

Antiglycation and antioxidant activity of a rare medicinal orchid *Dendrobium aqueum* Lindl.

Sourav Mukherjee¹, Devyani Phatak², Juhi Parikh², Suresh Jagtap¹, Shamim Shaikh²,
Rashmi Tupe^{2*}

¹Interactive Research School for Health Affairs, Bharati Vidyapeeth Deemed University, Pune-Satara Road,
Pune- 411 043, India.

²Biochemical Sciences Division, Rajiv Gandhi Institute of IT and Biotechnology, Bharati Vidyapeeth Deemed
University, Katraj, Pune- 411 046, India.

*Corresponding Author: rashmitupe@gmail.com

Received: 4 May 2012, Revised: 14 May 2012, Accepted: 15 May 2012

Abstract

Oxidative stress and glycation plays an important role in manifesting of diabetes and vascular complications. Agents with antiglycation and antioxidant properties may retard these pathological alterations. Aqueous extract of aseptically regenerated *Dendrobium aqueum* was used for *in vitro* estimation of antioxidant and antiglycation potential. Antioxidant activity was evaluated as DPPH radical scavenging activity, whereas the protein glycation inhibitory potential was evaluated using *in vitro* albumin/fructose glycation model. Glycation inhibition was estimated by different biochemical parameters i.e. fructosamine, protein carbonyl group, thiol content and Congo red binding; indicators of various glycation modification of albumin. *D. aqueum* extract showed a dose dependent DPPH free-radical scavenging potential and exhibited a significant antiglycation potential. Our finding might open a new arena of research in traditional herbal medicine with *D. aqueum* orchid which could be utilized in diabetes due to its antiglycation and antioxidant properties

Keywords: *Dendrobium aqueum*, diabetes, antioxidant, antiglycation

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS), including peroxides, super-oxides, hydroxyl radicals and nitrous oxide, generated in the living organisms by cellular metabolism, are known to play a vital role in oxidative cellular damage (Halliwell, 1997). Oxidative stress, resulting from these free-radicals play an important role in manifesting various disorders, including ageing and diseases like diabetes, cancer, cardiovascular diseases, Parkinson's and so on (Mukherjee et al., 2010; Mukherjee et al., 2011a, 2011b). Thus to alleviate such condition in the body, a defense mechanism becomes mandatory. Several types

of natural and artificial antioxidants are in regular use worldwide for such oxidative stress. Plants are known to produce natural antioxidants to control the oxidative stress. Hence, they are a potent source of useful new compounds with antioxidant activity for human consumption. The World Health Organization estimates that 80% of the world's inhabitants rely mainly on traditional medicines for their health care (Kalim *et al.*, 2010). Plants, either in the form of medicine or food supplements are widely used to maintain health and prevent oxidative stress-mediated diseases such as cancer, atherosclerosis, diabetes, inflammation and ageing (Kalim *et al.*, 2010).

Several studies have reported that advanced glycation end-products (AGEs) are generated in the diabetic milieu as a result of chronic hyperglycemia and enhanced oxidative stress (Chen *et al.*, 2011). AGEs via direct and receptor-dependent pathways, promote the development and progression of diabetic complications, including neuropathy, nephropathy, and cardiovascular disease (Chen *et al.*, 2011). Moreover, recent findings have suggested that metal-catalyzed oxidation reactions play a major role in accelerating the rate of AGEs formation (Chen *et al.*, 2011). Therefore, agents with antiglycation and antioxidant properties may retard the process of AGEs formation by preventing further oxidation of metal-catalyzed glucose oxidation (Chen *et al.*, 2011).

Dendrobium is well known as one of the largest genera in orchidaceae which include about 1000 species distributed from Himalayas, Asia, Australia, Tasmania and the pacific island (Kamemoto *et al.*, 1999). This genus is well appreciated for its ornamental beauty and for its medicinal values in traditional medicinal system. *Dendrobium aqueum* Lindl. is one of the rare exquisite epiphytic orchids on *Mangifera indica* L., *Syzygium cumini* (L.) Skeels, found in Western and Eastern Ghats (Robinson *et al.*, 2009). The plant produces pseudobulbs and on the onset of flowers the leaves are shaded off. The pseudobulbs are characterized by having a swollen in the middle portion and tapering at both ends.

