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An *In vitro* study on antidiabetic and anti-lipid peroxidation properties of polyphenol rich extract from the petals of nelumbo nucifera

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Abstract
The aim of this study was to evaluate the antidiabetic, antioxidant and phytochemical composition of polyphenol rich fraction of *Nelumbo nucifera*. Phytochemicals were analyzed through chemical tests, thin layer chromatography (TLC). Polyphenol rich fraction of *N. nucifera* was evaluated for antidiabetic, antioxidant potential via the inhibition of amylase and glucosidase. Polyphenol rich fraction of *N. nucifera* had the highest phenolic and flavonoid contents (67.80±1.62 µg GAE/g, 51,36±1,78 µg QE/g. Polyphenol rich fraction of *N. nucifera* considerably inhibited the amylase and glucosidase at a concentration of 100 µg/mL. The lowest EC_{50} (64.98 and 66.23 µg/mL) observed in the amylase and glucosidase assay. Positive correlations were observed between total phenolics, antidiabetic, antioxidant potential of the selected plant extracts, indicating a significant contribution of phenolic compounds in the plant extracts to these activities.

Keywords: *N. nucifer*, antidiabetic, antioxidant, polyphenol rich fraction

Introduction

The worldwide incidence of diabetes has risen in the past two decades. Type 2 diabetes is more common, and its prevalence is expected to rise more rapidly in the future because of increasing obesity and reduced activity levels. In spite of several new pharmacologically active agents have been developed for the management of diabetes, the treatment of diabetes with herbal remedies has also been increasing among practitioners. Ancient Indian literature has prescribed various herbs in the treatment of diabetes mellitus. Many indigenous drugs have been used by the practitioners of the Siddha and Ayurvedic system for the treatment of diabetes mellitus in India. Finding healing power in plants is an ancient idea (Middha *et al*., 2009) [7].

Biological actions of the plant products used as alternative medicines to treat diabetes are in relevance to their chemical composition. Herbal products or plant products are rich in flavonoids, phenolic compounds, coumarins, terpenoids and other constituents which help to reduce blood glucose levels (Jung *et al* ., 2006) [6]. Several species of herbal drugs with potential antidiabetic activity have been described in the scientific literature. Herbal drugs are prescribed due to their good effectiveness, fewer side effects in clinical experience and relatively low costs (Ren *et al*., 1997) [11]. Medicinal and natural herbal plant products are traditionally used from long time in many countries for the treatment of diabetes mellitus.

Type II diabetes mellitus is a heterogeneous disorder due to a combination of inherited and acquired factors that adversely affect glucose metabolism. It is thought that these factors lead to diabetes mainly by affecting β-cell function and tissue insulin sensitivity. If the amount of insulin produced is too little to allow for glucose to be used or stored, or if the insulin being produced does not work effectively, glucose accumulates in the blood. Hyper-glycaemia develops when rates of glucose release into the circulation exceed rates of tissue glucose uptake. This may occur because release is increased, because uptake is reduced, or due to a combination of factors such as increased release with a lesser increase in uptake (Gerich, 2000) [3]. In the normal individual, the concentration of glucose in blood is maintained at about 90 mg/dL of plasma. However, fasting blood glucose in diabetics may be 300-400 mg/dL and may even reach 1000 mg/dL (Johnson, 1998) [5]. Bio-flavonoids comprise a group of phenolic secondary plant metabolites that are widespread in nature. Major flavonoids that have well categorized structures and well defined structure function-relationships are: flavans, flavanones, flavones, flavonols, flavanols, flavanonols, cetechins, anthocyanidins and isoflavones. Bio-flavonoids are well-known for their multi-directional biological activities including anti-diabetic efficacy (Brahmachari, 2009) [2]. Numerous studies have been carried out to explore their potential role in the treatment of diabetes. A good number of studies have already demonstrated the hypoglycemic effects of flavonoids using different experimental models and treatments - the drug candidates have been shown to exert such beneficial effects against the disease manifestation, either through their capacity to avoid glucose absorption or to improve glucose tolerance.

