Research article

In vivo antidiabetic effects of phenolic compounds of spinach, mustard, and cabbage leaves in mice

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ABSTRACT

Leafy vegetables are considered to have health-promoting potentials, mainly attributed to bioactive phenolic compounds. The antidiabetic effects of spinach, mustard, and cabbage were studied by feeding their phenolic-rich aqueous extracts to alloxan-induced diabetic mice. The antioxidant, biochemical, histopathological, and hematological indices of the control, diabetic, and treated mice were studied. Phenolic compounds present in the extracts were identified and quantified using HPLC-DAD. Results showed ten, nineteen, and eleven phenolic compounds in spinach, mustard, and cabbage leave aqueous extracts, respectively. The body weight, tissue total glutathione (GSH) contents, fasting blood sugar, liver function tests, renal function tests, and lipid profile of the mice were affected by diabetes and were significantly improved by the extract treatments. Likewise, hematological indices and tissues histological studies also showed recovery from diabetic stress in treated mice. The study's findings highlight that the selected leafy vegetables potentially mitigate diabetic complications. Among the studied vegetables, cabbage extract was comparatively more active in ameliorating diabetic stress.

1. Introduction

Leafy vegetables are considered to have health-promoting potentials, mainly attributed to bioactive compounds [1]. It has been estimated that leafy vegetables contain thousands of individual phytochemicals [2]. Among these phytochemicals, phenolic compounds have been studied extensively due to their explicit health benefits, including acting as antioxidants [3], preventing chronic inflammatory responses [4], mitigating cardiovascular diseases [5], and reducing diabetic complications [6].

Diabetes mellitus (DM) is a prevalent chronic disease, which is generally characterized by a hyperglycemic response [7] followed by long-term diseases like micro/macro-vascular complications and hence leads to morbidity and mortality [8]. Prolonged hyperglycemia produces reactive oxygen species that ultimately generate oxidative stress coupled with cellular injury [9]. Oral medication to treat diabetes is also in practice; however, such medication may show significant side effects in the form of hypoglycemia [10], hypersensitivity, gastrointestinal distress [11], and body weight gain. Therefore, nowadays, researchers are trying to find natural resources, mainly phenolic compounds [12] and carotenoids [13], to treat such diseases [14]. Studies have revealed that these compounds may potentially treat diseases, particularly those which are developed mainly due to their responses against oxidative

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stress [15]. In vitro studies have shown that leafy vegetables, such as spinach [16] and cabbage [17], had antidiabetic activity. However, the selected green leafy vegetables have not yet been the subject of in vivo antidiabetic studies. Therefore, the main objective of this study was to demonstrate the in vivo antidiabetic properties of the aqueous extracts of spinach, mustard, and cabbage.

2. Materials and methods

2.1. Materials

Fresh samples of vegetables [Spinach (*Spinacia oleracea*), Mustard (*Brassica compestris*), and Cabbage (*Brassica oleracea*)] were collected from local fields of the botanical garden of the University of Malakand. Dr. Gul Rahim, Department of Botany, the University of Malakand, identified the plants. The voucher specimens were deposited and recorded in the Herbarium of the University, with codes H/UOM.BG.863, H/UOM.BG.864, and H/UOM.BG.865 for spinach, mustard, and cabbage, respectively. The leaves were thoroughly washed with distilled water and crushed with a blender to make a paste. Phenolic compounds were extracted using the reported method [18]. Subsequently, a paste (10 g) of each vegetable was added to 100 mL of deionized distilled water and shaken for 24 h. Each extract was then filtered through Whatman Millipore filter paper followed by ultra-filtration through an Agilent PFTE syringe filter (0.45 μm) and transferred to an HPLC vial to identify phenolic compounds. The filtrate of the vegetable extract was refrigerated as a treatment dose for diabetic mice.

2.2. Determination of phenolic compounds

The aqueous extract of each sample was analyzed for phenolic compounds using reversed-phase HPLC coupled to a DAD detector. An Agilent-1260 HPLC system was employed to yield accurate and precise identification of phenolic compounds in sample extract [19]. Chromatograms were obtained at 320 nm, whereas absorption spectra of each peak in the chromatogram were recorded at 200–600 nm. Peaks with purity higher than 90%, as was determined using ChemStation software (Agilent Technologies, Germany), were selected for identification. Calibration curves of standard phenolic compounds (gallic acid, p-hydroxybenzoic acid, caffeic acid, ferulic acid, sinapic acid, catechin, syringic acid, coumaric acid, quercetin, quercetin-3-glucose, quercetin-3-and rutinoside) were used for quantification of phenolic compounds. When the standard compound was unavailable, the calibration curves of those compounds with a relative response factor (RRF), such as rutin and quercetin-3-glucoside, were used. The amount of phenolic compounds was calculated and expressed as μg/mL of the extract.

2.3. Experimental animals

The experiments were carried out at the University of Malakand and as per approved guidelines to ensure the care of animals under experimentation. The protocols were approved by the Graduate Study Committee (GSC) of the Department of Biotechnology and finally approved by the Advanced Study and Research Board (ASRB) of the University of Malakand. For the in vivo antidiabetic study analysis, female Swiss albino mice (6 months old, weighing 40–50 g) were selected as samples. Mice were procured from the veterinary research institute in Peshawar, Pakistan, and were housed in proper polypropylene cages. They were fed a standard diet consisting of rice and wheat bran and fresh drinking water *ad libitum*. After the completion of the experiment, they were weighed again through digital balance to observe any change in body weight.

2.4. Induction of diabetes

Before the induction of diabetes in mice, they were allowed to acclimatize to the new environment for a week. To induce diabetes in animals, they were kept fasting for 16 h and injected with alloxan-monohydrate freshly prepared in 9.0% N-saline. Alloxan was administered at 150 mg/kg b/w with a single intra-peritoneal injection [20]. After a week, when the condition of diabetes was stabilized, fasting blood sugar (FBS) was measured. Animals having blood sugar levels above 210 mg/dl in fasting conditions were considered diabetic and used for the experiment.

2.5. Feeding of animals

The animals (n = 60) were placed in 6 major groups: SP (fed with spinach extract), BR (fed with mustard extract), CB (fed with cabbage extract), C (non-diabetic control group), D (diabetic control group), and G (fed with antidiabetic drug, i.e., Glucophage with a dose of 8.32 mg/kg body weight). The extract groups were further divided into 3 sub-groups consisting of 5 animals each: (SP1, BR1, CB1), (SP2, BR2, CB2), and (SP3, BR3, CB3). The aqueous extract of each sample was fed orally through a feeding tube with an O.D. dose for two weeks. Sub-group 1, 2, and 3 were fed with 0.5, 1.0, & 1.5 mL of dose, respectively. The concentration of SP was 5.77 mg/mL, BR was 4.69 mg/mL, and CB was 2.02 mg/mL.

