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Purslane (*Portulacaoleracea*) Seed Extracts Counteract Diabetic Nephropathy Oxidative Stress: Potential inhibition of DNA Damage and ROS-Mediated Apoptosis

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Abstract

The most frequent and dangerous consequence of diabetes mellitus is diabetic nephropathy (DN), which is the primary cause of chronic kidney damage. Oxidative stress is a key player in the pathophysiology of diabetic nephropathy. The purpose of this study was to employ naturally occurring products that have a strong ability to prevent oxidative stress, such as purslane (*Portulacaoleracea*) extracts. By using chromatographic techniques, seven compounds were isolated from the purslane seeds following a phytochemical examination. These compounds were identified through spectroscopic data analyses. Seventy rats were divided into seven groups including control, purslane seeds methanol and methylene chloride extracts, DN), DN plus each extracts and DN plus losartan. Purslane methanol and methylene chloride extracts against DN were evaluated to the DNA fragmentation, expression levels of diabetes and nephrotoxicity related genes, the activity of GPx, SOD, GST enzymes, NO level, the apoptosis, and ROS generation. DN rats showed increased in DNA fragmentation, rise in the expression levels of diabetes and nephrotoxicity related genes, decline in the tested antioxidant enzymes activity and elevation of the NO level and apoptosis. Treatment of DN rats with two extracts of purslane improved the harmful effects that appeared in the DN rats, but the efficiency of the methanolic extract was much higher than those in methylene chloride extract. These results suggested that the reduction of oxidative stress after treatment with *P. oleracea* extracts can be due to the presence of antioxidant compounds such as flavonoids and polyphenols which scavenge the free radical.

Keywords: Diabetic nephropathy; *P. oleracea* extracts; DNAfragmentation; Gene expression; Antioxidant capacity; ROS formation; Apoptosis.

1. Introduction

Over 90% of people with diabetes are diagnosed with type II diabetes mellitus, a metabolic and endocrine disorder an endocrine and metabolic condition carried on by a confluence of environmental and polygenic conditions **[1]**. Insulin resistance and/or a relative lack of insulin secretion are the hallmarks of this illness ***[1,2]***. The World Health Organization estimates that there are currently 200 million diabetic people worldwide, and by 2030, that number is predicted to reach to over 336 million**[2]**.

The main treatment for type 2 diabetes is oral hypoglycemia drugs (e.g. [biguanides**[3],** thiazolidinedione (TZDs**[4]**) or glucosidase **[5]** inhibitors. Although they are effective in controlling blood glucose levels, they may not protect against long-term complications (e.g., nephropathy)**,** and cardiovascular disorders. These drugs are also known to cause serious side effects over time, including: acarbose-related gastrointestinal disorders; granulocytopenic and hypoglycemic reactions (hypoglycemia) associated with the use of glibenclamide; Lactic acidosis associated with the treatment of metformin **[6,7]**.

In traditional Egyptian medicine, several plants are used to treat a lot of diseases such as diabetes and kidney diseases and there has been much clinical experience in its treatment over the years. The annual plant Purslane (*Portulaca olerace*) is known for its succulent leaves, which can be found growing on both the ground and in vertical orientation depending on the conditions of light, which spreads throughout the world and grows in a wide variety of geographical environments. Purslane, a C4 plant in the Portulacaceae family, is considered one of the most effective medicinal herbs. It has been acknowledged by the World Health Organization as a "Global Panacea". It is a. traditional Egyptian medicinal plant that is now widely distributed throughout the world Active ingredients include, coumarin, fatty acids, flavonoids, alkaloids, and polysaccharides**[8-11].**

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It can be used as a vegetable and for medicinal purposes in healthcare due to its quantity of antioxidant vitamins and omega-3 fatty acids, especially to prevent certain cardiovascular illnesses and support a healthy immune system. It is well recognized for its antimicrobial, anti-inflammatory, and antioxidant effects. It also controls how the body uses sugar and fat. *Portulacaoleracea* (*P. oleracea*) aqueous extract also protects against diabetes and its associated vascular consequences by preventing diabetic vasculitis, hyperglycemia, and diabetic endothelium dysfunction in type 2 diabetic db/db mice **[12, 13]**.This plant's crude polysaccharide extract has been demonstrated to lower blood glucose levels, regulate blood lipids and glucose metabolism in alloxan-induced diabetic mice, and lower total cholesterol, triglycerides, and fasting blood glucose in mice with type 2 diabetes**[12, 13]**.

Bioactive compounds derived from plants have distinct chemical structures, and their ability to be extracted or active in various model systems appears to depend entirely on their chemical structure. Therefore, different solvent systems can be used to extract antioxidants, and their selective regeneration varies depending on the nature of the plant materials as antioxidant. Furthermore, Antioxidant assays can be complicated by a variety of additional parameters, including non-antioxidant components and the polarity of extraction solvents **[14]**. Therefore, it is essential to evaluate how different solvents affect purslane's antioxidant properties and the extraction of bioactive compounds.

The work detailed in this preliminary report was done to assess how well two extracts (methanol and methylene chloride) performed in extracting the active ingredient from purslaneseeds and in preventing diabetes and nephrotoxicity. Use of purslane extracts for treatment of diabetes and kidney diseases is not strictly evaluated. In this study, purslane methanol, and methylene chloride extracts were separated and used for treatment of diabetic nephropathy (DN) rats to determine its effectiveness based on decrease of DNA fragmentation, decline the expression levels of diabetes and nephrotoxicity related genes, increase the activity of GPx, SOD and GST enzymes, decreases NO level and minimise the apoptosis and ROS generation in the DN rats.

2. Materials and methods

2.1. Plant material.

The Harraz Company for Agricultural Seeds, Spices, and Medicinal Plants, Cairo, Egypt, provided the purslane (*Portulacaoleracea* L.) seeds. The taxonomist Mrs.TressaLabib of the El-Orman Botanical Garden in Giza, Egypt, identified it.

2.1.1. General experimental procedure

Sephadex LH-20 polymer gel and silica gel 60 (Merck) were used in column chromatography. Silica gel (Merck) was used as the stationary phase in TLC. Chromatograms were first examined under a UV light, and after that, 20% sulfuric acid in $FeCl_3$ or ethyl alcohol spray reagent was sprayed on them. The NMR spectra were recorded using a JEOL EX 500 MHZ spectrometer. The TMS was used as the internal standard, and the chemical shifts were recorded on the δ-scale. However, the Shimadzu UV-240 spectrometer was used to obtain the UV spectra.

2.1.2. Extraction and isolation

After being cleaned and allowed to air dry for a few hours, the 1.5 kg of purslane seeds were extracted twice at room temperature using dichloromethane then with methanol were used to extract it. To obtain methylene chloride extract (10g) and methanolic extract (12g), the extraction procedure was repeated twice using the same solvents. Each extract was then evaporated under reduced pressure at a temperature not to exceed 40^{o}C. Rats were given the freshly made semi-solid extract residues as an oral suspension in distilled water for the experiments. The methylene chloride extract (10g) was chromated using a silica gel column. First, n-hexane and then n-hexane-ethyl acetate mixtures with increasing concentrations of ethyl acetate up to 15% were used to elute the solvents extract . 50 fractions in all, holding 100 milliliters each, were gathered. The fractions were sprayed with 20% sulfuric acid to examine them and were tracked using silica gel TLC plates with a chloroform-methanol (10:1) solvent system. Similarity was found between the fractions that eluted with 10% ethyl acetate. After these fractions were mixed, it was discovered that compound 1 was a significant component. **Compound 1**(100gm) was obtained by repeatedly chromatographing the combined fraction on a sephadex LH-20 Column with methanol as the eluent. A polymer gel Diaion HP-20 column (250g) was used to analyze the 12g of methanolic extract. Water was used to elute the column initially, and then an H_2O-MeOH mixture was added until 100% methanol was present. A total of sixty fractions, each containing fifty milliliters, were gathered. The fractions were then subjected to UV examination, TLC monitoring using the solvent systems $CHCl_3$-MeOH (5:1) and $CHCl_3$-MeOH-H_2O (60-30-5) and sprayed with $FeCl_3$ regnant. After eluting with 100% water, the fractions were combined. Following the solvent's evaporation, the residue was run through a sephadex column and eluted with 100% methanol, yielding **compound 2** (25 mg). **Compound 3** (15 mg) was obtained by repeatedly chromatographing the fraction eluted with 25% methanol on a sephadex LH-20 column with methanol serving as the eluent. The fractions were mixed after being eluted with 40% and 50% methanol in H_2O. Repeated preparative thin layer chromatography (PTLC) on silica gel plates with the solvent system $CHCl_3$-MeOH-H_2O (60-30-5) produced **compound 4** (20 mg) from the residue that was obtained. The HP-20 column's eluted