Nelumbo nucifera is a large aquatic rhizomatous herb consisting of slender, elongated, creeping stem with nodal roots. Lotus is perennial plant with both aerial and floating orbicular leaves. Aerial leaves are cup shaped and floating leaves have flat shape. Its petioles are considerably long and rough with distinct prickles. Flowers vary in color from white to rosy and are pleasantly sweet-scented, solitary, and hermaphrodite. Traditionally, the whole plant of lotus was used as astringent, emollient, and diuretic. It was used in the treatment of diarrhea, tissue inflammation, and homeostasis. The rhizome extract was used as antidiabetic and anti-inflammatory properties due to the presence of asteroidal

triterpenoid. Leaves were used as an effective drug for hematemesis, epistaxis, hemoptysis, hematuria, and metrorrhagia. Flowers were used to treat diarrhea, cholera, fever, and hyperdipsia. In traditional medicine practice, seeds are used in the treatment of tissue inflammation, cancer and skin diseases, leprosy, and poison antidote (Yu and Hu, (1997) [15].

Materials and Methods

Plant Collection and Preparation of Extracts

Nelumbo nucifera petals was obtained from Herbal garden of Government Siddha Medical College, Arumbakkam, Chennai, Tamil Nadu, India. A plant taxonomist authenticated the plant and samples were kept in the Medicinal Botany herbarium with voucher specimen numbers MB/GSMC-295/2021.

Phytochemical Analysis

The aqueous extract of *Nelumbo nucifera* were subjected to phytochemical screening to determine the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, glycosides, saponins and polyphenols using standard procedures (Harborne 1973; Trease and Evans 1983).

Thin Layer Chromatography

The polyphenol rich extract from the petals of *Nelumbo nucifera* were loaded on to pre coated TLC (60 F_2 54) and it was developed using solvent system in the ratio of Petroleum ether, Chloroform and methanol (1:0.5:0.1, V/V/V) was used for the development of the exudates on silica gel plates silica gel 60 F_{254} (10x20 cm, 0.2mmlayer). Visible and the non-visible spot given and it is fluorescent with UV light at 360nm and 240nm.

Glucose Uptake in Yeast Cells

The commercial baker's yeast in distilled water was subjected to repeated centrifugation (3,000×g, 5 min) until clear supernatant fluids were obtained and a 10% (v/v) of the suspension was prepared in distilled water. Various concentrations of the polyphenol rich extract from the petals of *Nelumbo nucifera* were added to 1mL of glucose solution (25 mM) and incubated together for 10 min at 37 °C. Reaction was started by adding 100 µL of yeast suspension followed by vortexing and further incubation at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2,500 × g, 5 min) and amount of glucose was estimated in the supernatant (Ou *et al* ., 2001) [9]. Metronidazole was used as standard drug. The percentage increase in glucose uptake by yeast cells was calculated using the following formula:

$$\text{Increase in glucose uptake (\%)} = \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \times 100$$

Abs sample

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample) and Abs sample is the absorbance of the test sample. All the experiments were carried out in triplicates.

α- Amylase Inhibition Activity

Alpha amylase is an enzyme that hydrolyses alpha-bonds of large alpha linked polysaccharide such as glycogen and starch to yield glucose and maltose. Alpha amylase inhibitory activity was based on the starch iodine method that was originally developed by (Ademiluyi and Oboh, 2013) [1] and later employed by others for determination of amylase activity in plant extracts with some modifications. In alpha amylase inhibition method 1ml substrate- potato starch (1% w/v), 1 ml of the polyphenol rich extract from the petals of *Nelumbo nucifera* of different concentration such as 25, 50, 75 and 100 µg/ml, 1ml of alpha amylase enzyme (1% w/v) and 2ml of acetate buffer (0.1 M, 7.2 pH) was added. NOTE- Potato starch solution, alpha amylase solution and drug solution was prepared in acetate buffer (820.3 mg Sodium acetate and 18.7mg sodium chloride in 100ml distilled water).

$$\text{Inhibition of alpha- Amylase (\%)} = \frac{\text{Abs sample} - \text{Abs control}}{\text{Abs sample}} \times 100$$

α-Glucosidase Inhibitory Activity

The α-glucosidase inhibitory activity was assessed by the standard method (Dong *et al*., 2012), with slight modifications. Briefly, a volume of 60µl of sample solution and 50 µl of 0.1 M phosphate buffer (pH 6.8) containing α-glucosidase solution (0.2 U/ml) was incubated in 96 well plates at 37 ºC for 20 min. After pre-incubation, 50 µl of 5 mM *p*-nitrophenyl-α-D-glucopyranoside (PNPG) solution in 0.1 M phosphate buffer (pH 6.8) was added to each well and incubated at 37 ºC for another 20 min. Then the reaction was stopped by adding 160 µl of 0.2 M $NaCO_3$ into each well, and absorbance readings (A) were recorded at 405 nm by micro-plate reader and compared to a control which had 60 µl of buffer solution in place of the extract. For blank incubation (to allow for absorbance produced by the extract), enzyme solution was replaced by buffer solution and absorbance recorded. The α-glucosidase inhibitory activity was expressed as inhibition % and was calculated as follows:

$$\text{Inhibition Percentage-} \frac{A_0 - A_1}{A_0} \text{ X100}$$

Where, ACO is absorbance of the control and at is absorbance of the sample the concentration of inhibitors required for inhibiting 50% of the α-glucosidase activity under the assay conditions was defined as the IC_{50} value.

