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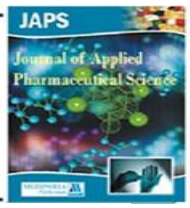

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Biochemical evaluation of anti-diabetic phytomolecule through bioactivity guided solvent fractionation and subfractionation from hydro-methanolic (2:3) extract of *Alligator pear* Fruit in streptozotocin induced diabetic rats

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ABSTRACT

The study was to evaluate the most effective fraction and subfraction having hypoglycemic activity from the hydro-methanolic (2:3) extract of the fruit of *Persea americana Mil*. through bioactivity guided investigation in streptozotocin induced diabetic rats. Out of different solvent fractions and subfractions, the effective fraction and subfraction of *Persea americana Mill.* were subjected to antidiabetic study in streptozotocin-induced diabetic rat. Serum insulin, glycosylated hemoglobin, transaminases, carbohydrate metabolic enzymes in liver, kidney, cardiac and skeletal muscle, were also assessed after 8 weeks of treatment and compared to the vehicle control. A significant recovery was noted in the level of serum insulin, glycosylated hemoglobin, activities of carbohydrate metabolic enzymes and serum transaminases after n-hexane fraction treatment in respect with other treated groups. Two sub fractions A and B were obtained using petroleum ether sub fractionation of which sub fraction B was more bioactive considering the above biosensors and was comparable with glibenclamide. HPTLC study focused four and two components (P1 and P2) in sub fraction A and B respectively where P1 is major, confirmed by HPLC study. The dose of sub fraction B was 1/4th of the n-hexane fractions dose. It has been concluded that sub fraction B (P1 and P2) which was obtained from n-hexane fraction had the antidiabetic activities in the streptozotocin-induced diabetic model.

Keywords: Hypoglycemic; phytomolecule; *Persea Americana Mill.*; Insulin; Transaminase.

INTRODUCTION

Diabetes mellitus, once considered a disease of minor significance to world health, is now a major threat to human health in the 21st century (Zimmet *et al*., 2001).A recent study by the World Health Organization (WHO) estimated that the worldwide prevalence of diabetes in2002 was 170 million, with the number predicted to grow to 366 million or more by 2030. The adoption of a sedentary lifestyle, the consumption of non-traditional foods and, a genetic predisposition to the disease are thought to be the major underlying causes of the epidemic.(Wild *et al*., 2004 and Leduc *et al*., 2006).Genetic predisposition to the disease is probably inhabited as an autosomal recessive trait. About 25% of family relatives of diabetics show diminished glucose tolerance as compared to 1% in the general population. It is a chronic endocrine disorder generally due to disordered essential energy nutrients metabolism, which is precipitated by insulin insufficiency and insulin inefficiency. This results in hyperglycemia and glycosuria, in particular (Chatterjea *et al*., 2002).

Thus it affects carbohydrate, fat and protein metabolism. It is associated with two to four fold increased risk of coronary artery disease. Studies indicate that patients with type 2 diabetes mellitus who have no history of coronary artery disease have the same risk for cardiac events as do non-diabetic patients with pre-existing coronary artery disease. This emphasizes the extensive but silent nature of coronary artery disease in patients with diabetes mellitus (Kreisberg, 1998). Therefore, search for a drug having twofold properties, that is lowering of blood lipids and glucose together is in great demand. Despite of remarkable advancement in the management of diabetes by synthetic drugs, there has been a renewed interest in medicinal plants because they do not elicit any side effects (Bastaki, 2005). *Alligator pear* [Persea americana Miller] (syn.P.gratissima Gaertn), belong to Lauraceae family commonly called *avocado*, is a small tree with a gray trunk. The fruit of this plant is used as an herbal medicine. The amount of simple sugars in avocado fruit is low, but in contrast, it contains appreciable levels of dietary fibers, DF (Bergh, 1992). This may resolve constipation, reduce fat absorption, lower glycemic index and plasma insulin levels, alter colon fermentation and microbial proliferation, and reduce plasma cholesterol (Kritchevsky *et al*., 1995). Therefore, adding recommended levels of DF to the diet is considered vital for normal intestine performance, good health, and for controlling major risk factors for diabetes, obesity, gall stones, hypercholesterolemia and heart disease (Gray, 1995). However, scientific evidences for the pharmacological properties of the avocado fruit are limited. Recently, we have evaluated the antidiabetic, antioxidant, antiulcerogenic and anti hyperlipidemic potential of avocado leaf and fruit extracts. The present investigation was aimed at to fractionate and subfractionate the active compound present in the fruit through bioactivity guided solvent fractionation.

MATERIALS AND METHODS

Collection and processing of avocado fruit

Samples of ripe avocado fruits were purchased from Supermarket, Chennai and authenticated and preserved from department of botany, University of Madras. The pulp required for the study was separated from the seed. The collected pulp was washed with clean tap water to remove dirt and then finally with deionized water. The cleaned pulp was dried completely at 40C in the drying oven. The dried pulp was pulverized separately in electrical grinder and was kept in the self-sealing polythene bags to avoid any contamination and for future experimental studies. Known amount of dry powder was repeatedly extracted by the process of maceration in an aspirator using 95% ethanol as menstruum. The extract was concentrated under reduced pressure by rotary evaporator to obtain thick syrup mass and stored at 4C. The yield was approximately 65% of fresh fruit. The aqueous extract was prepared freshly at the time of administration.