fractions containing 60% and 70% aqueous methanol were mixed. Using a CH_2Cl_2-MeOH (5:1) solvent system, preparative thin layer chromatography was applied to the residue. **Compounds 5** (15 mg) and **6** (20 mg) were obtained by repeatedly purifying the material from the two bands that were observed on a sephadex LH-20 column. **Compound 7** (50 mg) was obtained by repeating purification on a sephadex LH-20 column eluted with methanol after the fraction eluted from the HP-20 column with 100% methanol was subjected to a silica gel column eluted with CH_2Cl_2-MeOH (20:1 to 10:1) solvent system. Rats were given freshly made semi-solid extract residues as an oral suspension in distilled water for the experiments.

2.2. Animals

In this investigation, 180–200 g male Sprague-Dawley rats were used. Rat chow and tap water were freely available to the animals, who were kept in cages with a temperature control of 25 ± 2◦C and a 12:12 h light/dark cycle.

2.3. Toxicity study

Seven groups of fifty-six male Sprague-Dawley rats, weighing between 180 and 200 g each, were used to determine the acute toxicity of the two seed extracts under study. Based on studies on oral acute toxicity conducted by **Çelik et al.[15]**, different doses of purslane seeds (250, 500, and 1000 mg/kg b.wt) were chosen. After giving the tested extracts for 24 hours, the animals were observed for 15 days. The recorded LD50 was used to monitor the safety dose. The average body weight, organ weight, changes in the rate at which food and water were consumed, and various biochemical parameters of each group were recorded during the comparatively extended 42-day trial period.

2.4. Experimental design

Seventy rats were split up into the following seven groups, each with ten rats: **Group 1**: Throughout the experiment, these rats were used as controls and received daily injections of 0.1 ml of saline orally. **Groups 2 and 3:** Methanol (ME) and methylene chloride extracts (MCE) from *P. oleracea* seeds were given orally to control rats once a day for six weeks. The dose used was based on the acute toxicity study and was 250 mg/kg b.wt. For type 2 diabetic model (T2DM), animals were fed a high-fat diet (60% calories as fat) for 2 weeks, and then the animals received a single intraperitoneal injection of 30 mg/kg of streptozotocin**(STZ, Sigma-Aldrich Chemical Co., USA)** in 0.1 mol/L citrate buffer, pH 4.5, with recovery high-fat diet during the experiment **[16]**. All diabetic rats received drinking water supplemented with sucrose (15g/L) for 24 hours after the STZ injection according to **Kumar et al.[17]** to prevent hypoglycemic shock. Animals in **Group 4** were kept as positive control rats for DN, while **Groups 5 and 6** included DN rats that received methanol and methylene chloride extracts of *P. oleracea* seeds, respectively, for 6 weeks. **Group 7** included DN rats were received orally a dose of 2 mg/ Kg of Losartan as a reference drug for 6 weeks **[18].**

During weeks 1, 2, 4, and 6, blood samples were taken *via*retroorbital puncture while under a light diethyl ether anesthesia to assess molecular and biochemical markers associated with diabetes mellitus. Sera were kept at –20° C until they were needed after blood samples were centrifuged for 10 minutes at 3000 rpm. Animals were sacrificed at the end of the experiment, and the kidneys were removed and cleaned with isotonic saline. After the kidneys were minced, a homogenate was made in a homogenizer with 10% (w/v) phosphate-buffered (0.1 M, pH 7.4). The kidney homogenate was centrifuged for 10 minutes at 3000 rpm, and the supernatant was measured to determine the kidney's NO and SOD levels.

2.5. DNA fragmentation assay

2.5.1. DNA gel electrophoresis laddering assay

The laddering pattern of nuclear DNA, as reported by **Lu et al. [19]**, was used to qualitatively analyze apoptotic DNA fragmentation. In Tris/acetate/EDTA (TAE) buffer, the isolated DNA was electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The gels were exposed to ultraviolet transillumination to visualize and photograph the DNA fragments. A DNA ladder (Invitrogen, USA) was included as a molecular size marker.

2.5.2. Diphenylamine reaction procedure

The quantitative profile of the DNA fragmentation was determined using animal blood samples. After the samples were lysed in lysis buffer, they were centrifuged for 20 minutes at 4°C at 10,000 rpm. 0.5 ml of 25% tri-chloroacetic acid (TCA) was added to the pellets (P) and supernatants (S), and the mixture was incubated for 24 hours at 4°C. After centrifuging the samples for 20 minutes at 10,000 rpm at 4°C, the pellets were suspended in 80 milliliters of 5% TCA and incubated for 20 minutes at 83°C. Then, 160 milliliters of Diphenyl Amine (DPA) solution were added to each sample, and they were left to incubate for 24 hours at room temperature **[20]**. Using the following formula, the percentage of fragmented DNA was determined from absorbance measurements at 600 nm wavelengths:

$$\% \text{ Fragmented DNA} = [OD(S)/[OD(S) + OD(P)] \times 100$$

(OD: optical density, S: supernatants, P: pellets)

2.6. Expression of diabetes and nephrotoxicity related genes

2.6.1. Total RNA Isolation

Using a standard TRIzol® reagent extraction method (Invitrogen, Germany), total RNA was extracted from whole blood samples of male rats. After the separated RNA pellet was allowed to air dry, it was dissolved in water that had been treated with diethyl pyrocarbonate (DEPC) by repeatedly passing the mixture through a pipette tip *[21]*. Reverse transcription (RT) was conducted as soon as possible, and the remaining aliquots were stored at -80°C. The purity of total RNA was determined by measuring the ratio 260/280 nm (between 1.8 and 2.1).

2.6.2. Reverse transcription (RT) reaction

Using the RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Germany), total poly(A)+ RNA isolated from blood samples was reverse transcribed into cDNA in a total volume of 20 µl. Prior to being used for quantitative real-time polymerase chain reaction (RT-PCR) DNA amplification, test tubes holding RT preparations were kept at -20ºC.

2.6.3. qRT-PCR analysis

To ascertain the copy number of DNA in animal samples, Applied Biosystems' StepOne^{TM} real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA) was employed. 25 µL reaction mixtures containing 12.5 µL 1× SYBR® Premix Ex TaqTM (TaKaRa, Biotech. Co. Ltd.), 0.5 µL 0.2 µM sense primer, 0.5 µL 0.2 µM antisense primer, 6.5 µL distilled water, and 5 µL of cDNA template were used for PCR reactions. There was distilled water control in every experiment. Primer3 software was used to design the gene-specific primer sequences (Col1a1-F: TCA AGA TGG TGG CCG TTA CT, Col1a1-R: CAT CTT GAG GTC ACG GCA TG; Tgfb1-F: TCG CTT TGT ACA ACA GCA CC, Tgfb1-R: ACT GCT TCC CGA ATG TCT GA; Cox6c-F: TAA GTT TGG CGT GGC TGA AC, Cox6c-R: TTC AGG AAC ACA GGT CAG CA;β-actin-F: TGT GTT GTC CCT GTA TGC CT, β-actin-R: R: AAT GTC ACG CAC GAT TTC CC). The designed primers have the following NCBI references: NM_053304.1, NM_021578.2, NM_019360.2, and EF156276.1. To assess the quality of the primers utilized in each qRT-PCR, a melting curve analysis was carried out at 95 °C *[22]*. The 2−ΔΔCT method was used to determine the target for comparison.

