**In vitro** anti-oxidant property and reduction of hyperglycemia-induced oxidation by hydroalcoholic extract of *Phyllanthus emblica* in cultured mesangial cell lines

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**Abstract.** The pathology of diabetic nephropathy (DN) is due to long-term poor blood sugar control leading to changes in the filtration unit of the kidneys. However, drugs used to offer renal protection cause many side effects. Therefore, it is necessary to develop a new drug or food supplement that acts on multiple factors of DN. Studies have confirmed that *Phyllanthus emblica* (PE) has anti-diabetic, free radical scavenging, anti-inflammatory, immune regulation, antipyretic, analgesic, wound healing, anti-atherosclerosis, liver protection, kidney protection, and neuroprotection properties. In this study, we explored the antioxidant activity and ability to maintain redox balance in MES-13 cell line. We performed DPPH and FRAP assay to detect the antioxidant property of PE. SDS-PAGE was performed to understand the changes in the types of protein of the glomerular cells treated (24 hr) with high sugar (25 mM) with/without PE extract (10 & 100 µg/ml). Oxidative stress in cells was detected by RedCC-1 staining. Significant antioxidant activity was shown by the extract (IC₅₀ – 108.9 µg/ml) compared to Trolox (IC₅₀ – 60.9 µg/ml). The protein pattern of high glucose (25 mM) treated cells significantly reduced compared to normal and PE treated cells. Mitochondrial dysfunction and cellular oxidative stress produced by high glucose (intensity - 222.04 units) supplementation was significantly reduced by PE (intensity - 38.67 units). In conclusion, PE extract possesses an antioxidant property that can reduce the glomerular damage and oxidative stress in the cells caused by high glucose exposure.

1. **Introduction**

Diabetes mellitus is a metabolic disease characterized by patient's blood sugar which will be higher than the standard value for a prolonged period of time [1]. Major cause of diabetes includes inability of pancreas to produce enough insulin (Type I), or the cells are resistance to insulin mediated glucose uptake (Type II) [2]. Diabetes is a disorder which induces a number of complications in the major organs through glycosylation of functional proteins that are involved in cellular signaling. Long-term complications include cardiovascular diseases, stroke, chronic kidney disease, diabetic foot, retinopathy, neuropathy etc. [3, 4] At present, the main mechanisms of diabetes medications are to increase insulin sensitivity, promote insulin secretion, and supplement insulin [5].

Among the diabetes induced complications, more than 25% of the cases were reported to suffer from nephropathic complications which includes glomerular, tubular and renovascular abnormalities leading to chronic loss of kidney function due to diabetes [6]. Glomerular damage occurs at the early onset of nephropathy which causes the urine to excrete a large amount of albumin (Albuminuria), resulting in a
decrease in serum albumin, which leads to systemic swelling (edema), and syndromes of nephropathy increase the risk of death, especially by cardiovascular abnormalities [7, 8].

Management of nephropathy and pertaining complications has been challenging over the years [2]. Patients with diabetic nephropathy are usually supplemented with a combination of drugs and treatment strategies focusing on multiple predisposing factors. However, mild side effects such as dry cough, patient resistance to drugs and causing serious damage in the system are still being reported during the treatment of nephropathy [9]. Continuous explorations are conducted to develop a drug that acts on multiple causes of diabetes which can avoid all the hyperglycemia induced complications including nephropathy [10, 11].

Plant based medicine or bioactive phytocompounds and nutraceutical are well-known to reduce or completely reverse diabetes and its complications [11]. Among the plant materials, *Phyllanthus emblica* (PE), commonly known as Indian gooseberry, is an important medicinal plant in the traditional Indian Ayurvedic medical system. It has several bioactivities such as antibacterial, antiviral, anti-inflammatory, anti-tumor, anti-oxidant, immune enhancement, blood sugar lowering, blood lipid lowering, liver protection, etc. [12] In this study we used the glomerular cell line (MES-13) as the experimental model to understand the possible effect of *Phyllanthus emblica* and constituted phytocompounds, on scavenging the free radicals, reducing the ROS production and restoring mitochondrial imbalance created by high glucose supplementation to the cells which mimic the diabetic condition induced glomerular damage.