Inhibition of Lipid Peroxidation Activity

Lipid peroxidation induced by Fe^{2+}ascarbate system in egg yolk was assessed as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa *et al* . (1979) [8]. The experimental mixture contained 0.1 ml of egg yolk (25% w/v) in Tris-HCl buffer (20 mM, pH 7.0); KCl (30 mM); $FeSO_4$ $(NH_4)_2SO_4.7H_2O$ (0.06 mM); and different concentrations of the polyphenol rich extract from the petals of *Nelumbo nucifera* in a final volume of 0.5 ml. The experimental mixture was incubated at 37°C for 1 h. After the incubation period, 0.4 ml was collected and treated with 0.2 ml sodium dodecyl sulphate (SDS) (1.1%); 1.5 ml thiobarbituric acid (TBA) (0.8%); and 1.5 ml acetic acid (20%, pH 3.5). The final volume was made up to 4.0 ml with distilled water and then kept in a water bath at 95 to 100 °C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1 v/v) were added to the reaction mixture, shaken vigorously and centrifuged at 4000 rpm for 10 min. The absorbance of

butanol-pyridine layer was recorded at 532 nm in Deep Vision (1371) UV–Vis Spectrophotometer) to quantify TBARS. Inhibition of lipid peroxidation was determined by comparing the optical density (OD) of test sample with control. Ascorbic acid was used as standard.
Inhibition of lipid peroxidation (%) by the each extracts was calculated according to 1-(E/C) × 100, where C is the absorbance value of the fully oxidized control and E is absorbance of the test sample.

Nitric Oxide Radical Scavenging Activity
Nitric oxide scavenging ability of the polyphenol rich extract from the petals of *Nelumbo nucifera* was measured according to the method described by Green *et al*. (1982) [4]. 0.1 ml of sodium nitroprusside (10 mM) in phosphate buffer (0.2 M, pH 7.8) was mixed with different concentration of extracts and incubated at room temperature for 150 min. After treated period, 0.2 ml of Griess reagent (1% Sulfanilamide, 2% Phosphoric acid and 0.1% N-(1-Naphthyl) ethylene diamine dihydrochloride) was added. The absorbance of the experimental sample was read at 546 nm against blank. All readings were taken in triplicate and ascorbic acid was used as standard. The percentage of inhibition was calculated by following equation:

$$\% \text{ Nitric oxide radical scavenging capacity} = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 was the absorbance of control and A_1 was the absorbance of flavonoid rich fraction.

Phytochemical Screening
The phytochemical screening of the aqueous extract from the *Nelumbo nucifera studied* presently showed the presence of alkaloids, flavonoids, phenol, Terpenoids, glycosides and saponin, and absence of glycosides and tannin (Table-1).

Table 1: Phytochemical screenings of aqueous extract from the petals *Nelumbo nucifera*

Sl. No.	Phytochemical Constituents	Observation	Aqueous extract of *Nelumbo nucifera*
1	Alkaloids		
	-Dragendorff's test	Orange /red precipitate	+
	-Mayers test	Cream pie ppt	+
2.	Flavonoids		
	-Alkalai Reagent	Intense yellow colour	+
	-Lead aceate test	Precipitate formed	+
3.	Glycosides		
	-Keller-Killiani test	Pink colour (Ammonia layers)	+
4.	Tannin		
	-$FeCl_3$ test	Blue-black colour	+
5.	Saponins		
	-Frothing test	Foam	-
6.	Terpenoids		
	-Salkowski test	Reddish brown colour ring formed in interface	-
7.	Polyphenols		
	-Ferrozine test	Raddish blue	+
8.	Anthocyanin		
	-Ammonia test	Pink color in ammonia layer	+

+ Positive result; - Negative result

The Partial Characterization of Polyphenol Rich Extract From the Petals of *Nelumbo Nucifera* by TLC
The polyphenol rich extract from the petals of *Nelumbo nucifera* loaded on Pre-coated TLC plates (60 F_2 54 Merck) and developed with a solvent system of petroleum ether, chloroform and methanol in the ratio of 1:0.5:0.1 were efficient to extract the antidiabetic, antioxidant and anti-inflammatory compound it is used for further studies. The developed plate was viewed under UV 240nm and 360nm (Table-2 and Fig-1).