2.7. Apoptosis assay

Using **Khalil et al. [23]** method, blood cells were homogenized in single-cell suspensions. The Annexin V/PI Apoptosis Detection Kit was used in flow cytometry (FCM) to determine the apoptosis of the cells level. 200 µL of ice-cold binding buffer were used to suspend a single cell suspension (1 x 106 cells/ml). Next, 10 µL of horseradish peroxidase FITC labeledAnnexin V and 5 µL of propidium iodide (PI) were added. The cell suspension was incubated for fifteen minutes at room temperature in the dark. By using flow cytometry to distinguish between normal cells (FITC- and PI-negative) and apoptotic or necrotic cells (FITC- and PI-positive), the rate of apoptosis was ascertained.

2.8. Determination of ROS formation

After single-cell suspensions were prepared according to **Khalil et al. [24]**, the amount of intracellular ROS production in blood cell tissues was measured using a flow cytometer fitted with an oxidation-sensitive DCFH-DA fluorescent probe. The flow cytometer (with excitation at 488 nm and emission at 525 nm) was used to detect fluorescence. Three replicates of the experiment were run, with 1×10^5 cells counted for each treatment.

2.9. Determination of Glutathione peroxidase and Glutathione S-transferase activities in serum

A **Miranda et al.** *[25]* protocol was used to measure the activities of glutathione S-transferase (GST) and glutathione peroxidase (GPx). The reaction mixture contained the following components: pH 5.5, 40 mMguaiacol, 50 mM sodium acetate buffer, and 8 mM H_2O_2. Every 30 seconds, the oxidation of guaiacol caused a change in absorbance at 470 nm, which was recorded. Under standard test conditions, the amount of enzyme that raises the optical density (OD) by 1.0/min was designated as one unit of glutathione peroxidase and glutathione-S-transferase activity.

2.10. Determination of Superoxide dismutase activity and Nitric oxide level in kidney homogenate

Superoxide dismutase (SOD) activity and Nitric oxide (NO) levels were measured using commercially available kits (Bio-diagnostic, Egypt) in kidney homogenate, according to manufacturer's protocol.

2.11. Statistical analysis

The Generalized Models (GLM) technique of the Statistical Analysis System **[26]** was employed to analyze all the data. The Scheffé test was then used to determine whether there were any significant differences between the groups. The values are given as mean ± SEM. Based on the probability of $P < 0.05$, all significance expressions were created.

3. Results

3.1. Chromatography

The phytochemical examination of the purslane seeds led to the isolation of the following seven compounds (**Figure 1**).**Taraxeryl acetate (1)** was isolated from methylene chloride while rest of compounds (**from compound 2-7**) was isolated from methanol.

Taraxeryl acetate (1): white crystals, H^1-NMR (500MHz, $CDCl_3$) 0.83 (3H, S, H -25), 0.87 (3H, S, H-23), 0.91 (3H, S, H-24), 0.92 (3H, S, H-26), 0.93 (3H, S, H-30), 0.93 (3H, S, H-29), 1.07 (3H, S, H-27), 2.03 (3H, S, OAC), 4.46 (1H, dd, J: 11.0, 6.4Hz, H-3), 5.51 (1H, dd, J: 8.5, 3.7 Hz, H-15). ^{13}C NMR (125MHz, CDCl3) δ: 37.8 (C-1), 23.6 (C-2), 81.1 (C-3), 37.8 (C-4), 55.7 (C-5), 18.8 (C-6), 33.4 (C-7), 39.1 (C-8), 49.2 (C-9), 37.5 (C-10), 17.4 (C-11), 35.4 (C-12), 37.8 (C-13), 160.6 (C-14), 116.9 (C-15), 33.8 (C-16), 35.5 (C-17), 48.9 (C-18), 41.5 (C-19), 28.7 (C-20), 35.5 (C-21), 37.4 (C-22), 28.1 (C-23), 16.7 (C-24), 15.7 (C-25), 26.2 (C-26),29.4 (C-27), 29.8 (C-29), 21.4 (C-30), 171.0 ($COCH_3$), -$COCH_3$ (21.4). The spectral data was consistent to the reported literature values ***[27].***

Gallic acid (2): white amorphous powder, ^{1}HNMR (500 MHz, acetone –d_6) δ: 7.2 (2H, S, H- 2, 6), ^{13}CNMR (125 MHz, acetone –d_6), δ: 167.09 (-COOH), 145.1 (C-3, 5), 137.7 (C-4), 121.2 (C-1), 109.2 (C-2,6).The spectral data was consistent to the reported literature values **[28]**.

3,4dihydroxyBenzoicacid (**3**): Brown amorphous powder, ^{1}HNMR (500 MHZ, DMSO) δ: 7.3 (^{1}H, br s, H-2), 7.24 (^{1}H , dd, J = 7.6, 2.0 Hz), 6.74 (^{1}H, d, J = 7.6 Hz), ^{13}CNMR (125 MHx, DMSO) δ: 122.4 (C-1), 117.1 (C-2), 145.4 (C-3), 150.5 (C-4), 115.6 (C-5), 122.4 (C-6), 167.8 (C-7). The spectral data was consistent to the reported literature values***[29].***

Kaempherol (**4**): Amorphus yellow powder, UV λmax nm (Meo11): 254 sh, 268, 322sh, ^{1}HNMR (500 MHz, acetone – d_6) δ: 12.04 (1H, S, OH -5), 8.02 (24, d, J = 8.9 Hz, H-′2, ′6), 6.86 (2H, d, J = 8.9 Hz, H- ′3, ′5), 6.41 (1H, d, J= 2 Hz, H-8), 6.14 (1H, d, J= 2.0 Hz, H-6). The spectral data was consistent to the reported literature values***[30].***

Quercetin (**5**): Amorphus yellow powder, UV λmax nm (MeOH): 266sh, 299sh, 360. ^{1}HNMR (500, DMSO) δ: 12.4 (1H, s, OH-5), 7.62 (1H, d, J= 2.0 Hz, H- ′2), 7.50 (1H, dd, J= 6.7, 2.0 Hz, H- 6), 6.85 (1H, d, J= 6.7 Hz, H-′5), 6.36 (1H, d, J=1.5 Hz, H-8), 6.14 (1H, d, J= 1.5, H-6). The spectral data was consistent to the reported literature values [***30]***.