2. Materials and methods

2.1. Chemicals

All the chemicals and reagents used in this study were purchased from Merck Taiwan Co., Ltd. Red CC-1 staining kit was purchased from Molecular Probes, Eugene, Oregon, USA. MES-13 cell lines were purchased from Bioresource Collection and Research Centre, Food Industry Research and Development Institute, Taiwan.

2.2. Preparation of water extract of *Phyllanthus emblica*

*Phyllanthus emblica* fruit sample (PE) was gently washed twice with 95% alcohol and air dried. A dried fruit sample of 500g was homogenized and the powdered sample was taken in a bottle, mixed with 1 L of 70% alcohol, and placed in a 40 °C water bath overnight. The concoction was filtered using the Buchner funnel and filter paper (Whatmann No-1). Then, by vacuum concentrator (BUCHI R210), the sample was concentrated (water bath is set to 42° C & Pressure was set at 175 mbar) into a green, paste-like thick substance. The final weight of the extract was 39.71 g and the yield was approximately 8%. Final extract was aliquoted into 6 tubes (15 ml), each weighed from 5 to 9.43 g.

2.3. Anti-oxidant activities

2.3.1. DPPH free radical scavenging activity: PE samples with 5 different concentrations (0 – 300 μg/mL) were used to check its DPPH free radical scavenging activity [13]. Trolox was used as the standard. In a 96-well plate, 50 μL sample/trolox and 50 μL of DPPH in methanol (0.16 mM) were added to each well, mixed thoroughly. The experiment was performed in 3 replicates, with methanol (50%) as the background control. After incubating for 20 minutes in the dark, the absorbance was measured at 517 nm. The results were expressed as the % scavenging ability of the sample and it was calculated by the formula given below.

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\text{DPPH radical scavenging activity (\%)} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100
\]

Absorbance of Sample
2.3.2. **Ferric reducing antioxidant power (FRAP) assay:** PE samples with 7 different concentrations (0 – 100 μg/mL) were used to check its Ferric ion reducing power [13]. Trolox was used as the standard. FRAP working solution was prepared using acetate buffer (0.3 M), TPTZ (10 mM) and FeCl\textsubscript{3}.6H\textsubscript{2}O (20 mM) mixed at a ratio of 10:1:1. In a 96-well plate, 6 μL of sample was added with 180 μL of FRAP working solution to each well and mixed thoroughly. The experiment was performed in 3 replicates with water as the background control. After incubating for 5 minutes at room temperature in dark, the absorbance was measured at 593 nm. The amount of Fe\textsuperscript{2+} formed was calculated using standard graph generated with 7 different concentrations of FeSO\textsubscript{4} against absorbance value.

2.4. **Cell culture method**

SV40-transformed Murine mesangial cells (MES-13) were obtained from the Bioresource Collection and Research Centre, Food Industry Research and Development Institute, Taiwan, and maintained in DMEM and Ham’s F-12 medium (3:1) containing 5% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), HEPES (14 mM), and glucose (100 mg/dl) at 37°C in an atmosphere containing 5% CO\textsubscript{2}.

2.5. **SDS-PAGE**

Cells were seeded (1 x 10\textsuperscript{4} cells per well) in a 24 well plate and incubated for 1 day at 37°C with 5% CO\textsubscript{2}. Then the cells were exposed to serum-free medium for 2 days and then exposed to different glucose concentrations with and without PE for 24 hr. After incubation, cells were trypsinised and the cell pellet was washed with lysis buffer containing 1 mM Tris–HCl, 0.1 mM EDTA, pH 8.0 and 200 mM PMSF, 1 mg/ml aprotinin, and supernatant was collected and run on an SDS-PAGE to observe the protein patterns of PE treated and untreated cells. After running the gel, it was stained with Coomassie brilliant blue dye to observe the protein bands.

