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Research article

IN VITRO ANTIOXIDANT AND ANTIDIABETIC ACTIVITY OF HYDROALCOHOLIC EXTRACT OF *PLUMERIA PUDICA* JACQ

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ABSTRACT

The aim of the present study was to investigate the *in vitro* antioxidant and antidiabetic activity of hydroalcoholic extract of *Plumeria pudica* Jacq. In the present study we conducted bioassay of the extract using DPPH (α, α- Diphenyl- β-Picryl Hydrazyl) radical scavenging activity, Reducing power assay and Nitric oxide scavenging assay to find out the antioxidant activity. The results were compared with the standard ascorbic acid. Antidiabetic activity was proved by various methods like carbohydrate enzyme inhibition, determination of glucose uptake by cell lines and effect on glucose diffusion. Primary phytochemical screening of the plant extract showed the presence of carbohydrates, alkaloids, glycosides, flavonoids, phenolics etc. The hydroalcoholic extract showed a concentration dependent activity in all bioassays. By the study it was concluded that hydroalcoholic extract of *P.pudica* possess pharmacologically important phytoconstituents like phenolic compounds and flavonoids which impart strong antioxidant and free radical scavenging activities. Result indicated the potential of the plant in modulation of oxidative stress. And the extract also showed antidiabetic properties may be due to the antioxidant activity. Further investigation of which is suggested by fractionation and other scientific methods including *in vivo* antidiabetic studies.

Key words: *Plumeria pudica*, antioxidant activity, antidiabetic acivity, DPPH, Reducing power assay, nitric oxide scavenging assay, carbohydrate enzyme inhibition, glucose uptake, glucose diffusion.

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INTRODUCTION

Whenever a cell's internal environment is perturbed by infections, disease, toxins or nutritional imbalance, mitochondria diverts electron flow away from itself, forming reactive oxygen species (ROS) and reactive nitrogen species (RNS), thus lowering oxygen consumption (Maritim AC *et al.*, 2003). This oxidative shielding acts as a defense mechanism for either decreasing cellular uptake of toxic pathogens or chemicals from the environment, or to kill the cell by apoptosis and thus avoid the spreading to neighboring cells. Therefore, ROS formation is a physiological response to stress (Tiwari AK *et al.*, 2004)

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Increased oxidative stress at the cellular level can come about as a consequence of many factors, including exposure to alcohol, medications, trauma, cold, infections, poor diet, toxins, radiation, or strenuous physical activity (Tiwari A K *et al*., 2001)

The term oxidative stress has been used to define a state in which ROS and RNS reach excessive levels, either by excess production or insufficient removal. Being highly reactive molecules, the pathological consequence of ROS and RNS excess is damage to proteins, lipids and DNA (Mark P *et al.,* 1998) This oxidative damage to DNA, proteins, and other macromolecules are involved in the pathogenesis of various diseases through cell death and pathological dysfunction. The main diseases are diabetes, cancer, neurodegenerative diseases like Alzheimer's disease Parkinsons disease and aging of organism (Hamid AA *et al*., 2010)

An antioxidant is a molecule capable of slowing or preventing the oxidation of other molecules, and thus attracting the cells. Antioxidants terminate the chain reaction of formation of free radicals by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves (Sheikh M and Chehade *et al*.,2011)

Plants are rich in phytoconstituents which are having pharmacological activities and can be used against many diseases. These phytoconstituents are also possessing antioxidant activities (Chew AL *et al*.,2012). The term oxidative stress has been used to define a state in which ROS and RNS reach excessive levels, either by excess production or insufficient removal. Being highly reactive molecules, the pathological consequence of ROS and RNS excess is damage to proteins, lipids and DNA. This oxidative damage to DNA, proteins, and other macromolecules are involved in the pathogenesis of various diseases through cell death and pathological dysfunction. The main diseases are diabetes, cancer, and aging of organism (Fernandez MC *et al*., 2013)

Diabetes mellitus (DM) describes a metabolic disorder of multiple etiology. DM is a leading cause of morbidity and mortality world over. It is estimated that approximately 1% of population suffers from DM. It occurs when pancreas does not produce enough insulin, or when the body cannot utilize the insulin it produces, or both, leading to an increased concentration of glucose in the body (hyperglycaemia). It is characterized by chronic hyperglycaemia, disturbances in carbohydrate, fat and protein metabolism like hyper lipaemia, glycosuria, negative nitrogen balance and sometimes ketonaemia.