Rutin (**6**): Amorphus yellow powder , UV λmax nm (MeOH): 358.6 257.8, ^{1}HNMR (500 MHz, DMSO-d_6) 12.56 (1H, S, 5-OH), 7.53 (2H, d, J= 8.0Hz H- ′2, ′6), 6.83 (1H, d, J= 8.4 Hz, H-′5), 6.36 (1H, S, H-8), 6.17 (1H, d, J= 1.6 Hz, H-6), 5.32 (1H, d, J= 7.2 Hz, H- ′′1), 4.39 (1H, brs, H′′′), 0.97 (3H, d, J=6.0 Hz, CH_3-6′′′) ^{13}CNMR (125 MHz, DMSOd_6) δ: 177.7 (C-4) 164.6 (C-7), 161.6 (C-5), 156.8 (C-2), 157.0 (C-9), 148.8 (C-′4), 145.1 (C-′3), 133.7 (C-3), 122.0 (C-′6), 121.5 (C-′1), 116.3 (C-′5), 115.6 (C-′2), 104.35 (C-10), 101.6 (C-′′1), 101.1 (C-′′′1), 99.1 (C-6), 94.0 (C-8), 76.87, (C-′′5), 76.33 (C-′′3), 70.4 (C-′′4), 74.5 (C-′′2), 67.4 (C-′′6), 70.80 (C-′′2), 70.98 (C-′′′3), 72.27 (C-′′′4), 68.67 (C-′′′5), 18.1 (C-′′′6).The spectral data was consistent to the reported literature values [***30].***

β-sitosterol 3-gulcoside (7): white amorphous powder, ^{1}HNMR (500 MHZ, DMSO-d_6) δ: 5.29 (1H, brs, H-6), 3.60 (1H, m, H-3), 0.87 (3H, d, H-21), 0.75 (3H, d, J= 6.0 Hz, H_3-26), 0.77 (3H, d, J= 6.0 HZ, H- 29), 4.18 (1H, d, J= 7.6 Hz, H-′1). 13CNMR ($_{125}$MHz, DMSO-d6) δ: 36.77 (C-1), 29.32 (C-2), 77.5 (C-3), 42.41 (C-4), 141.03(C-5), 121.69 (C-6), 31.93 (C-7), 31.99 (C-8), 50.19 (C-9), 36.7 (C-10), 20.6 (C-11), 38.89 (C-12), 42.4 (C-13), 56.74 (C-14), 24.40 (C-15), 28.31 (C-16), 56.02 (C-17), 12.2 (C-18), 19.16 (C-19), 33.94 (C-20), 19.16 (C-21), 32.0 (C-22), 26.0 (C-23), 45.0 (C-24), 29.5 (C-25), 19.6 (C-26), 19.5 (C-27), 20.23 (C-28), 12.30 (C-29), 101.4 (C-′1), 74.04 (C-′2), 77.28 (C-′3), 70.70 (C-′4), 77.30 (C-′5), 61.69 (C-′6).The spectral data was consistent to the reported literature values***[31].***

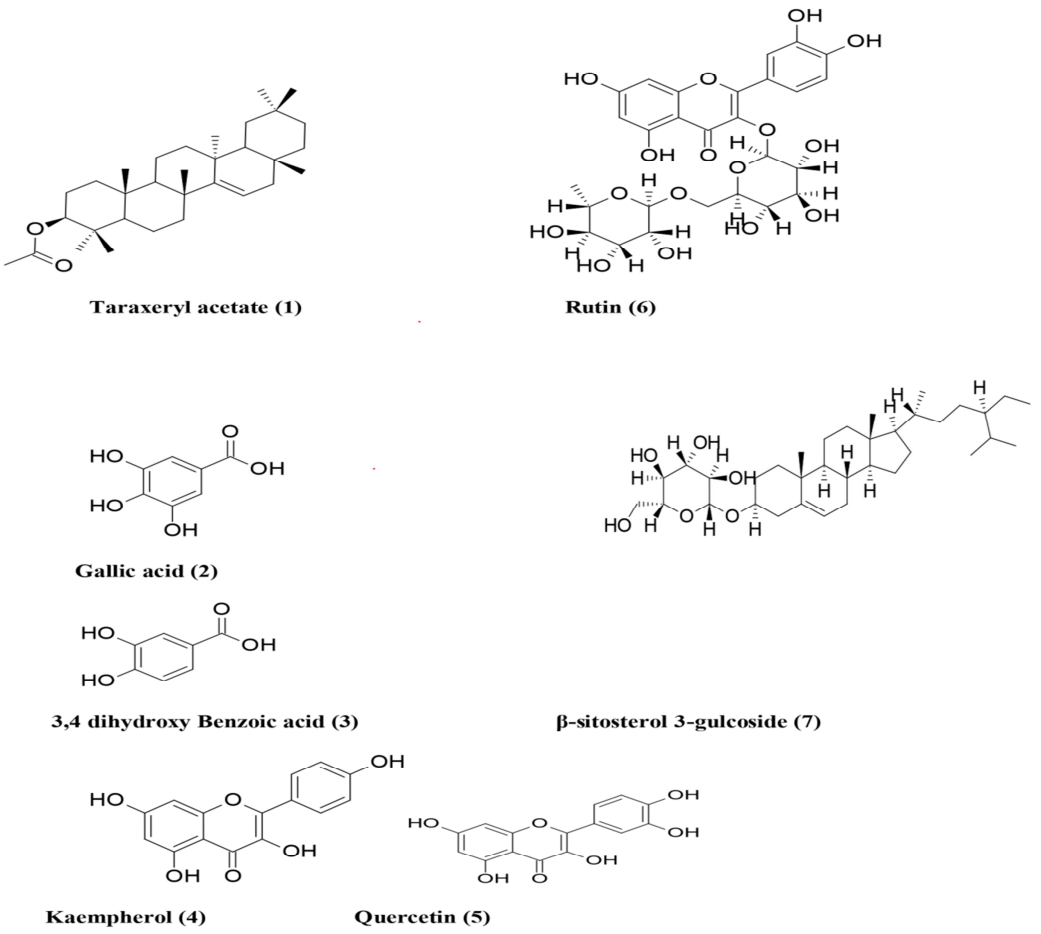

Figure 1: Compounds isolation for purslane seeds.

3.2. Toxicity study

During the experimental period, no dead animals were reported so we decided to use lower dose (250 mg/kg body weight) for biological analyses. Along with the renal function markers, body weight, organ weight, and all the measured biochemical parameters (serum levels of ALT, AST, total cholesterol, and triglycerides) estimated in the above groups were mentioned in a parallel work that was submitted but has not yet been published.

3.3. DNA fragmentation

Table 1 and Fig. 2 show the effect of *P. oleracea* seeds methanol (ME) and methylene chloride extracts (MCE) against DNA fragmentation induction in DN rats. In the first week after STZ injection, the rate of DNA fragmentation in DN rats was increased (26.5±1.26) significantly compared with control rats (8.3±0.41). On the other hand, when DN-induced rats were treated with ME, the rate of DNA fragmentation was significantly ($P<0.01$) lower in those rats (17.2±0.92) than in DN rats. Furthermore, the rate of DNA fragmentation (19.6±0.64) in DN rats was significantly ($P<0.05$) lower after receiving MCE treatment than in DN rats. At the second, fourth and sixth week after STZ injection, the rate of DNA fragmentation in DN rats was elevated in ascending manner (29.4±1.42, 31.1±1.51 and 32.4±1.33, respectively) compared with control rats. However, DN rats treated with ME decreased significantly ($P<0.01$) the rate of DNA fragmentation at 2^{nd},4^{th} and 6^{th} week (19.7±0.76, 21.2±0.86 and 20.8±1.11, respectively) compared with those in DN rats. Moreover, the rate of DNA fragmentation in DN rats treated with MCE was significantly ($P<0.05$) lower than that of DN rats (23.8±0.91, 21.7±0.98, and 25.9±1.52, respectively). Using Losartan as a reference medication, treatment of DN-induced rats resulted in a significant ($P<0.05$) decrease in the rate

of DNA fragmentation at all 4-week intervals (18.7±0.98, 20.5±1.12, 21.7±0.98, and 21.5±1.14, respectively) when compared to DN rats.