2.6. Liver and kidney histopathology

After feeding the treated dose for two weeks, mice were kept fasting for 12 h, and a blood sample (2.0 mL) was taken through the cardiac puncture, followed by slaughtering. Vital organs, i.e., the liver and kidney, were collected and preserved in a 10%

formaldehyde solution. Histological slides were prepared from the tissues and were observed, and interpreted according to a standard histopathological protocol as described earlier [21].

2.7. Hematology and biochemistry

Hematology and HbA1c analysis was performed using *Sys-Max,* a fully automated hematology analyzer from the blood collected in EDTA tubes. Serum biochemical analysis (FBS, ALT, ALP, AST, TG, TC, HDL, LDL, Urea, and creatinine) was performed through *Micro-Lab 300,* using standard reagent kits, from the blood samples taken in a gel tube.

2.8. Determination of tissue GSH contents

Tissue total reduced glutathione (GSH) contents were determined using the method of Owens and Belcher [22] with some modifications. Then, 2.0 mL of phosphate buffer (pH 7.5) was added to 100 mg of grinded tissue, followed by 8.0 mL ice-cold phosphoric acid. The reaction mixture was shaken for 30 min, and then 0.03 mL dithio-nitro-benzene (DTNB) reagent was added. After incubating for 2 min, the absorbance of the mixture was taken at 412 nm against the reference blank. The amount of GSH was expressed as μmol/g of tissue.
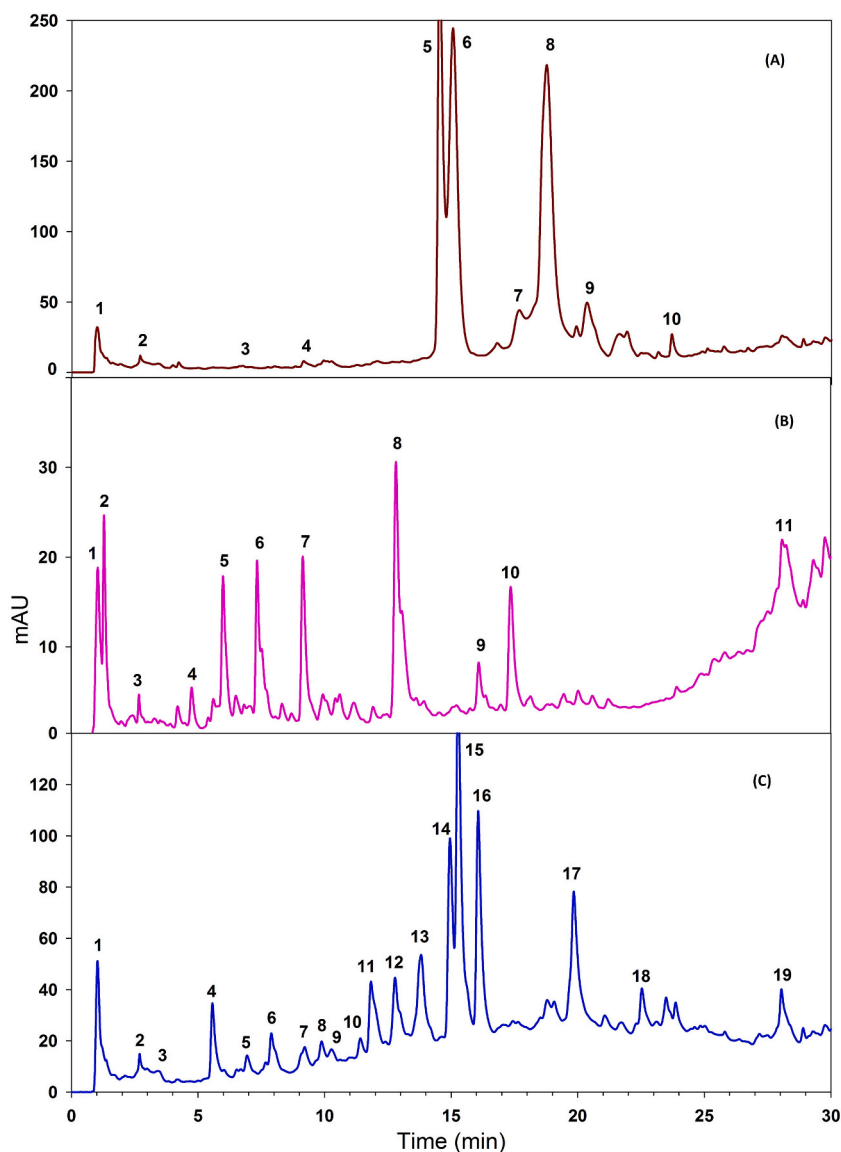

Fig. 1. HPLC-DAD chromatograms of the leaves extracts at 320 nm. (**A**) Spinach, (**B**) cabbage, and (**C**) mustard.

2.9. Statistical analysis

Statistical analysis was performed using ordinary one-way ANOVA with multiple comparisons of variables using Dunnett's multiple comparison test.

3. Results

3.1. Phenolic profile

Fig. 1 shows the HPLC chromatograms of extracts of the selected leafy vegetables. In spinach extract, ten phenolic compounds were identified and quantified. The major compounds in spinach were diosmetin-7-rutinoside (1259.2 μg/mL), quercetin-3-caffeoylglucoside-6-malonylglucoside (1033.4 μg/mL), isorhamnetin-3-caffeoyl-7-glucoside (942 μg/mL), p-hydroxybenzoic acid (268.8 μg/mL), quercetin-3-(p-coumaroyl-diglucoside)-7-glucoside (210.9 μg/mL), kaemferol-3-(p-coumaroyl-diglucoside)-7-glucoside (178.7 μg/mL), and vanillic acid hexoside (157 μg/mL), as shown in Table 1. In mustard leaves extract, nineteen compounds were identified and quantified. Kaempferol-3-(caffeoyldiglucoside)-7-glucoside (425.4 μg/mL), isorhamnetin-3-caffeoyl-7-rhamnoside (411.2 μg/mL), and kaempferol-3-(caffeoyldiglucoside)-7-rhamnoside (303.6 μg/mL) were the major compounds. The amount of caffeoylhexose, apigenin-7-glucoside, dihydrokaempferoyl hexoside, luteolin-3-glucoside, 4-feruloylquinic acid, and sinapic acid glucoside were less than 200 μg/mL. In cabbage leaf extract, 11 phenolic compounds were identified and quantified. The compounds with concentrations higher than 100 μg/mL were kaempferol, epicatechin-3-(4-methyl) gallate, quercetin, sinapic acid, syringic acid, and kaempferol-3-glucuronide. The total phenolics were higher in Spinach (4271.2 μg/mL), followed by mustard (3660.29 μg/mL),

Table 1
Phenolic compounds present in the extract of spinach, mustard, and cabbage leaves.