Several works have proven that polysaccharides are major active constituents in *Dendrobium* species (Luo *et al.*, 2011). Voluminous work on these polysaccharides from some *Dendrobium* species, such as *Dendrobium nobile* Lindl (Luo *et al.*, 2009; Wang *et al.*, 2010). *Dendrobium huoshanense* (Zha *et al.*, 2007) and *Dendrobium chrysotoxum* Lindl. (Zhao *et al.*, 2007) have been reported to have beneficial effects on antioxidation, immunity stimulation and antitumor activities. However, no work has been so far accomplished on the *D. aqueum* species in relation to its *in vitro* antioxidant potential and anti-glycation potential.

As part of the endeavor for search of medicinal properties in local floristic resources we herein report a study of antioxidant and preliminary anti-glycemic activity of the pseudobulbs of *Dendrobium aqueum* Lindl. orchid germinated aseptically *in vitro*.

Materials and Methods

Chemicals

All chemicals used for assays were of analytical grade. 2,2-diphenyl-1-picrylhydrazyl (DPPH), Bovine serum albumin (BSA), Nitroblue tetrazolium, 2,4-dinitrophenylhydrazine (DNPH), Dithiobis Nitrobenzoic acid (DTNB), Congo red powder were procured from Sig-

ma-Aldrich, USA. Fructose, Potassium dihydrogen orthophosphate (KH₂PO₄), Dipotassium hydrogen phosphate (K₂HPO₄), sodium azide, Trichloroacetic acid (TCA), hydrochloric acid (HCl), Urea, Ethyl alcohol, Ethyl acetate, sodium chloride were procured from Qualige-ns Pvt. Ltd., Mumbai, India.

Collection of plant material and in vitro germination

Initially immature (green) and undehisced pods of the orchid was collected from its natural habitat *i.e.* Western Ghats of India during 2010-2011. Seeds were allowed to germinate *in vitro* in optimum medium. Aseptically grown callus and regenerated plantlets served as the source of plant material every time for the *in vitro* assays.

Preparation of plant extracts

Aqueous extracts of *D. aqueum* was prepared by mixing 10% (w/v) plant material in ultra pure water by homogenizing using a mortar and pestle. Extract was filtered through Whatman filter paper (No. 1) and the filtrate was centrifuged (10000 rpm, 10°C, 10 min) to obtain a clear supernatant. Its yield was determined and 10 mg/ml stock solution prepared, which was stored in amber coloured bottles at 4°C till further studies.

In vitro antioxidant potential measurement

DPPH Free-radical scavenging assay

Complementarities of the antioxidant capacity of the formulation was confirmed by the DPPH scavenging assay according to the protocol as described elsewhere (Mukherjee et al., 2011b) with slight modification. Different concentrations (0.01 to 0.1 mg/ml) of the extracts and ascorbic acid (standard) were thoroughly mixed with 4 ml of methanolic DPPH solution (33 mg/L, 0.08 mM) in test-tubes and the resulting solution was kept standing for 10 minutes at 37°C before the absorbance was measured at 517 nm. The measurement was repeated with three sets and an average of the reading was considered. The percentage radical scavenging activity was calculated from the following formula:

$$\% \text{ scavenging [DPPH]} = [(A_0 - A_1)/A_0] \times 100$$

Where, A₀ was the absorbance of the control and A₁ was the absorbance in the presence of the samples.

IC₅₀ value was determined from the graph obtained using standard ascorbic acid by using the “ $y = mx + c$ ” formula from the slope of the graph.

In vitro antiglycation potential measurements

In vitro glycation of albumin

Albumin glycation was performed as mentioned by McPherson *et al.* (1988) with slight modifications. Initially, to prepare glycated BSA samples, BSA (10 mg/ml) was incub-

ated with fructose (250 mM) in potassium phosphate buffer (200 mM, pH 7.4 containing 0.02% Sodium azide) along with aqueous plant extracts in dark at 37 °C for 5 days in sealed tubes. Positive control (BSA + Fructose) was maintained under similar conditions. Before incubation, all the solutions were filtered through 0.22 µm membrane filters in sterile plastic-capped vials to maintain sterility and strict asepsis was maintained during the entire process. All the incubations were performed in triplicates. After the incubation period, it was ensured that all the reaction mixtures were free of microbiological contamination. The unbound fructose from the solutions was removed by dialysis against the phosphate buffer (200 mM, pH 7.4) and then subsequently stored at 4 °C until further analysis. The dialysates were used to determine the antiglycation activity of plants by estimation of four parameters such as – i) Fructosamines adducts, ii) Protein carbonyls, iii) Protein thiols iv) Congo Red absorbance.