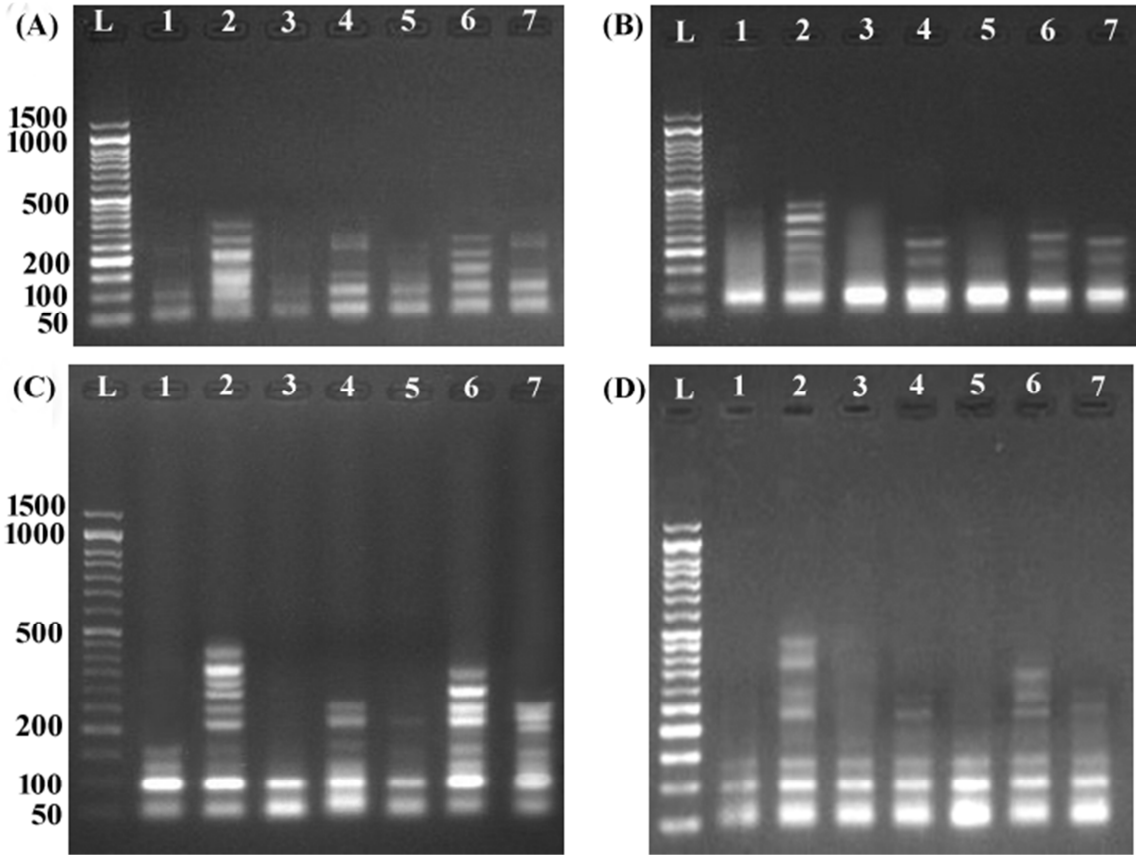

Figure 2: DNA fragmentation detected with agarose gel of DNA isolated from blood samples of diabetic nephropathy (DN) induced rats treated with *P. oleracea* seeds extracts for first week (A), second week (B), third week (4) and fourth week (5). Lane M represents DNA marker. Lane 1 represents control group. Lane 2 represents DN rats. Lane 3 represents rats treated with Methanolic extract (MEP). Lane 4 represents DN rats treated with MEP. Lane 5 represents rats treated with Methylene chloride Extract (MCP). Lane 6 represents DN rats treated with MCP. Lane 7 represents DN rats treated with reference drug.

3.4. Expression of nephrotoxicity related genes

Expression analysis of Col1a1, Tgfb1, Cox6c genes as nephrotoxicity markers in blood samples of DN rats treated with methanolic extract (ME) and methylene chloride extract (MCE) of *P. oleracea* seeds for 6 weeks are illustrated in **Figs.3A-3C.** The results showed that expression levels of Col1a1, Tgfb1, Cox6c genes were significantly over-expressed [Col1a1: 1^{st}W (2.17±0.09), 2^{nd}W (2.85±0.10), 4^{th}W (3.36±0.11) and 6^{th}week (3.29±0.08); Tgfb1: 1^{st}W (3.51±0.12), 2^{nd}W (3.94±0.09), 4^{th}W (4.38±0.13) and 6^{th}week (4.45±0.09); Cox6c: 1^{st}W (4.47±0.13), 2^{nd}W (5.39±0.14), 4^{th}W (5.68±0.11) and 6^{th}week (5.33±0.15)] in DN rats compared with those in control rats at all-time intervals. However, the expression levels of Col1a1, Tgfb1, Cox6c genes at all-time intervals were similar in rats treated with ME and MCE of *P. oleracea* seeds compared with control rats. Additionally, treatment of DN rats with ME [Col1a1: 1^{st}W (1.64±0.06), 2^{nd}W (2.16±0.07), 4^{th}W (2.38±0.09) and 6^{th}week (2.33±0.09); Tgfb1: 1^{st}W (2.37±0.08), 2^{nd}W (2.51±0.09), 4^{th}W (3.22±0.10) and 6^{th}week (3.12±0.10); Cox6c: 1^{st}W (3.29±0.11), 2^{nd}W (3.81±0.13) , 4^{th}W (4.21±0.12) and 6^{th} week (3.95±0.14)] and MCE [Col1a1: 1^{st}W (1.71±0.07), 2^{nd}W (2.39±0.08), 4^{th}W (2.51±0.08) and 6^{th}week (2.47±0.10); Tgfb1: 1^{st}W (2.76±0.09), 2^{nd}W (3.19±0.10), 4^{th}W (3.44±0.08) and 6^{th}week (3.52±0.09); Cox6c: 1^{st}W (3.62±0.12), 2^{nd}W (4.27±0.11) , 4^{th}W (4.64±0.14) and 6^{th}week (4.31±0.10)] of *P. oleracea* seeds decreased significantly the Col1a1, Tgfb1, Cox6c genes expression levels compared with those in DN rats.The Col1a1, Tgfb1, Cox6c genes expression levels in the group of DN rats treated with reference drug were relatively similar to those in DN rats treated with MCE **(Figs. 3A-3C).**

Table 1: Effect of *P. oleracea* seeds methanol (ME) and methylene chloride extracts (MCE) against DNA fragmentation induction in DN rats

Treatment	DNA Fragmentation % (M ± SEM)			
	Week1	Week2	Week4	Week6
Control	8.3±0.41^{c}	8.4±0.67^{d}	8.2±0.62^{d}	8.3±0.73^{d}
Diabetic nephropathy (DN)	26.5±1.26^{a}	29.4±1.42^{a}	31.1±1.51^{a}	32.4±1.33^{a}
Methanolic extract (ME)	8.1±0.57^{c}	8.3±0.32^{d}	8.1±0.92^{d}	8.4±0.62^{d}
DN+ME	17.2±0.92^{b}	19.7±0.76^{c}	21.2±0.86^{c}	20.8±1.11^{c}
Methylene chloride Extract (MCE)	8.4±0.89^{c}	8.6±0.44^{d}	8.5±0.27^{d}	8.4±0.48^{d}
DN+MCE	19.6±0.64^{b}	23.8±0.91^{b}	25.7±1.61^{b}	25.9±1.52^{b}
DN+Reference drug	18.7±0.98^{b}	20.5±1.12^{bc}	21.7±0.98^{c}	21.5±1.14^{c}