Sample	Peak	Retention time (min)	Compound	Absorption spectra (nm)	Concentration (μg/mL)
Spinach	1	1.0	p-Hydroxybenzoic acid	265	268.8 ± 3.7
	2	2.7	Vanilic acid hexoside	292, 258	157 ± 2.2
	3	6.7	Caffeic acid	323, 298	25.5 ± 1.3
	4	9.1	5-O-Caffeoylquinic acid	326, 240	52 ± 2.4
	5	14.5	Quercetin-3-caffeoylglucoside-6-malonylglucoside	338, 268	1033.4 ± 5.1
	6	15.1	Isorhamnetin-3-caffeoyl-7-glucoside	337, 268	942 ± 6.5
	7	17.6	Quercetin-3-(p-coumaroyl-diglucoside)-7-glucoside	316	210.9 ± 2.1
	8	18.7	Diosmetin-7-rutionside	342, 270	1259.2 ± 8.9
	9	20.3	Kaemferol-3-(p-coumaroyl-diglucoside)-7-glucoside	315, 267	178.7 ± 9.2
	10	23.6	Genistein 8-C-glucoside-O-sulfate	304, 250	141.7 ± 2.4
	Total amount				**4271.2 (74.02%)**
Mustard	1	1.0	Gallic acid	271	284.0 ± 7.0
	2	2.6	p-Hydroxybenzoic acid	256	67.8 ± 2.6
	3	3.4	Caffeic acid	323	40.1 ± 1.9
	4	5.6	4-Caffeoylquinic acid	331, 279	214.7 ± 6.0
	5	6.9	Ferulic acid	330, 240	106.8 ± 6.5
	6	7.9	Sinapic acid	323, 240	249.1 ± 5.8
	7	9.2	Rosmarinic acid	330, 270	197.8 ± 4.1
	8	9.9	(+)-Catechin	280	54.6 ± 1.6
	9	10.2	Caffeoylmalic acid	328, 240	42.2 ± 1.8
	10	11.4	Caffeoylferuloylquinic acid	324, 236	80.8 ± 2.0
	11	11.8	Sinapic acid glucoside	328	141 ± 1.4
	12	12.7	4-Feruloylquinic acid	322, 266	174.3 ± 1.4
	13	13.8	Caffeoylhexose	330, 244	238.4 ± 2.0
	14	14.9	Apigenin-7-glucoside	336, 268	239.7 ± 2.1
	15	15.2	Isorhamnetin-3-caffeoyl-7-rhamnoside	330, 267	411.2 ± 9.4
	16	16.1	Kaempferol 3-(caffeoyldiglucoside)-7-rhamnoside	330, 268	303.6 ± 2.6
	17	19.8	Kaempferol-3-(caffeoyldiglucoside)-7-glucoside	330, 268	425.4 ± 2.4
	18	22.5	Luteolin-3-glucoside	350, 248	180 ± 1.1
	19	28	Dihydrokaempferoyl hexoside	292, 250	208.9 ± 1.8
	Total amount				**3660.29 (78.03%)**
Cabbage	1	1.0	Syringic acid	274	117.7 ± 3.7
	2	1.3	Sinapic acid	266	132.1 ± 2.4
	3	2.7	Coumaric acid	255	15.7 ± 1.7
	4	4.8	Sinapoyl hexoside	275	26.6 ± 1.0
	5	6.1	Ferulic acid	325, 279	110.4 ± 3.4
	6	7.3	Caffeic acid	320, 299	91.1 ± 2.9
	7	9.1	Quercetin	372, 256	153.3 ± 4.8
	8	12.8	Kaempferol	338, 266	383.7 ± 3.1
	9	16.1	Quercetin-3-glucoside	354, 256	92.1 ± 4.3
	10	17.3	Kaempferol-3-glucoronide	336, 271	191.8 ± 2.4
	11	28.2	Epicatechin-3-(4-methyl) gallate	276	268.4 ± 4.8
	Total amount				**1582.9 (78.36%)**

and lowest in cabbage leaves (1582.9 μg/mL). This revealed that phenolic compounds identified in spinach were more than 74%, and 78% in mustard and cabbage, respectively, of the dried extract.

3.2. Effects on weight change

It has been observed that diabetic mice lost body weight significantly (P < 0.05) during the entire period of treatment. However, the treated groups may have significantly reduced the loss of body weight when compared to the diabetic group. Moreover, it has been observed that a high dose may reduce the risk of body weight loss more efficiently than the lower feeding dose for each treated group, as shown in Fig. 2.

3.3. Effects on serum biochemical parameters

Table 2 shows a significant increase in the biochemical parameters, including fasting blood sugar (FBS), liver function tests (LFTs), renal function tests (RFTs), lipid profile, and HbA1C levels of the diabetic group (D) versus control (C) group. The positive control (G) group and the treated groups with vegetable extract did not show any major increase in these parameters compared to the control group (C). In Spinach treated group (SP), there was a significant decrease observed in FBS, ranging from 135.3 (SP1) to 113.3 mg/dl (SP3). These results are comparable to the FBS level of the G (104.6 mg/dl) and C (80.34 mg/dl) groups. However, the diabetic (D) group was of potentially raised FBS level (201.6 mg/dl).

In the Brassica treated group, i.e. *Brassica Compestris* (BR) and *Brassica oleracea* (CB), the biochemical parameters were also found to be essentially close to the C and G groups. For example, the FBS level of the BR-treated group: BR1-BR3 (156.3–120.3 mg/dl), and CB-treated group: CB1-CB3 (125.6–99 mg/dl), was nearer to the FBS level of G and C groups. In the same context, the level of other biochemical parameters of the treated groups was also comparable to G and C. For example, the lipid profile of the BR1 group, the TG, total cholesterol, LDL, and HDL (129.3, 128.6, 113.3, 40.6 mg/dl), respectively, were found to be importantly lower when compared to the D group, i.e., TG, cholesterol, HDL (161.3, 142.3, 39 mg/dl), however, LDL was momentously driven up in D group 135.3 mg/dl. Similarly, the RFT, i.e., blood urea nitrogen (BUN) and creatinine (CRE) of the CB3 group (33, 0.73 mg/dl), respectively, was substantially lower as compared to the D group (94 & 2.1 mg/dl), respectively. These results indicate the antidiabetic role of selected vegetable extracts. It was observed that by increasing the dose of the feeding extract, the antidiabetic potentials are also significantly improved.