Preparation of hydro-methanolic extract of Avocado

Aqueous extract of avocado fruits (5000 g) were taken into 20 L percolator and maceration was carried out with 10L hydromethanolic solution (H_2O: MeOH :: 40:60) at 25 °C to avoid any degradation or deactivation of the active compound (s). The slurry was stirred intermittently for 1h and left for overnight. The extract was collected on the second day after 24 hr of extraction process and then freshly prepared 5L hydromethanolic solution was added to the extraction chamber and the slurry was stirred again with glass rod. The same procedure was repeated again on the third day with another 5L solvent mixture and last extract was collected on the fourth day. The extract was filtered first by cotton filter and then by Whatman filters paper (No.1). The filtrate was evaporated under reduced pressure by Rotavapour (BUCHI–R124; Switzerland) at 40°C for complete removal of methanol. Finally plain aqueous filtrate (9.5 L free from methanol) was lyophilized on VirTis bench top K lyophilizer. The lyophilized extract (920 g) was collected and put into the amber colored glass containers which were finally stored in the refrigerator under vacuum for subsequent fractionation and experimental studies. The lyophilized extract was a mixture of dark brownish sticky layer and light brownish solid powder (slightly hygroscopic in nature).

Bioactivity guided fractionation

In 5L separating flask, 600 g of lyophilized extract of *Avocado* fruit was dissolved with 2L of hydromethanolic (H2O: MeOH :: 40:60) solution and solvent fractionation was carried out using solvents (n-Hexane,Chloroform, Ethyl acetate and n-Butanol) with increasing polarity. TLC was carried out to monitor the progress in fractionation. All fractionates were collected separately and dried under reduced pressure (20-200mbar) using rotavapour instrument at 40°C. Finally from 600g of lyophilized extract of *Avocado* fruit, 2.5 g n-hexane,fraction, 19.4 g chloroform fraction, 56.8 g ethyl acetate fraction and 138.5
g n-butanol fractions were obtained. All the fractions were administered orally through gavage twice a day.

Subfraction of n-hexane fraction by petroleum ether

One g of n-hexane fraction was taken into an eppendorf tube (2 ml) and 1ml of petroleum ether was added to it. The solution was then vortexed for 2 mins for proper mixing. Finally the said solution was centrifuged at 5000 r.p.m for 5 mins. After centrifugation, it was observed that the said solution was separated into two distinct layers. The upper portion was liquid and oily in nature with greenish color (subfraction A) whereas the lower portion was white colored solid compound (subfraction B). Finally from 1g of n-hexane fraction 669 mg subfraction A and 328 mg subfraction B were obtained. Subfraction A and B were administered orally through gavage.

Chemicals and reagents

Streptozotocin (Sigma-Aldrich Diagnostic Ltd. USA), glibenclamide (Medicare Pharmacy, India), glucose estimation kit (Clic diagnostics, India) ALT and AST kits (Clic diagnostics, India) were purchased. All other chemicals and reagents used for the study were of analytical grade obtained from E.Merck and HIMEDIA, India.

Experimental animals

The animal experiments were designed and conducted according to the ethical norms approved by Indian Government and Institutional Animal Ethical Committee (IAEC) for the investigation of experimental pain in conscious animals. Before beginning the experiments, the albino rats were allowed to acclimatize to animal house condition for a period of one week. Male Wistar Albino rats weighing 180±20g were used. Throughout the experimental period, the rats were fed with balanced commercial pellet diet with composition of 5% fat,21% protein, 55% nitrogen free extract and 4% fiber (w/w) with adequate mineral and vitamin levels for the animals. Diet and water were provided *ad libitum.*

Induction of diabetes mellitus

Rats were fasted overnight and experimental diabetes was induced by single intra peritoneal injection of STZ with a dose of 50mg/kg body weight. STZ was dissolved in a freshly prepared 0.1M cold citrate buffer of pH 4.5 (Rakieten *et al*., 1963). Control rats were similarly injected with 0.1M cold citrate buffer (pH4.5). As STZ is capable of introducing fatal hypoglycemia as a result of massive pancreatic insulin release, STZ –administrated rats were provided with 10% glucose solution after 6hrs for the next 24hrs to prevent hypoglycemia. Neither death nor any other adverse effect was observed. After 3 days for the development and aggravation of diabetes, rats with moderate diabetes (i.e., blood glucose concentration >250mg/dl) that exhibited glycosuria were selected for the study (Canep *et al*., 1990).

Toxicity and Dosage Fixation Studies

Acute toxicity studies with P.americana fruit extract were performed in experimental rats. Graded doses of ethanol extract of avocado fruits (100,250,500 and 1000mg/kg body weight) were administered orally, and the animals were subsequently observed for 2 weeks. Changes in body weight, food consumption, hematological, macroscopic and clinical-biochemical findings, including the activities of enzymes, were noted. Dosage fixation studies were carried out by virtue of unequally long administration of graded doses of P.americana fruit extract (100,250,500 and 1000mg/kg body weight) given to rats introduced into STZ-induced hyperglycemia; it was found that the fruit extract shows its maximal hypoglycemic effect at the concentration of 300mg/ body weight administered orally for 28 days. Hence, the dosage was fixed at 300mg/ body weight/rat/day and pursued for 28 days.

Experimental design

Screening for hypoglycemic activity of the different fractions of fruit of Avocado

The rats were divided into seven groups and each group consisted of 6 rats

Group I - vehicle control.

Group II - diabetic control (50 mg STZ in 0.1ml citrate buffer/100g body weight. of rat).

Group III - diabetic rats treated with 300mg/kg.b.w n-hexane fraction twice a day.

Group IV - diabetic rats treated with 300mg/kg.b.w chloroform fraction twice a day.

Group V - diabetic rats treated with 300mg/kg.b.w ethyl acetate fraction twice a day.

Group VI - diabetic rats treated with 300mg/kg.b.w n-butanol fraction twice a day.

