The *in-vitro* antidiabetic activity of *Phoenix roebelenii* leaf extract

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Abstract

Aim: The aim of the study was to evaluate *in-vitro* α-amylase inhibitory activity, α-glucosidase inhibitory activity and glucose diffusion inhibition activity of various leaf extracts of *Phoenix roebelenii*. **Materials and Methods:** *P. roebelenii* leaf extracts were prepared by maceration in ethanol, methanol, water, acetone, and petroleum ether. The extracts were used for evaluating their α-amylase and α-glucosidase inhibitory activity using dinitrosalicylic acid method. The results were compared with standard drug acarbose. Glucose diffusion was studied using dialysis membrane method. **Results:** The ethanolic extract showed maximum inhibitory activity for both α-amylase ($75.5 \pm 0.66\%$) and α-glucosidase ($77.5 \pm 1.07\%$) at 400 µg/ml in a concentration dependent manner. Methanolic extract showed nearly similar activity. Acarbose showed $80.7 \pm 0.74\%$ inhibition for α-amylase and $80.2 \pm 0.23\%$ for α-glucosidase at 400 µg/ml. The ethanolic and methanolic extracts also showed a significant amount of inhibition of glucose across a dialysis membrane. **Conclusion:** The plant can be further studied to isolate the compound(s) responsible for the antidiabetic properties.

Key words: α-amylase inhibitory activity, α-glucosidase inhibitory activity, antidiabetic, glucose diffusion, medicinal plants, *Phoenix roebelenii*

INTRODUCTION

Type 2 diabetes or diabetes mellitus is a chronic metabolic disorder which affects people of all ages across the globe. This disease is characterized by hyperglycemia, that is, increase in the blood glucose level. This can mainly be attributed to the lack of insulin production. In India, this disease is fast reaching epidemic proportions and India is becoming the diabetes capital of the world,[1] by accounting for nearly 20% of the diabetic global patients.[2] Studies have shown that in 2000, approximately 31.7 million people were affected by diabetes. It is estimated that by 2030, nearly 79.4 million Indians will be suffering from this disease.[3] This is a global epidemic which has affected 171 million people in 2000 and expected to rise to 366 million by 2030.[4] For these reasons, there is dire need to develop newer and better medication for the control and cure of this deadly disease.

The treatment of Type 2 diabetes or noninsulin-dependent diabetes mellitus has two main parameters – preventing the breakdown of dietary complex carbohydrates into glucose and preventing the diffusion of the glucose through the intestinal membrane into blood stream. The inhibition of carbohydrate hydrolysing enzymes is helpful is reducing postprandial blood glucose levels.[5,6] The two main carbohydrate hydrolysing enzymes responsible for the breakdown of dietary polysaccharides are α-amylase and α-glucosidase.[7] The first step in digestion of dietary starch is catalyzed by the pancreatic α-amylase, which converts the starch into a mixture of small oligosaccharides. After this step α-glucosidase further degrades the oligosaccharides into glucose. This glucose then diffuses through the intestine wall into the blood stream, increasing postprandial blood glucose levels.

Medicinal plants have been used for the treatment of several diseases since ancient times.[8] In recent times, many pharmaceutical companies are looking forward on researching the bioactive compounds obtained from traditionally used medicinal plants and synthesizing them.[9,10] Throughout history, several medicinal plants such

as the *Aloe vera*,[11] *Arnica montana*,[12] *Allium sativum*,[13] and *Azadirachta indica*,[14] were used to treat diseases such as stomach ailments, liver problems, diabetes, urinary dysfunctions, wound healing, inflammation, fever, cough and cold, jaundice, cardiovascular diseases, and several other.[15,16] Herbal medicines have been proven to have less side effects as compared to synthetic drugs.[17] For this reason, they are being increasingly used in the modern health-care system as an alternate to the standard synthetic drugs.[18]