3.4. Effects on hematological parameters

Table 3 shows that the D group has been found to have significantly lower RBCs and WBCs count compared to C or other treated groups (SP, BR, CB). However, no significant changes were observed in other hematological parameters of C or others treated groups. A
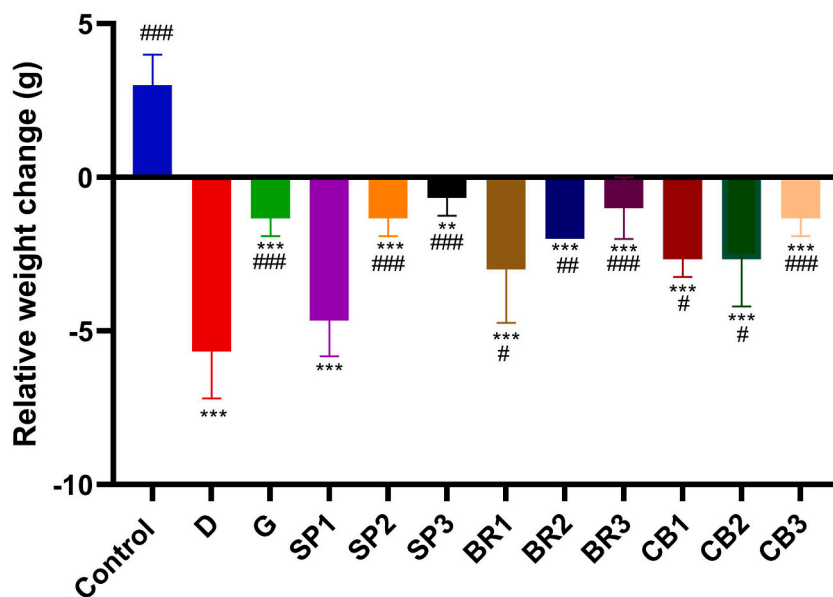

Fig. 2. Effect of spinach, mustard, and cabbage leaves extracts on the body weight of mice. Abbreviations: C (non-diabetic control group), D (diabetic control group), and G (fed with antidiabetic drug i.e., Glucophage with a dose of 8.32 mg/kg body weight), SP (fed with spinach extract), BR (fed with mustard extract), CB (fed with cabbage extract), and 1, 2, and 3 of SP, BR, and CB are different doses. Data are the mean of replicate measurements with standard deviation (n = 5). * = vs control (C) group, # = vs diabetic (D), * = 0.03, ** = 0.002, *** < 0.001, # = 0.03, ## = 0.002, ### < 0.001 using Dunnett's multiple comparison test.

Table 2

Effect of spinach, mustard, and cabbage phenolic extracts on serum biochemical parameters. Abbreviations: C (non-diabetic control group), D (diabetic control group), and G (fed with antidiabetic drug i. e., Glucophage with a dose of 8.32 mg/kg body weight), SP (fed with spinach extract), BR (fed with mustard extract), CB (fed with cabbage extract), and 1, 2, and 3 of SP, BR, and CB are different doses.

Sample	FBS (mg/dL)	ALT (U/L)	ALP (U/L)	AST (U/L)	TG (mg/dL)	TC (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	Urea (mg/dL)	Creatinine (mg/dL)	HbA1C (%)
C	$80.3 \pm 5.5^{\#\#\#}$	$49.3 \pm 4.5^{\#\#\#}$	$99 \pm 1.4^{\#\#\#}$	$48.3 \pm 6.5^{\#\#\#}$	$111.6 \pm 2.5^{\#\#\#}$	$107.34 \pm 8.5^{\#\#\#}$	45 ± 3.6	$86.6 \pm 8.9^{\#\#\#}$	$21 \pm 2.0^{\#\#\#}$	0.6 ± 0.1	4.07 ± 0.15
D	$201.6 \pm 8.9^{***}$	$139 \pm 4.0^{***}$	$201.6 \pm 4.6^{***}$	$161.6 \pm 3.3^{***}$	$161.3 \pm 2.1^{*}$	$142.3 \pm 3.3^{***}$	39 ± 2.0	$135.3 \pm 6.0^{***}$	$94 \pm 3.6^{***}$	2.1 ± 0.2	5.0 ± 0.1
G	$104.6 \pm 7.0^{***\#\#\#}$	$81.7 \pm 11.6^{***\#\#\#}$	$132.7 \pm 10.5^{***\#\#\#}$	$67.6 \pm 4.0^{***\#\#\#}$	$136 \pm 1.5^{*\#\#\#}$	$133.6 \pm 4.0^{***}$	42.3 ± 1.5	$116 \pm 1.0^{***\#\#\#}$	$38 \pm 2.0^{**\#\#\#}$	0.85 ± 0.05	4.2 ± 0.2
SP1	$135.3 \pm 8.2^{***\#\#}$	$103.6 \pm 6.1^{***\#\#\#}$	$128.7 \pm 11.5^{***\#\#\#}$	$85.6 \pm 7.0^{***\#\#\#}$	$124 \pm 9.5^{*\#\#\#}$	$131.6 \pm 3.1^{***}$	41 ± 2.0	$118 \pm 3.6^{***\#\#\#}$	$35.7 \pm 3.1^{**\#\#\#}$	0.9 ± 0.26	4.2 ± 0.1
SP2	$133 \pm 5.1^{***\#\#\#}$	$96.3 \pm 5.1^{***\#\#\#}$	$115.3 \pm 6.5^{**\#\#\#}$	$68.3 \pm 3.0^{***\#\#\#}$	$122.3 \pm 4.0^{\#\#\#}$	$129.3 \pm 1.5^{***\#}$	41.3 ± 2.5	$112 \pm 4.0^{***\#\#\#}$	$43.6 \pm 3.5^{***\#\#\#}$	0.6 ± 0.15	4.25 ± 0.05
SP3	$113.3 \pm 5.1^{***\#\#\#}$	$78.3 \pm 6.0^{***\#\#\#}$	$106.6 \pm 6.8^{\#\#\#}$	$70.3 \pm 5.6^{***\#\#\#}$	$120.6 \pm 8.5^{\#\#\#}$	$127.3 \pm 5.1^{***\#\#}$	43.6 ± 3.5	$107.3 \pm 5.1^{***\#\#\#}$	$25.6 \pm 4.1^{\#\#\#}$	0.7 ± 0.1	4.13 ± 0.2
BR1	$156.3 \pm 9.1^{***\#\#\#}$	$90.7 \pm 10.9^{***\#\#\#}$	$181 \pm 6.5^{***\#\#\#}$	$90.6 \pm 7.0^{***\#\#\#}$	$129.3 \pm 4.5^{*\#\#\#}$	$128.6 \pm 6.8^{***\#}$	40.6 ± 1.5	$113.3 \pm 9.0^{***\#\#\#}$	$39.6 \pm 5.1^{***\#\#\#}$	0.8 ± 0.1	4.4 ± 0.3
BR2	$131.5 \pm 4.5^{***\#\#\#}$	$66.5 \pm 2.5^{**\#\#\#}$	$132.5 \pm 16.2^{***\#\#\#}$	$56.5 \pm 7.7^{\#\#\#}$	$136 \pm 3.0^{*\#\#\#}$	$122.5 \pm 4.5^{**\#\#\#\#}$	40.5 ± 2.1	$108.5 \pm 2.5^{***\#\#\#}$	$29.5 \pm 2.1^{\#\#\#}$	0.65 ± 0.07	4.2 ± 0.07
BR3	$120.3 \pm 5.7^{***\#\#\#}$	$79.7 \pm 8.6^{***\#\#\#}$	$123.6 \pm 9.2^{*\#\#\#}$	$68.6 \pm 6.5^{***\#\#\#}$	$132.6 \pm 6.0^{*\#\#\#}$	$128.3 \pm 7.0^{***\#}$	43.3 ± 4.0	$111 \pm 7.2^{***\#\#\#}$	$33.3 \pm 2.6^{*\#\#\#}$	0.66 ± 0.15	4.15 ± 0.1
CB1	$125.6 \pm 9.0^{***\#\#\#}$	$125.3 \pm 5.2^{***\#}$	$133.3 \pm 6.1^{***\#\#\#}$	$98.6 \pm 6.3^{***\#\#\#}$	$133.7 \pm 3.5^{*\#\#\#}$	$132.7 \pm 8.1^{***}$	39.6 ± 2.0	$119.3 \pm 9.5^{***\#\#}$	$38.3 \pm 4.0^{*\#\#\#}$	0.8 ± 0.1	4.3 ± 0.2
CB2	$110.3 \pm 9.8^{***\#\#\#}$	$92.3 \pm 9.6^{***\#\#\#}$	$126 \pm 6.3^{***\#\#\#}$	$78.3 \pm 7.5^{***\#\#\#}$	$112.3 \pm 3.5^{\#\#\#}$	$126 \pm 6.5^{***\#\#}$	42.3 ± 1.5	$105.7 \pm 6.5^{***\#\#\#}$	$39 \pm 3.0^{***\#\#\#}$	0.76 ± 0.15	4.2 ± 0.2
CB3	$99 \pm 3.2^{***\#\#\#}$	$86.3 \pm 6.4^{***\#\#\#}$	$116.3 \pm 6.2^{**\#}$	$76 \pm 5.2^{*\#}$	$128.7 \pm 6.2^{*\#\#\#}$	$136 \pm 7.0^{***}$	43.6 ± 2.5	$117 \pm 6.4^{***\#\#\#}$	$33 \pm 5.6^{***\#\#\#}$	0.73 ± 0.15	4.1 ± 0.2

