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Functional relevance of Gedunin as a bona fide ligand of NADPH oxidase 5 and ROS scavenger: An *in silico* and *in vitro* assessment in a hyperglycemic RBC model

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ABSTRACT

Clinical evidence suggests that type 2 diabetes therapy can greatly benefit from the suppression of reactive oxygen species generation and the activation or restoration of cellular antioxidant mechanisms. In human, NADPH oxidase (NOX) is the main producer of reactive oxygen species (ROS) that supress the activity of endogenous antioxidant enzymes. In the present study, the antioxidant potential of Gedunin was studied. *In silico* findings reveal its strong binding affinity with NOX5 C terminal HSP90 binding site that disrupts NOX5 stability and its ability to generate ROS, leading to restoration antioxidant enzymes activities. It was found that Gedunin suppressed hyperglycaemia induced oxidative stress in an *in vitro* RBC model and markedly reversed glucose induced changes including haemoglobin glycosylation and lipid peroxidation. A significant restoration of activities of cellular antioxidant enzymes; superoxide dismutase, catalase and glutathione peroxidase in the presence of Gedunin revealed its ability to reduce oxidative stress. These results substantiated Gedunin as a bona fide inhibitor of human NOX5 and a ROS scavenging antioxidant with promising therapeutic attributes including its natural origin and inhibition of multiple diabetic targets.

1. Introduction

RBCs are continuously under the assaults of oxidative stress that generates endogenous and exogenous reactive oxygen species (ROS) [1]. The antioxidant system of RBC that includes non-enzymatic and enzymatic antioxidants neutralizes these deleterious effects of oxidative stress. In hyperglycaemia, the elevated glucose level of blood accelerates redox disequilibrium which imposes alterations in structural, functional and biochemical properties of RBC. Scientific researches have established the claim that augmented ROS generation is the preparator of diabetic symptoms [2]. Normal cellular homeostasis is maintained by the balance between endogenous pro-oxidant enzymes (NADPH oxidase, xanthine oxidase, and mitochondrial respiratory chain enzymes), oxidants and endogenous antioxidants. When pro-oxidant enzyme activity and ROS generation exceeds the antioxidant capacity the balance is shifted towards oxidative stress. In human, the sole function of Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) isoform 5 (NOX5) is the generation of ROS, mainly superoxide. NADPH oxidase present in RBC generates ROS in normal and disease condition. Though at normal concentration ROS acts as an important messenger in cellular signalling, excess ROS formation is implicated in disease pathophysiology. In sickle cell anaemia, implication of NADPH oxidase present in RBC has been documented [3]. Hyperglycaemia induced ROS generation is a common phenomenon in diabetes which is attributed to augmented activity of NOX5. Post diabetic complications are reported to be a consequence of NOX5 overexpression [4–6]. Overproduction of ROS and elevated oxidative stress enhance RBC membrane fragility, haemolysis, glycation of haemoglobin and alterations in endogenous non-enzymatic and enzymatic defence systems.

The current knowledge on the relevance of oxidative stress in the pathophysiology of diseases like diabetes and cancer has shifted the focus of therapeutic use of antioxidants [7]. Various *in vivo* and *in vitro* studies have supported the view that the endogenous antioxidant defence system can be strengthened by natural bioactive supplements [8].

Natural antioxidants generally act as a ROS-scavengers and play a protective role against elevated free radicals [9]. Despite such a wealth of studies, there has been no distinction made between a ROS scavenging antioxidant, and a true NOX binding ligand that modulates ROS formation and redox reactions [10,11]. Inhibition of hNOX5(Human)

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can represent an additional strategy to interfere with diabetes. Recently importance of shifting therapeutic paradigm towards targeting these enzymatic ROS sources by natural compounds, without affecting the physiological redox state, is gaining momentum in diabetic regime. In this study we investigated how Gedunin, a tetranortriterpenoid isolated from *Azadirachta indica* influences NOX5 activity and antioxidant defence system through *in silico* and *in vitro* approach using RBC as bioindicator.