Phoenix roebelenii is not popularly known for its medicinal values. It has not been explored for its medicinal properties. *P. roebelenii*, also known as pygmy date palm belongs to the *Arecaceae* family. It is found in Southeast Asia, commonly in Laos, Vietnam and Yunnan region of China.[19] It is a dwarf plant, growing 60-120 cm tall, having a majestic crown. It is commonly used for landscaping purposes as a potted plant.[20]

Our main objective was to determine the α-amylase and α-glucosidase inhibitory activity of the various extracts of *P. roebelenii*. If the bioactive compounds present in the leaf sample can inhibit the carbohydrate hydrolyzing enzymes, then the breakdown of dietary starch and other complex carbohydrates will be inhibited. Again, we are also exploring the ability of the extracts to prevent glucose diffusion across a membrane. Both these effects combined, will enable us to reduce blood glucose level.

MATERIALS AND METHODS

Chemicals and Reagents

The chemicals α-amylase, α-glucosidase, soluble starch, para-nitrophenyl glucopyranoside, dinitrosalicylic acid (DNSA), and dialysis membrane were purchased from Hi Media Laboratories, Mumbai, India. The solvents were of AR grade and were purchased from SRL Pvt. Ltd., Mumbai, India. All other chemicals and reagents used in this study were purchased from local manufacturers.

Glassware and Apparatus

The glassware, such as test tubes, conical flasks, and beakers were purchased from Borosil Glass Works Ltd. The plastic wares such as centrifuge tubes, micropipette tips, and eppendorf tubes were obtained from Tarsons Products Pvt. Ltd. Orbitek Orbital Shaker was used for Maceration. For spectrophotometry, Shimadzu UVmini-1240 ultraviolet (UV) – visible spectrophotometer was used. For volumetric measurement, EPPENDORF Research-Plus M30533BB 100-1000 μl; 10-100 μl micropipettes were used. A SHIMADZU AUW220D Microbalance was used for weight measurements.

Extract Preparation

The leaf samples of *P. roebelenii* were collected and authenticated by VIT Horticulture Department. The leaves were washed thoroughly with distilled water. Leaves showing signs of browning, curling or any other signs of damage were discarded. The selected leaves were then shade dried for 5 days, then powdered using a mechanical grinder. The powder obtained was stored in a sterilized airtight container.

For extract preparation, the method of maceration was used. 10 g of powdered leaf sample was put into a 250 ml sterile conical flask. 100 ml of the respective solvent was poured into each of the conical flasks. The solvents used were: Methanol, ethanol, distilled water, acetone, and petroleum ether. The solvents were sterilized under an UV light for 5 min to remove any microbial contamination. The conical flasks were properly sealed, marked and loaded onto the orbital shaker at 180 rpm and kept for 72 h at room temperature.

The solvent phase was collected by filtration using a Whatmann No. 2 filter paper. The extract thus obtained was concentrated using vacuum rotatory evaporator. The solvents were further evaporated by leaving the extracts in a hot air oven at 40°C. The crude extracts obtained were stored under dry, air tight condition for further use.[21]

Assay for α-amylase Inhibitory Activity

Four different concentrations were prepared – 400, 300, 200, and 100 μg/ml for each of the five extracts in test tubes. Similarly, four different concentrations were prepared for acarbose to be used as a standard drug for comparison. A control test tube was prepared which did not contain any plant extract or drug. A 0.5 mg/ml solution of α-amylase was prepared in a buffer solution, which consists of 0.2 M sodium phosphate buffer at 6.9 pH with 0.006 M NaCl. A 1% starch solution is prepared my mixing 1 g starch in 100 ml buffer solution.

To each of the 25 test tubes, 1 ml of the earlier prepared α-amylase solution is added. The test tubes are allowed to incubate for 10 min at room temperature. After this, 0.5 ml starch solution is added to all the test tubes and left for incubation at 25°C for 10 min. Then, 1 ml DNSA is added to each of the test tubes and incubated in boiling water for 5 min to stop the reactions.