Data are the mean of triplicate with standard deviation. $*$ = vs control (C) group, $\#$ = vs diabetic (D), $*$ = 0.03, $**$ = 0.002, $***$ < 0.001, $\#$ = 0.03, $\#\#$ = 0.002, $\#\#\#$ < 0.001 using Dunnett's multiple comparison test.

Table 3

Effect of spinach, mustard, and cabbage phenolic extracts on hematology indices of mice. Abbreviations: C (non-diabetic control group), D (diabetic control group), and G (fed with antidiabetic drug i.e., Glucophage with a dose of 8.32 mg/kg body weight), SP (fed with spinach extract), BR (fed with mustard extract), CB (fed with cabbage extract), and 1, 2, and 3 of SP, BR, and CB are different doses.

Parameters	Treatments											
	C	D	G	SP1	SP2	SP3	BR1	BR2	BR3	CB1	CB2	CB3
RBC(× 10^6 µL)	8.85 ± 0.04	4.9 ± 0.2	7.72 ± 0.2	6.22 ± 0.6	7.03 ± 0.4	7.68 ± 0.5	5.14 ± 0.5	6.96 ± 0.6	8.14 ± 0.2	6.31 ± 0.07	6.82 ± 0.04	7.75 ± 0.4
HB (g/dl)	12.4 ± 0.3	9.2 ± 0.3	11.8 ± 0.4	10.3 ± 1.0	11.1 ± 1.1	11.5 ± 0.3	9.7 ± 1.7	10.1 ± 1.6	10.5 ± 0.6	10.5 ± 0.3	10.8 ± 0.8	11.6 ± 0.9
HCT %	30.8 ± 1.7	37.5 ± 2.1	38.9 ± 0.6*	41.5 ± 1.7***	39.0 ± 1.4*	28.2 ± 0.6##	42.3 ± 1.9***	27.5 ± 0.6###	34.3 ± 2.9	37.8 ± 1.1*	35.5 ± 2.3	24.6 ± 2.1###
MCV (fL)	52.6 ± 1.0	57.2 ± 1.0	57.9 ± 1.3	57.5 ± 1.1	55.1 ± 2.6	49.6 ± 1.4#	52.0 ± 2.0	59.1 ± 0.5	59.8 ± 0.7*	55.1 ± 1.2	52.8 ± 2.3	72.2 ± 2.0***###
MCH (pg)	13.2 ± 1.0	15.5 ± 0.8	14.9 ± 1.5	17.0 ± 2.3	15.7 ± 1.1	12.1 ± 0.7	14.4 ± 0.2	16.9 ± 0.8	15.5 ± 0.2	16.8 ± 1.4	15.0 ± 0.1	24.5 ± 1.0***##
MCHC (g/dL)	31.0 ± 2.5#	23.2 ± 1.6*	28.7 ± 0.8	25.6 ± 1.2	27.5 ± 1.6	28.5 ± 1.5	27.7 ± 0.4	28.6 ± 1.7	29.9 ± 2.0	26.4 ± 0.9	28.5 ± 0.7	30.8 ± 2.6#
PLT(× 10^6/µL)	812 ± 9.1###	286 ± 3.1***	787 ± 2.4***###	556 ± 5.6***###	573 ± 6.7***###	603 ± 6.7***###	404 ± 8.5***###	451 ± 4.8***###	512 ± 6.5***###	387 ± 4.3***###	414 ± 3.7***###	695 ± 9.8***###
WBC (× 10^3 µL)	8.7 ± 0.2	4.9 ± 0.2	8.2 ± 0.9	7.6 ± 0.7	5.3 ± 0.4	5.0 ± 0.8	5.3 ± 0.2	7.7 ± 1.0	8.2 ± 0.4	4.6 ± 0.8	6.4 ± 0.2	8.1 ± 0.8
LYM (%)	78.1 ± 2.8###	36.8 ± 3.2***	57.7 ± 0.9***###	60.5 ± 3.0***###	43.4 ± 4.1***	67.7 ± 2.8***###	45.8 ± 0.6***##	71.5 ± 3.9###	81.2 ± 4.6###	78.4 ± 2.4###	53.9 ± 2.1***###	67.2 ± 1.8***###
NEU (%)	57.1 ± 1.7###	17.8 ± 1.6***	35.5 ± 2.6***###	32.9 ± 1.5***###	50.1 ± 2.1*###	26.3 ± 1.0***##	47.2 ± 0.9***###	22.3 ± 0.2***	13.5 ± 0.3***	16.3 ± 0.9***	41.5 ± 0.9***###	25.3 ± 1.2***#
EOS (%)	2.3 ± 0.3	2.7 ± 0.3	2.9 ± 0.3	4.2 ± 0.7	2.3 ± 0.5	3.0 ± 0.2	2.5 ± 0.0	4.8 ± 0.1	2.3 ± 0.2	2.5 ± 0.3	2.21 ± 0.2	3.4 ± 0.4
MON (%)	3.1 ± 0.3	1.2 ± 0.3	2.4 ± 0.2	2.1 ± 0.4	3.2 ± 0.3	1.0 ± 0.1	3.1 ± 2.0	2.3 ± 0.2	2.1 ± 0.1	1.7 ± 0.2	1.6 ± 0.1	2.3 ± 0.2