Group VII - diabetic rats treated with 20mg/kg.b.w glibenclamide (reference drug) twice a day.

Every day, the first oral dose of the said fractions was given 1 hour before the supply of animal feeds in the morning (at 8 AM) and second oral dose was administered after 2 hours of the cleaning of feed box atafternoon (5 PM). Feeds were supplied again to the animals after 1 hr of second oral administration of the said fractions. Animals of vehicle control group (Group I) and diabetic control group (Group II) were subjected to gavage of 0.5 ml 2% w/v Tween 80 for 49 days at the time of fractions and glibenclamide administration to the animals of (Group III- Group VII), to keep all the animals under the same experimental condition and stress imposition, if any, due to administration of fractions and animal handling. Starting from first day of extract administration to diabetic rats, fasting blood glucose levels (12 hrs after feed delivery) in all the groups were measured on every 7 days interval. On the 56th day of experiment, peripheral blood was drawn by retro orbital puncture, under the light anesthesia at 12 hour fasting state and fasting glucose level was monitored.

All the animals were sacrificed at fasting state by light ether anesthesia followed by decapitation after recording the final body weight. Rest blood was collected from dorsal aorta by a syringe and the serum was separated by centrifugation at 5000 rpm for 5 min for the estimation of serum insulin and serum toxicity study. The liver, kidney, skeletal muscle and cardiac muscle were dissected out and stored at -20°C for the quantification of hepatic glycogen, assessment of the activities of the carbohydrate metabolic enzymes – hexokinase, glucose-6- phosphate dehydrogenase, glucose-6-phosphatase and lactate dehydrogenase. Blood was used for the quantification of glycated hemoglobin.

Evaluation of the antidiabetic effect of petroleum ether sub fractions (sub fraction A and sub fraction B) for 28 days in STZ diabetic rats

The rats were divided into 5 groups and each group consisted of 6 rats

Group I - vehicle control

Group II - diabetic control.

Group III - diabetic rats treated with 150 mg fraction A/kg.b.w/day for 28 days.

Group IV - diabetic rats treated with 150 mg fraction B/kg.b.w/day for 28 days.

Group V - diabetic rats treated with 20 mg glibenclamide/kg.b.w/day for 28 days.

Fraction A or B or glibenclamide was administered into animals of the respective groups every day morning (at 8 AM) for 21 days (first 7 days was stabilization period) by gastric intubation with a force feeding needle. All the 5 groups were sacrificed on the 29th day after an overnight fasting by light ether anesthesia and then blood was collected from dorsal aorta by a syringe for fasting blood glucose level.

Measurement of Fasting Blood Glucose Level

At the time of grouping of the animals, fasting blood glucose (FBG) level was measured. After every six days interval (on every 7th day) of treatment, FBG was further recorded from all the animals of all groups. The blood samples were withdrawn from the animals by retro orbital puncture under light anesthesia. The plasma glucose estimation was done by glucose oxidase / peroxidase (GOD/POD) (Ragavan *et al*., 2006) method using a standard kit.

Biochemical measurement

Serum insulin levels were measured (Brugi *et al*., 1998) using rat insulin enzyme linked immuno sorbent assay (ELISA) kit obtained from Millipore Corporation, Billerica, MAO 1821. Glycosylated hemoglobin level was measured using a glucose memory test (Chandalia *et al*., 1980). Hepatic glycogen level was measured according to the standard methods.(Kemp *et al*., 1954). The activities of carbohydrate metabolic enzymes such as of hexokinase, glucose-6-phosphate dehydrogenase, glucose-6-phosphatase and lactate dehydrogenase were assayed (Langdon, 1966; Chou *et al*., 1975; Swanson, 1955 and Anon 1970). The measurement of AST and ALT activities was performed by (Henry, 1960) using semi auto analyzer kit.

Acute toxicity studies of n-hexane fraction

Healthy adult Wistar albino rats, starved overnight were divided into four groups (n = 6) and were orally fed with the n-hexane fraction of *Avocado* in escalating dose levels of 100, 500, 1000 and 3000 mg/kg body weight suspended in water with 2% w/v Tween 80.(Ghosh, 1984) The rats were pragmatic continuously for 2 h for behavioral, neurological and autonomic profiles and after a period of 24 and 72 h for any lethality or death. (Turner, 1985)

Statistical analysis

All the data were evaluated statistically using one-way analysis of variance (ANOVA) followed by multiple comparison two tail 't' test by using the Origin Lab (Ver. 6.0) software. P values of less than 0.05 were considered to indicate statistical significance. Data were presented as mean ± S.E.M.

RESULTS

Effect of the different fractions on body weight

Final body weight of vehicle control group was significantly increased than at the beginning of the experiment. On the contrary a significant decrease in body weight was observed in diabetic control group in respect to the vehicle control. On the other hand, the administration of n-hexane, chloroform, ethyl acetate, n-butanol fraction of *Avocado* was resulted an increase in body weight. The changes in body weights were most effective in n-hexane fraction in comparison to other fractionation groups and the change was compared to glibenclamide (Reference drug) treated group (Table 1).

Table 1.Comparative study of different fractions of fruit of *Avocado* on body weight in male albino rat: Duration dependent response.