The test tubes are diluted to 10 ml by the buffer solution. Their absorbance was observed using the UV – visible spectrophotometer at 540 nm. The % inhibition of α-amylase activity was calculated using the following formula:

$$[(OD_{Control} - OD_{Sample})/OD_{Control}] \times 100$$

The entire process was triplicated.[21]

Assay for α-glucosidase Inhibitory Activity

The five extracts were taken and for each of them, four different concentrations were prepared – 400, 300, 200, and 100 µg/ml. The same procedure was followed for acarbose, which is the standard drug. A control test tube was prepared which did not contain any plant extract or drug. A 0.075 unit/ml solution of α-glucosidase was prepared, and 1 ml of the solution was added to each of the test tubes.

A 3 mM solution of p-nitrophenyl glucopyranoside was prepared which was used as substrate. 0.5 ml of this solution was added to each of the test tubes to start the reaction. Then, the test tubes were incubated for 25 min at 37°C. To stop the reaction, 1 ml 0.02 M solution of sodium bicarbonate, Na_2CO_3 was added to all the test tubes and incubated in room temperature for 10 min.

The test tubes were made up to 10 ml. Their absorbance was observed using the UV – visible spectrophotometer at 400 nm which is due to the release of p-nitrophenol. The % inhibition of α-glucosidase activity was calculated using the following formula:

$$[(OD_{Control} - OD_{Sample})/OD_{Control}] \times 100$$

The entire process was repeated thrice.[21]

Glucose Diffusion Inhibition Assay

For this assay, two different concentrations of each of the five extracts, as well as the standard drug acarbose, were prepared – 400 and 200 µg/ml in test tubes. A control was prepared for reference. A 0.15 M NaCl with 0.22 mM glucose was prepared. To each of the test tubes, 1 ml of this solution was added.

To test the diffusion of glucose across a membrane, 3 cm long strips of 12000 MW dialysis membrane was taken. Each of 13 samples was loaded into separate membrane strip. Both ends of the strips were sealed properly to prevent any leakage. 100 ml glass beakers were taken and filled with 40 ml 0.15 M NaCl and 10 ml distilled water.

Each of the strips was immersed in separate beakers and kept in the Orbital Shaker at 150 rpm. 1 ml of the solution in the beaker was taken out every 30 min for 3 h. This solution was tested for glucose concentration using the DNSA method and compared with the control. The entire process was repeated three times.[21]

Inhibitory Concentration 50% (IC$_{50}$) Value Calculation

The concentration of the extract or drug which inhibits 50% of enzyme activity is termed as the IC$_{50}$. Acarbose was used as the standard drug for reference and comparison. For each of five extracts, a standard dose response curve was plotted at all the different concentrations. From the plotted curves, the IC$_{50}$ value for each of the extract was calculated using Graph Pad Prism version 6.0 Software.

Statistical Calculations

For the two-way ANOVA calculations for all the sets of data obtained, SPSS version 21.0 was used. The IC$_{50}$ values were calculated using Graph Pad Prism version 6.0 Software. All the values in the table are given in the form of mean ± standard deviation.

RESULTS

Assay for α-amylase Inhibitory Activity

Table 1 shows the results of α-amylase inhibitory action of the various leaf extracts of *P. roebelenii* at the four different concentrations. Among the five extracts, ethanolic and methanolic extracts have shown the highest inhibitory activity. The maximum inhibition of the ethanolic extract was 75.5 ± 0.66% at 400 µg/ml, while for methanolic extract it was 70.4 ± 0.62%, also at 400 µg/ml. For acarbose, the maximum inhibition was 80.7 ± 0.74% at 400 µg/ml.

Figure 1 shows the comparison of the α-amylase inhibitory activity of the extracts with that of acarbose. Since acarbose is considered the reference, the inhibitory activity of acarbose has been considered as 100%. Based on that the relative inhibitory activity has been calculated.