Data are the mean of triplicate with standard deviation. * = vs control (C) group, # = vs diabetic (D), * = 0.03, ** = 0.002, *** < 0.001, # = 0.03, ## = 0.002, ### < 0.001 using Dunnett's multiple comparison test.

significant decrease was also observed in the platelets count (812–286 $\times 10^6/\mu l$) of C and D groups, respectively. No significant differences were observed in eosinophils (EOS) and monocytes (MON) counts among all the treatments. No indicative changes were recorded between the G, SP, BR, and CB groups, and their values were close to that of the hematological parameters of the C group, indicating the beneficial effect of various treatments.

3.5. Effects on GSH contents

Fig. 3 A&B shows the effect of extracts treatments on the tissue GSH contents of the liver and kidney. It has been observed that liver GSH contents of the D group have been considerably reduced ($P < 0.001$) compared to C or G group. The GSH levels of the treated groups have also been significantly increased ($P < 0.001$) in a dose-dependent manner. The kidney GSH level, however, showed a significantly lower quantity of GSH in C and G groups. However, the extract-treated groups resulted in a considerably higher quantity of kidney GSH amounts. Moreover, the GSH contents of the animal tissues have greatly improved when treated with CB extract, followed by SP and BR extract. These findings suggested that leafy vegetables have the potential phytochemicals to raise the GSH contents of diabetic tissues.

3.6. Effects on liver histopathology

Fig. 4 A shows the radial arrangement of the Hepatic lamina (arrow) and the Hepatic Sinusoid (row) of the control group (C), having a well-organized central vein (CV) with normal morphology. Fig. 4B represents histo-pathological findings of the diabetic (D) group, which showed an absence of radial arrangement of the hepatic lamina (HL) and hepatic sinusoid (HS). Also, it has degenerated central vein (CV), increased Kupfer cells (KC) count, and lymphocytic infiltration between hepatic lamina, indicating a diabetic stress situation. Fig. 4C shows the G group's liver histology, revealing the radial arrangement of HL and HS having a well-organized CV and the presence of KC between hepatic lamina. Fig. 4D, E, & F are the histo-pathological findings of mice liver treated with various
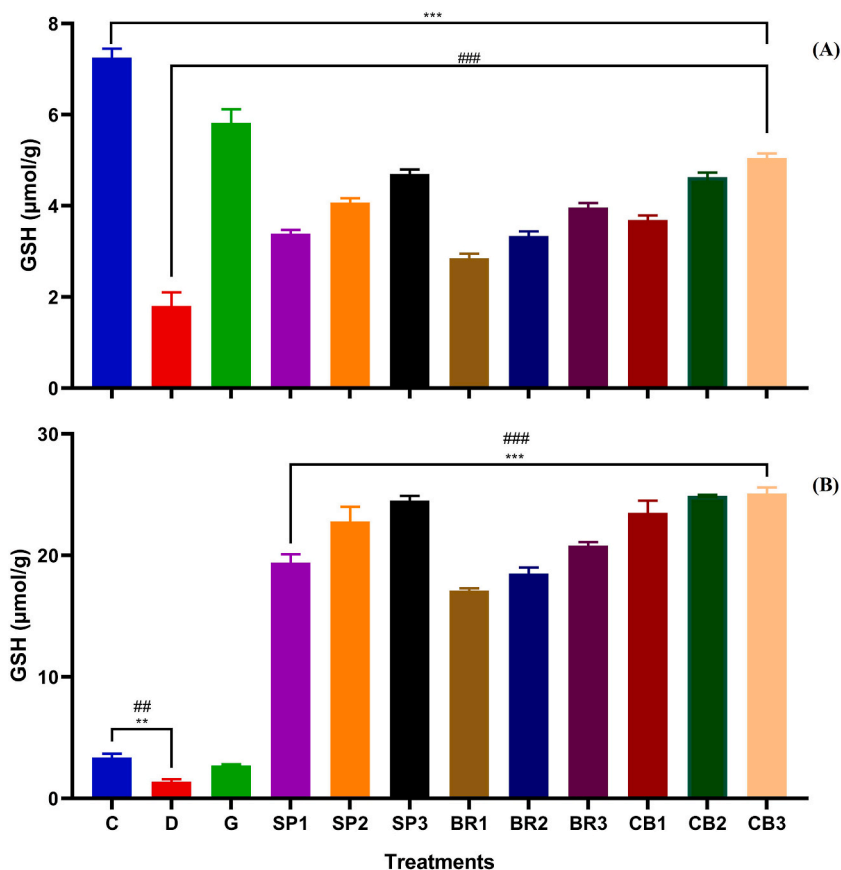
Fig. 3. Effect of spinach, mustard, and cabbage leaves extracts on the total glutathione levels (GSH) (A) liver, and (B) kidneys of mice. Abbreviations: C (non-diabetic control group), D (diabetic control group), and G (fed with antidiabetic drug i.e., Glucophage with a dose of 8.32 mg/kg body weight), SP (fed with spinach extract), BR (fed with mustard extract), CB (fed with cabbage extract), and 1, 2, and 3 of SP, BR, and CB are different doses. Data are the mean of triplicate measurements with standard deviation. * = vs control (C) group, # = vs diabetic (D), ** = 0.002, *** < 0.001, ## = 0.002, ### < 0.001 using Dunnett's multiple comparison test.
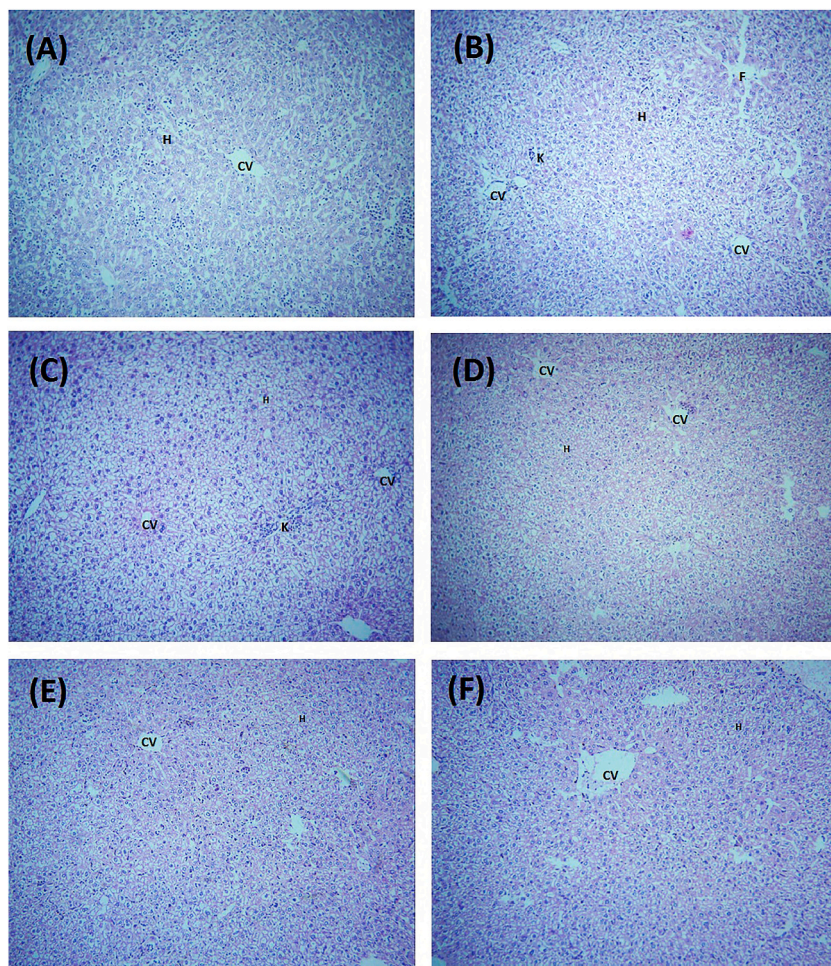
Fig. 4. Effect of spinach, mustard, and cabbage leaves extracts on the liver of mice. (A) Control, (B) diabetic, (C) positive control, (D) Spinach extract, (E) mustard, and (F) cabbage. Abbreviations are CV, central vein; H, hepatocytes; K, Kupfer cells; and F, fats.