Gedunin is a known HSP90 inhibitor and NOX5 being a client of HSP90, there is reason to believe that Gedunin may have a modulatory role on NOX5. In this novel study, for the first time NOX5α C domain with HSP90 or FAD/NADPH binding site has been targeted to study if Gedunin is a bonafide ligand of NOX5 and its relation to antioxidant activity.

2. Materials and methods

2.1. Chemicals and reagents

Gedunin was purchased from Santa Cruz Biotechnology Inc,USA, Reduced glutathione (GSH), oxidised glutathione (GSSG), Nicotinamide adenine-dinucleotide hydrogen phosphate (NADPH) and epinephrine were obtained from Sigma Chemicals, St. Louis, U.S.A. All other chemicals used were of highest analytical grade.

2.2. Molecular modelling of NOX5 C-Domain

Since there is no specific 3D structure available for NOX5α isoform, we used NOX5α protein sequence (Uniprot id: Q96PH1-3) [https://ebi12.uniprot.org] for modelling the C domain residues ranging from aa 490–707 known as, NOX5α CD. The homology modelling was performed in Swiss Model workspace based on the homologous template (PDB Id: 5O0x), which is the crystal structure of dehydrogenase domain of organism *Cylindrospermum stagnale* NOX5 and a close homolog of human NOX5 dehydrogenase domain [12]. This template is the only available known dehydrogenase domain structure with resolution 2.2 and 49.28% sequence identity to the query sequence suggested by Swiss model and BLAST.

2.3. Structural evaluation and analysis of the predicted model

The predicted model has been subjected to structure optimization using various tools like YASARA, 3DRefine and RAMPAGE. The quality of predicted models was analysed with SAVES https://servicesn.mbi.ucla.edu/SAVES/] and MolProbity [13].

2.4. Molecular dynamics simulation

Molecular dynamics simulation was carried out using GROMACS 2020.1 program [14] to evaluate the stability and conformation of the obtained NOX5α C domain over time. NOX5α C domain was subjected to a molecular dynamics simulation. OPLS –AA/L all atom force field [15] was used to generate the topology of system. A triclinic box was used for solvating the protein with SPCE water model. The system was neutralised by adding appropriate counter ions using genion command in the program and energy minimised using steepest descent to eliminate clashes and bad contacts. The energy minimised protein model underwent two levels of conventional equilibration stages like NVT and NPT. The NVT was carried out at 300 K based on a modified Berendsen thermostat method. NPT was set for 1 atm pressure based on Parrinello-Rahman pressure coupling approach. The bonds were constrained using LINCS algorithm [16]. Particle Mesh Ewald [17]was applied for long-range electrostatics. The production runs with no constraints were conducted for 50 ns using a leap-frog integrator and the coordinates were saved for every 10.0 ps The simulated structure was used for studying the molecular interaction between the two ligands (Gedunin, Celastrol) to expose Hsp 90 or FAD/NADPH binding site.

2.5. Molecular docking

The simulated NOX 5α CD was subjected to Molecular Docking. The ligands Gedunin (CID:12,004,512) and Celastrol (CID:122,724) were retrieved from PubChem database. AutoDock tools 4.2 were used for preparing the protein and ligands for preparing the grid parameters. The docking was performed by using AutoDockVina. The grid box dimensions were set as 22 × 26 x 24 with a grid spacing of 1.0 Å and which is suitable for performing in Vina program.

2.6. Preparation of RBCs

Blood was collected in EDTA tubes from healthy volunteers after informed consent. After centrifugation, plasma and buffy coat were removed and RBCs washed thrice with ice cold phosphate buffered saline (PBS), pH 7.4. The RBC pellet was diluted to 10% using PBS and used to set up the experiments [18].

2.7. Estimation of haemolysis

Separate experiments with diluted RBCs and PBS containing 5 and 10 mM of glucose to mimic normal (90 mg/dl) and diabetic (180 mg/dl) blood conditions were treated with 0, 5, 10 and 20 μM of Gedunin. Complete haemolysis was induced by addition of distilled water. All experimental tubes were incubated for 24 h at 37 °C with penicillin as antibiotic. The tubes were centrifuged at 3000 rpm for 10 min and the supernatant was read at 540 nm for determination of haemolysis [19]. The percent haemolysis was calculated based on the optical density of 100% haemolysis control.