S.No.	Groups	Changes in body weight in grams	
		Initial	final
1	Vehicle control	179.50±6.98 [a]	195.65±6.25 [a]
2	Diabetic control	184.62±7.17 [a]	152.32±5.49 [a]
3	Diabetic + n-hexane fraction	180.40±6.01 [a]	195.42±5.65 [a]
4	Diabetic + chloroform fraction	182.65±7.12 [a]	192.70±5.99 [c]
5	Diabetic + ethyl acetate fraction	182.40±6.12 [a]	192.70±5.99 [d]
6	Diabetic + n-butanol fraction	179.40±6.31 [a]	192.70±5.99 [d]
7	Diabetic + glibenclamide fraction	181.61±4.98 [a]	193.10±6.14 [a]

Data were expressed as mean ± SEM, n=6. ANOVA followed by multiple comparisons two tail "t" test. Values with different superscripts (a, b, c, d) differ from each other significantly ($p<0.05$)

Effect of the different fractions on FBG

There was a significant decrease (75.96%) in FBG levels in diabetic treated rat after the administration of n-hexane fraction at the dose of 100mg/kg. b.w twice a day whereas only 59.05%, 44.51% and 35.31% reduction were observed in fasting blood glucose levels of diabetic treated after the administration of chloroform, ethyl acetate and n-butanol fractions at the above said dose. The n-hexane has more potent action than the glibenclamide (62.01%) in this concern (Table 2).

Effect of the different fractions on serum insulin

Serum levels of insulin were decreased significantly in untreated diabetic test animals as compared with non- diabetic control animals. Administration of n-hexane fraction of *Avocado* to diabetic animals resulted to a significant corrective effect than other said fractions on the levels of insulin (Table 2).

Table 2. Protective effect of n-hexane, chloroform, ethyl acetate and n-butanol on Fasting blood Glucose level,

S. No.	Groups	Fasting Blood Glucose (mg/dl)	Serum Insulin (µIU/ml)	HbA_1C in %
1	Vehicle control	90±9.54 [a]	14.5±1.5 [a]	4.8±0.45 [a]
2	Diabetic control	240±14.85 [a]	5.9±0.46 [b]	7.7±0.69 [b]
3	Diabetic + n-hexane fraction	120±9.68 [a]	9.1±0.67 [a]	5.4±0.51 [a]
4	Diabetic + chloroform fraction	123±10.05 [a]	6.3±0.65 [c]	6.8±0.61 [c]
5	Diabetic + ethyl acetate fraction	146±13.84 [a]	6.2±0.61 [c]	6.6±0.64 [c]
6	Diabetic + n-butanol fraction	135±12.05 [a]	6.1±0.64 [c]	6.6±0.64 [c]
7	Diabetic+ glibenclamide fraction	105±9.01 [a]	8.0±0.78 [d]	6.0±0.59 [d]

Values are expressed as Mean±SEM, n=6 for each group. ANOVA followed by multiple comparisons two tail't' test. Values with different supersctipts (a,b,c) differ from other significantly at the level of $p<0.05$.

Effect of the different fractions on glycosylated hemoglobin

Glycosylated hemoglobin level is one of the important indicators of diabetic condition, and the level of the parameter was increased significantly in diabetic group in comparison with the vehicle control group. After administration of n-hexane, chloroform, ethyl acetate, n-butanol of *Avocado* in STZ-induced diabetic rat, the level of this parameter was recovered towards the control level. This recovery was most significant after administration of n-hexane fraction (Table 2).

Effect of the different fractions on glycogen

The levels of hepatic and skeletal muscle glycogen were decreased in the untreated diabetic group in comparison with the vehicle control group. The administration of n-hexane or chloroform or ethyl acetate or n-butanol fraction to diabetic animal resulted to a significant recovery in this parameter, but the administration of n-hexane produced the most significant recovery in the test animals in comparison with those animals treated with any other individual fraction (Fig. 1).

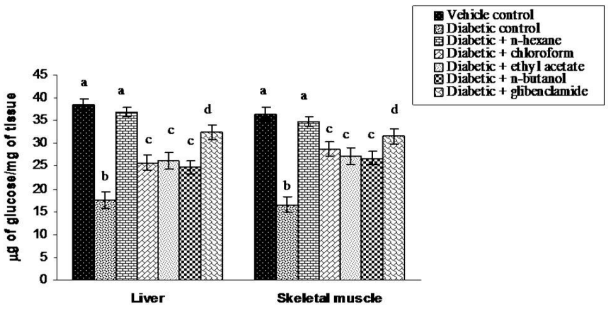

Fig. 1. Comparative analysis of different fractions of *P.americana* fruit on liver and skeletal muscle glycogen levels in control and experimental rats.

Each bar represents Mean±SEM, n=6. ANOVA followed by multiple comparison two tail 't' test. Bar diagrams with different superscripts (a,b,c,d) differ from other significantly, $p<0.05$.

Effect of the different fractions on carbohydrate metabolic enzyme

The activities of hexokinase and glucose-6-phosphate dehydrogenase in hepatic, kidney, skeletal muscle and cardiac muscle were decreased, but the activities of glucose-6-phosphatse and lactate dehydrogenase in said tissues were increased in the untreated diabetic group in comparison with the vehicle control group. The administration of n-hexane, chloroform, ethyl acetate and n-butanol to diabetic animal was resulted a significant recovery in these metabolic reactions but the administration of n-hexane produced a most significant recovery of normalcy in comparison with those animals treated with any of the said individual fraction (Table 3)

Effect of the different fractions on AST and ALT in serum

Activities of AST and ALT in serum were increased in diabetic group compare to the vehicle control group. After administration of n-hexane fraction there was a significant recovery in the levels of these parameters shown in (Fig. 2).

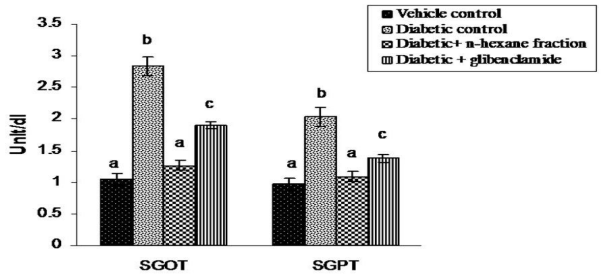

Fig. 2. Resettlement on the activities of serum AST and ALT after administration of n-hexane and glibenclamide in STZ-induced diabetic rat.