Several pathological processes are involved in the development of DM which results in the destruction of the beta cells of the pancreas with consequent insulin deficiency and others that result in resistance to insulin action. The abnormalities of carbohydrate fat and protein metabolism are due to deficient action of insulin on target tissues resulting from insensitivity or lack of insulin (Tripathi KD, 2008)

Hyperglycaemia and free fatty acid intake are among the causes for oxidative stress conditions and also diabetic patients will be having a decrease in antioxidant defense mechanism. And also oxidative stress affect two major mechanisms that failing during diabetes; insulin resistance and insulin secretion.

Oxidative stress process in insulin resistance

ROS and RNS affect the insulin signaling cascade and leads to insulin resistance in diabetes. Disturbances in the cellular redistribution of insulin signaling components may alter the insulin cascade. And there will be insulin action impairment by cytokines in response to metabolic stress.

Oxidative stress process in insulin secretion

Pancreatic beta cells are especially sensitive to ROS and RNS, because their natural enzymatic antioxidant defenses are lower as compared to other tissues such as liver. And also they lack the ability to adapt their low enzyme activity levels in response to stress such as high glucose or high oxygen.

Oxidative stress in diabetic complications

Oxidative stress plays a main role in the development of diabetic complications at both microvascular and macrovascular level. The main tissues affected at the microvasculature levels are retina, glomerulus, and peripheral nerves. And also associated with accelerated atherosclerotic disease affecting arteries that supplies the heart, brain and lower extremities.

Plumeria pudica Jacq. which is also known as Wild plumeria, bridal bouquet, gilded spoon Plant is indigenous to Panama,Columbia and Venezuela and grows well in subtropical countries like India. It is grown throughout the plains of India as ornamental plant for its attractive flowers (Shinde PR *et al*., 2014)

It is an evergreen to semi-deciduous shrub with white flowers that continue to develop whenever the plant is in active growth. This profuse bloomer has an unusual spoon shaped leaves and its flowers are white with a yellow centre (Jaydeep S *et al.,* 2013) The use of this plant is not well explored but relative species were used as remedy for pain

and inflammation, parasite infestation etc (Heliana BF *et al.*,2015)

MATERIALS AND METHODS

Collection and identification of plant material

The leaves of *Plumeria pudica* Jacq were obtained from Punnamparambu, Thekkumkara, Trissur, Kerala, (India) in the month of November 2015 and authenticated by Jacob Abraham, Associate professor, Department of Botany, St.Thomas College, Thrissur, Kerala. A voucher specimen APSC/COL/06/2015 was deposited in Department of Pharmacology, Academy of Pharmaceutical Sciences, Pariyaram Medical College, Kannur, Kerala,India.

After authentication the leaves were collected, cleaned and dried in shade at room temperature. The dried leaves were pulverized in a mechanical grinder to obtain coarse powder.

Preparation of extract

The powdered plant (500g) was sieved through sieve No. 10 and the powder was subjected to defatting with petroleum ether for 6 hours. The filtered powder was then subjected to cold maceration with ethanol: water (70:30) for 7 continuous days. The hydroalcoholic extract was prepared by mixing with the help of a sonicator. It was then filtered through a muslin cloth and marc was discarded. The filtrate was concentrated using rotary vacuum evaporator and stored in a refrigerator for further use.