The hemolysate was used for haemoglobin glycosylation studies and estimation of enzymic and non-enzymic antioxidants.

2.8. Estimation of haemoglobin glycosylation

Non-enzymic glycosylation of haemoglobin was estimated by using the method described by Parker et al. [20].

2.9. Estimation of enzymatic and non-enzymatic antioxidants

2.9.1. Estimations of lipid peroxide(LPx) [21], reduced glutathione(GSH) [22] levels and assay of activities of catalase [23], superoxide dismutase (SOD) [24], glutathione reductase (GRD) [25] and were carried out

Lysate protein was estimated by the method of Folin-Lowry [26].

2.10. Statistical analysis

Results are represented as mean ± SD for three independent experiments. The statistical significance was calculated by unpaired student's t-test.

3. Results

3.1. In silico study

The sequence alignment between NOX5 human alpha CD and *C. stagnale* showed similarity and identity of the two sequences (Fig. 1a). The secondary structure information of the modelled C domain segment of the protein showing 416–735 amino acids generated using ESPript 3.0 program is highlighted in Fig. 1b. However, 490–550 amino acids are known to be involved in Hsp90 binding whereas 689–707 are FAD/NADPH binding domain. We have modelled the protein considering our focus on these two distinct binding sites. The MolProbity score of the model is 1.62 which indicates the improved quality of the structure.

The Ramachandran plot indicated the phi and psi angle distribution

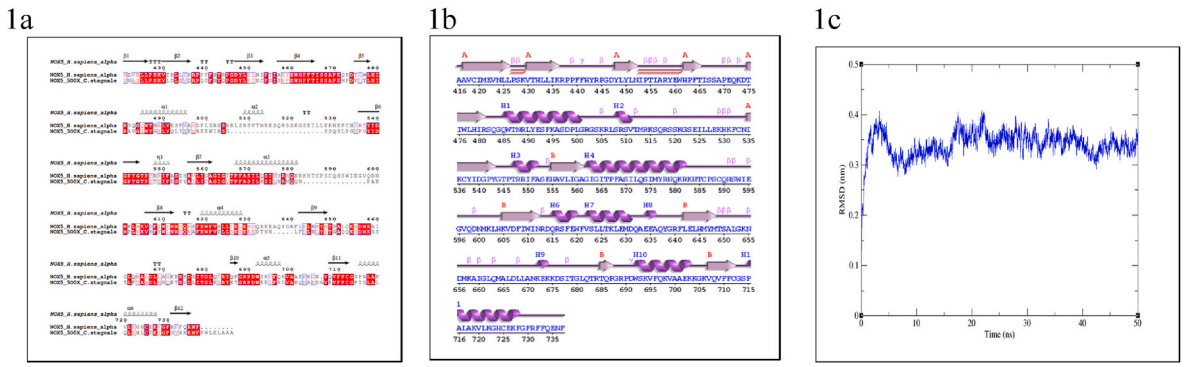

Fig. 1. a: The sequence alignment between human NOX5 alpha sequence and C. stagnale (PDB Id: 5o0x)
Fig 1b: secondary structure assignment of NOX5α CD based on PDB Sum
Fig. 1c: RMSD plot showing the α carbon backbone of NOX5α CD protein.

of the C domain with 92.81% amino acids in the allowed region and 1.88% in the outliers. The RMSD plot in Fig 1c shows the stability of NOX5α CD protein backbone and compactness during 50 ns simulation time. The protein attained stability around 0.35 nm and retains the same till the end of the simulation.

Molecular docking study was conducted focusing on the two binding sites (Hsp90, FAD/NADPH) of NOX5α CD protein. We have selected a standard drug Celastrol for comparing the specificity towards the target. Interestingly, we have obtained the binding of Gedunin and Celastrol at theHsp90 binding site encompassing amino acid residues 511 to 531. Fig. 2 shows the docked conformation of Gedunin at the site. Gedunin showed binding affinity of −6.8 kcal/mol and K_i values of 8.0 μM. Amino acids Lys531 and Arg518 play a crucial role in forming H-bond in the binding site. However, Arg514 formed pi-sigma bond with the pentane ring of Gedunin. These interactions were observed near the vicinity of serine rich region of the binding site. Furthermore, Ser516, Ser517 and Ser520 formed van der Waals interactions to further stabilise the complex.