Each bar represents Mean±SEM, n=6 for each group. ANOVA followed by multiple comparison two tail 't' test. Values of bar diagrams with different superscripts (a,b,c) differ from other significantly at the level of $p<0.05$.

Acute toxicity studies of n-hexane fraction

Treatment of in rats with n-hexane fraction in rat did not produce any significant change in the autonomic, behavioral or neurological responses up to doses of 3000 mg/kg body wt.. Acute toxicity studies revealed the non-toxic nature of the n-hexane fraction of *Avocado*.

Hypoglycemic activity of sub fraction A and B

FBG levels of untreated diabetic rats were significantly higher than those in normal rats. After the treatment of subfraction A or subfraction B or glibenclamide there was a significant reduction of blood glucose level in diabetic group but subfraction B was more effective in this concern than subfraction A or glibenclamide treated group (Fig. 3). Serum level of insulin was decreased significantly in untreated diabetic group as compared with vehicle control group. Administered of subfraction A or subfraction B or glibenclamide to diabetic animals resulted a significant corrective effect through subfraction B is more effective in this concern (Fig. 4). The level of hepatic glycogen was decreased in the untreated diabetic group in compare with the vehicle control group. The administration of subfraction A or subfraction B or glibenclamide to diabetic animal resulted a significant recovery in the level of liver glycogen, but the administration of subfraction B was more effective in comparison with those animals treated with subfraction A or glibenclamide (Fig. 5). The activities of hexokinase and glucose-6-phosphate dehydrogenase in liver were decreased, but the activities of glucose-6-phosphatse and lactate dehydrogenase in said tissue were increased in the untreated diabetic group in comparison with the vehicle control group of animals. The administration of subfraction A or subfraction B or glibenclamide to diabetic animal resulted a significant recovery in the metabolic reactions but the administration subfraction B resulted a more significant recovery in the levels of these parameters towards normal in the test animals in comparison with those animals treated with subfraction A (Table 4). Activities of AST and ALT in serum were increased in diabetic group compare to the vehicle control group. After administration of subfraction A or subfraction B or glibenclamide there was a significant recovery in the levels of these parameters. Subfraction B was most effective than subfraction A or glibenclamide (Table 5).

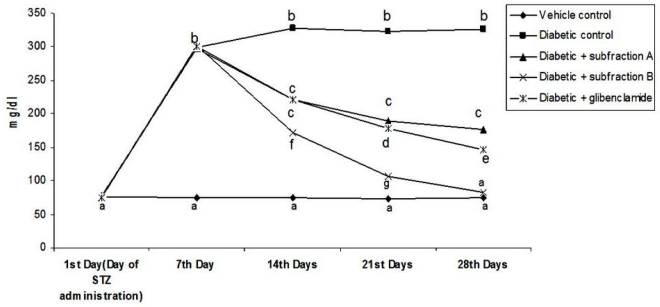

Fig. 3. Resettlement on the levels of serum FBG after administration of subfraction A or subfraction B or glibenclamide in STZ-induced diabetic rat.

Each line represents Mean±SEM, n=6 for each group. ANOVA followed by multiple comparison two tail 't' test. Values of the line diagrams with different supersctipts (a,b,c,d,e,f,g) differ from other significantly, $p<0.05$.

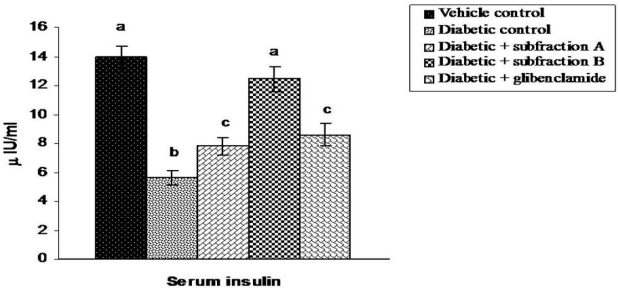

Fig. 4. Protective effect of subfraction A or subfraction B or glibenclamide on serum insulin level in STZ-induced diabetic rat.

Each bar represents Mean±SEM, n=6 for each group. ANOVA followed by multiple comparison two tail 't' test. Bar diagrams with different supersctipts (a,b,c) differ from other significantly,$p<0.05$.

Table 3. Protective effect of different fractions of P.americana fruit extract on carbohydrate metabolic enzymes in liver, kidney, skeletal muscle and cardiac muscle.

