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Original article

Ameliorative impact of *Morus alba* leaves' aqueous extract against embryonic ophthalmic tissue malformation in streptozotocin-induced diabetic rats

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ABSTRACT

Background: Diabetes mellitus (DM) is becoming a serious threat to human health. *Morus alba* var. multicaulis (Perr.) Loudon (Moraceae) showed a bright future in DM therapy.
Objective: The study evaluates the antioxidant activity of *Morus alba* leaves aqueous extract (MLAE) and anti-diabetic properties of MLAE in streptozotocin-induced diabetic rats focusing on the ameliorative effects against embryogenesis defects.
Materials and methods: MLAE was assayed for bioactive compounds, and antiradical potential. MLAE (100 mg/kg body weight) was orally administered to albino rats. DM was induced by intraperitoneal injection of STZ (60 mg/kg). The pregnant rats were arranged into 4 groups including control pregnant (C), MLAE-treated group (M), experimental diabetic group (D), and combined diabetic with MLAE-treated group (D-MLAE). The experiment performed in about six months.
Results: TPC in MLAE accounted for 11 mg GAE/g dry weight (dw) while vitamin C and β-carotene amounts were 144 and 0.1 mg/100 g, respectively. MLAE exhibited DPPH·, NO· and $O\cdot_2^-$ radical scavenging activities. Treatment of diseased-rats with MLAE resoluted serum glucose levels (378 mg/dL), wherein glucose recorded the highest level (830 mg/dL) in DM mothers. DM rats recorded the highest level of TC, TG, HDLc, LDLc, and CK, while MLAE treatment reduced those levels. DM rats recorded the highest level of MDA, H_2O_2, SOD, CAT, GST, GSPase, GSH, GOT, GPT, albumin, bilirubin, arginase, and α-L-fucosidase, while MLAE reduced those levels. Histological photomicrographs of maternal retina showed degenerated ganglionic cells, and neovascularization of nerve fiber layer with edematous inner plexiform layer, and partial loss outer plexiform layer in DM rats.
Conclusion: MLAE could be used to ameliorate DM. Thus, it might be considered as useful dietary supplements in diabetic patients.

1. Introduction

Diabetes mellitus (DM) is one of major chronic diseases worldwide. By 2025, the World Health Organization (WHO) predicted that there will be around 300 million people with diabetes [1–3]. DM is classified into type 1 (T1DM) and type 2 (T2DM). T1DM involves the autoimmune destruction of insulin-producing pancreatic β-cells *via* autoaggressive T-cells, and pancreatic macrophage infiltration [4]. T2DM is the most endocrine disorder worldwide, covering 90–95% of all DM cases. T2DM classification and pathogenesis involves lipid metabolism, abnormalities in glucose, inadequate insulin secretion from pancreatic β-cells and resistance to insulin activity [5]. Oxidative stress plays a pivotal role in the DM pathogeneses [6]. DM is linked with male reproductive dysfunction [7]. In addition, DM can induce apoptosis in the testicular germ cells and decreases plasma testosterone levels as well as sperm count in rats. DM hyperinsulinemia can increase re-absorption of uric acid in the proximal tubules, while its overproduction due to an increased activity of xanthine-oxidase usually takes place [8,9]. In diabetic pregnancies, lipid metabolism alterations and hyperglycemia are associated with maternal and fetal complications [10].

Despite the current preoccupation with chemistry as a vehicle to manufacture drugs, the contribution of medicinal plants to disease prevention is still enormous [11,3,12,13]. According to the World Health Organization (WHO) more than 150 medicinal plants and herbs

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are utilized for DM treatment [14]. Mulberry [*Morus alba* var. multicaulis (Perr.) Loudon (Moraceae)], a multipurpose tree, is cultivated for its leaves [food for silkworms (*Bombyx mori*, Bombycidae)] [15–17]. Consumption of *M. alba* leaves as infusion and juice is widespread in Japan and Korea [18]. Leaves are used as an alternative medicine in China and Japan [19,20]. Bazylak and Olszewska-Slonina [21] reported that *M. alba* leaves contain high levels of digestive proteins, carbohydrates, micro- and macronutrients, polyphenols, free amino acids, and organic acids. *M. alba* contains many bioactive compounds including phenolic compounds, polysaccharides, 1-deoxynojirimycin (DNJ) and anthocyanins, which associated with its biological functions such as antiobesity, antidiabetes, antioxidation, antiinflammation and antiatherosclerosis [22–24].

