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# Phytochemical Screening, *In-vitro* and *In-vivo* Anti-diabetic Activity of *Nelumbo nucifera* Leaves against Alloxan-Induced Diabetic Rabbits

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## ABSTRACT

A recent survey has demonstrated that traditional healers and herbalists frequently use *Nelumbo nucifera* plant to treat diabetes. The plant investigated here has been previously reported as natural folk medicine, but until now no scientific investigation of potential anti-diabetic effects have been reported. Plant leaves of *Nelumbo nucifera* were screened out for quantitative analysis of phytochemicals, *in-vitro* anti-diabetic activity (Alpha-amylase inhibitory assay) and *In-vivo* anti-diabetic assay of animals (rabbits). Quantitative analysis indicates that different phytochemicals were present in plant leaves extract/fractions. *In-vitro* antidiabetic analysis results poses that butanol fraction is significantly more active than other fractions or extract in antihyperglycemic activity with minimum $IC_{50}$ value of 8.56µg/ml. For *In-vivo* anti-diabetic assay, animals (rabbits) were examined and animals were divided into 10 groups and except blank group all were treated with standard and plant extracts/fractions doses (25mg and 50mg per Kg body weight) as an aqueous suspension. Their blood sugar level was thoroughly recorded with a glucometer before the administration of sample and standard drug. Alloxan Monohydrate was administrated orally (as an ice-cold water solution) at dose rate 150mg/kg body weight for one week to make animals hyperglycemic. *In-vivo* analysis indicates that butanol fraction is the most active fraction in antihyperglycemic activity. So Leaves of *N. nucifera* can be used as antidiabetic remedy. So, it is strongly recommended that *N. nucifera* leaves should be elucidated further for isolation of active phytochemicals.

**Key words:** Alpha amylase, Blood sugar level, Hyperglycemia, Hypoglycemia.

## INTRODUCTION

Medicinal plants are considered as a gift of nature and are used to cure a number of diseases of human beings because of presence of naturally occurring phytochemicals. These chemicals are alkaloids, tannins, flavonoids and phenolic compounds. This is why, flora are used for the cure of different diseases Abdelaaty *et al.* (2018). *Nelumbo nucifera Gaertn* (*Nelumbonaceae*) is known by numerous common names like sacred lotus, Indian lotus, bean of India, and simply lotus. The Linnaean binomial system currently recognized the name of this specie as *N. nucifera Gaertn* Bhardwaj *et al.* (2015). Nelumbo nucifera is an aquatic herb with white or red coloured flowers. Their seed have demonstrated antioxidant and antifertility action Deepa *et al.*(2008). *N. nucifera* water leaves extract has anti-tumor activity and has significant potential as a chemotherapeutic agent Yang *et al.* (2011), hot water extract of different parts (flowers, roots, seed and leaf) of plant *N. nucifera* show anti-wrinkle and skin whitening effects, leaf extracts can be used as a source of natural antioxidant for potential preventative therapies against angiogenesis-related disease Kim *et al.* (2011). Every year, millions of people get affected by diabetes. The population of diabetic patient increasing worldwide and it is estimated that about 336 million peoples are diabetic and about 552 million peoples are expected to have diabetes in 2030 Sharma and Rhyu (2014). This disease increases 25% chance of blindness, 20% renal