S.No.	Groups		Vehicle control	Diabetic control	Diabetic + n-hexane	Diabetic + chloroform	Diabetic + Ethyl acetate	Diabetic + n-butanol	Diabetic +glibenclmide
1.	Hexokinase	Liver	23± 2.50 [a]	7± 0.65 [b]	21± 2.03 [a]	15± 1.65 [c]	15.3± 1.61 [c]	15.5± 1.62 [c]	17± 1.54 [d]
		Kidney	21± 1.95 [a]	6.8± 0.51 [b]	18.5± 1.94 [a]	14.0± 1.64 [c]	14.2± 1.03 [c]	14.1± 1.32 [c]	14.4± 1.64 [d]
		Skeletal muscle	27.4± 2.05 [a]	15.6± 1.84 [b]	24.9± 2.34 [a]	20.1± 2.61 [c]	20.2± 2.05 [c]	19.3± 1.99 [c]	20.8± 2.01 [d]
		Cardiac muscle	28.6± 2.65 [a]	18.1± 2.00 [b]	27.1± 2.51 [a]	21.7± 2.94 [c]	21.9± 2.19 [c]	21.8± 2.07 [c]	24.6± 2.34 [d]
2	Lactate dehydrogenase	Liver	32.4± 3.22 [a]	59.7± 5.24 [b]	34.7± 2.96 [a]	48.2± 3.62 [c]	48.4± 4.00 [c]	48.5± 5.32 [c]	39.9± 2.89 [d]
		Kidney	38.8± 3.41 [a]	63.4± 6.24 [b]	39.4± 3.51 [a]	54.5± 5.61 [c]	54.6± 5.31 [c]	54.7± 5.34 [c]	44.7± 4.12
		Skeletal muscle	31.1± 2.99 [a]	54.6± 5.68 [b]	33.2± 3.16 [a]	45.2± 4.31 [c]	45.3± 4.61 [c]	45.7± 4.61 [c]	36.5± 3.18 [d]
		Cardiac muscle	28.4± 2.51 [a]	51.7± 4.96 [b]	29.6± 2.45 [a]	42.3± 4.95 [c]	42.4± 4.23 [c]	42.4± 4.35 [c]	35.0± 3.08 [d]
3	Glucose 6 phosphatase	Liver	28.9± 3.00 [a]	44.2± 4.51 [b]	30.3± 3.51 [a]	36.4± 3.61 [c]	36.5± 3.61 [c]	36.7± 3.54 [c]	35.1± 3.61 [d]
		Kidney	23.4± 2.16 [a]	37.2± 4.00 [b]	25.0± 2.54 [a]	32.4± 3.51 [c]	32.5± 3.51 [c]	32.6± 3.68 [c]	28.2± 2.65 [d]
		Skeletal muscle	19.5± 1.50 [a]	30.0± 2.89 [b]	20.0± 2.00 [a]	24.3± 2.94 [c]	24.4± 2.61 [c]	24.6± 2.91 [c]	22.7± 2.35 [d]
		Cardiac muscle	18.3± 1.62 [a]	31.4± 3.97 [b]	18.5± 1.64 [a]	23.4± 2.51 [c]	23.3± 2.91 [c]	24.1± 2.65 [c]	23.2± 2.34 [d]
4	Glucose 6 phosphate dehydrogenase	Liver	79.2± 7.65 [a]	39.8± 3.51 [b]	78.9± 7.34 [a]	59.4± 4.99 [c]	59.5± 5.66 [c]	59.4± 5.38 [c]	63.7± 6.24 [d]
		Kidney	90.0± 10.0 [a]	48.2± 4.51 [b]	86.4± 7.96 [a]	72.1± 7.21 [c]	71.9± 7.31 [c]	71.7± 7.38 [c]	78.2± 6.97 [d]
		Skeletal muscle	60.8± 6.21 [a]	40.0± 4.00 [b]	60.4± 6.38 [a]	52.3± 5.06 [c]	52.1± 5.21 [c]	52.1± 5.28 [c]	48.2± 3.99 [d]
		Cardiac muscle	65.3± 6.84 [a]	39.7± 3.46 [b]	56.4± 4.99 [a]	45.4± 5.01 [c]	45.2± 4.31 [c]	45.1± 4.68 [c]	54.5± 5.09 [d]

Data were expressed as Mean±SEM, n=6 for each group. ANOVA followed by multiple comparison two tail 't' test. Values with different supersctipts (a,b,c,d) differ from other significantly at the level of $p<0.05$.

Table 4. Protective effect of subfraction A or subfraction B or glibenclamide on the activities of hepatic hexokinase, glucose-6-phosphatase, glucose-6-phosphate dehydrogenase and lactate dehydrogenase in STZ-induced diabetic rat.

S.No.	Groups	Hexokinase	Lactate dehydrogenase	Glucose-6-phosphatase	Glucose-6-phosphate dehydrogenase
1	Vehicle control	24.6±1.9 [a]	30.0±2.51 [a]	76.1±6.32 [a]	32.4±3.51 [a]
2	Diabetic control	8.1±0.76 [b]	46.2±4.95 [b]	40.0±3.95 [b]	59.7±5.00 [b]
3	Diabetic + Subfraction A	18.4±1.1 [c]	38.5±3.51 [c]	59.8±6.01 [c]	40.3±4.09 [c]
4	Diabetic + Subfraction B	24.2±2.21 [a]	30.2±2.86 [a]	78.3±6.82 [a]	34.5±3.51 [a]
5	Diabetic + glibenclamide	19.1±1.69 [c]	34.7±2.99 [c]	61.5±5.79 [c]	40.1±4.16 [c]

Each bar represents Mean±SEM, n=6 for each group. ANOVA followed by multiple comparison two tail 't' test. Bar diagrams with different supersctipts(a,b,c) differ from other significantly, $p<0.05$

Table 5. Resettlement on the activities of serum AST and ALT after administration of subfraction A or subfraction B or glibenclamide in STZ-induced diabetic rat.

S. No.	Groups	AST (Units/dl)	ALT (Units/dl)
1	Vehicle control	1.2±0.01 [a]	1.1±0.01 [a]
2	Diabetic control	2.9±0.03 [b]	2.4±0.02 [b]
3	Diabetic + Subfraction A	2.2±0.02 [c]	1.5±0.02 [c]
4	Diabetic + Subfraction B	1.3±0.01 [a]	1.2±0.02 [a]
5	Diabetic + glibenclamide	1.95±0.02 [c]	1.5±0.01 [c]

Values were expressed as Mean±SEM, n=6 for each group. ANOVA followed by multiple comparison two tail 't' test. Values with different supersctipts (a,b,c) differ from other significantly, $p<0.05$.