Primary phytochemical screening

For primary phytochemical screening freshly prepared hydroalcoholic extract of leaves were tested for the presence and absence of phytoconstituents such as carbohydrates, alkaloids, glycosides, tannins, flavonoids, phenolic compounds, steroids by using standard methods (Evans, WC *et al., 2003)*

IN VITRO DETERMINATION OF ANTIO XIDANT ACTIVITY

Reducing power assay

Different concentrations of extract and 1 ml of distilled water was mixed with phosphate buffer (2.5 ml, 2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%).The mixture was incubated at 50 0C for 20 min. A portion (2.5 ml) of trichloroacetic acid (TCA, 10%) was added to the mixture which was then centrifuged at 1500 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl3 (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Ascorbic acid was used as standard.

$$\text{Reducing power (\%)} = \frac{Ac-At}{Ac} \times 100$$

Where Ac is the Absorbance of the control and At is Absorbance of the test/standard.The tests were carried out in triplicate. The extract concentration providing 50% inhibition (IC50) was calculated from the graph of inhibition percentage plotted against extract concentration.

Nitric oxide radical scavenging assay

In this assay 0.5 ml of Sodium nitro prusside (10mmolL-1) in phosphate buffered saline pH 7.4, was mixed with different concentration of the extract and incubated at 25°C for 150 minutes. A control without the test compound, but an equivalent amount of methanol was taken. After 3 hrs, 0.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthyl ethylene diamine dihydrochloride) was added and incubated for 30 minutes for color development. Absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with N-1- naphathyl ethylene diamine dihydrochloride was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard. The scavenging activity on the nitric oxide was expressed as inhibition percentage using the following equation,

$$\text{Nitric oxide scavenging (\%)} = \frac{Ac-At}{Ac} \times 100$$

Where Ac is the absorbance of the control and At is the absorbance od test/standard.

The tests were carried out in triplicate. The extract concentration providing 50% inhibition (IC50) was calculated from the graph of inhibition percentage plotted against extract concentration (Rajamanikandan S *et al.*, 2011). .

DPPH radical scavenging assay

1 ml of various concentrations of the extract and standard ascorbic acid in methanol were added to 1 ml of a methanolic solution of DPPH (0.135 mM). The mixture was vigorously shaken and then allowed to stand at room temperature for 30 min in the dark. The absorbance of the mixture was measured at 517 nm by using a double-beam UV-Visible spectrophotometer. A mixture of 1 ml of methanol and 1 ml of DPPH solution was used as the control. The scavenging activity on the DPPH radical was expressed as inhibition percentage using the following equation:

$$\text{Scavenging (\%)} = \frac{Ac-At}{Ac} \times 100$$

Where Ac is the absorbance of the control and At is the absorbance of the test/standard.

The tests were carried out in triplicate. The extract concentration providing 50% inhibition (IC50) was calculated from the graph of inhibition percentage plotted against extract concentration **(**Jamuna S *et al.*,2012).

***In vitro* determination of anidiabetic activity**

Inhibition of carbohydrate digesting enzymes

Inhibition of α-amylase activity

Starch solution (0.1% w/v) was obtained by stirring 0.1 g of potato starch in 100 ml of 16mM of sodium acetate buffer. A reaction mixture containing 500µl of plant extract, 500µl 0.02M sodium phosphate buffer (pH 6.9 with 0.006M sodium chloride) and 500µl porcine α-amylase enzyme solution was incubated at 25^0C for 10 min. After pre incubation,500µl of 1% starch solution 0.02M sodium phosphate buffer (pH 6.9 with 0.006M sodium chloride) was added to each tube at 5s intervals. Reaction mixture was then incubated at 25^0C for 10min. The reaction was stopped by adding 1.0ml of dinitrosalicylic acid color reagent. The test tubes were then incubated in boiling waterbath for 15min and cooled to room temperature and absorbance was measured at 540nm **(**Nair S S *et al*.,2013)· Control represent 100% enzyme activity and were conducted in similar way by replacing the extract with vehicle. Experiment was repeated with standard Acarbose also. Results were compared