2.6. **Processing of coverslips**

Coverslips were washed with 1 M HCl followed by subsequent washes with water and ethanol separately. The dried coverslips were coated with poly-D-lysine and washed with double distilled water and ethanol separately. The poly-D-lysine coated coverslips were air-dried and used for Red CC-1 assay.

2.7. **Red CC-1 assay**

Cells were seeded to Poly-D-lysine coated cover slips in 24 well plate at 1 x 10\textsuperscript{4} cells per coverslip and incubated for 1 day. Cells were then moved to serum-free medium for 2 days, before they were changed to serum free media containing normal sugar (5 mM) or high sugar levels (25 mM), with or without extract. Red CC-1 and Mito-tracker double staining was used to detect oxidation and mitochondria, respectively. Cells were then fixed with 4% Paraformaldehyde for 15 minutes and stained with DAPI staining. Photographic analysis was performed under a Zeiss Axiocam CCD camera mounted on Zeiss Axioscope.

3. **Results**

3.1. **Antioxidant activities**

Establishing the antioxidant potential is the key step in our study, since we propose to inhibit the high glucose-induced glomerular toxicity through restoration of cellular redox status. Here we report the antioxidant potential of PE through exhibiting its ability to inhibit ROS generation and scavenge the generated free radical.

3.1.1. **DPPH free radical scavenging activity:** Free radical scavenging ability was checked using DPPH assay (Figure 1A-D). Our results suggested that PE showed logarithmic increase in activity until 200 μg/ml (80%) concentration and saturated beyond that (Figure 1B & D). Extract containing various
phytoconstituents scavenges free radicals is comparable to the pure Trolox (Figure 1A & C), which provides almost 90% of the free radical scavenging ability around 200 µg/ml.

![Figure 1. DPPH free radical scavenging activity of Trolox and PE.](image)

A: Standard curve of DPPH scavenging activity obtained after addition of different concentration of Trolox; B: Standard curve of DPPH scavenging activity obtained after addition of different concentration of PE extract; C: DPPH scavenging activity of Trolox; D: DPPH scavenging activity of PE. Both Trolox and PE showed increase in scavenging activity with increase in the sample concentration, while PE showed saturation at 300 µg/ml.

3.1.2. FRAP assay
Ferric reducing power of the PE was found to be significant and it increased with increasing concentration of the extract (Figure 2A-E), as the polyphenolic compounds’ concentration would have increased. FRAP assay reports the ability of antioxidant compounds to reduce Fe$^{3+}$ to Fe$^{2+}$ which will be calculated with the help of measuring the Fe$^{2+}$ concentration formed in the solution, using the linear curve plotted with FeSO$_4$ (Figure 2A). Higher the Fe$^{2+}$ level greater is the reducing power of the sample, which was observed in case of PE extract which showed up to 200 µg/ml of Fe$^{2+}$ formation with 100 µg/ml of PE extract (Figure 2C & E). However, similar to the DPPH activity, pure Trolox showed much better reducing power (Figure 2B & B), but PE extract showed comparable ferric reducing power to Trolox.
Figure 2. Ferric reducing power of Trolox and PE.
A: Standard curve of FeSO$_4$; B: Standard curve of Fe$_2$SO$_4$ concentration obtained after addition of different concentration of Trolox; C: Standard curve of Fe$_2$SO$_4$ concentration obtained after addition of different concentration of PE extract; D: Ferric reducing power of Trolox measured with amount of FeSO$_4$; E: Ferric reducing power of PE measured with amount of FeSO$_4$. Trolox and PE showed significantly increasing conversion of Fe$^{3+}$ to Fe$^{2+}$ with increase in the concentration.

