. Defects in insulin secretion effects the lipid metabolism. In the present study, high levels of triglyceride, LDL, VLDL and cholesterol was observed in HFD fed obese mice. Administration of PTAE (100mg/kg) to HFD mice showed significantly reduced levels of cholesterol, triglycerides, LDL, VLDL when compared with the obese control. Furthermore HDL is known to have anti atherogenic properties. HDL is involved in transport of cholesterol from peripheral tissues to liver and thereby reducing the amount stored in the tissue and possibility of developing atherosclerotic plaques [45]. HDL level was significantly decreased in HFD fed mice when compared to normal mice. Its level increased in PTAE (100mg/kg) treated HFD mice compared to obese control which is shown in Table 6. The results are in agreement with Santos *et al*., 2012; Saikia and Lama, 2011; Ahmed *et al*., 2012 [46-48]. This study demonstrated that daily supplementation with PTAE had pronounced antiobesity and beneficial metabolic effect in HFD fed obese mice as well as HFD + STZ diabetic mice. It may be due to the presence of hypoglycemic effect of flavonoids, phenols and saponins present in PTAE.

The histological study of pancreas the ultra structure of diabetic pancreas showed considerable reduction in the beta cell number and shrinkage in the islets of langerhans but in HFD fed group the islets of langerhans showed more beta cell proliferation when compared to normal control which may be the reason of increase in plasma insulin in this group. This is in agreement with the findings of Ellenbroek *et al*., 2013 [49] where they reported high fat diet induces the rapid proliferation of pancreatic β cells that increases the insulin level in the obese mice. PTAE treatment restored the size of the islets and number of the pancreatic beta cells in obese mice. In PTAE treated groups (100 mg/kg b.w and 200 mg/kg), in the pancreatic islets, beta cell proliferation was observed and was comparable to the normal control. Furthermore, the number of beta cells and islets diameter increased in PTAE treated group compared to STZ treated diabetic group. The beneficial effect of PTAE on glycemic regulation and beta cell mass are at least partially mediated via increased GLP-1signaling. These lines of evidence supported a role of incretins in the hypoglycemic effect of PTAE. However this study focuses on the doubtful role of GIP as incretin and its role in diabetes treatment.

CONCLUSION: In conclusion, this study provides evidences that PTAE can exert an incretin effect which might contribute at least, in part, to a better glucose homeostasis model. To our

knowledge this is the first study demonstrating an incretin effect of *Phlogacanthus thyrsiflorus*. The study regarding medicinal plants modulating entero insular axis is very limited. The study proved that the hyperglycaemic activity of the PTAE is may be due to the modulation of the entero-insular axis in the hyperglycaemic mice, is a new field of study and will provide major breakthrough to develop herbal drugs to control hyperglycaemia.

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