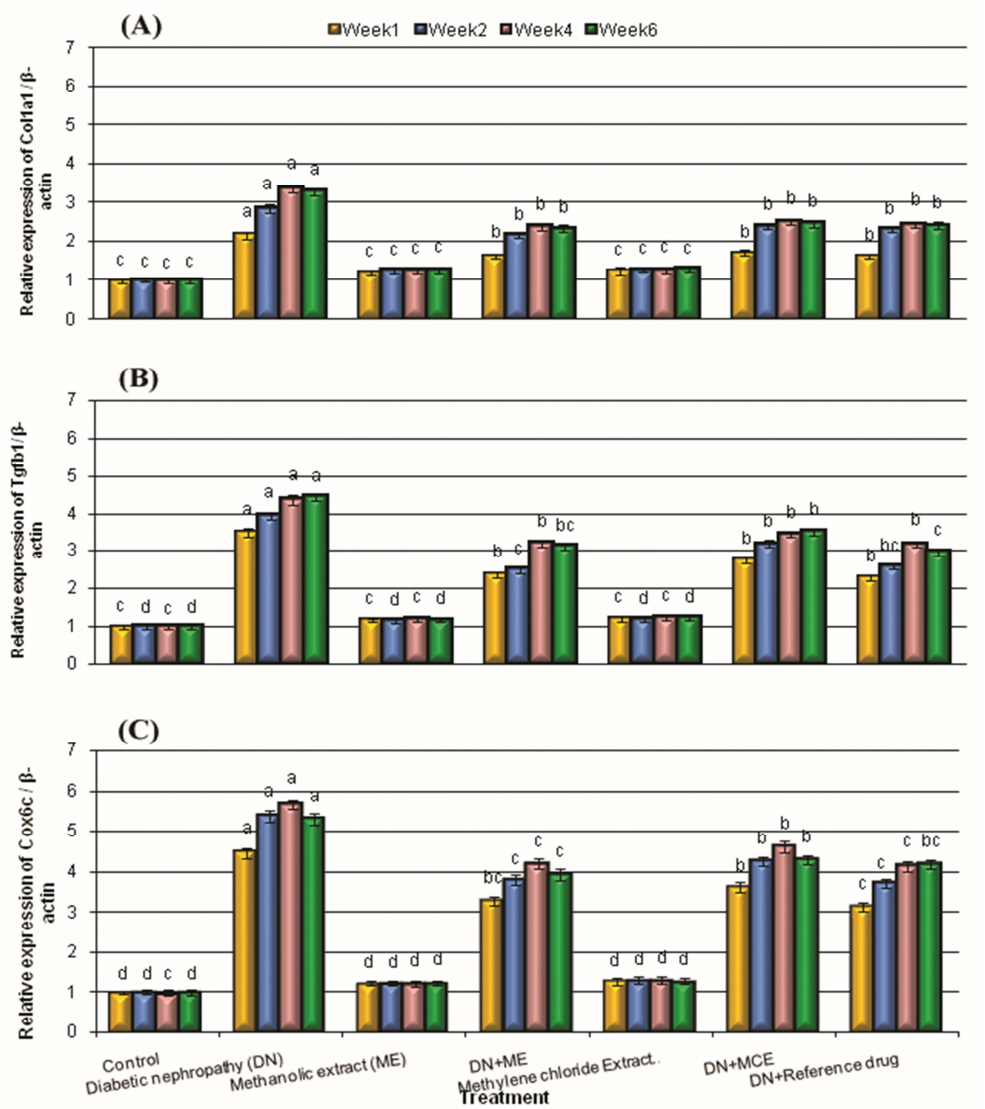

Figure 3: The expression alterations of TGFβ1 (A); Col1a1 (B) and Cox6c (C) genes in blood samples of diabetic nephropathy (DN) induced rats treated with methanolic extract (ME) and methylene chloride extract (MCE) of *P. oleracea* seeds for 6 weeks. Means with different superscripts (^{a,b,c,d}) between groups in the same treatment week are significantly different at P<0.05 Data are presented as mean ± SEM.

3.5. Measurement of Apoptosis

Fig. 4A shows the effect of *P. oleracea* seeds methanol and methylene chloride extracts on rates of necrotic/apoptotic in blood cells of DN rats. The amount of necrosis/apoptosis in blood cells of DN rats was significantly increased in all time intervals [1st (18.5±0.52), 2nd (21.4±0.71), 4th (23.8±0.44) and 6th week (24.4±0.82)] compared with those in control rats [1st (9.1±0.33), 2nd (9.2±0.26), 4th (9.4±0.43) and 6th week (9.0±0.38)]. So, the percentage of necrotic /apoptotic rates in DN rats at 1st, 2nd, 4th and 6th were increased by 203.3, 232.6, 261.5 and 271.1%, respectively, compared with control rats. Nevertheless, the necrotic /apoptotic rates in blood cells of rats treated with ME and MCE were relatively similar to those in control rats **(Fig. 4A).** Additionally, DN rats treated with ME [1st (13.2±0.52), 2nd (15.5±0.49), 4th (16.1±0.65) and 6th week (16.7±0.43)] and MCE [1st (14.7±0.65), 2nd (17.3±0.53), 4th (18.2±0.83) and 6th week (19.1±0.75)] revealed significant decrease in the necrotic /apoptotic rates compared with those in DN rats. Similarly, treatment of DN rats with the reference drug declined considerably the necrotic /apoptotic rates at all-time intervals in comparison to those in DNA rats.

3.6. Intracellular ROS levels

The effect of *P. oleracea* seeds methanol and methylene chloride extracts on intracellular ROS changes in blood cells of DN rats is demonstrated in **Fig. 4B**. The levels of intracellular ROS in DN rats were augmented significantly at all-time intervals [1st (325±7.2), 2nd (365±8.1), 4th (388±8.6) and 6th week (396±7.4)] compared with those in control rats [1st (88±3.5), 2nd (90±4.2), 4th (91±3.7) and 6th week (89±4.3)]. The intracellular ROS levels in DN rats at 1st, 2nd, 4th and 6th were increased by 369.3%, 405.6, 426.4 and 444.9, respectively, compared with control rats. In contrast, intracellular ROS levels in rats treated with ME and MCE were quite similar to those in control rats **(Fig. 4B).** Furthermore, DN rats treated with ME [1st (212±6.5), 2nd (227±7.4), 4th (239±7.8) and 6th week (242±7.3)] and MCE [1st (244±7.3), 2nd (261±8.5), 4th (272±7.7) and 6th week (279±8.2)] showed significant decline intracellular ROS levels compared with those in DN rats. Likewise, treatment of DN rats with the reference drug reduced considerably the intracellular ROS levels at all-time intervals in comparison to those in DNA rats **(Fig. 4B)**.

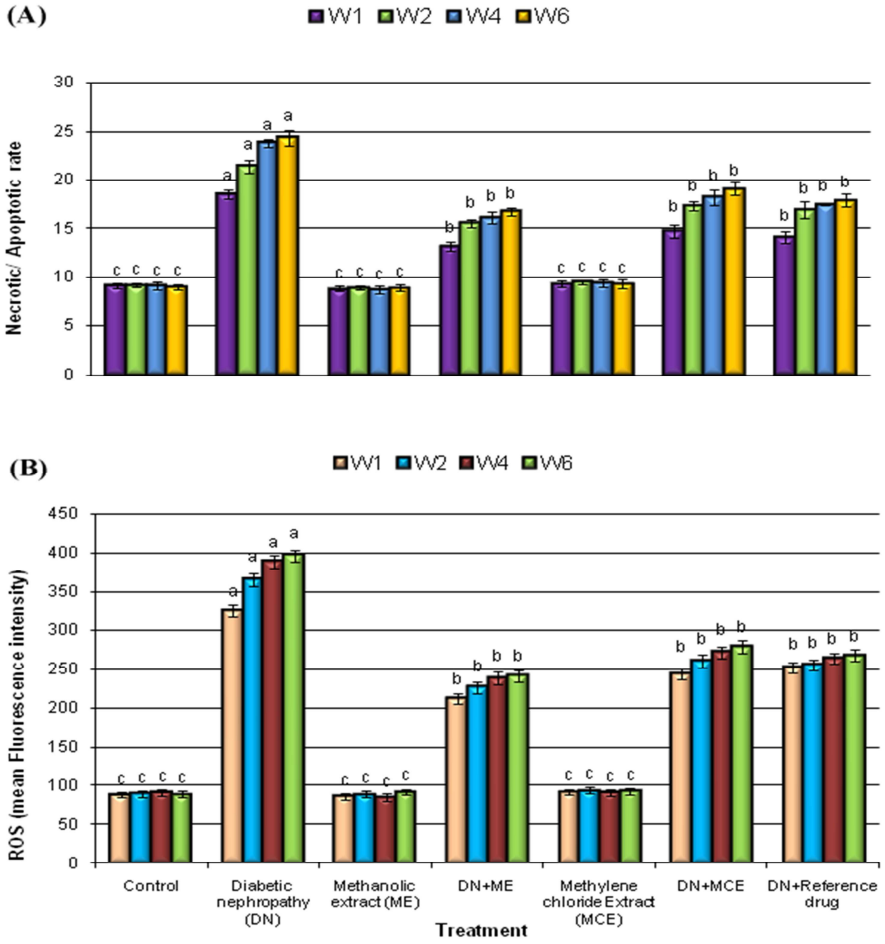

Figure 4: Necrotic/apoptotic rates (A) and Intracellular ROS levels alteration (B) in blood cells of DN rats treated with *P. oleracea* seeds methanol and methylene chloride extracts for 6 week. Results are expressed as the Mean ± SEM. [a,b,c] Mean with different letters, within tissue, differ significantly ($p \leq 0.05$).