Assay for α-glucosidase Inhibitory Activity

The results of α-glucosidase inhibitory action of the various leaf extracts of *P. roebelenii* at the four different concentrations have been summarized in Table 2. In this case also, ethanolic and methanolic extracts have shown
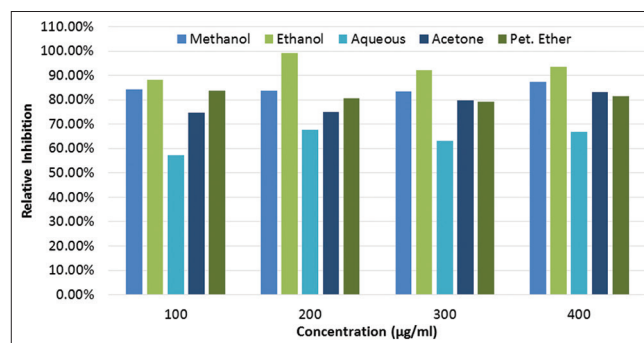

Figure 1: Relative inhibition of α-amylase activity for different extracts at different concentrations as compared with acarbose

Table 1: Inhibition of α-amylase activity for different extracts at different concentrations

Concentration µg/ml	Standard drug acarbose	Inhibition (in %)				
		Methanol	Ethanol	Aqueous	Acetone	Petroleum ether
100	66.8±0.30	56.3±0.36*	59.0±0.26*	38.3±0.76*	49.9±0.55*	56.0±0.65*
200	71.1±0.93	59.5±0.70*	70.6±0.55	48.2±0.90*	53.4±0.67*	57.4±1.96*
300	79.1±1.04	65.9±0.60*	72.9±1.26*	49.9±0.18*	63.2±2.00*	62.6±0.30*
400	80.7±0.74	70.4±0.62*	75.5±0.66*	53.9±0.74*	67.2±0.61*	65.8±0.31*

The values are mean±standard deviation for three observations. *$P<0.001$

Table 2: Inhibition of α-glucosidase activity for different extracts at different concentrations

Concentration µg/ml	Standard drug acarbose	Inhibition (in %)				
		Methanol	Ethanol	Aqueous	Acetone	Petroleum ether
100	66.0±0.44	59.6±0.46*	61.3±0.37*	37.3±0.28*	48.7±0.36*	50.2±0.07*
200	71.8±0.40	70.2±0.25*	72.1±0.37	45.1±0.50*	54.3±0.36*	56.4±0.48*
300	76.3±0.13	73.1±0.63*	74.3±0.67*	49.9±0.29*	60.6±0.32*	59.2±0.60*
400	80.2±0.23	75.5±0.09*	77.5±1.07*	55.3±0.43*	66.7±0.32*	63.1±0.13*

The values are mean±standard deviation for three observations. *$P<0.001$

Table 3: Diffusion of glucose across the membrane for extracts at 400 µg/ml at different time intervals

| Time (min)	Control	Glucose concentration (in mg/ml)				
		Methanol	Ethanol	Aqueous	Acetone	Petroleum ether
30	3.44±0.04	1.12±0.06	1.06±0.04	2.09±0.06	1.57±0.05	1.42±0.02
60	3.91±0.01	1.33±0.04	1.24±0.02	2.50±0.05	1.91±0.05	1.53±0.01
90	4.4±0.03	1.47±0.02	1.43±0.05	2.62±0.04	2.23±0.05	1.75±0.02
120	5.00±0.02	1.53±0.04	1.51±0.05	2.91±0.09	2.35±0.11	1.94±0.04
150	5.57±0.02	1.87±0.05	1.65±0.07	3.39±0.25	2.60±0.12	2.29±0.05
180	6.19±0.06	2.17±0.04	1.87±0.05	3.80±0.32	2.69±0.17	2.96±0.04

The values are mean±standard deviation for three observations. All values are significant at $P<0.001$

the highest inhibitory activity. The ethanolic extract has shown the maximum inhibitory activity of 77.5 ± 1.07% at 400 µg/ml. The methanolic extract has shown the maximum inhibitory activity of 75.5 ± 0.09% at 400 µg/ml. For acarbose, the maximum inhibitory activity was found to be 80.2 ± 0.23% at 400 µg/ml.

Similarly, Table 4 shows the relative inhibitory activity of each of the extracts. For comparison, the inhibitory activity of the standard drug acarbose has been considered to be 100%.