The antioxidant potential of *M. alba* leaves extracts (MLE) is linked to their phenolics content. MLE are rich source of phenolic acids and flavonoids, such as caffeic acid, kaempferol-3-*O*-(6-malonyl) glucoside, quercetin-3-*O*-(6-malonyl)-β-D-glucopyranoside and quercetin-3-*O*-glucoside [18,1]. Sánchez-Salcedo et al. [16,17] reported that MLE are rich in flavonols (3.7–9.8 mg/g), and caffeoylquinic acids (6.8–8.5 mg/g). Wanyo et al. [25] identified *p*-coumaric acid, (+)-catechin, chlorogenic acid, ferulic acid, and gallic acid in MLE. Hunyadi et al. [26] reported that the hot aqueous extract of *M. alba* leaves containing phenyl-propane derivatives, can increase the glucose consumption of adipocytes. Polysaccharides in *M. alba* leaves attracted attention due to their multiple biological activities [23,27,28,24]. Yuan et al. (2015) reported that *M. alba* leaves polysaccharides exhibited Fe^{2+} chelating activity, and antiradical action against 1,1-diphenyl-2-picrylhydrazyl (DPPH·), superoxide hydroxyl and 2,2-azinobis-(3-ethyl-benzothiazolin-6-sulfonic acid) radicals.

MLE varies depending on mulberry variety and the extraction method [29–32,1]. MLE exhibited hypoglycaemic, hypolipidaemic, and antiatherogenic effects in animal models and in humans [19,33,34,8,9,1,16,17,35]. Wu et al. [36] reported that MLE can reduce the accumulation of hepatic lipid through activation of the AMP-activating protein kinase signaling pathway. Park et al. [31] also indicated that MLE could be used to prevent diseases characterized by chronic inflammation.

M. alba leaves are a valuable, and low-cost material that can be utilized in the treatment of T2DM, diseases of the cardiovascular system, urinary system, and nervous system (*i.e.*, Alzheimer's disease) as well as in weight loss [1,16,17]. *M. alba* leaves aqueous extract (MLAE) is already used in the treatment of hyperglycaemia and DM [37,38]. Antidiabetic traits of MLE in STZ-induced diabetes in rats was reported [39]. Jeszka-Skowron et al. [1] evaluated the antidiabetic impact of MLE in diabetic rats fed high-fat diet. *M. alba* leaves ethanol extract with high level of phenolics was more active than dry leaves or acetone extract in increasing insulin level and lowering of blood glucose. However, the mechanisms by which *M. alba* leaves and their extracts could normalize hyperglycaemia or increase insulin levels are still not completely understood. Moreover, the contribution of oxidative stress due to diabetic in testicular abnormalities development has not been clarified.

Searching for new antidiabetic agents that retain therapeutic efficacy without side effects is needed. Also, it is strongly significant to pay attention to traditional medical therapeutics for DM treatment in pregnant and lactating mothers. Our main objective in this study was to explore the potential of MLAE as dual-target phytotherapeutics to treat DM. To the best of our knowledge, there is few data on antidiabetic traits of MLAE against embryogenesis defects in STZ-induced diabetic rats [10]. Therefore, the aim of the study was to evaluate the antioxidant potential and antidiabetic properties of *M. alba* leaves aqueous extract (MLAE) in STZ-induced diabetic rats with focusing on the ameliorative effects against embryogenesis defects.