3.6. Activity determination of GPx and GST in blood sample

Tables 2 and 3 demonstrate the GPx and GST antioxidant enzymes activity levels in diabetic nephropathy (DN) rats treated with *P. oleracea* seeds methanol and methylene chloride extracts. The activity levels of GPx in blood samples of DN rats were reduced significantly at all-time intervals [1st (4.8±0.02), 2nd (3.6±0.03), 4th (3.5±0.01) and 6th week (3.1±0.03)] compared with those in control rats [1st (7.2±0.03), 2nd (7.3±0.05), 4th (7.1±0.04) and 6th week (7.2±0.02)]. However, activity levels of GPx in blood samples of rats treated with ME and MCE were relatively similar to those in control rats **(Table 2).** Furthermore, treatment of DN rats with ME [1st (5.4±0.02), 2nd (5.7±0.01), 4th (6.1±0.05) and 6th week (6.2±0.04)] and MCE [1st (5.1±0.04), 2nd (5.2±0.06), 4th (5.5±0.05) and 6th week (5.7±0.03)] elevated significantly the activity levels of GPx in blood samples starting from the 2nd week compared with those in DN rats [1st (4.8±0.02), 2nd(3.6±0.03), 4th (3.5±0.01) and 6th week (3.1±0.03)]. Also, DN rats' model treated with the reference drug significantly raises the GPxactivity levels starting from the 2nd week in comparison to those in untreated DN rats. The activity levels of GST in blood samples of DN rats were decreased significantly at all-time intervals [1st (1.2±0.03), 2nd (1.1±0.02), 4th (0.9±0.03) and 6th week (0.8±0.04)] compared with those in control rats [1st (2.4±0.02), 2nd (2.3±0.03), 4th (2.4±0.04) and 6th week (2.3±0.03)]. In contrary, activity levels of GST in blood samples of rats treated with ME and MCE were relatively similar to those in control rats (Table 3). Likewise, treatment of DN rats with ME [1st (1.8±0.05), 2nd (1.6±0.06), 4th (1.5±0.04) and 6th week (1.4±0.03)] and MCE [1st (1.6±0.03), 2nd (1.5±0.05), 4th (1.3±0.06) and 6th week (1.3±0.05)] increased significantly the activity levels of GST in blood samples at all-time intervals compared with those in DN rats [1st (1.2±0.03), 2nd (1.1±0.02), 4th (0.9±0.03) and 6th week (0.8±0.04)]. Additionally, treatment of DN rats modes with the reference drug significantly increased the GST activity levels at all-time intervals in comparison to those in DNA rats.

Table 2: Effect of *P. oleracea* seeds methanol (ME) and methylene chloride extracts (MCE) on Glutathione peroxidase enzyme activity levels in DN rats

Treatment	**Glutathione peroxidase activity** (U/mL)			
	Week1	Week2	Week4	Week6
Control	7.2±0.03[a]	7.3±0.05[a]	7.1±0.04[a]	7.2±0.02[a]
Diabetic nephropathy (DN)	4.8±0.02[b]	3.6±0.03[c]	3.5±0.01[c]	3.1±0.03[c]
Methanolic extract (ME)	7.1±0.05[a]	7.2±0.03[a]	7.1±0.04[a]	7.3±0.06[a]
DN+ME	5.4±0.02[b]	5.7±0.01[b]	6.1±0.05[b]	6.2±0.04[b]
Methylene chloride Extract (MCE)	6.9±0.07[a]	6.8±0.04[a]	7.0±0.05[a]	6.9±0.06[a]
DN+MCE	5.1±0.04[b]	5.2±0.06[b]	5.5±0.05[b]	5.7±0.03[b]
DN + Reference drug	5.3±0.05[b]	5.4±0.03[b]	5.7±0.06[b]	5.8±0.02[b]

Data are presented as mean ± SEM. Means with different superscripts (a,b,c,d) between groups in the same treatment week

Table 3: Effect of *P. oleracea* seeds methanol (ME) and methylene chloride extracts (MCE) on Glutathione S-transferase enzyme activity levels in DN rats

Treatment	Glutathione-S-transferase activity (U/mL)			
	Week1	Week2	Week4	Week6
Control	2.4±0.02[a]	2.3±0.03[a]	2.4±0.04[a]	2.3±0.03[a]
Diabetic nephropathy (DN)	1.2±0.03[c]	1.1±0.02[c]	0.9±0.03[c]	0.8±0.04[c]
Methanolic extract (ME)	2.3±0.05[a]	2.4±0.03[a]	2.3±0.02[a]	2.2±0.06[a]
DN+ME	1.8±0.05[b]	1.6±0.06[b]	1.5±0.04[b]	1.4±0.03[b]
Methylene chloride Extract (MCE)	2.2±0.02[a]	2.3±0.03[a]	2.2±0.05[a]	2.1±0.03[a]
DN+MCE	1.6±0.03[b]	1.5±0.05[b]	1.3±0.06[b]	1.3±0.05[b]
DN+Reference drug	1.7±0.03[b]	1.4±0.01[bc]	1.3±0.04[b]	1.2±0.03[b]

Data are presented as mean ± SEM. Means with different superscripts (a,b,c,d) between groups in the same treatment week

3.7. Activity determination of Nitric oxide and SOD in kidneytissues

Tables 4 demonstrates the activity of SOD and the level of NO in diabetic nephropathy (DN) rats treated with *P. oleracea* seeds methanol and methylene chloride extracts. The activity of SOD in kidney tissue of DN rats was reduced significantly ($3.42^{c} \pm 0.84$) compared with those in control rats ($21.27^{a} \pm 2.34$). However, activity of SOD in kidney tissue of rats treated with ME and MCE were relatively like that in control group (**Table 4).** Furthermore, DN rats' model treated with ME ($18.02^{b} \pm 2.30$) and MCE($19.90^{ab} \pm 2.03$) elevated significantly the activity of SOD compared with those in untreated DN rats. Also, treatment of DN induced rats with the reference drug significantly increased the SOD activity in comparison to those in untreated DN rats. In contrast, NO level in kidney tissue of DN rats was increased significantly ($3039^{a} \pm 67.17$) compared with those in control rats($246^{g} \pm 12.94$). However, activity of NO in kidney tissue of rats treated with ME and MCE were nearly normalized to those in control group rats **(Table 4).** Furthermore, treatment of DN rats with ME ($1932^{b} \pm 20.37$) and MCE ($1055^{c} \pm 16.18$) reduced significantly the activity level of NO compared with those in DN rats **(Table 4)**.