Glucose Diffusion Inhibition Assay

The results for glucose diffusion inhibition are given in Tables 3 and 4 for 400 µg/ml and in Tables 5 and 6 for 200 µg/ml. For calculating the relative inhibition of movement of glucose, the glucose diffusion for the control sample was considered to be 100%. Maximum inhibition of glucose was shown by ethanolic extract for both 400 µg/ml (69.77 ± 1.00%) and 200 µg/ml (58.72 ± 0.43%) after 180 min. Methanolic extract also showed a significant amount of inhibition for 400 µg/ml (64.87 ± 0.9%). For
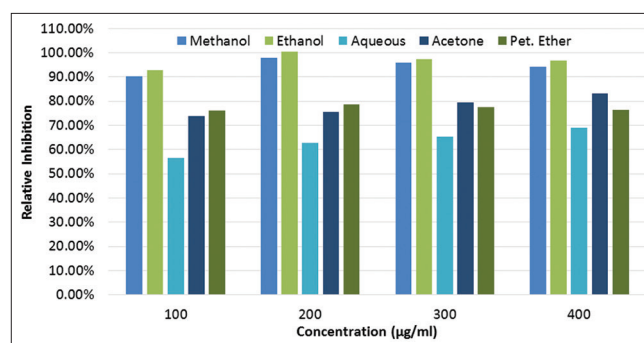
Figure 2: Relative inhibition of α-glucosidase activity for different extracts at different concentrations as compared with acarbose

200 µg/ml, the inhibition of the ethanolic (57.11 ± 0.56%) and methanolic extracts (57.05 ± 0.93%) after 180 min was nearly similar.

The acetone and petroleum ether extracts showed moderate amounts of inhibition at both 400 and 200 µg/ml. However, the aqueous extract showed poor inhibition.

Table 4: Relative movement of glucose across bio-membrane for extracts at 400 µg/ml

Time (min)	Relative inhibition of movement of glucose (in %)				
	Methanol	Ethanol	Aqueous	Acetone	Petroleum ether
30	67.35±1.35	69.27±1.27	39.15±1.8	54.45±1.53	58.62±0.88
60	66.10±0.98	68.23±0.48	36.20±1.18	51.28±1.42	60.99±0.23
90	67.06±0.67	67.81±1.10	41.24±1.08	49.92±1.28	60.63±0.54
120	69.46±0.91	69.67±0.97	41.80±1.75	53.06±2.36	61.14±0.68
150	66.53±0.76	70.41±1.30	39.28±4.43	53.43±2.21	59.00±0.75
180	64.87±0.90	69.77±1.00	38.54±4.78	56.51±2.84	52.15±0.92

The values are mean±standard deviation for three observations

Table 5: Diffusion of glucose across bio-membrane for extracts at 200 µg/ml at different time intervals

Time (min)	Control	Glucose concentration (in mg/ml)				
		Methanol	Ethanol	Aqueous	Acetone	Petroleum ether
30	3.44±0.04	1.64±0.04	1.42±0.00	2.58±0.04	2.12±0.01	2.03±0.01
60	3.91±0.01	1.76±0.03	1.51±0.03	3.01±0.02	2.24±0.02	2.27±0.04
90	4.45±0.03	1.95±0.03	1.74±0.04	3.14±0.02	2.74±0.03	2.74±0.08
120	5.00±0.02	2.20±0.03	1.99±0.05	3.45±0.02	3.04±0.02	2.93±0.02
150	5.57±0.02	2.43±0.02	2.25±0.03	4.11±0.04	3.51±0.05	3.07±0.04
180	6.19±0.06	2.65±0.02	2.65±0.04	4.67±0.05	3.87±0.04	3.46±0.04

The values are mean±standard deviation for three observations. All values are significant at $P<0.001$

Table 6: Relative movement of glucose across bio-membrane for the extracts at 200 µg/ml