% inhibition = (Ac-As)/Ac x 100

Where Ac is the absorbance of control and As is the absorbance of sample

Inhibition of α-glucosidase activity

1mg protein equivalent to 10 units of α-glucosidase incubated with different concentrations of extracts for 5 min before initiating the reaction with substrates sucrose (37mM), in a final reaction mixture of 1 mL of 0.1 M phosphate buffer pH 7.2. The reaction mixture was incubated for 20 and 30 min at 37 °C and reaction was stopped by incubating in boiling water bath for 2 minutes. A tube with phosphate buffer and enzyme was maintained as control. The tubes were added with 250µl of glucose reagent and incubated for 10 minutes followed by measuring absorbance at 510nm. Experiment was repeated with both control and standard (Rengasamy KRR *et al*., 2013).

% inhibition = (Ac-As)/Ac x 100

Where Ac is the absorbance of control and As is the absorbance of sample

Measurement of glucose uptake by cells

Measurement of glucose uptake in HepG2 cells

Hep G2 hepatic cells was maintained in Dulbecco's modified eagles media supplemented with 10% FBS and grown to confluency at 37°c in 5 % CO_2 in a humidified atmosphere in a CO_2 incubator. The cells were trypsinized (500µl of 0.025% Trypsin in PBS/ 0.5mM EDTA solution for 2 minutes and passaged to T flasks in complete aseptic conditions. The cells were then sub cultured to a 24 well plate. After attaining 80% confluency, cells were kept in DMEM without glucose for 24 hours. Extracts were added to grown cells at a final concentration of 25 µg, 50 µg and 100 µg from a stock of 1mg/ml and incubated for 24 hours in DMEM containing 300mM glucose. An untreated control with high glucose was also maintained. After incubation cells were isolated by spinning at 6000 rpm for 10 minutes. Supernatant was discarded and 200µl of cell lysis buffer (1MTris Hcl,0.25M EDTA, 2M Nacl, 0.5% Triton) was added.The incubation was done for 30 minutes at 4°C and the glucose uptake was estimated using DNSA method. 100µl of sample was mixed with 100µl of DNSA and boiled for 10 minutes. After cooling it was diluted to 1 ml of distilled water. The OD was read at 600nm using UV visible spectrophotometer. All experiments were repeated in triplicates and mean average was used for calculation **(**Dorin D *et al.*, 2015).

Increase in glucose uptake = (As- Ac)/As × 100

Where As is the absorption of sample and Ac is the absorption of control

Determination of *in vitro* inhibition of glucose diffusion

25ml of 20mM of glucose solution and the plant extract of concentration 1% were dialyzed in dialysis bags against 200mL of distilled water at 37^0C in a shaker waterbath. Glucose content in the dialysate was determined at 30, 60, 120 and 180 min using glucose oxidase peroxidase diagnostic kit. A control test was carried out without sample (Ahmed F et al., 2011)

Glucose dialysis retardation index (GDRI) was calculated by

GDRI % =

$$\frac{\text{100 - glucose content with addition of sample}}{\text{glucose content of the control}} \times 100$$

RESULTS AND DISCUSSION

Preliminary phytochemical screening

Preliminary phytochemical screening of the leaf extract showed the presence of alkaloids,

glycosides, carbohydrates, flavonoids, phenolic compounds, proteins, steroids.

Reducing power assay

Reducing power activity is often used to evaluate the ability of natural antioxidants to donate electron. Many reports have revealed that there is a direct correlation between antioxidant activities and reducing power of certain plant extracts.

In the present study, the percentage of reducing power of the extract increased as the concentration of extract increased from 50µg/ml to 250µg/ml. Extract showed an IC50 value of 125µg/ml. (Table 1 and Figure 1).