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failure and 2-3% heart diseases. It may produce either by non-proper functioning of beta-cells of the pancreas or by inheritance, excessive use of drug and by malnutrition Ahmed *et al.* (2010). At present two types of diabetes are here *i.e*. Type-1 diabetes and Type-2 diabetes. Type-1 produce due to complete non-secretion of insulin from beta cells of the pancreas and type-2 appear due to insufficient or non-action of insulin. Type-1 is the most common chronic condition in childhood or adolescence and affect 0.3-0.6% young individuals of the age of 20 Rodin *et al.* (2002). At present, different formulated drugs are being used to control hyperglycemia conditions like insulin sensitizers, insulin secret a gogues, inhibitors of sugar cleavage sulfonylureas, thiazolidinediones, biguanides, dipeptidyl peptidase IV inhibitors and α-glucosidase inhibitors Sharma and Rhyu (2014). All of these drugs control the homeostasis of blood sugar by different mechanisms. But there are some drawbacks/side effects which limit them for longer use such drugs. So there is a need of some those chemicals which show effective anti-diabetic results and low side-effects. Plants are a good source of such naturally occurring compounds which show better anti-diabetics effects with minimum side effects. The phytochemicals reported in leaf of Nelumbo nucifera are "Quercetin- 3-O-D- glucuronide, Tartaric acid, Quercetin, Roemerine, Nuciferine, Nornuciferine, Armepavine, Acetic acid, Pronuciferine, N-nornuciferine, Anonaine, Liriodenine, Gluconic acid, Malic acid, Ginnol, Nonadecane, Succinic, Quercetin 3-O-Dxylopyranosyl-D galactopyranoside, Rutin, Hyperin, Isoquercitrin, Keshav *et al*. (2015), hydnocarpin B and hydnocarpin D, isohydnocarpin A, flavolignan Jong *et al.* (2014).

## MATERIALS AND METHODS

### Plant collection

The fresh leaves of plant *N. nucifera* were collected from different areas of district Multan in April 2015. The plant leaves were identified and authenticated by local Plant Botanist, Department of Botany, BZU, Multan, Pakistan and Kept under in herbarium of the same department vocuhur name NN-1. Approval required for collection/cutting is not applicable.

### Preparation of plant extract/fractions

The leaf powder (100g) was soaked in 300mL, 95% ethanol for 48 hours and the filtrate was collected after 48 hours and the filtrate was concentrated at 34°C with the help of Vacuum Rotary Evaporator and partitioned with n-Hexane, ethyl acetate, butanol to get their fractions.

### Quantitative analysis for phytochemicals

Chemical tests for screening and identification of phytochemicals in the experimental plant extract and fractions were carried out using the standard procedures for Alkaloids, Phenols, Flavonoids, Saponins and Tannins as described by (Hussain *et al.*2011; Abidemi 2013).

### *In-vitro* anti-diabetic assay

Alpha-amylase inhibitory assay was carried out according to the method described by Rege and Chowdhary (2014) with little modifications. In labelled small test tubes an assay mixture (containing 100 µl phosphate buffer (of PH 6.8), 20 µl alpha-amylase and 40 µl of different concentrations of plant leaf extract/fractions) was pre-incubated for 10 minutes at room temperature and then 40µl soluble starch was added in a assay mixture tubes as a substrate and incubated further at 37°C for 15 minutes. The reaction was stopped by adding 40 µl 1N HCl followed by the addition of 200 µl iodine reagent (which was prepared by mixing 5mM KI and 5mM $I_2$). The absorbance was recorded at 620nm by spectrophotometer. Each experiment was repeated three times along with appropriate blanks. The negative control (blanks) samples were prepared without plant extract/fraction. Acorbose at various concentrations (125, 250, 500 and 1000µg/ml) was used as the reference alpha amylase inhibitor. The percentage inhibition was calculated from given formula:

$$\text{Inhibition (\%)} = \frac{\text{Abs (negetive control) - Abs (sample)}}{\text{A (negetive control)}} \times 100$$

The $IC_{50}$ values were determined from plots of percent inhibition versus log inhibitor concentration.

### *In-vivo* anti-diabetic assay

### Experimental design

White healthy 60 rabbits of 20-25 week of age (1- 1.5Kg body weight) were purchased from local market, from a single supplier and were divided into 10 groups and named as a blank group, Tolbutamide untreated (negative control) group, Tolbutamide treated (positive control) groups, Ethanol extract treated groups, Ethyl acetate fraction treated groups and butanol fraction treated groups. Each group contains 6 rabbits. All the animals were kept in neat and clean animal room at Bahauddin Zakariya University, Multan Pakistan. The fresh green barseem and water was provided to them three times per day. All the animals except blank group were treated with standards and plant extracts/fractions dose 25mg and 50mg per Kg body weight as an aqueous suspension and blood sugar level was thoroughly