Time (min)	Relative inhibition of movement of glucose (in %)				
	Methanol	Ethanol	Aqueous	Acetone	Petroleum ether
30	52.32±1.51	58.72±0.43	25.09±1.04	38.27±0.97	41.08±0.31
60	55.11±0.69	61.33±0.80	23.17±0.73	42.76±0.55	41.99±0.98
90	56.21±0.81	60.86±0.89	29.41±0.05	38.33±0.43	38.39±2.11
120	56.07±0.43	60.27±0.83	31.06±0.71	39.13±0.53	41.46±0.52
150	56.42±0.23	59.59±0.62	26.36±0.93	37.12±1.01	44.89±0.62
180	57.11±0.56	57.05±0.93	24.57±0.62	37.38±1.24	44.01±0.96

The values are mean±standard deviation for three observations

Table 7: The IC_{50} values for α-amylase and α-glucosidase inhibitory activities

Sample	IC_{50} value (in mg/ml)	
	α-amylase	α-glucosidase
Acarbose	22.45	21.60
Methanolic extract	60.94	40.37
Ethanolic extract	42.68	34.63
Aqueous extract	276.30	282.67
Acetone extract	115.09	121.72
Petroleum ether extract	50.49	99.31

IC_{50}: Inhibitory concentration 50%

Table 7 shows the IC50 values of the extracts for α-amylase and α-glucosidase inhibitory activity

DISCUSSION

Metformin[22] and acarbose[23] are two popular drugs used in the treatment of diabetes mellitus. However, several studies have shown that these drugs have certain side effects. Acarbose has been reported to cause frequent gastrointestinal complications, such as diarrhea and flatulence.[24] Metformin causes side effects such as gastrointestinal upset, lactic acidosis, and loss of weight.[25] On the other hand, bioactive compounds isolated from medicinal plants have much less side effects and are thus being preferred over synthetic drugs in our modern health-care system. Thus, there is need to explore various medicinal plants which can be effectively used for the control and treatment of diabetes.

In our present study, we have examined the *in-vitro* antidiabetic activities of the five extracts at four concentrations, comparing

them with the standard drug acarbose. From Tables 1 and 2, we can see that both methanolic and ethanolic extracts have shown significant inhibition of carbohydrate hydrolyzing enzymes.

By comparing the results, we can see that the α-amylase inhibitory activity of the ethanolic extract at 400 μg/ml was 93.56% of that of acarbose. For methanolic extract, it was slightly less 87.24%, but still significant. For the α-glucosidase inhibitory activity, the ethanolic extract, at 400 μg/ml had an inhibitory activity of 96.63% as compared with acarbose. For the methanolic extract, the inhibition was 94.14%, which is very much significant.

On the other hand, the petroleum ether and acetone extract showed a significant degree of inhibitory activity of both α-amylase and α-glucosidase. But they were not as high as that of the ethanolic and methanolic extracts. However, the aqueous extract showed poor inhibitory activity. Thus, we can conclude that the crude ethanolic and methanolic extracts are very much effective as compared with acarbose.

In this study, we have used a crude extract. On performing Benedict's test and Fehling's test on the extracts, we have observed the presence of carbohydrate. Hence, when we are using the extracts in the enzyme inhibitory assays, the samples with the extracts will be having a slightly more amount of carbohydrate as compared with the acarbose sample. Thus, if we fractionate the extracts to remove the carbohydrate, we should get a higher inhibitory activity.

Tables 3-6 show the inhibition of glucose diffusion across the dialysis membrane. In this case, also, we see that the ethanolic and methanolic extracts are very much effective in inhibiting the movement of glucose across the membrane, for as long as 180 min. For the other extracts, the pattern observed is very much similar to that of the previous assays. Acetone and petroleum ether have shown moderate inhibition, whereas the aqueous extract has shown very poor inhibition.

CONCLUSION

The results of our study indicate that the ethanolic, as well as the methanolic leaf extracts of *P. roebelenii* can effectively inhibit the breakdown of complex carbohydrates as well as reduce the rate of absorption of glucose, thus reducing postprandial hyperglycemia. Further studies need to be done on isolating the bio-active compound responsible for the antidiabetic activities. The results obtained in this study needs to be further verified using *in-vivo* animal model studies.

ACKNOWLEDGMENTS

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