vegetable extracts, spinach, mustard, and cabbage. These Figures showed a better recovery in the CV and normalized radial arrangement of hepatocytes, indicating a beneficial treatment effect during diabetic conditions.

### 3.7. Effects on kidney histopathology

Fig. 5 A shows normal Glomerular corpuscle (GC) and balance in the urinary and vascular poles of the control (C) group. Also, the proximal (PCT) and distal convoluted tubules (DCT) have normal morphology. However, in the diabetic group (D), the kidney showed glomerular corpuscle damage (arrow) and thickening of the mesangial layer or glomerular basement membrane (line). Degenerated PCT and DCT were also reported, as shown in Fig. 5B.

The G group has shown normal glomerular corpuscle (GC), balanced in urinary and vascular pole, and normal PCT and DCT as shown in Fig. 5C. Fig. 5D, E & F represent kidney histology of the treated groups with vegetable extract SP, BR, and CB, respectively. From the figures, it was noticed that such treatments had shown better results in ameliorating diabetic complications. These micrographs showed normal PCT and DCT, a balance in the urinary and vascular pole, and a recovery in epithelial tissues of kidney tubules.

4. Discussion

The phenolic compounds in leafy vegetables are important secondary metabolites and possess medicinal properties. These include antioxidant [23] and anti-diabetic activity [24]. The present study observed that aqueous extracts of the selected leafy vegetables contain essential phenolic compounds. The total phenolic compounds identified were higher in Spinach, followed by mustard, and lowest in cabbage, but cabbage still has retained a comparatively high antidiabetic activity. This might be attributed to the presence of phenolic acids such as syringic acid [26] and coumaric acid [27] in cabbage, which could exacerbate the diabetic condition. Likewise, p-Hydroxybenzoic acid [25] and other phenolic acids have been reported in spinach, mustard, and cabbage leaves of Portuguese and
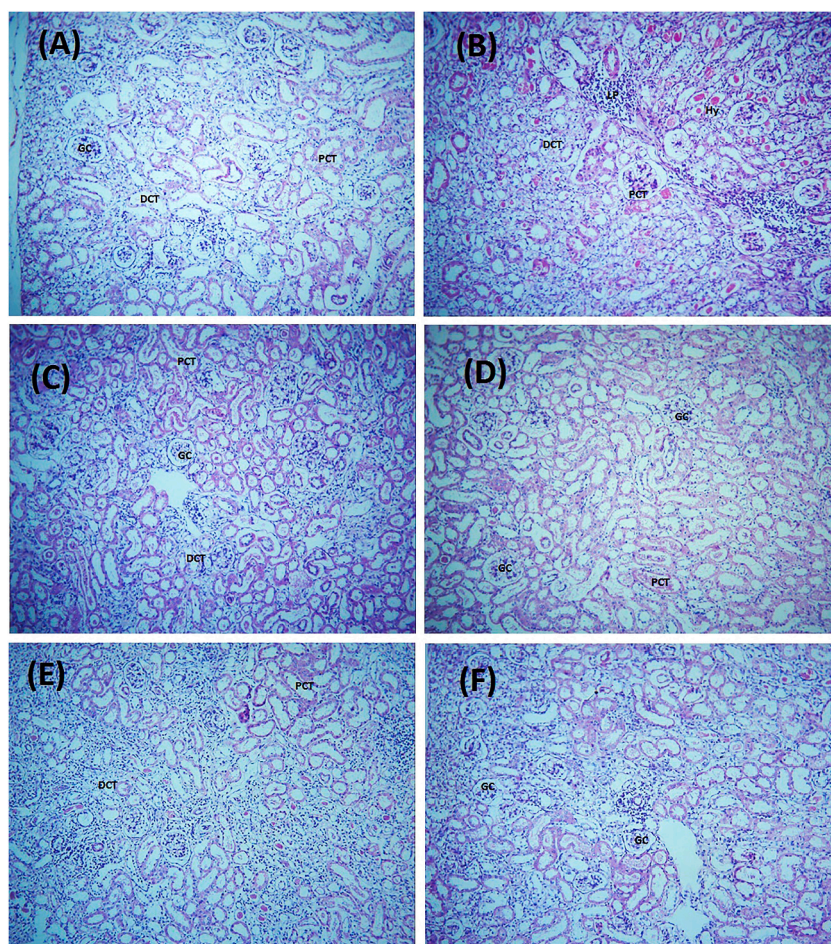
Fig. 5. Effect of spinach, mustard, and cabbage leaves extracts on the kidney of mice. (A) Control, (B) diabetic, (C) positive control, (D) Spinach extract, (E) mustard, and (F) cabbage. Abbreviations are GC, Glomerular corpuscle; Hy, hyalinization of kidney tubules; LP, lymphocytic proliferation; DCT, Distal convoluted tubules; PCT, Proximal convoluted tubules; and CV, central vein.