Estimation of fructosamine

This was carried out by using Nitroblue tetrazolium assay as mentioned by Baker *et al.* (1994). Briefly, 40 µL aliquots of glycated samples and positive control were added to the 0.8 mL of nitroblue tetrazolium (0.75 mM) in sodium carbonate buffer (100 mM, pH 10.35) and incubated at 37°C for 30 min. The absorbance was measured at 530 nm (Thermo Scientific, UV-10 Spectrophotometer, USA) and the percent inhibition of fructosamines by plant extracts was calculated using the following equation:

$$\text{Inhibitory activity (\%)} = [(A_0 - A_1) / A_0] \times 100$$

Where A_0 is the absorbance value of the positive control at 530 nm and A_1 is absorbance at 530 nm of the glycated albumin samples co-incubated with plant extracts.

Carbonyl group estimation

Protein carbonyls were estimated by the method of Uchida *et al.* (1988). Briefly, (i) mixing of 0.5 mL glycated albumin samples and positive control with an equal volume of 2,4-dinitrophenylhydrazine (DNPH) (10 mM) in 2.5 M-HCl, (ii) incubation at room temperature for 60 min (iii) precipitation of proteins by adding 0.5 mL of trichloroacetic acid (20%), (iv) washing precipitate thrice with 1 ml of mixture of ethanol: ethyl acetate (1:1, v/v), and finally (v) dissolving in 1 mL of 6 M Urea. The absorbance was read at 365 nm and protein carbonyl concentration was calculated by using the molar extinction coefficient ($\epsilon_{365\text{nm}} = 21 \text{ mM per cm}$). The results were expressed as % inhibition as calculated by the following formula:

$$\text{Inhibition \%} = [1 - (A_0 - A_1) / (A_0)] \times 100$$

Where, A_0 is the absorbance of protein carbonyls of the positive control, and A_1 is the absorbance of glycated albumin samples coincubated with plant extracts.

Protein Thiol Estimation

Thiol groups of glycated albumin samples and positive control were measured according to Ellman (1959) assay using DTNB. Briefly, 250 µL samples and control were incuba-

ted with thrice the volumes of 750 μL DTNB (0.5 mM) for 15 min. The absorbance was measured at 410 nm. The free thiol concentration was calculated using standard curve performed with various BSA concentrations (0.8 to 4 mg/mL) as nM thiol /mg protein. The results were expressed as percentage protection calculated by the following formula:

$$\text{Protection \%} = [1 - (A_0 - A_1) / (A_0)] \times 100$$

Where, A_0 is the absorbance of protein thiols of the positive control, and A_1 is the absorbance of protein thiols of the glycated albumin samples co-incubated with plant extracts.

Binding of Congo red

Congo red binding to amyloid cross- β structure was estimated by measuring absorbance at 530 nm as mentioned by Klunk *et al.* (1999). Briefly, glycated samples (500 μL) were incubated with 100 μL of Congo red (100 μM) in PBS with 10% (v/v) ethanol for 20 minute at room temperature. Absorbance was recorded for the Congo red-incubated samples as well as for Congo red background. The results were expressed as percentage inhibition calculated by the formula:

$$\text{Inhibition \%} = [1 - (A_0 - A_1) / (A_0)] \times 100$$

Where, A_0 is the absorbance at 530 nm of positive control, and A_1 is the absorbance of the glycated albumin samples co-incubated with plant extracts.

Statistical analysis

Data were expressed as the mean and standard deviations of triplicate values. The statistical analysis was carried out using the Microsoft Excel software package (Microsoft Corp.).

Result and Discussion

DPPH free radical scavenging activity

In vitro, aqueous extract of *D. aqueum* has shown increase in the percentage free-radical scavenging potential in a dose dependent manner (Figure 1) with highest activity of 49% at a dose of 100 $\mu\text{g/ml}$. However, this activity was significantly less than that of standard at the same concentration (93.3% at 100 $\mu\text{g/ml}$).