The molecular docking of Celastrol and f NOX5α CD is shown in Fig. 3. The site of binding was quite similar to Gedunin. However, the binding energy and K_i values of Celastrol were found to be −6.4 kcal/mol and 16.5 μM receptively. Celastrol's terminal hydroxyl group formed H-bonds with Ser516 and Arg518. van der Waals interactions were also observed with Lys515, Gln517 and Val511 whereas, Arg514, Lys521, Lys531 formed alkyl and Pi-Alkyl interactions with the ligand.

3.2. *In vitro study*

3.2.1. *Estimation of haemolysis*

A distinct increase in RBC haemolysis, almost 1.2 times in 10 mM of glucose when compared to non-diabetic glucose (5 mM) level was observed which was reversed by Gedunin in a dose dependent manner. There was nearly 4 times decrease in percentage haemolysis in 10 mM glucose in the presence of 20 μM Gedunin (Fig. 4a).

3.2.2. *Estimation of haemoglobin glycosylation*

In the Hb glycosylation inhibition assay, Gallic acid was used as standard inhibitor. After 72 h of incubation with 10 mM glucose, the inhibition was 85% by 20 μM of Gedunin. The comparative effect of Gedunin with time against a known glycosylation inhibitor Gallic acid clearly indicates higher percentage inhibition by Gedunin in comparison to standard. (Fig. 4b).

3.2.3. *Estimation of lipid peroxidation*

Fig. 4c represents the content of lipid peroxide in erythrocytes incubated with glucose as compared to those which were concomitantly incubated with glucose and Gedunin. With the increase in glucose concentration there is an increase in thiobarbituric acid reactive substances (TBARS) level. Gedunin significantly ($P < 0.001$) lowers the lipid peroxide content in a dose dependent manner and there is notable reduction (76%) in TBARS at higher glucose (10 mM) levels.

3.2.4. *Estimation of cellular first line defence antioxidant enzyme activities*

Table 1 shows a tendency towards reduction in activity of antioxidant enzymes SOD and Catalase with hyperglycaemia. This trend was reversed in the presence of Gedunin with maximum activity restoration (82%) at 20 μM. Even at normal physiological glucose load, addition of 5 μM Gedunin brought twofold enhancement in the activity of catalase and 20 μM of Gedunin approximately four times.

From Table 2 it is evident that the activity of GSH and GRD was not very significantly different in samples treated with 5 or 10 μM Gedunin at 5 mM glucose, compared to that of control. The enhancement of

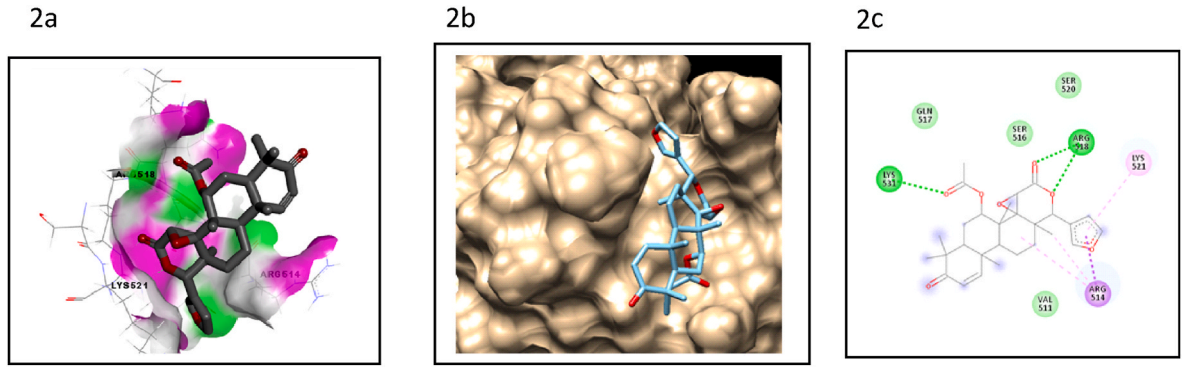

Fig. 2. a) Molecular docking of Gedunin in the binding site of NOX 5C domain, violet colour indicates H-bond donors, green colour represents H-bond acceptors, b) Gedunin present in the molecular surface of the binding site. c) 2D plot of interactions between Gedunin and NOX 5α CD.