Table 4: Effect of *P. oleracea* seeds methanol (ME) and methylene chloride extracts (MCE) on Nitric oxide level and SOD activity

Groups **Weeks**	Nitric oxide **(umol/g tissue)**	SOD **(U/g tissue)**
Control	$246^{g} \pm 12.94$	$21.27^{a} \pm 2.34$
Diabetic nephropathy (DN)	$3039^{a} \pm 67.17$	$3.42^{c} \pm 0.84$
Methanolic extract (ME)	$548^{e} \pm 11.7$	$21.67^{a} \pm 2.64$
DN+ME	$1932^{b} \pm 20.37$	$18.02^{b} \pm 2.30$
Methylene chloride Extract (MCE)	$330^{f} \pm 28.09$	$21.54^{a} \pm 2.59$
DN+MCE	$1055^{c} \pm 16.18$	$19.90^{ab} \pm 2.03$
DN + Reference drug	$978^{d} \pm 15.4$	$19.25^{ab} \pm 1.10$

Data are presented as mean ± SEM. Means with different superscripts (a,b,c,d) between groups

4. Discussion

In the current research, we explored the anti- Diabetic nephropathy (anti-DN) effects of purslane methanol, and methylene chloride extracts. The study's findings showed that the DNA fragmentation rate in DN experimental model was increased significantly compared with control rats and the highest DNA fragmentation rate was found at the fourth week after DN induction. Numerous detrimental intracellular events, including damage to DNA in the nucleus, can be brought on by oxidative stress. In the end, this damage may result in dysfunction, cell death, and a persistent risk of oncogenesis. Oxidative DNA damage manifested as oxidized bases can happen in the human body up to a million times a day as a result of normal metabolism and other environmental factors **[32, 33].**

The peripheral lymphocytes of individuals with type 1 and type 2 diabetes are more vulnerable to DNA breaks caused by oxidative stress **[34].** The same phenomenon has been observed in gestational diabetes, which is particularly interesting because of its association with renal complications **[35]**. Research has focused on the impact of ROS production on DNA damage in type 1 diabetes models **[36]**. These studies provide evidence for the theory that oxidative stress-induced damage occurs in the diabetic kidney. Consistent with these findings, our study found that DN rats had high ROS formation, especially in the fourth and sixth weeks. Thus, DNA fragmentation induced in DN rats can be attributed to the mechanism of ROS generation. On the other hand, alteration in the gene expression of DN related genes is an important pathway associated with DN induction. The present study showed that expression levels of Col1a1, Tgfb1, Cox6c genes were significantly over-expressed in DN rats compared with those in control rats at all-time intervals. In agreement, with our findings, **Zhang *et al.* [37]** reported that expression levels of Col1a1, Cox6c, and Tgfb1 were significantly up-regulated in the DN group of rats. According to their hypothesis, the oxidative phosphorylation pathway, the TGF-b pathway, the ECM receptor interaction, and the renin-angiotensin system could all play a role in the development of DN. The present study showed that the activity of GPx, and GST in DN rats were reduced significantly at all-time intervals while SOD activity decreased, and NO level increased significantly in kidney homogenate compared with those in control rats. In the same line, **Bessa et al. [38]** found that diabetic patients with and without nephropathy had significantly lower levels of reduced glutathione (GSH), glutathione S-transferase (GST), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) activities, and GSH when compared to control. They arrived at the conclusion that erythrocyte GST and GPx could serve as useful biomarkers for distinguishing between distinct nephropathy groups. Cell defense and detoxification are carried out by the glutathione enzyme

superfamily. These enzymes' primary task is to use an electrophilic center to conjugate GSH with toxic hydrophobic compounds. So, toxins can be more easily inactivated and removed in greater quantities through the kidneys **[39].**

DN rats' blood cells showed significantly higher rates of necrosis and apoptosis than control rats did at all time intervals, peaking at weeks four and six. So, the percentage of necrotic /apoptotic rates in DN rats at 4^{th} and 6^{th} were increased by 261.5 and 271.1, respectively, compared with control rats. **Sriwastawa and Kumar [40]** reported that the pathogenesis of diabetic sensory neuropathy may be influenced by mitochondrial dysfunction and the involvement of apoptosis.

In the current work we have used purslane extracts to minimize as possible the harmful effects of diabetes and kidney diseases in the DN rats. We found that treatment of DN induced rats with ME and MCE significantly decreased the rate of DNA fragmentation at all-time intervals in comparison to those in DN rats. Evidence suggests that the antioxidant activities of *P.*oleracea extracts are likely linked to their constituents, which include gallotannins and/or kaempferol; omega-3 fatty acids, quercetin, apigenin& -tocopherols; flavonoids (quercecetiner), ascorbic acid and glutathione **[41,42]**. In addition to reducing the generation of free radicals, ascorbic acid and glutathione can scavenge free radicals and prevent DNA damage and nucleic acid peroxidation**[43,44]**. Considering the components of P. oleracea aqueous extract, our findings indicate that P. oleracea extracts function as a barrier to stop hydrogen peroxide-induced oxidative DNA damage in human lymphocytes. P. oleracea may therefore be useful in the treatment and prevention of oxidative stress-related illnesses.

The current findings demonstrated that DN rats treated with ME and MCE of *P. oleracea* seeds significantly decreased the expression levels of Col1a1, Tgfb1, Cox6c genes compared with those in DN rats. **Baiet al.[45]** reported that*P.Oleracea* extract has been found to significantly decrease the tumor necrosis factor (TNF-α) and interleukin-6 (IL-6) levels in rats with diabetes. Further evidence supporting the association between the genes TNF-alpha, TGF-beta1, IL-10, and IL-6 and diabetes prevention came from the study of **Tsiavou et al.** ***[46]***. Thus, suppression of gene expression levels of Col1a1, Tgfb1, Cox6c, TNF-α, and IL-6 after treatment with P. oleracea extract may be associated with the reduction of diabetes and kidney disease in DN rats.

In this study, the activity levels of GPx and GST in blood samples of rats treated with ME and MCE were elevated starting from the 2^{nd}week compared with those in DN rats.**Uddin et al. [47]** noted that purslane is a very likely candidate for the treatment of many diseases, including kidney disease and diabetes, due to its high content of omega-3 fatty acids and antioxidants (vitamins A and C, alpha-tocopherol, beta-carotene, and glutathione). Additionally, **Palaniswamy et al. [48]** reported that omega-3 fatty acids can increase the activity of Na(+)-K(+) and Mg(2+) ATPases in tetrachlorodibenzo-p-dioxin (TCDD)-induced kidney disease in rats with kidney disease by improving antioxidant status. Thus, purslane extracts can improve GPx and GST activity in DNA rats because they contain omega-3 fatty acids.

In many physiological and pathological conditions of multicellular organisms, apoptosis is an active suicide mechanism that kills unwanted or potentially harmful cells**[48]**. The present study proved that DN rats treated with ME and MCE declined the apoptosis and intracellular ROS levels compared with those in DN rats. According to **Lee*et al.[49]*** indicated that *P. oleracea*extracts are effective in protecting human epithelial keratinocytes and fibroblasts from UVB-induced apoptosis, which is consistent with our findings. Furthermore, **Ohet al. [50]** proved that crude extracts of *P. oleracea* abundant in phenolic content are playing an important role in decreasing the ROS formation through blocking the activation of Matrix Metalloproteinase (MMPs). These results suggest that the decrease in ROS production in cells treated with *P. oleracea* L. extracts might result from the presence of substances that act as antioxidants, including flavonoids, polyphenols and alkaloids that scavenge free radicals**.**

5. Conclusion

In conclusion, diabetes had a deleterious effect on rats' kidney functions, DNA fragmentation, increased expression of genes linked to nephrotoxicity and diabetes, decline in theGPx, SOD and GST enzymes activities and elevation of the NO level and apoptosis. Treatment of DN rats with methanol and methylene chloride extracts of purslane improved the harmful effects that appeared in the DN rats, but the efficiency of the methanolic extract was much higher than those in methylene chloride extract. These results suggested that the reduction of oxidative stress after treatment with *P. oleracea* extracts can be due to the presence of antioxidant compounds such as flavonoids and polyphenols which scavenge the free radical.

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