Nitric oxide radical scavenging assay

Nitric oxide free radical is generated from the sodium nitroprusside in aqueous solution at physiological pH. This nitric oxide interacts spontaneously with the oxygen to produce stable products (nitrate and nitrite), which can be determined using Griess reagent. The antioxidant molecules or the free radical scavengers compete with the oxygen leading to the reduced production of nitrite. The extract showed good scavenging effects with IC50 value of 138.21µg/ml when compared with standard ascorbic acid. The scavenging activity was concentration dependent. (Table 2 and Figure 2)

DPPH radical scavenging assay

This method is based on the reduction of DPPH (2, 2-Diphenyl-1-picrylhydrazyl), a stable free radical and any molecule that can donate an electron or hydrogen to DPPH and thereby bleach the DPPH absorption. Because of its odd electron, DPPH gives a strong absorption maximum at 517 nm by visible spectroscopy (purple color). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, that is, a free radical scavenging antioxidant, the absorption strength is decreased and the resulting decolorization is stoichiometric with respect to the number of electrons captured. The scavenging reaction between (DPPH.) and an antioxidant (H-A)can be written as

$$\underset{\text{(Antioxidant)}}{\text{(DPPH)}} + \underset{\text{(Yellow)}}{\text{(H-A)}} \longrightarrow \underset{\text{(Purple)}}{\text{DHAP-H}} + \text{(A)}$$

HAPP showed a dose dependent radical scavenging activity on $DPPH^{\cdot}$ with an IC50 value of 271.64µg/ml. Percentage scavenging was purely concentration dependent from 50µg/ml to 250µg/ml. Activity was compared with standard ascorbic acid.

Inhibition of α amylase activity

In humans, the digestion of starch involves several stages. Initially, partial digestion by the salivary amylase results in the degradation of polymeric substrates into shorter oligomers. Later on in the gut these are further hydrolyzed by pancreatic amylases into maltose, maltotriose and small malto-oligosaccharides. The digestive enzyme (α-amylase) is responsible for hydrolyzing dietary starch (maltose), which breaks down into glucose prior to absorption. Inhibition of α-amylase can lead to reduction in post prandial hyperglycemia in diabetic condition.

Extract showed dose dependent inhibition of α-amylase when compared to a standard, Acarbose with an IC_{50} value of 660.69 µg/ml and358.48µg/ml respectively.

Inhibition of α-glucosidase activity

Hydroalcoholic extract showed concentration dependent reduction in percentage inhibition of α-glucosidase with an IC_{50} value of 650.83µg/ml. Acarbose showed an IC_{50} value of 439.48 µg/ml.

α-glucosidase is a membrane bound enzyme located on the epithelium of the small intestine, catalyzing the cleavage of disaccharides to form glucose. Inhibitors can retard the uptake of dietary carbohydrates and suppress post-prandial hyperglycemia. Therefore, inhibition of α-glucosidase could be one of the most effective approaches to control diabetes.

Determination of glucose uptake in cells

Diabetes mellitus is associated with insulin deficiency and decreased glucose uptake in skeletal muscles. The increased plasma free radicals observed in DM may impair insulin action thus contributing to the generation of hyperglycaemia. Skeletal muscle is the major tissue for blood glucose utilization and primary target tissue for insulin action. So if there is any impairment in this glucose uptake by the cells will leads to hyperglycaemia is the basis for measuring the glucose uptake by different cells. In this study we used HepG2 cells and glucose uptake by those cells were measured. [69] Metformin was taken as the standard for comparison. Hydroalcoholic extract of *Plumeria pudica* showed a concentration dependent increase in glucose uptake when compared with standard. (Table 6 and Figure 6)

Effect of extract on glucose diffusion

Another *in vitro* method which was adopted to demonstrate the antidiabetic activity was the effect of hydroalcoholic extract of *Plumeria pudica* on glucose diffusion. GDRI is a useful *in*

vitro index to predict the effect of a fiber on the delay in glucose absorption in the gastrointestinal tract. The rate of glucose diffusion was found to increase with time from 30 to 180 min. In the present study, the movement of glucose across the dialysis membrane was monitored once in 30 min till 180 min and it was found that, both the samples of plant extract demonstrated significant inhibitory effects on movement of glucose into external solution across dialysis membrane compared to control.(Table 7, 8 and Figure 7).