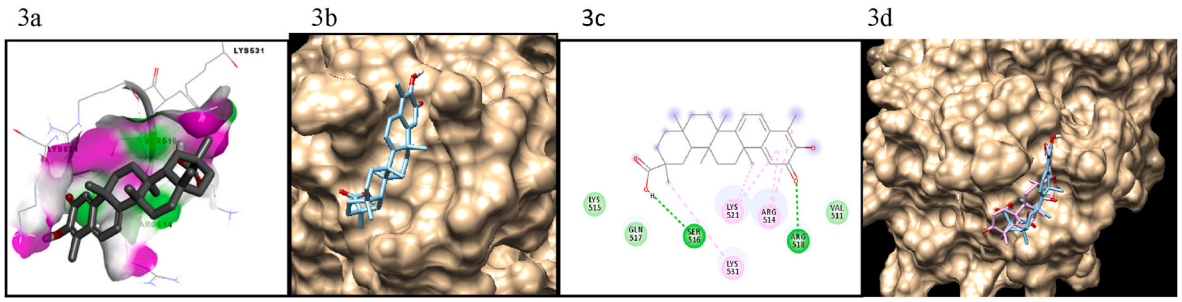

Fig. 3. a) Celastrol shown in the binding site of protein, green surface colour indicates H-bond acceptors, and violet colour indicates H-bond donors. b) shows Celastrol in molecular surface of binding pocket c) 2D plot of molecular interactions between Celastrol and NOX5α CD d) Super imposition of Gedunin and Celastrol. Gedunin (purple) and Celastrol (blue) occupy the same site in NOX5α CD protein.

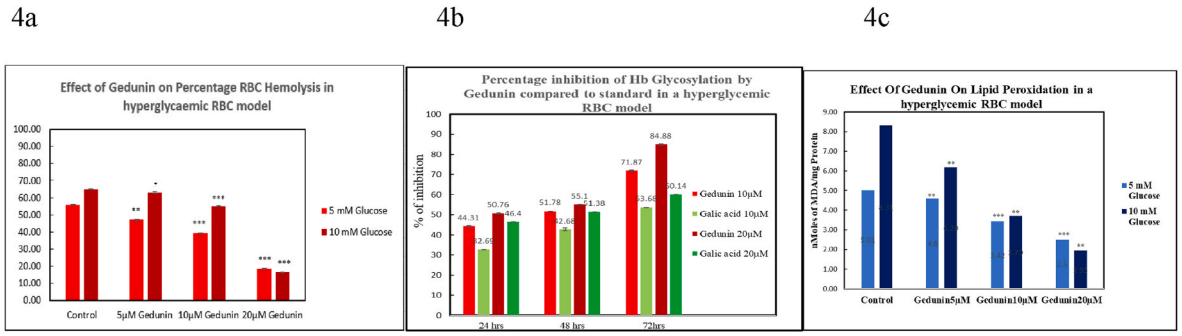

Fig. 4. a. Effect of Gedunin on percentage RBC haemolysis in hyperglycaemic RBC model,Fig 4b.Percentage inhibition of Hb Glycosylation by Gedunin compared to standard in hyperglycaemic RBC model, Comparisons are made between standard (Gallic acid) and glucose + Gedunin of same group.Fig 4c. Effect of Gedunin on lipid peroxidation in hyperglycaemic RBC model. The values represent mean ± SD for 3 independent experiments. Significant difference between the two groups is expressed as ***P < 0.001, **P < 0.005, *P < 0.05.

Table 1
Effect of Gedunin on activity of SOD and Catalase of RBC.