**Table 1:** Quantitative Phytochemical analysis of *Nelumbo nucifera* leaf sample (gm/1g sample).

| Samples | Alkaloids | Saponnins | Tannins | Flavonoids | Phenols |
|---|---|---|---|---|---|
| Ethanol Extract | 0.025 ± 0.040 | 0.340 ± 0.010 | 0.050 ± 0.02 | 0.320 ± 0.040 | 0.086 ± 0.052 |
| Butanol Fraction | 0.001 ± 0.001 | 0.303 ± 0.005 | 0.340 ± 0.030 | 0.180 ± 0.020 | 0.000 |
| EthOAc Fraction | 0.00 | 0.120 ± 0.017 | 0.140 ± 0.040 | 0.280 ± 0.040 | 0.040 ± 0.010 |
| Hexane Fraction | 0.009 ± 0.02 | 0.060 ± 0.020 | 0.0006 ± 0.00 | 0.004 ± 0.001 | 0.00 |
| P-Value | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |

recorded with a glucometer before the administration of sample and standard drug. For experimental work, the experimental animals (rabbits) were approved by the ethical committee of Bahaudin Zakaria University, Multan Pakistan.

**Induction of diabetes**

Alloxan Monohydrate was used to make all the experimental animals (rabbits) hyperglycemic. Alloxan Monohydrate was administrated orally (as an ice-cold water solution) at dose rate 150mg/kg body weight for one week. Alloxan Monohydrate affects the pancreas of experimental animals and inhibit the production of insulin, as a result animals become artificially hyperglycemic. All the rabbits showed the symptoms of diabetes like increased thrust, urination and laziness. The rabbits having blood glucose level 130 mg/dl or above, were considered as hyperglycemic. On 8$^{th}$ day, after administration of Alloxan Monohydrate for a week, blood glucose level was recorded and 8$^{th}$ day was considered as a zero day for the launching of drug treatment (Tolbutamide, plant extract/fraction). During the drug period, blood glucose level was regularly measured on 0, 3, 6, 9, 12, 15 days with glucometer, and blood was drawn from the marginal ear veins of the rabbits Sultan *et al.* (2016).

## RESULTS AND DISCUSSION

Medicinal plants are effective for treating as well as for management of many intractable human diseases because they contain several phytochemical constituents. These compounds incorporate with primary and secondary

**Table 2:** Alpha amylase activity of various extract/fractions of *N. Nucifera* Leaves.

| Extract/ fractions | Concentration (µg/ml) | Inhibition (%) | $IC_{50}$ (µg/ml) | P-value |
|---|---|---|---|---|
| | 125 | 52 ± 2 | | |
| Acarbose | 250 | 61 ± 3 | | |
| (standard) | 500 | 73 ± 1 | 140.66 | 0.000 |
| | 1000 | 94 ± 4 | | |
| | 125 | 46 ± 2 | | |
| Ethanol | 250 | 57 ± 3 | | |
| Extract | 500 | 65 ± 1 | 40.96 | 0.000 |
| | 1000 | 81 ± 2 | | |
| | 125 | 48 ± 2 | | |
| Butanol | 250 | 61 ± 3 | | |
| Fraction | 500 | 74 ± 2 | 78.56 | 0.000 |
| | 1000 | 93 ± 3 | | |
| | 125 | 49 ± 2 | | |
| Ethyl acetate | 250 | 55 ± 3 | | |
| Fraction | 500 | 62 ± 1 | 83.33 | 0.000 |
| | 1000 | 71 ± 3 | | |
| | 125 | 49 ± 2 | | |
| Hexane | 250 | 55 ± 4 | | |
| Fraction | 500 | 63 ± 3 | 110.13 | 0.000 |
| | 1000 | 76 ± 1 | | |