3.2. Comparison of the antioxidant activities
Based on the linear regression curve plotted with scavenging activity against concentration of the sample (PE/Trolox) we could see at 50 µg/ml, the scavenging activity of PE was around 1/3 of the activity displayed by Trolox (Figure 3A). On the other hand, the Ferric reducing power of PE was observed to be 1/4 of the activity exhibited by Trolox at 80 µg/ml (Figure 3B).
Figure 3. Comparison of antioxidant activities between Trolox and PE.
A: DPPH free radical scavenging activity; B: Ferric reducing power. PE extract showed significantly comparable activity in both assays towards Trolox.

3.3. Change in proteomic profile in SDS-PAGE
The ability of PE to reduce and scavenge the ROS will affect significant number of cellular signalling and hence the proteomic profile of cells should be upregulated significantly. We intended to show the change in proteomic pattern through SDS-PAGE analysis (Figure 4), where we could see high glucose exposure has significantly reduced the amount and pattern of proteins in the cells compared to the cells supplied with normal glucose concentration.

Interestingly, an improved proteomic pattern was observed when the high glucose (25 mM) treated cells were supplied with PE at 10 µg/ml concentration and are found to be almost similar to the normal glucose treatment. However, 100 µg/ml PE supplementation showed a reduced protein profile similar to high glucose treatment without PE, which might be due to several reasons including the extract itself interfering with the electrophoretic separation process and so we can infer that, 100 µg/ml of PE is not suitable for further testing.

3.4. Red CC-1 assay for mitochondrial imbalance
The major observation of our study is to expose the MES-13 cells to high glucose and check the redox status of the cells with and without PE treatment. From Figure 5A & 5B, it was observed that oxidative
stress was induced in the cells (Figure 5B) with positive Red CC-1 stain, while normal cells (Figure 5A) showed an insignificant number of red stains.

Figure 5C-F indicated oxidative stress and mitochondrial health in cells. High glucose supplemented cells treated with PE showed significantly reduced Red CC-1 stain (Figure 5D, 5F) compared to untreated cells (Figure 5C & E) indicating the restored redox balance.

Figure 5G shows the level of red staining observed in cells and an increased in the red stain intensity was clearly seen in the high glucose supplemented cells compared to cells supplemented by normal glucose levels. However, the intensity of red stain was measured to be significantly less in the case of high glucose supplemented cells added with PE extract than the cells without PE extract.

![Figure 5](image)

**Figure 5.** Red CC-1 fluorescence staining of MES-13 cells, treated with high glucose and supplemented with and without PE extract.
A: Normal glucose treatment (5 mM); B: High glucose treatment (25 mM); C & E: High glucose treatment (25 mM) without PE; D & F: High glucose treatment (25 mM) with PE (1000 µg/ml). The results were statistically analysed by students t-test (n=3) and the comparisons were made as Normal glucose Vs High glucose (*); High glucose Vs High glucose + PE (#). Increased oxidative stress in the untreated cells were observed while PE has reduced the oxidative stress and improved mitochondrial homeostasis.

### 4. Discussion

Complications that arise in the system due to chronic diabetic condition make the management of this disorder very difficult [14]. A lot of research has been conducted for years to treat diabetes and its complications, but still, medications that provide successful cure with minimal side effects were not identified [15, 16]. Phytochemicals were explored for the management of diabetic nephropathy that includes several polyphenolic compounds from medicinal plant parts and functional foods [17, 18]. PE is one of the medicinally renowned plant species, rich in terpenoids and several other bioactive compounds. In our study, we prepared an hydroalcoholic extract from PE fruit and exhibited its antioxidant potential. We have also proved its ability to reduce the ROS generation and scavenge the free radicals to restore the mitochondrial redox balance, which is considered as the main event among several metabolic abnormalities induced by high glucose levels in the system.