Antiglycation potential

The effect of *D. aqueum* aqueous plant extract on albumin glycation was determined by estimating various parameters which are indicatives of different stages of glycation. As seen in Table 1, based on four complementary assays *i.e.* inhibition to fructosamine, protein carbonyls, protection to thiols and amyloids (Congo red), it was found that the plant has pote-

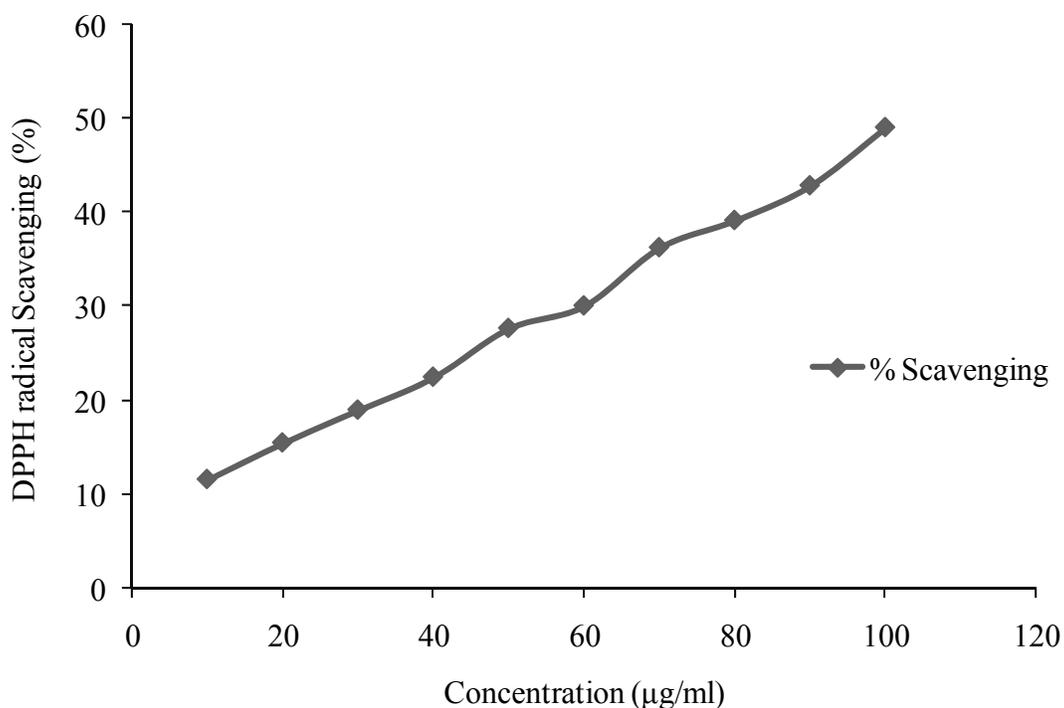


Figure 1. DPPH free radical scavenging with increasing concentration of *D. aqueum* aqueous extract.

ntial to inhibit initial stages of glycation reaction. As seen from results there is variation in inhibition of different glycation modifications. *D. aqueum* can inhibit the initial glycation product *i.e.* fructosamine by about 42.45%, while the intermediate modification in terms of carbonyls was observed to be reduced by 10%, whereas at the latter phases in glycation as amyloid formation no inhibition was observed. The plant extract has shown remarkable potential in protecting the protein thiols (68%) from oxidation during glycation modifications.

Plants with antioxidant activities have been reported to possess free radical scavenging activity (Mukherjee *et al.*, 2011b). Several plants are known to be a potent antioxidant and significant free-radical scavenging capacity is documented in our previous studies (Mukherjee *et al.*, 2010, Mukherjee *et al.*, 2011a, 2011b). The *in vitro* DPPH scavenging activity in this study indicated that aqueous extract of *D. aqueum* might contain some compounds that are capable of donating hydrogen to a free radical in order to remove odd electron, which is responsible for the radical's reactivity (Olayinka and Anthony, 2010).