2. Material and methods

2.1. Chemicals and plant material

M. alba leaves were plucked from healthy plants in June 2014 from the Nile delta (Egypt). Plants were verified by professors of botany at Mansoura University (Egypt). A voucher specimen number 1523 was used, wherein the specimen was lodged at Department of Botany, Faculty of Agriculture, Zagazig University (Egypt). The leaves were washed, freeze-dried, powdered and packed in polythene covers. β-Carotene, butylated hydroxytoluene (BHT), and DPPH· were obtained from Sigma (USA). All chemicals and solvents were of the highest purity available.

2.2. Preparation of M. alba leaves aqueous extract (MLAE)

Powder of *M. alba* leaves was extracted using distilled water, wherein the extraction was performed by placing known weight of powder in water then heating at 85 °C for 5 min followed by cooling. The mixture was filtered, evaporated in vacuum evaporator [10] then lyophilized to obtain freeze-dried MLAE which used during the experiment. The applied protected dose of extract was 100 mg/kg body weight (bw) which orally administered prior to DM induction every other day until the end of the experiment.

2.3. Bioactive compounds and radical scavenging potential of MLAE

Total phenolic compounds (TPC), β-carotene, and vitamin C contents were estimated in MLAE according to Ramadan and Moersel [40]. MLAE was defatted by extraction in a Soxhlet extractor using *n*-hexane for 6 h. The defatted material was extracted with ethanol (95%) for 72 h. The extract was concentrated using vacuum evaporator and the residue was stored for subsequent experiments in a desiccator. The antiradical potential of five different concentrations (50–250 μg/mL) of MLAE against DPPH·, nitric oxide radical NO· [41], and superoxide radical $O\cdot_2$ [42] was measured.

2.4. Animals and experimental protocol

One hundred fertile male and virgin female albino rats (1 male: 3 females) weighing *ca.* 125 g were used in the experiment. Animals were obtained from breading farm, Ministry of Health (Giza, Egypt). Free access of standard diet composed of 50% grinded barley, 20% milk, 10% grinded maize and 10% vegetables were supplied. Free excess of water was allowed *ad-libitum.* The applied protected dose of MLAE was 100 mg/kg bw which orally administered prior to induction of DM every other day till the end of experiment. All animal procedures and the protocol were conducted according to EU Directive [43] 2010/63/EU for animal experiments (2010). All necessary efforts were carried out to decrease the number of animals used and their suffering. Animals were housed in temperature (20–25 °C), humidity (45–60%), and 12 h light/dark. Females were mated in a special cage (1 male: 3 females) overnight and copulation was identified in the next morning by the presence of sperm in the native vaginal smear. The day of conception was considered to be the first day of pregnancy. Vaginal smears were carried out to give a precise determination of the zero day of gestation. The experiment was carried out in about 6 months.

2.5. Induction of diabetes

Pregnant rats of the control group (C) were fed on standard diet free from excess fats with free excess of food and water. Rats were allowed *ad libitum* throughout the experimental period. Experimental DM was induced in all rats by a single intraperitoneal injection (i.p.) of STZ (60 mg/kg) in citrate buffer (0.05 M, pH 4.5) at the 5th day of gestation for two consecutive days [44]. Control animals were treated with

physiological saline as vehicle. Presence of DM in rats was confirmed by measuring fasting blood glucose (FBG) concentration in blood samples withdrawn from the tail tip after 48 h using a glucometer.

The pregnant rats' groups were arranged into 4 groups (10 animals in each group) as follows:

1. Control pregnant (C).
2. MLAE-treated group (M).
3. Experimental diabetic group (D).
4. Combined diabetic and MLAE-treated group (D-MLAE).

At parturition, the pregnant rats of control and experimental groups were anesthetized by an i.p. injection of sodium pentobarbital solution (50 mg/kg bw), sacrificed, and analyzed for biochemical assessments, histological investigation of maternal tissues and their newborn, effects on pregnancy and offspring, analysis of ocular disease, and the single cell gel electrophoresis (Comet assay).