Table 2: Partial characterization of polyphenol rich extract from the petals of *Nelumbo nucifera* by TLC

S.No	Polyphenol rich extract from the petals of *Nelumbo nucifera*		
	UV 240 nm Rf value	UV 360 nm Rf value	Visible Rf value
1	0.89	0.89	0.89
2	0.85	0.85	0.85
3	0.73	0.73	0.73
4	0.64	0.64	0.64
5	0.52	0.52	0.52

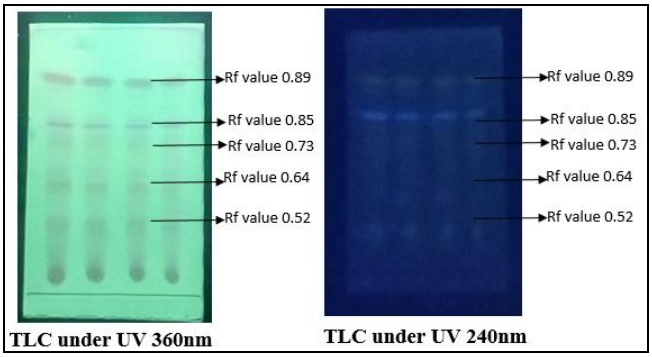

Fig 1: Partial characterization of polyphenol rich extract from the petals of *Nelumbo nucifera* by TLC

Glucose Uptake in Yeast Cells of Polyphenol Rich Extract from the Petals of *Nelumbo Nucifera*

The rate of glucose transport across cell membrane in yeast cells system is presented in Fig-2. The amount of glucose remaining in the medium after a specific time serves as an indicator of the glucose uptake by the yeast cells. The rate of uptake of glucose into yeast cells was linear in all the three glucose concentrations. The polyphenol rich extract from the petals of *Nelumbo nucifera* exhibited significantly higher activity than at all concentrations. However the highest uptake of glucose was seen in 20mM Glucose concentration. The result showed the lower uptake of glucose by the yeast cells which conformed the highest activity.

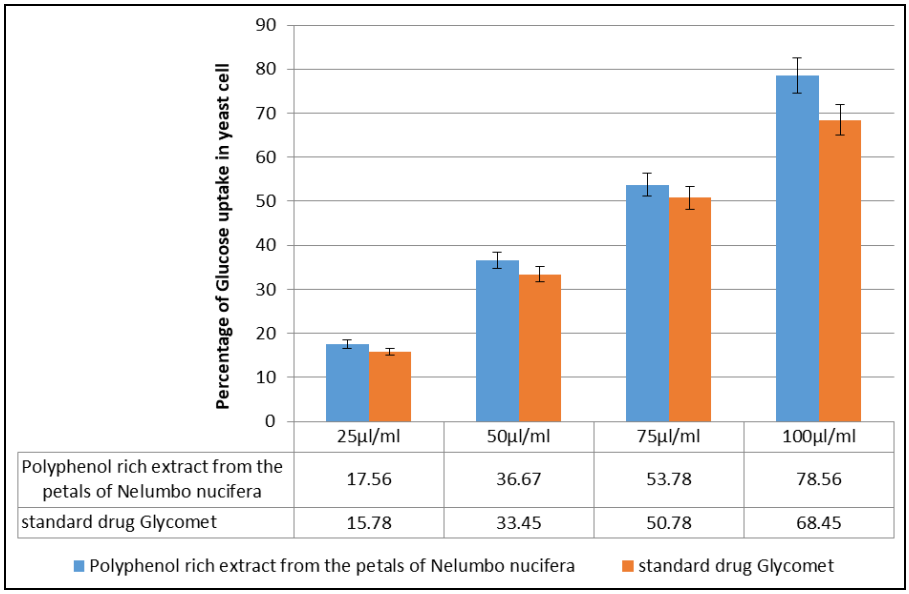

Fig 2: Glucose Uptake in Yeast Cells of Polyphenol Rich Extract from the Petals of *Nelumbo Nucifera*

α-Amylase Inhibition of Polyphenol Rich Extract From the Petals Of *Nelumbo Nucifera*