Table 1. Reducing power assay

	50	20.51	
	100	36.24	
HAPP	150	63.23	125.36
	200	84.04	
	250	93.97	
	50	28.88	
	100	51.81	
Ascorbic acid	150	79.51	
	200	94.66	93.05
	250	95.98	

Table 2. Nitric oxide radical scavenging assay

	50	7.38	
	100	38.03	
HAPP	150	55.42	138.21
	200	80.53	
	250	94.11	
	50	16.03	
	100	40.29	
Ascorbic acid	150	65.24	123.31
	200	88.36	
	250	95.15	

Table 3. DPPH radical scavenging assay

	50	8.65	
	100	23.53	
HAPP	150	30.27	271.64
	200	36.29	
	250	45.67	
	50	13.34	
	100	30.49	
Ascorbic acid	150	34.09	
	200	44.06	209.9
	250	61.80	

Table 4. Inhibition of α-amylase enzyme

	125	12.16	
	250	31.47	
HAPP			660.69
	500	45.84	

	1000	66.62	
	125	23.86	
	250	48.08	
Acarbose			359.48
	500	73.05	
	1000	81.37	

Table 5. Inhibition of α-glucosidase

	125	8.54	
	250	21.99	
HAPP			650.48
	500	43.20	
	1000	73.46	
	125	20.09	
	250	38.73	
Acarbose			439.48
	500	63.47	
	1000	86.06	

Table 6. Glucose uptake by HepG2 cells

	25	14.10
	50	28.05
HAPP		
	100	43.72
	200	63.13
	25	27.20
	50	46.14
Metformin		
	100	57.59
	200	72.40

Table 7. Effect on glucose diffusion

Groups ***Groups***	Glucose content in dialysate (mmol/L) ***Glucose content in dialysate(mmol/L)***			
	30 min ***30min***	60 min ***60min***	120 min ***120min***	180 min ***180min***
Control Control	0.88± 0.01 0.88±0.01	1.36± 0.01 1.36±0.01	1.68±0.01 1.68±0.01	1.87±0.02 1.87±0.02
HAPP HAPP	0.56±0.01 0.56±0.01	0.94±0.02 0.94±0.02	1.22±0.01 1.22±0.01	1.40±.01 1.40±.01

Table 8. Glucose dialysis retardation index (GDRI)

Groups **Group**	GDRI(%)			
	30 min **30min**	60 min **60min**	120 min **120min**	180 min **180min**
HAPP HAPP	36.36 36.36	30.88 30.88	27.38 27.38	25.13 25.13