Spanish samples [26,27]. Ferulic acid and flavonoids such as kaempferol and quercetin showed antioxidant, anti-inflammatory, and anti-arthritic activities in the animal model [28]. Vinayagam et al. [29] showed that simple phenolic acids increased glucose uptake and glycogen synthesis; improve glucose and lipid profiles in certain diseases. Kaempferol and its glycosides have been reported as significant compounds in cabbage from Portugal [30]. Kaempferol and derivatives have been found to promote insulin secretion and improve liver protein kinase B (Akt) activity by regulating mitochondrial calcium uptake, thus reversing the up-regulation of gluconeogenesis, down-regulation of glycogen synthesis, and glucose uptake caused by Akt inactivation [31]. In conclusion, the selected leafy vegetables were rich in these phenolic compounds and thus may be responsible for the anti-diabetic properties.

During diabetes, body weight loss is usually observed. For example, the study of Owens, Belcher [22] reported a significant weight loss in male CD1 albino mice during the induced diabetic conditions. Weight loss was also observed during this work, which was ameliorated by supplementing the extracts.

Abnormal lipid metabolism is also linked to diabetes which plays a crucial role in the development of atherosclerosis and cardiovascular disease [22]. Dyslipidemia is one of the most causative agents in developing diabetic macro-vascular complications [32]. By improving glycemic control during diabetes, the risk of diabetic complications may be minimized [33]. Our findings agree with Peng et al. [34], who observed a significant increase in serum TG, cholesterol, and LDL-C and a significant decline in HDL-C in diabetic male mice versus the control group. Creatinine (CR) is a significant indicator of diabetic nephropathy. Ma et al. [35] observed a significantly increased level of CR in diabetic mice, with which our present results coincided. The present work is also in agreement with Adesokan et al. [36], who found a significant increase in renal profile parameters, BUN, and CR in diabetic male rats versus the control group.

Similarly, the glycated hemoglobin HbA1C, which measures an average glycemic control over approximately 8 weeks, is affected by factors including RBC's life span, hemodialysis, and anemia [37]. Therefore HbA1C may not predict a precise glycemic control. However, our findings also agree with Bhonsle et al. [38], who observed a comparatively high HbA1C level in diabetic mice.

The association between liver dysfunction and type 2 DM has been reported in different studies [39]. The liver performs a key role

in glucose homeostasis by exacerbating liver insulin resistance [40] since it has been observed that insulin resistance and T2D may disturb liver function [40]. The study conducted by Adesokan et al. [36] observed a significant increase in liver enzyme ALT, ALP, and AST in alloxan-induced diabetic male albino rats. Their results are consistent with our findings of elevated levels of these parameters.

The GSH has been widely studied as a vital biomolecule countering oxidative stress and cellular injuries [41], which may trigger the onset of several diabetic complications [35]. The GSH also scavenge free radicals by initiating a radical scavenging response during biological damage or oxidative stress condition [42]. A high GSH is quite helpful in countering oxidative stress [43]. In the present study, we have observed significantly decreased levels of liver and kidney GSH in diabetic mice versus in control group C. The decreased level of GSH in the diabetic group indicated the utilization of GSH during diabetic stress [43]. Kim et al. [44] also reported a decline in the GSH level of diabetic rats. In this study, the liver and kidney GSH contents have been improved, particularly in the treated groups. This may be due to the presence of important phenolic compounds viz coumaric acid [45], ferulic acid [46], and quercetin [47,48], which could ameliorate the tissue stress by improving the GSH. Antidiabetic drugs may also restore the liver GSH level [49,50].

Liver histopathology of diabetic mice showed degenerated central vein (CV), absence of hepatic lamina (HL) and hepatic sinusoid (HS), an increase in Kupfer cell count, and infiltration of lymphocytic, which indicated liver damage. However, the treated mice have shown better liver texture showing the in vivo antidiabetic effect of the treated extracts.

During diabetes, the expansion of the kidney mesangial cells could lead to reducing the bowman's space. Such an expansion has been described by Teoh et al. [51]. Our findings also coincide with the study of Prabhakar et al. [52], who reported extensive mesangium and basement membrane thickening in diabetic rats. The study of Fioretto, Mauer [53] also confirmed that the thickening of the mesangial layer is a core structure leading to diabetic nephropathy.

These results suggest that extracts of the selected leafy vegetables have antidiabetic activity, mainly when their extract was fed at a high dose. They may have the potential to neutralize diabetic stress and improve the biochemical, histo-pathological, and hematological indices of the treated organism. The main limitation of this study is the analysis of molecular targets of each of these phenolic compounds present in selected leafy vegetables for the treatment of diabetes. However, it is concluded that among the treated vegetables, cabbage can minimize diabetic complications more effectively when compared to the other studied vegetables.

Author contribution statement

Arif Mehmood: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Alam Zeb: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Muhammad Khalil Ateeq: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Data availability statement

Data will be made available on request.

Ethics approval statement

The experiments were carried out according to the approved guidelines for the care and experimentation of animals, at the University of Malakand. The protocols were approved by the graduate study committee (GSC) of the Department of Biotechnology and finally approved by the advanced study and research board (ASRB) of the University of Malakand in its 58th meeting held on November 30, 2020.

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Declaration of competing interest

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

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