**Table 3:** Blood glucose readings of experimental animals treated with *N. nucifera* extract and fractions.

| Study animals | BSL (Blood Sugar level) during Induction of Alloxan monohydrate | | | | BSL after treatment with plant leaves extract/fraction | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Day 0 | Day 3 | Day 6 | Day 7 | Day 0 | Day 3 | Day 6 | Day 9 | Day 12 | Day 15 |
| Blank Group | 84±0.02 | 83±0.02 | 83±0.03 | 84±0.045 | 84±0.04 | 84±0.043 | 85±0.043 | 86±±0.04 | 84±0.043 | 88±0.03 |
| Tolbutamide untreated group | 80±0.03 | 87±0.04 | 102±0.04 | 121±0.06 | 121±0.05 | 116±0.04 | 114±0.04 | 113±0.03 | 111±0.04 | 110±0.03 |
| Tol. Treated group (25mg) | 78±0.04 | 85±0.06 | 99±0.03 | 127±0.03 | 127±0.03 | 120±0.03 | 103±0.05 | 102±0.04 | 90±0.04 | 79±0.04 |
| Tol. Treated group (50mg) | 82±0.04 | 89±0.03 | 101±0.04 | 125±0.04 | 125±0.03 | 118±0.05 | 109±0.04 | 101±0.03 | 93±0.03 | 84±0.06 |
| EtOH Extract treated group (25mg) | 83±0.03 | 88±0.04 | 104±0.04 | 121±0.06 | 121±0.04 | 118±0.06 | 107±0.06 | 103±0.06 | 94±0.04 | 85±0.05 |
| EtOH Extract treated group (50mg) | 84±0.02 | 92±0.03 | 100±0.05 | 122±0.04 | 122±0.05 | 116±0.04 | 108±0.03 | 102±0.05 | 100±0.03 | 92±0.04 |
| Ethyl acetate fraction group (25mg) | 88±0.04 | 99±0.04 | 117±0.03 | 124±0.05 | 124±0.06 | 118±0.06 | 102±0.04 | 107±0.04 | 100±0.05 | 98±0.04 |
| Ethyl acetate fraction group (50mg) | 85±0.03 | 95±0.05 | 109±0.02 | 118±0.03 | 118±0.05 | 112±0.05 | 110±0.06 | 108±0.06 | 103±0.04 | 99±0.03 |
| ButOH fraction group (25mg) | 70±0.04 | 76±0.04 | 99±0.05 | 119±0.05 | 119±0.03 | 109±0.023 | 90±0.03 | 89±0.05 | 80±0.06 | 72±0.04* |
| ButOH fraction group (50mg) | 72±0.03 | 78±0.06 | 98±0.04 | 118±0.03 | 118±0.05 | 108±0.05 | 98±0.05 | 92±0.03 | 80±0.03 | 77±0.03** |

*P< 0.01 as compared to antidiabetic drug (dose 25mg/Kg body weight).

**P< 0.05 as compared to antidiabetic drug (dose 50mg/Kg body weight).

metabolism of plants and are present in all parts like root, stem, bark, leaves, flowers and fruits or plant exudates. Even though ever rising advancement in field of molecular diagnosis and medicine, it is estimated that approximate 80% world population from developing countries still depends on natural products derived from medicinal plants Jyoti Rani *et al.* (2017). Different plants are used for the treatment of diseases like Mango Ginger plant can be used as anti-inflammatory, hypotriglycerideemic and hypoglycemic Ayodele *et al.* (2019). Quantitative analysis of plant leave extract indicates that one gram of ethanol leaf extract and its (1gram) fractions contains different amounts of phytochemicals which are given in Tabe 1. Table 1 indicates that alkaloids, saponins, flavonoids and phenols are present inhigh amount in Ethanol extract while tanins are present in high amount in butanol fraction than other fractions. The results of alpha amylase assay (*In-vitro antidiabetic assay*) indicates that butanol fraction of leaves of the plant *N. nucifera* has highest ability (P<0.05 as compared with Acarbose) to inhibit the working of alpha amylase than the remaining fractions or extract of the same plant. Butanol fraction contain high amount of tannins which inhibit the working of alpha amylase. The $IC_{50}$ values of Ethanolic extract, butanolic fraction, ethyl acetate fraction and Acarbose are 140.66, 78.56. 83.33. 110.13 and 40.96 µg/ml, respectively, as shown in Table 2. The smaller the $IC_{50}$ value of a drug (extract or fraction) higher will be the activity.