Fig 1. Reducing power assay

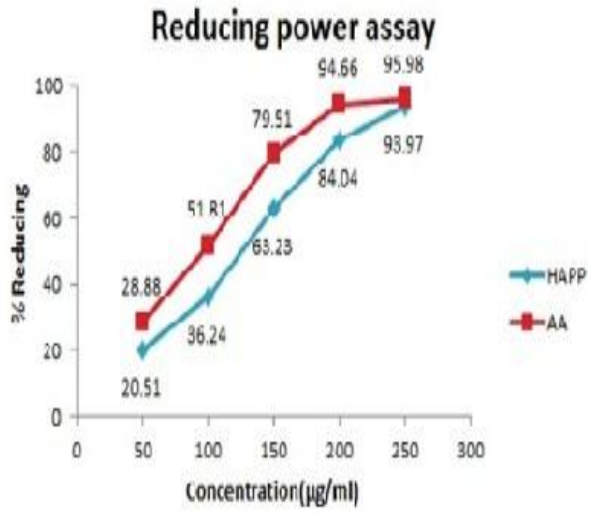


Fig 2. Nitric oxide scavenging assay

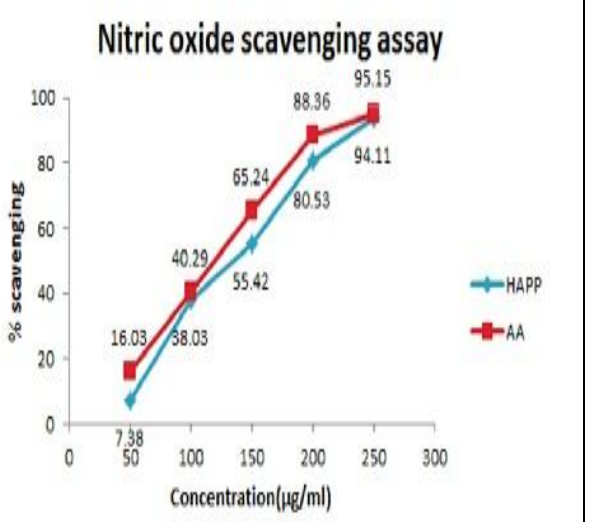


Fig 3. DPPH radical scavenging assay

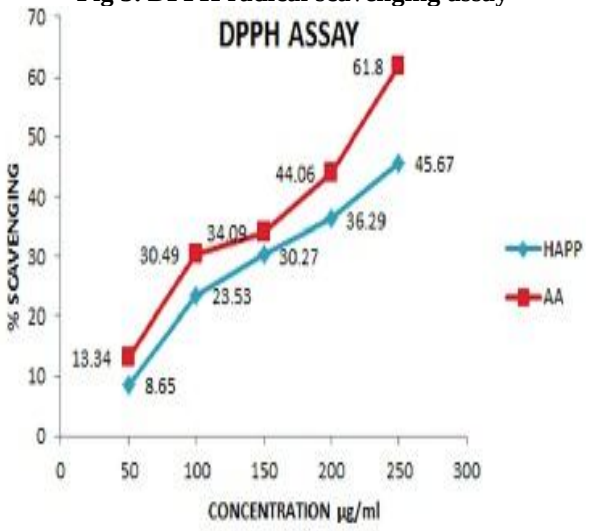


Fig 4. Inhibition of α-amylase enzyme

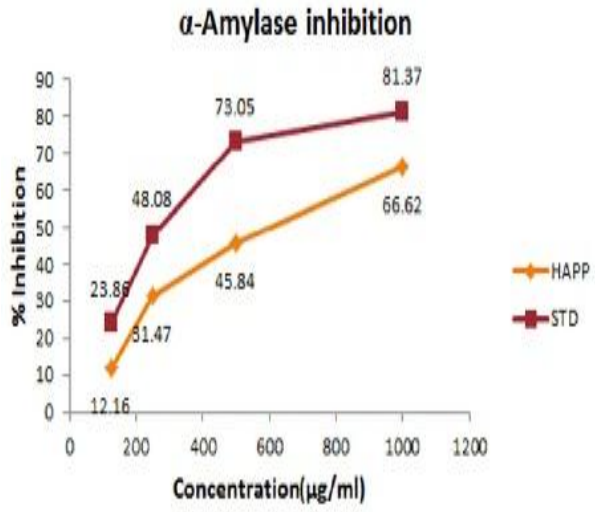


Fig 5. Inhibition of α-glucosidase

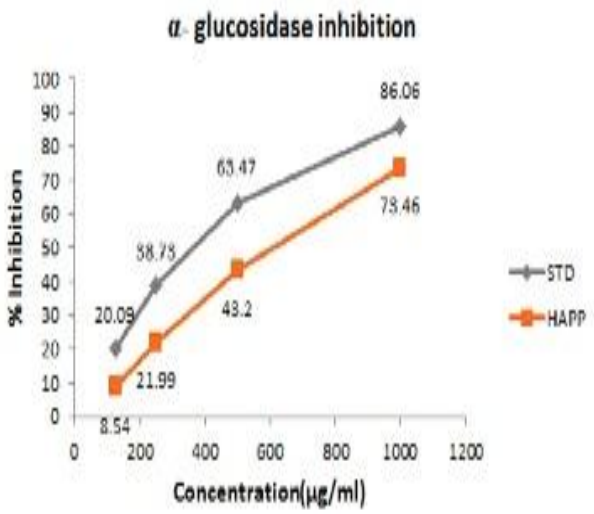


Fig 6. Glucose uptake by HepG2 cells

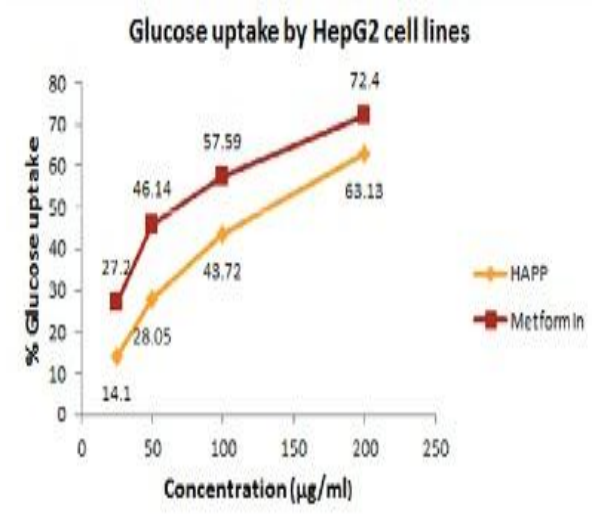
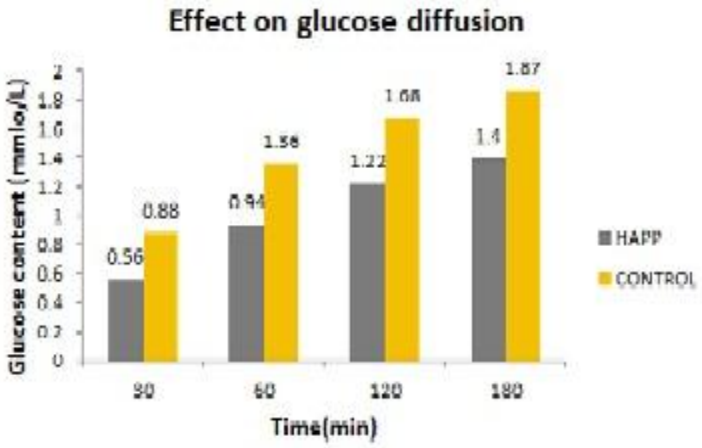


Fig 7. Effect on glucose diffusion



CONCLUSION

In conclusion, the results suggests that the hydroalcoholic extract of *Plumeria pudica* Jacq exhibits a significant antioxidant potential which is proved by the techniques such as DPPH scavenging assay method, reducing power assay and hydrogen peroxide assay. The antioxidant properties elicited by the extract may be due to the presence of flavonoids and phenolic compounds. So the leaf extract is a potent source of natural antioxidant, which might be helpful in preventing the progress of various oxidative stresses. Some studies suggest that flavonoid compounds may improve and stabilize the secretion of insulin from pancreatic beta cells. That is they possess insulinomimetic activity. They may result in a new antidiabetic drug with less toxicity and potential for synergism with currently existing anti-diabetics in market. Therefore anti-diabetic activity of the plant extract may be due to the presence of flavonoids.

Further studies are suggested for isolation and identification of individual component and *in vivo* studies are needed for understanding their mechanism as an antioxidant prior to clinical use.

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CONFLICT OF INTEREST

No interest.

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