	Activity of SOD (Units/mg protein)		Activity of Catalase (μmol of H_2O_2 consumed/min/mg protein)	
Sample	With 5 mM Glucose	With 10 mM Glucose	With 5 mM Glucose	With 10 mM Glucose
Control	274.05 ± 14.01	260.61 ± 10.12	0.212 ± 0.01	0.153 ± 0.061
5μMGedunin	307.67 ± 22.37^{NS}	311.20 ± 11.55**	0.420 ± 0.025***	0.173 ± 0.017NS
10μMGedunin	366.95 ± 38.37*	396.23 ± 40.18**	0.574 ± 0.065***	0.407 ± 0.028**
20μMGedunin	371.73 ± 23.64**	474.47 ± 6.97***	0.745 ± 0.024***	0.563 ± 0.013***

Values are expressed as mean ± SD for three independent experiments. Comparisons are made between control and glucose + Gedunin of same group. Significant difference between the two groups is expressed as *p < 0.05, **p < 0.005, ***p < 0.001, NS = not significant.

Table 2
Effect of Gedunin on the level of lysate GSH and activity of GRD from RBC.

	Level of GSH		Activity of GRD	
Sample	With 5 mM Glucose	With10mM Glucose	With 5 mM Glucose	With 10 mM Glucose
Control	0.70 ± 0.112	0.12 ± 0.003	0.125 ± 0.016	0.107 ± 0.001
5μMGedunin	0.73 ± 0.050NS	0.32 ± 0.020****	0.179 ± 0.042NS	0.270 ± 0.030***
10μMGedunin	0.96 ± 0.008*	0.43 ± 0.020***	0.310 ± 0.093*	0.421 ± 0.040***
20μMGedunin	1.45 ± 0.012***	1.40 ± 0.06***	0.402 ± 0.009***	0.586 ± 0.105**

Values are expressed as mean ± SD for three independent experiments. Significant difference between the two groups is expressed as *p < 0.05, **p < 0.005, ***p < 0.001, NS = not significant. Comparisons are made between control and glucose + Gedunin of same group.

enzyme activity was prominent (P < 0.001) in diabetic samples even at lowest concentration of Gedunin (5 μM). Use of 20 μM concentration of the bioactive compound boosted the activity of GRD by more than 5-fold and GSH by 12 times when compared to control.

4. Discussion

NOX5 is a member of family of transmembrane proteins that transfer electrons by catalysing the reduction of O_2 to produce superoxide ($O_2^{\cdot-}$), which in turn dismutates to generate hydrogen peroxide (H_2O_2).

The NOX5α C domain structure is not available till date and we have modelled the structure based on homologous template. The sequence alignment between the query sequence and the template (Fig. 1a) indicated that the selected homologous template was ideal candidate for modelling the NOX5α protein. The RMSD, the Ramachandran plot and MolProbity score of this model gave reasons to select the model further for docking studies. Celastrol was selected as standard inhibitor because it is also a pentacyclic triterpenoid isolated from plant and shares structural similarity with Gedunin. Both are known Hsp90 inhibitors and similar functionality had been reported in other studies. Celastrol is also a reported NOX5 inhibitor [27] though its binding with NOX5 is obscure as no *in silico* study has been documented so far.

Interestingly, we observed that both Gedunin and Celastrol are binding at same site. The result revealed a novel binding site of the NOX5α CD probed by Gedunin and Celastrol, which is in the Hsp90 binding site of NOX5 protein, surrounded by conserved serine residues. Gedunin showed better binding affinity and inhibition constant than