It is known that there exists a controversial relation between antioxidant and antiglycation potentials. The hyperglycemic state, commonly occurring in diabetes mellitus is repor-

Table 1. Antiglycation activities of *D. aqueum* aqueous extract

Sample	Fructosamine inhibition*	Protein Carbonyls inhibition*	Protein Thiols protection*	Protein amyloids inhibition*
<i>D. aqueum</i> extract	42.45%	10.76%	68.12%	ND

* % inhibition/protection as compare to positive control; ND= Not detected

ted to be associated with the development of diabetes-specific microvascular complications and accelerated macrovascular disease (Chen *et al.*, 2011, Hudson *et al.*, 2002). Evidence implicates the formation and subsequent effects of AGEs as a contributing cause for such a complication. Further, recent data have pointed out to an increased oxidative damage in the vicinity of the glycated residues of histones (Chen *et al.*, 2011, Guedes *et al.*, 2010). Thus it could be expected that initial glycation and thereby oxidation may interact synergistically in the development of diabetic complications (Chen *et al.*, 2011).

The glycation reaction involves a series of non-enzymatic reactions between the carbonyl group on reducing sugars and the amino group on proteins, leading to the formation of AGEs, which are involved in the pathogenesis of diabetic and aging-related complications (Rahbar and Figarola, 2002). Therefore, targeting glycation should have a broad and beneficial effect on treating diabetes and also on aging or age-related skin damage. Several traditionally used herbal medicines have been shown to possess not only *in vitro* antioxidant but also anti-inflammatory as well as antiglycation effects (Povichit *et al.*, 2010).

Several medicinal formulations are known to be prepared from this genus in traditional Chinese medicine. '*Herba Dendrobii*' (*Shihu*) is a very commonly used medicine derived from the pseudobulbous stem of several orchid species belonging mainly to the genus *Dendrobium* (Lau *et al.*, 2001). Findings have also suggested that *D. chrysotoxum* has a potential utility in treating patients who require enhanced antioxidation, immune function and/or hypoglycemic activity. Moreover, oral administration of *D. chrysotoxum* produced a significant reduction in blood glucose level in alloxan-induced diabetic mice (Zhao *et al.*, 2007).

Our study, for the first time reported the *in vitro* antioxidant and antiglycation potential of another member of this medicinal orchid genus. Aqueous extracts of *D. aqueum* has shown a dose dependent *in vitro* free-radical scavenging (DPPH) potential, although, the efficacy was significantly lesser than that of ascorbic acid. Similarly, the plant has been found to possess a significant *in vitro* antiglycation with thiol group protection property as evident by the biochemical assays.

In conclusion, it could be mentioned that the genus *Dendrobium* is well appreciated in Chinese Traditional medicine for possessing several medicinal values. Our finding might open a new arena of research in traditional herbal medicine with *D. aqueum* orchid which could be utilized in age-related diseases and even to combat diabetes due to its antiglycation and antioxidant properties. However, further detailed *in vivo* model would throw more light in this direction.

Acknowledgement

The authors are sincerely thankful to G. D. Sharma, Principal, Rajiv Gandhi Institute of IT and Biotechnology, Bharati Vidyapeeth Deemed University for providing facilities to conduct the research. We are also thankful to Prof. P. K. Ranjekar, Director, Interactive Research School for Health Affairs, Bharati Vidyapeeth Deemed University for providing opportunity to work and for constant encouragement in research.