In *In vivo* antidiabetic assay, the change in blood sugar level (BSL) of all the experimental animals was observed during drug treatment period (from zero to 15$^{th}$ day) and it is mentioned in Table 3. The present investigation indicates that alloxan monohydrate administration at dose rate 25mg

**Table 4:** Change in blood sugar level of experimental animals treated with Tolbutamide and various extract/fractions of *N. nucifera* leaves.

| Experimental groups | Change in BSL (%) |
|---|---|
| Blank group | 4.545 ± 0.05 |
| Tolbutamide untreated group | 6.771 ± 0.73 |
| ***Tolbutamide*** | |
| Tol. Treated group (25mg) | 37.793 ± 0.19 |
| Tol. Treated group (50mg) | 32.814 ± 1.32 |
| ***Ethanol extract*** | |
| Ethanol extract treated group (25mg) | 29.762 ± 1.89 |
| Ethanol extract treated group 50mg) | 24.603 ± 1.22 |
| ***Ethyl acetate fraction*** | |
| EtOAc fraction treated group (25mg) | 20.951 ± 3.05 |
| EtOAC fraction treated group (50mg) | 16.112 ± 1.83 |
| ***Butanol fraction*** | |
| ButOH fraction treated group (25mg) | 39.512 ± 1.50 |
| ButOH fraction treated group (50mg) | 32.724 ± 1.79 |
| P-value | 0.000 |
| F-value | 63.803 |