Our exploration started with exhibiting the ability of PE extract to scavenge the free radicals by DPPH scavenging activity and through FRAP assay we aimed to show the effect of PE in reducing ROS generation. DPPH is a stable free radical and upon addition of an antioxidant compound that can donate...
electrons results in a colourless solution due to scavenging of DPPH free radicals [19]. PE extract showed a significant reduction in the DPPH free radical, with increasing concentration which indicates the presence of several anti-oxidant molecules that can reduce the free radical molecules. The ability to reduce the radical compounds is one of the prime requirements in our study as continuous generation of ROS is reported due to mitochondrial dysfunction during diabetic kidney disease which must be quenched to avoid interfering with several successive abnormal signalling [20, 21]. Simultaneously reduction of ROS generation is also required to be inhibited to counter that redox imbalance, hence we tested the potential of PE in the conversion of ferric ion (Fe$^{3+}$) to ferrous state (Fe$^{2+}$) which indirectly shows the ability of PE to reduce ROS generation. Generation of hydroxyl radicals act on several biomolecules especially lipids, to form lipid peroxides that further helps in accelerated lipid peroxidation reactions [22]. PE extract increased conversion of Fe$^{3+}$ to Fe$^{2+}$ with increasing concentration which indicates its ability to slow down that ROS generation in the cells. We have compared the anti-oxidant activities of PE with Trolox as positive control and it was observed that PE showed 1/3rd of the DPPH scavenging activity of Trolox. In the case of Ferric reducing power, PE extract showed a quarter of the ability shown by Trolox.

Our further exploration with cellular protection of PE is carried out on MES-13 cells, for mimicking the glomerular toxicity induced due to high glucose exposure. Generally, imbalance in the cellular metabolism largely affect the signalling pathways and this can modify the proteomic profile of the cells [23, 24]. We intended to observe that with SDS-PAGE experiment and we found that high glucose supplementation affected the protein separation pattern in the gel, compared to the untreated cells. This could be due to interference in normal signalling pathways and protein synthetic process which could be slowed down in order to channelize the cellular processes towards protection and restoration of homeostasis rather than cell growth and energy production [25]. This was reflected in the proteomic pattern where high glucose treatment has significantly reduced number of proteins than normal cells. However, PE supplementation has seemed to restore that cellular imbalance as indicated by lane 4 of the gel (Figure 4) showing nearly identical separation pattern comparable to normal cells. Higher concentration of the PE however did not support this observation due to reasons like impurities in the sample due to extract or increased number of compounds that did not allow the active compounds to protect the cells. But our study proves the ability of PE to interfere with cellular toxicity caused by high glucose levels. This is a very promising observation as glucose-induced processes like AGE formation, inflammatory signalling, apoptosis, etc were presumed to be reduced [26-28].

Cells exposed to abnormal glucose levels undergo several changes and the key step that initiates these cell-damaging processes is the generation of ROS due to mitochondrial dysfunction [21]. Shi et al., (2018) [2] has discussed various signalling molecules upregulated during diabetic nephropathy condition that includes, advanced glycation end products, diacylglycerol, protein kinase C, NF-κB, caspases, TGF-β, etc [2]. However, the major culprit was seeming to be the generation of ROS due to high glucose level induced abnormal glycosylation of functional protein, including the mitochondria-derived proteins [28]. Hence, inhibition of ROS-induced stress was understood as the primary target for reducing glucose-induced glomerular toxicity. In our study, MES-13 cells exposed to high glucose showed poor redox status compared to normal cells and PE supplemented cells. This shows PE was able to inhibit the ROS generation as discussed above which resulted in reduced mitochondrial dysfunction and redox imbalance.

5. Conclusion
To summarize, the ability of PE to scavenge and inhibit free radicals, showed great promise towards its cellular protection. Cells supplemented with PE showed significantly improved cellular redox status and mitochondrial health. This shows that the active compounds in the PE extract can reduce the glucose induced cellular damage not only in the glomerular region, but in other regions of nephron and exhibits its potency against diabetic nephropathy. Further studies on specific proteins regulation during PE supplementation leading to reduced mitochondrial damage and cellular ROS levels has to be done along
with exploration of single active compounds from the extract that can help in identification of a potent medication for diabetes induced complications.