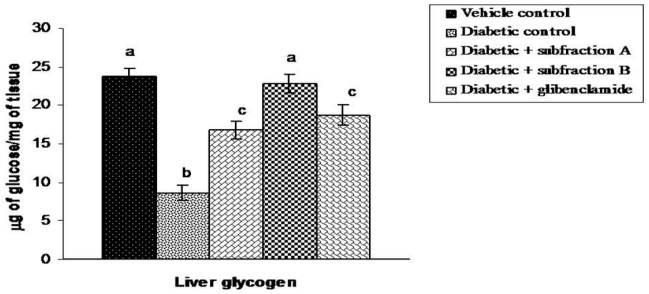

Fig. 5. Effect of subfraction A, subfraction B and glibenclamide on liver glycogen levels in STZ-induced diabetic rat.
Each bar represents Mean±SEM, n=6 for each group. ANOVA followed by multiple comparison two tail 't' test. Values of bar diagrams with different supersctipts (a,b,c) differ from other significantly, $p<0.05$.

HPTLC finger printing of most effective fraction and subfraction

To identify the number of compounds present in the active n-hexane fraction of *Avocado*, HPTLC fingerprinting was performed using chloroform: methanol: ethyl acetate: 90:5:5 v/v as a most suitable solvent system deduced from pilot study. From this study it was finally observed that the said fraction was a mixture of six different compounds. HPTLC finger printing was also carried out to examine the number of compounds present in most active in subfraction B. After HPTLC study it was observed that subfraction B contained two different compounds in which one was very prominent (P1, Rf = 0.22) on chromatographic plate and other was very faint (P2, Rf = 0.42) (Fig. 6).

HPLC analysis of most effective fraction and subfraction

Analytical RP-HPLC was performed to find out the number of compounds present in the solid subfraction of the petroleum ether (subfraction B) of the n-hexane fraction of fruit of TI show in Fig. 7. This study was also carried out to establish the purity percentage of the isolated compound as well the λ max values. HPLC analysis was performed on Water Alliance HPLC system equipped with 2695 separation module 2996 Photodiode Array Detector, performance plus 4 channels in line Degasser auto injector, quaternary pump and column oven. The HPLC analysis was carried out on Thermo Hypersil BDS C18 (4.6X250 mm, 5 µm), Column using a premixed solvent system, as mobile phase with a flow rate of 1 ml/min and Isocratic Illusion Technique. The column temperature was maintained at 300°C and detection was performed at 254 nm. For carrying out HPLC study, the white colored solid layer was separately dissolved in a mobile phase (Ethyl acetate: Hexane: Isopropyl alcohol:: 60:30:10). The solution was sonicated for 15 mins and then filtered through Millipore Millex Syringe filter unit (0.45 µm). The sample solution of 10µl injected through auto-injector fitted with 200 µl syringe and 100 µl sample loop. The separated compounds using scanned were the entire UV range on 3D spectral mode to record the PDA spectra. HPLC chromatograms of isolated compound were recorded at 254 nm. All the data of Water Alliance HPLC system were acquired and processed using Water Millennium32 software.

DISCUSSION

The present study was conducted to identify the possible active principle(s) having antidiabetic activity present in the n-hexane fraction followed by subfraction of *Avocado* fruit. The n-hexane fraction has shown more significant reduction than other fractions in blood glucose levels in STZ-induced diabetic rats. The n- hexane fraction produced a maximum antidiabetic activity which has been established from the comparative study with of glibenclamide in the diabetic rats. The levels of blood glucose, serum insulin, and glycosylated hemoglobin were noted in normal and experimental groups of rats. There was a significant elevation in blood glucose and glycosylated hemoglobin, while the level of serum insulin decreased in diabetic group when compared with the vehicle control group. (Kasetti *et al*., 2010) Administration of n-hexane fraction of *Avocado* (group- III – group VII) resulted a significant recovery near normal values as that of standard drug glibenclamide treatment. Restoration in glycogen level with fraction treatment may be explained by the recovery of insulin and insulin control the activity of glycogen synthetase (Sridevi *et al*., 2007). Such recovery of insulin has been indicated by plasma insulin level assessment. For the assessment of antidiabetic potency of the plant extract, we have measured the activity of hepatic glucose-6-phosphatase, an important enzyme for glycogenolysis (Aiston *et al*., 2003). Similarly glucose-6-phosphate dehydrogenase, hexokinase are two enzymes which are under the positive control of insulin (Kruszynska *et al*., 1998). In diabetic state the activities of these three enzymes were deviated as reported by others (Rajasekaran *et al*., 2004). The said fraction can able to recover the activities of these enzymatic biosensors significantly and comparable to glibenclamide which may be due to the recovery of insulin. Lactate dehydrogenase is the bifunctional enzymes involved in the glycolytic pathway. The lactate dehydrogenase system reflects the NAD+/NADH ratio indicated by the lactate/pyruvate ratio of hepatocyte cytosol. In different fraction of *Avocado* or glibenclamide treated group of rats, the reduction in the LDH activity is probably due to the regulation of NAD+/ NADH ratio by the oxidation of NADH. There was no toxicity of this extract as proved by serum AST and ALT activities as these enzymes are important sensors for toxicity assessment (Ghosh *et al*., 2001). In respect to LD_{50} values and maximum non-fatal doses studies revealed the non-toxic nature of the n-hexane fraction of this plant. There was no lethality or any toxic reactions found at any of the doses selected until the end of the study period. According to a toxicity classification (Loomis, 1968), the n-hexane fraction of *Avocado* was nontoxic. To find the number of active biomolecule for the management of STZ-induced