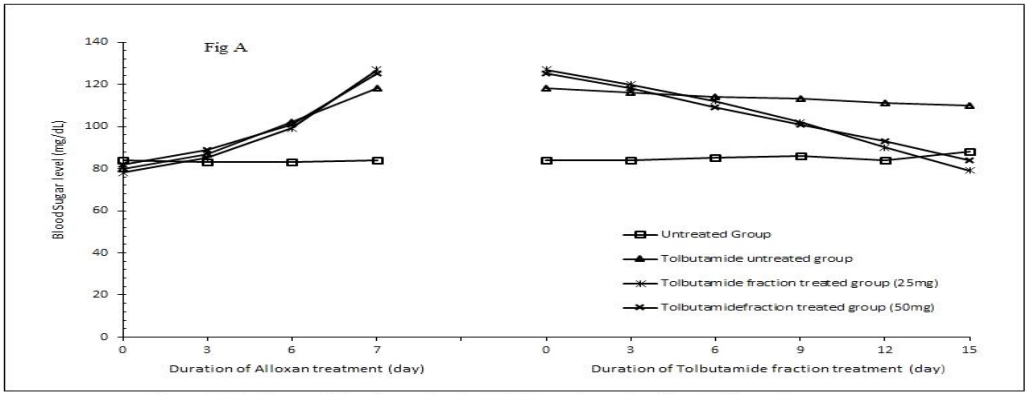



**Fig 1(a):** Change in blood sugar level of Rabbits with standard drug Tolbutamide treatment.

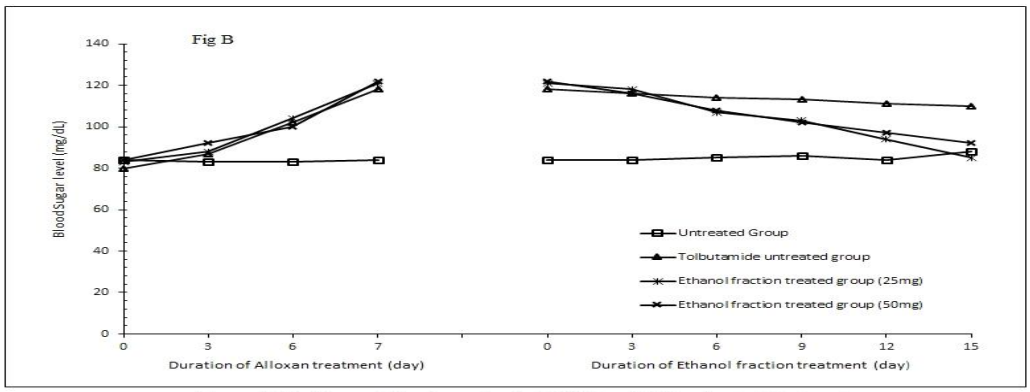



**Fig 1(b):** Change in blood sugar level of Rabbits with Ethanol extract treatment.

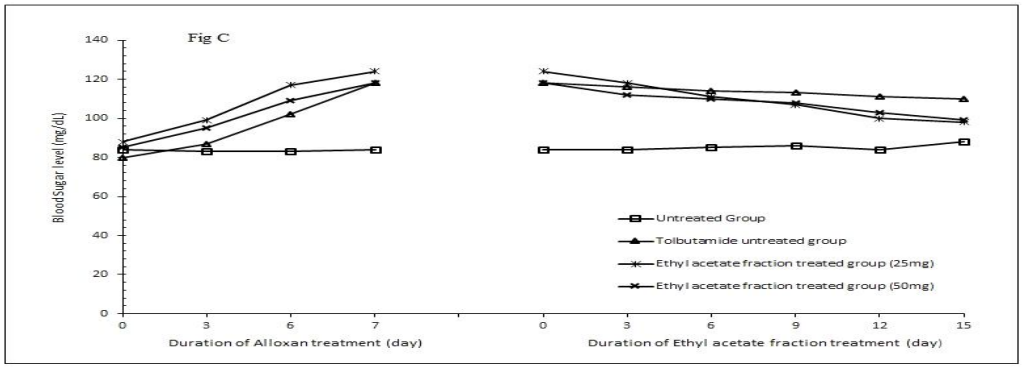



**Fig 1(c):** Change in blood sugar level of Rabbits with ethyl acetate fraction treatment.

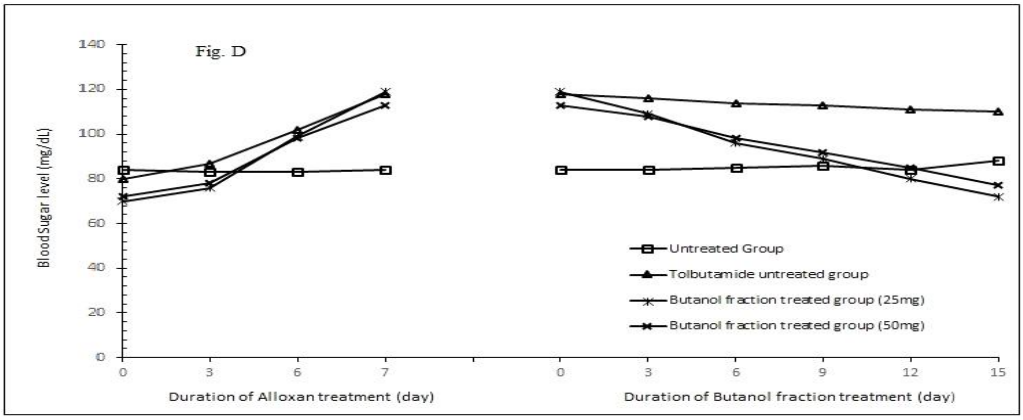



**Fig 1(d):** Change in blood sugar level of Rabbits with butanol fraction treatment.

and 50mg per Kg body weight significantly ($P<0.05$) increases the blood sugar level compared with the rabbits not treated with Aloxan monohydrate as shown in Table 3, 4 and Fig 1(a). Table 3 and 4 also indicates that butanolic fraction (25mg/Kg body weight) significantly ($P<0.01$) reduces the blood sugar lavel of rabbits as compared with standard drug Tolbutamide at same dosage rate than 50mg/ Kg body weight dosage and less significant ($P<0.06$) results were observed with all other extract and fractions as shown in Table 3 and 4. Table 4 also show that ethanolic extract has 29.76%, and 24.60%, ethylacetate fraction has 30.95% and 16.11% ability to decreas the BSL with respect to tolbutamide drug, in 25mg and 50mg dosage respectively. Our result also indicate that tolbutamide is more effective in 25mg/kg body weight than 50mg dose.

## CONCLUSION

*Nelumbo nucifera* is an aquatic, ornamental plant found all over the world. It is widely used as a food stuff as well as medicine. From present investigation, it is concluded that leaves of *N. nucifera* contain some active phytochemicals like saponnins, tannins, flavonoids, which are mainly present in butanolic fraction, can reduce the blood sugar level maximally than other fractions. So, leaves of *N. nucifera* can be used as diabetes remedy. So, it is strongly recommended that *N. nucifera* leaves should be elucidated further for isolation of active phytochemicals.

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## Disclosure Statement

The authors report no declarations of interest.

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