Celastrol though they share many common bonding amino acid residues like Gln517, Ser516, Lys531, Lys521, Arg518, and Arg514. Involvement of 75% same binding residues explains similar mode of action on hNOX5 by both ligands. It has also been reported that the C-terminal region of NOX 5 protein contains a group of serine and threonine residues responsible for phosphorylation with respect to various kinases [28]. Previous studies reported that Celastrol inhibits NOX2 by phosphorylated modulation. The mechanism underlying PMA induced activation of NOX 5 indicates direct phosphorylation of serine residues like S516 within the C-terminal region which in turn accelerates PCK mediated NOX5 activation and enhanced ROS generation [29]. The same residue S516 is also involved in bond formation between NOX5 and Gedunin or Celastrol. Another study documented that presence of Hsp90 inhibitors reduced the binding of Hsp90 to NOX5 which is required for the stability and oligomerization of the protein [30]. This postulates that after binding at the HSP90 binding site, Gedunin allosterically triggers a conformational change through blocking serine phosphorylation which can restrict HSP90 protein recognition of the site and destabilises it. Hence, the enzyme is unable to generate ROS (superoxide and H_2O_2). The results of computational model in the premise of Gedunin's role on ROS generation by NOX5 and ability to maintain the endogenous redox homeostasis was further validated using *in vitro* methods.

Haemolysis is one of the manifestations of hyperglycaemia induced oxidative stress. Free radical formation in hyperglycaemia through glucose auto-oxidation, formation of advanced glycated end products and increased polyol pathway activation with concomitant increase in NADPH level and cellular lipid peroxidation causes RBC haemolysis. Reversal of haemolysis with increasing concentrations of Gedunin was a finding that pointed towards protective role of Gedunin in hyperglycaemia induced oxidative stress. Being a lipophilic compound, it can easily incorporate into the membrane and inhibit lipid peroxidation thereby resisting haemolysis. Gedunin ameliorated haemolysis probably by modulating the oxidative stress via suppressing ROS generation or scavenging the free radicals. Earlier studies have reported such attenuation of glucose induced haemolysis by antioxidants like Vitamin E and plant phytochemicals [19,31]. Since most of the reactions causing RBC membrane damage are oxidative in nature, inhibition of redox reactions by antioxidants play a role in membrane protection.

Glycation is a major pathway for formation of advance glycation end products (AGEs) in type 2 diabetic complications. Accumulation of reactive di-carbonyls leads to structural and functional modifications of proteins and lipids, and vascular damages which can be ameliorated by phytochemicals [32]. In present study, ther was a dose and time dependent decline in glycated haemoglobin generation (Fig. 4b). The ability of *Azadirachta indica* (AI) leaf extract to react with carbonyls is proposed to be the major mechanism for protein glycation inhibition [33]. The antiglycation activity of Gedunin could be attributed to its ability to scavenge reactive carbonyls.

Lipid peroxidation is a marker for oxidative stress assessment in progressive diabetes [34]. In the present study an increase in TBARS formation is an indicator of glucose induced lipid peroxidation (Fig. 4c) which was inhibited by addition of Gedunin. The result is in agreement with other data where administration of AI leaf and bark extract reversed the elevated MDA level of diabetic rats [8].

The mobilization of cellular enzymatic and non-enzymatic antioxidant system is the most plausible mechanism for the protection against oxidative stress. Hence the influence of Gedunin on the activities first line defence antioxidant enzymes SOD, catalase and GRD were determined under hyperglycaemic conditions.

Partial inactivation of SOD, catalase and GRD and depletion of GSH in RBC incubated with 10 mM glucose (Tables 1 and 2) may be a consequence of increased production of H_2O_2 and hydroxyl radicals by the auto oxidation of excess glucose in blood that cause non-enzymatic glycation of enzymes [35]. It could be inferred that Gedunin restored the activity of all the enzymes by inhibiting ROS production. The results are in agreement with other studies where the phytoactives restored enzyme activity [36,37]. The restoration of GSH may be a consequence of increased activity of GRD revitalized by Gedunin. These restorations can be interpreted as a combined effect of pro antioxidant response and inhibition of ROS formation by Gedunin. The present study for the first time established the Neem limonoid Gedunin as a legitimate ligand of NOX5 and recognises its antioxidant potential. Thus, Gedunin exhibits antioxidant property with dual mode of action: as an ROS scavenger and NOX5 inhibitor. Further analysis would throw light on the pharmacodynamics of Gedunin and optimize its employment in the management of diabetes.

Declaration of competing interest

The authors declare that they have no known competing financialinterestsor personal relationships that could have appeared to influence the work reported in this paper.

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