References

[1] Okur M E, Karantas I D and Siafaka P I 2017 Acta Pharm. Sci. 55 61-82
[2] Shi G J, Shi G R, Zhou J Y, Zhang W J, Gao C Y, Jiang Y P, Zi Z G, Zhao H H, Yang Y and Yu J Q 2018 Biomed. Pharmacother. 101 510-27
[3] Neelofar K and Ahmad J 2017 Glycoconj. J. 34 575-84
[4] Fournet M, Bonté F and Desmoulière A 2018 Aging Dis. 9 880-900
[5] Gulseth H L, Wium C, Angel K, Eriksen E F and Birkeland K I 2017 Diabetes Care 40 872-8
[6] Zheng Y, Ley S H and Hu F B 2018 Nat. Rev. Endocrinol. 14 88-98
[7] Persson F and Rossing P 2018 Kidney Int. Suppl. 8 2-7
[8] Zeni L, Norden A G, Cancarini G and Unwin R J 2017 J. Nephrol. 30 701-17
[9] Xue R, Gui D, Zheng L, Zhai R, Wang F and Wang N 2017 J. Diabetes Res. 2017 1839809
[10] Kandhare A D, Mukherjee A and Bodhankar S L 2017 Chem.-Biol. Interact. 278 212-21
[11] Dehdashtian E, Pourhanifeh M H, Hemati K, Mehrzadi S and Hosseinzadeh A 2020 Diabetes Metab. Res. Rev. 36 e3336
[12] Yadav S S, Singh M K, Singh P K and Kumar V 2017 Biomed. Pharmacother. 93 1292-302
[13] Lee M J, Rao Y K, Chen K, Lee Y C, Chung Y S and Tzeng Y M 2010 J. Ethnopharmacol. 132 497-505
[14] Ighodaro O and Adeosun A 2018 Kidney 4 16-9
[15] Kasole R, Martin H D and Kimiywe J 2019 Evid Based Complement Alternat Med 2019 2835691
[16] Raval N, Kumawat A, Kalyane D, Kalia K and Tekade R K 2020 Drug Discov. Today 25 862-78
[17] Parveen A, Jin M and Kim S Y 2018 Phytomedicine 39 146-59
[18] Gong X, Li X, Xia Y, Xu J, Li Q, Zhang C and Li M 2020 Trends Food Sci. Technol. 103 304-20
[19] Yeo J and Shahidi F 2019 J. Agric. Food Chem. 67 7526-9
[20] Galvan D L, Green N H and Danesh F R 2017 Kidney Int. 92 1051-7
[21] Wei P Z and Szeto C C 2019 Clin. Chim. Acta 496 108-16
[22] Kaczmarczyk-Sedlak I, Folwarczna J, Sedlak L, Zych M, Wojnar W, Szumińska I, Wyględowska-Promieńska D and Mrukwa-Kominek E 2019 Arch Med. Sci. 15 1073-80
[23] Kang G G, Francis N, Hill R, Waters D, Blanchard C and Santhakumar A B 2020 Int. J. Mol. Sci. 21 140
[24] Tarazona O A and Pourquïé O 2020 Dev. Cell 54 282-92
[25] Sarkar N, Das B, Bishayee A and Sinha D 2020 Antioxid. Redox Signal 33 1230-56
[26] Prasad K and Tiwari S 2017 Curr. Pharm. Des. 23 937-43
[27] Wang P, Chen F, Wang W and Zhang X D 2019 Mediators Inflamm. 2019 8908960
[28] Sifuentes-Franco S, Padilla-Tejeda D E, Carrillo-Ibarra S and Miranda-Diaz A G 2018 Int. J. Endocrinol. 2018 1875870