In the present study, polyphenol rich extract from the petals of *Nelumbo nucifera* showed a significant inhibition of αamylase enzyme activity in a concentration dependent manner. Polyphenol rich extract from the petals of *Nelumbo nucifera* at the concentrations 25, 50, 75 and 100 µg/ml showed 74.45% inhibition of α-amylase enzyme activity, respectively with an EC_{50} value 64.98 µg/ml. The Glycomet used as a reference standard at the same concentrations showed 71,39% inhibition of α-amylase activity with an EC_{50} value 71.23 µg/ml (Fig-3). Many bioactive compounds from different plants have been reported to have hypoglycemic effect, in that mostly phenolics and triterpenoids such as oleanane, ursane, lupane, and flavonoids have a positive correlation as antidiabetic agents (Sales *et al* ., 2012) [13].

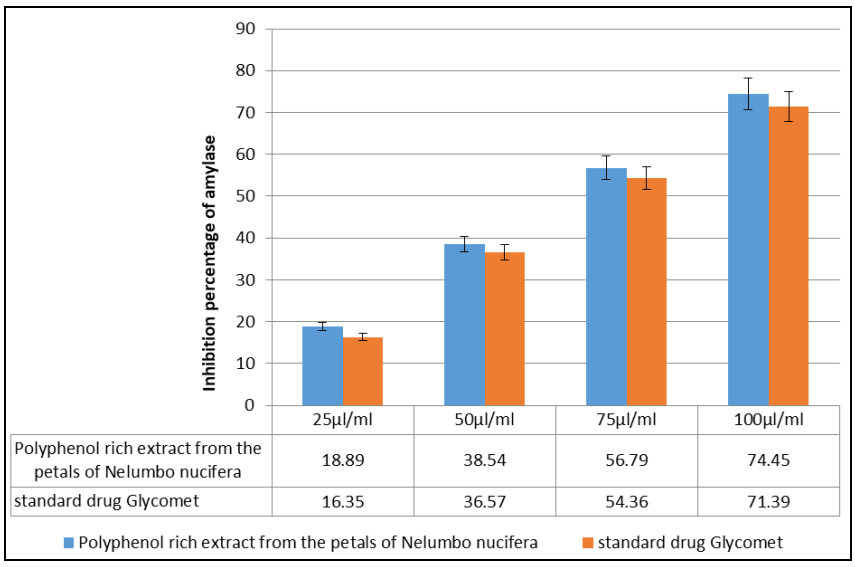

Fig 3: α-Amylase Inhibition of Polyphenol Rich Extract from the Petals of *Nelumbo Nucifera*

α-Glucosidase Inhibitory Activity of Polyphenol Rich Extract From the Petals of *Nelumbo Nucifera*

The results of *in-vitro* α-glucosidase inhibitory study are showed in Fig-4. The polyphenol rich extract from the petals of *Nelumbo nucifera* showed a concentration-dependent inhibition of enzyme. The highest concentration of 100 µl/ml tested showed a maximum inhibition of nearly 73.89% (EC_{50} 66.23µg/ml) in polyphenol rich extract from the petals of *Nelumbo nucifera* seems to be less potent in α-glucosidase inhibitory potential compared to Glycomet. It may be that α-glucosidase is more sensitive towards glycomet with the concentration required for 50% inhibition

(EC_{50}) found to be 68.79µg/ml. Apart from that polyphenolic compounds were found in fraction 2, may interact or inhibit specific positions in enzymes thereby reducing the potency of α-amylase and α-glucosidase.

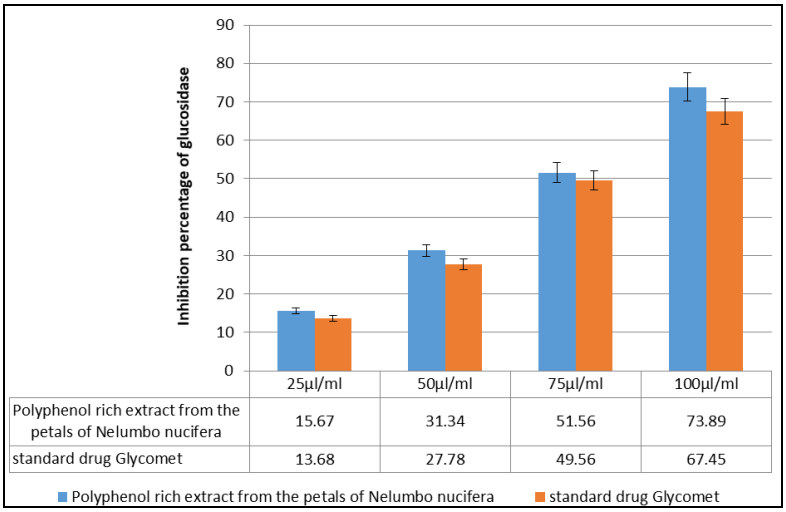

Fig 4: α-Glucosidase Inhibitory Activity of Polyphenol Rich Extract From the Petals of *Nelumbo Nucifera*