References

- Baker, JR, Zyzak, DV, Thorpe, SR, Baynes, JW. (1994) Chemistry of the fructosamine assay: D-glucose is the product of oxidation of Amadori compounds. *Clinical Chemistry* 40, 1950–1955.
- Chen YF, Roan HY, Lii CK, Huang YC, Wang TS. (2011). Relationship between antioxidant and antiglycation ability of saponins, polyphenols, and polysaccharides in Chinese herbal medicines used to treat diabetes. *Journal of Medicinal Plants Research* 5, 2322-2331.
- Ellman GL. (1959). Tissue Sulfhydryl Groups. *Archives of biochemistry and biophysics* 82, 70-77.
- Guedes S, Vitorino R, Domingues MR, Amado F, Domingues P. (2011). Glycation and oxidation of histones H2B and H1: in vitro study and characterization by mass spectrometry. *Analytical and Bioanalytical Chemistry* 399, 3529-39.
- Halliwell B. (1997). Anti-oxidants and human diseases: a general introduction. *Nutrition Reviews* 55, S44-S55.
- Hudson BI, Hofmann MA, Bucciarelli L, Wendt T, Moser B, Lu Y, Qu W, Stern DM, D'Agati V, Yan SD, Yan SF, Grant PJ, Schmidt AM. (2002). Glycation and diabetes: The RAGE connection. *Current Science* 30, 1515-1520.
- Kalim M D, Bhattacharyya D, Banerjee A and Chattopadhyay S. (2010). Oxidative DNA damage preventive activity and antioxidant potential of plants used in Unani system of medicine. *BMC Complementary and Alternative Medicine* 10, 77-87.
- Kamemoto H, Amore T D and Kuehnle A R. (1999). Breeding *Dendrobium* orchids in Hawaii. Canada, University of Hawaii Press
- Klunk WE, Jacob RF, Mason RP. (1999). Quantifying amyloid by Congo red spectral shift assay. *Methods in Enzymology* 309, 285–305.
- Lau DTW, Shaw PC, Wang J, But PPH. (2001). Authentication of Medicinal *Dendrobium* Species by the Internal Transcribed Spacer of Ribosomal DNA. *Planta Medica* 67, 456-460.
- Luo A, Ge Z, Fan Z, Luo A, Chun Z and He X J. (2011). in vitro and in vivo antioxidant activity of a water-soluble polysaccharide from *Dendrobium denneanum*. *Molecules* 16, 1579-1592.
- Luo, A.X.; He, X.J.; Zhou, S.D.; Fan, Y.J.; He, T.; Chun, Z. (2009). In vitro antioxidant activities of a water-soluble polysaccharide derived from *Dendrobium nobile* Lindl. Extracts. *International Journal of Biological Macromolecules* 45, 359-363.
- McPherson JD, Shilton BH, Walton DJ. (1988). Role of fructose in glycation and cross-linking of proteins. *Biochemistry* 27, 1901–1907.
- Mukherjee S, Dugad S, Bhandare R, Pawar N, Jagtap S, Pawar P, Kulkarni O. (2011b). Evaluation of free-radical quenching potential of *Bramhi* (*Bacopa monnieri*) and *Mandookparni* (*Centella asiatica*). *AYU* 32, 258-64.
- Mukherjee S, Jagtap S, Kuvalekar A, Kale Y, Kulkarni O, Harsulkar A and Pawar P. (2010). Demonstration of the potential of *Hibiscus cannabinus* L. flowers to manage oxidative stress, bone related disorders and free radical induced DNA damage. *Indian Journal of Natural Products Resources* 1, 322-327.
- Mukherjee S, Pawar N, Kulkarni O, Nagarkar B, Thopte S, Bhujbal A, Pawar P. (2011a). Evaluation of free-radical quenching properties of standard Ayurvedic formulation Vayasthapana Rasayana. *BMC Complementary and Alternative Medicine* 11, 38-43.
- Olayinka AA, Anthony IO. (2010). Preliminary phytochemical screening and In vitro antioxidant activities of the aqueous extract of *Helichrysum longifolium* DC. *BMC Complementary and Alternative Medicine* 10:21.
- Povichit N, Phrutivorapongkul A, Suttajit M, Chaiyasut C, Leelapornpisid P. (2010). Phenolic content and in vitro inhibitory effects on oxidation and protein glycation of some thai medicinal plants. *Pakistan Journal of Pharmaceutical Sciences*, 23, 403-408.

- Uchida KM, Kanematsu K, Sakai T. *et al.* (1998). Protein-bound Macrolein: potential markers for oxidative stress (covalent modification of protein/antibody/atherosclerosis). *Proceedings of the National Academy of Sciences USA* 95, 4882–4887.
- Wang JH, Luo JP, Zha XQ. (2010). Structural features of a pectic polysaccharide from the stems of *Dendrobium nobile* Lindl. *Carbohydrate Polymers* 81, 1-7.
- Zha XQ, Luo JP, Luo SZ, Jiang ST. (2007). Structure identification of a new immunostimulating polysaccharide from the stems of *Dendrobium huoshanense*. *Carbohydrate Polymers* 69, 86-93.
- Zhao Y, Son YO, Kim SS, Jang YS, Lee JC. (2007). Antioxidant and Anti-hyperglycemic Activity of Polysaccharide Isolated from *Dendrobium chrysotoxum* Lindl. *Journal of Biochemistry and Molecular Biology* 40, 670-677.