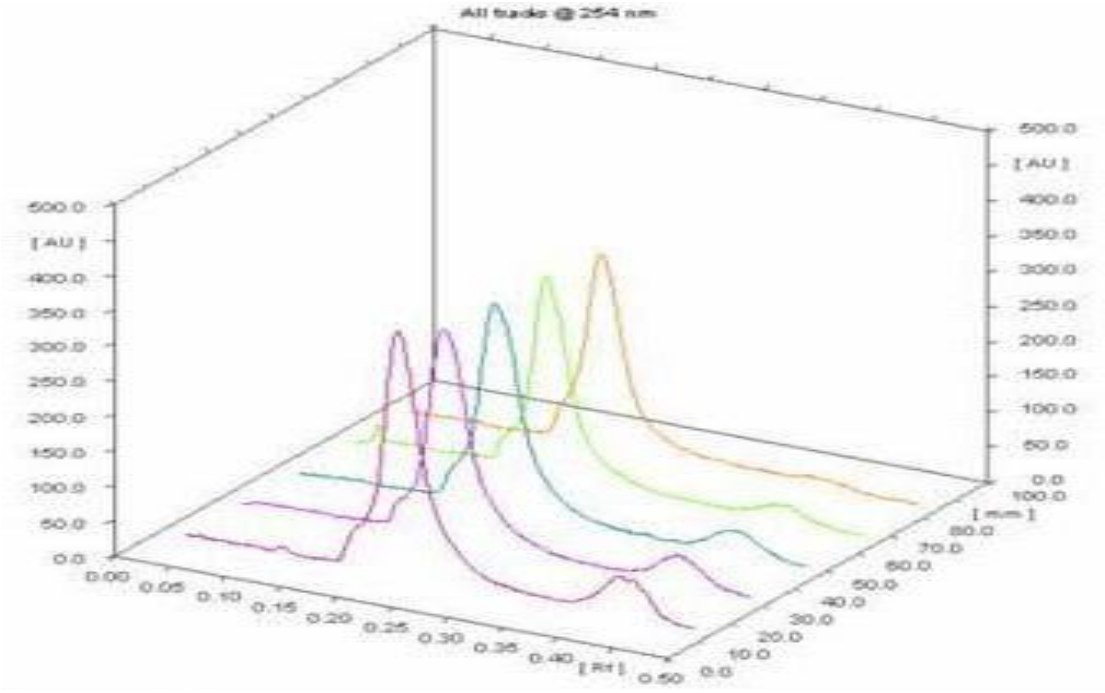

Fig. 6. Scan at 254 nm of different concentration of subfraction B (Three dimensional).

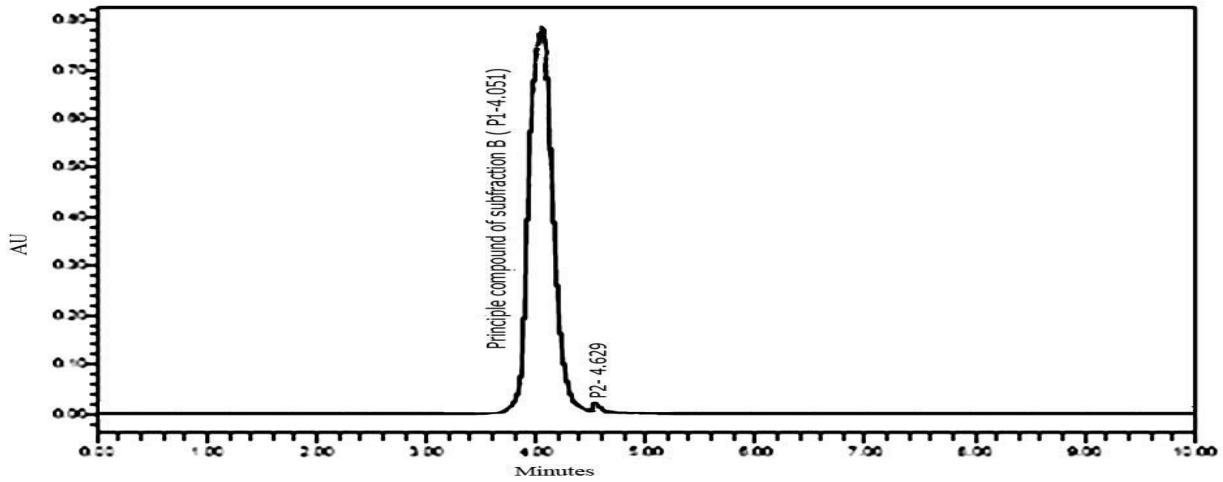

Fig. 7. Representative HPLC chromatogram of petroleum ether subfraction B from n-hexane fraction.

diabetic subfraction study using petroleum ether was conducted. Subfraction B was more effective than of with a dose which 1/4th of the dose of n-hexane fraction. From HPLC it has been justified that there are two components named as P1 and P2 where P1 is more effective and also present in more than in the petroleum ether subfraction. So, it may be concluded that possibly the single phytomolecule P1 may be in the causative phytomolecule for the management of diabetes mellitus from the fruit of *Avocado*. Further work is necessary for characterization of the biologically active phytoingredient present in the fruit of *Avocado*.

CONCLUSION

Our results concluded that the fraction and sub-fraction of the fruit of *Avocado* tested for antidiabetic activity have shown appreciable results in ameliorate the blood glucose level and related other complications. Our findings also may open the door for a new, alternative, leading drug for treating diabetic patients in future.

CONFLICT OF INTEREST

The authors do not have any conflict of interest.

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