Inhibition of Lipid Peroxidation Activity of Polyphenol Rich Extract From the Petals of *Nelumbo Nucifera*

The polyphenol rich extract from the petals of *Nelumbo nucifera* also inhibited the lipid peroxidation induced by ferrous sulfate in egg yolk homogenates. Maximum inhibition was recorded in polyphenol rich extract from the petals of *Nelumbo nucifera* (71.56%) and lowest inhibition percentage of Vitamin-C was found in 66.67% (Table-3). As it is identified that lipid peroxidation is the net result of any free radical attack on membrane and other lipid components present in the system, the lipid peroxidation may be enzymatic (Fe/NADPH) or non-enzymatic (Fe/ascorbic acid). In the present study, egg yolk was used as substrate for free radical mediated lipid peroxidation, which is a non-enzymatic method. Normally, the mechanism of phenolic compounds for antioxidant activity includes neutralizing lipid free radicals and preventing decomposition of hydroperoxides into free radicals.

Table 3: Inhibition of lipid peroxidation activity polyphenol rich extract from the petals of *Nelumbo nucifera*

Different concentration of extract	Inhibition of Lipid peroxidation activity	
	Polyphenol rich extract from the petals of *Nelumbo nucifera*	Standard Vitamin-C
25 µl/ml	19.89±1.78	17.89±1.56
50 µl/ml	37.76±2.56	34.78±0.89
75 µl/ml	55.89±2.90	53.16±2.67
100 µl/ml	71.56±0.89	66.67±1.78
EC_{50} Value	65.89	72.89

[a] Results are expressed as percentage inhibit of lipid peroxidation with respect to control. Each value represents the mean+SD of three experiments.

Nitric Oxide Radical Scavenging Assay of Polyphenol Rich Extract from the Petals of *Nelumbo Nucifera*

Nitric oxide radical quenching activity of polyphenol rich extract from the petals of *N. nucifera* were identified and compared with the standard ascorbic acid. The polyphenol rich extract from the petals of *N. nucifera* also caused a moderate dose-dependent inhibition of nitric oxide with an EC_{50} of 62.34 µg/ml and 71.34 µg/ml respectively (Table-4). Vitamin-C was used as a reference compound and 69.31 µg/ml was needed for 50% inhibition. The EC_{50} value of the extract was less than that of the standard. At 100 µg/ml, the percentage inhibition of the polyphenol rich extract from the petals of *N. nucifera* were 76.47% whereas that of Vitamin-C was 73.67%. Effect of free radicals on DNA can be minimized by the use of combination therapies that act at sequential steps in the DNA destruction process. Inhibition of DNA strand-breaks can reduce the mutagenicity and further carcinogenicity. Antioxidants have direct effects on transcription through antioxidant response elements present in the promoters of many genes (Palmer & Paulson, 1997) [10].

Table 4: Nitric oxide radical scavenging assay of the polyphenol rich extract from the petals of *Nelumbo nucifera*

Different concentration of extract	Percentage of Nitric oxide radical scavenging activity	
	Polyphenol rich extract of *Nelumbo nucifera*	Ascorbic acid (+ve control)
25 µl/ml	22.45±1.45	18.45±2.67
50 µl/ml	41.67±3.56	38.56±1.56
75 µl/ml	58.95±2.78	52.61±2.89
100 µl/ml	76.47±1.67	73.67±1.89
EC_{50} Value	62.34	71.34

[a] Results are expressed as percentage of Nitric oxide radical activity with respect to control. Each value represents the mean+SD of three experiments.

Conclusion

In conclusion, results specify that the polyphenol rich extract from the petals of *N. nucifera* possess antidiabetic and antioxidant properties at varying levels. Petals of *N. nucifera* showed higher antidiabetic, alpha amylase and glucosidase inhibition activity. Pearson's correlation studies showed that there were significant correlations between estimated antidiabetic and antioxidant properties. Results indicate that these antidiabetic and antioxidant activities may be due to the occurrence of bioactive phenolic compounds in these petals of *N. nucifera*.

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