2.6. Biochemical assessments

Five pregnant rats were sacrificed, and the blood was collected in non-heparinized tube then centrifuged at 224 × *g* (2000 rpm), and the serum was collected. Total cholesterol (TC), triglycerides (TG), and HDL-cholesterol (HDLc) concentrations were determined in the serum using standard kits. In case of LDLc, it was calculated from TC, HDLc, and TG according to Friedewald et al. [45].

2.7. Effect of MLAE on pregnancy and offspring

There were no significant differences in the body weight gain between control group and other groups during the study (data not shown). The STZ injection did not affect the weight among the treated groups. The total number of aborted, and pregnant mothers a well as numbers of their offspring, and pattern of congenital malformations were determined in all groups. The body weights (g) were also recorded. Offspring of both control, and experimental groups were fixed in 10% formal saline followed by treatment with 2% KOH for 5 days till ossified areas were clearly visible through the soft tissue.

2.8. Histological investigation of maternal tissues and their newborn

The eye of mothers, and their offspring were fixed in 10% phosphate-buffered formalin for 24 h, dehydrated in ascending grades of ethanol, cleared in xylene then mounted in molten paraplast at 58–62 °C, and processed in a routine manner to generate 5 µm thick paraffin sections [46]. Sections were stained with hematoxylin and eosin (H & E) and used in microscopic examination.

2.9. Analysis of ocular disease

The offspring of all groups were examined morphologically *via* processing of histological section, and incidence of cataract was observed.

2.10. Single cell gel electrophoresis (Comet assay)

Retina of newly born offspring were separated, and stored at −20 °C for Comet assay. Specimens of control, and experimental groups were homogenized [47]. Eye homogenate (6 µL) was suspended on low melting agarose (0.5%), and sandwiched between a layer of 0.6% normal melting agarose, and a top layer of 0.5% low melting agarose on fully frosted slides then immersed in a lysis solution for 10 min at 0 °C to allow DNA to unwined. Electrophoresis was performed at 300 mA and 1 V/cm for 10 min. Cells (100) were analyzed on each slide using the comet assay II automatic digital analysis system (perspective tail length, µm). The tail moment is the product of the tail length, and the fraction of total DNA in the tail (Tail moment = tail length X% of DNA in the tail). Tail length, and tail intensity were determined by image analysis software.

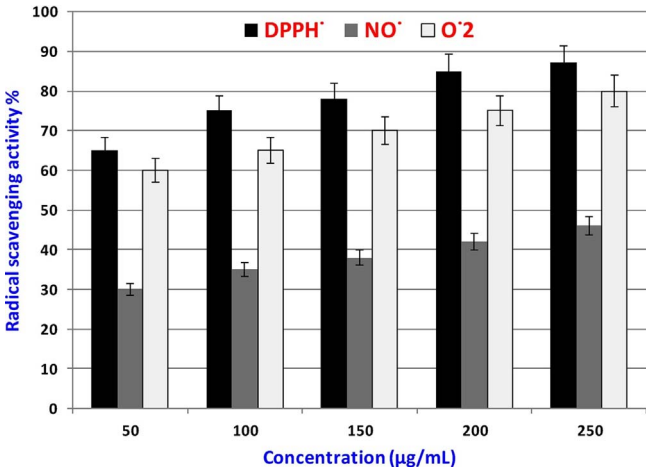

Fig. 1. Antiradical action of MLAF against DPPH·, NO·, and O_2·.

2.11. Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical comparisons between the groups were done using one-way analysis of variance (ANOVA) followed by Tukey's test. Correlation between variables was analyzed using Pearson's correlation coefficient. The significance level was set at $p \leq 0.05$. Statistica 9.0 program (StatSoft Inc., Tulsa, OK, USA) was used for analyses.

3. Results

3.1. MLAE antioxidants' content and activity

Total phenolic content (TPC) in MLAE was 11 mg GAE/g dry weight (dw). Phenolic compounds are effective antioxidants with biological traits that have been reported as the main phytochemicals in *M. alba* leaves [19,1]. This value agrees with a recent study showed that the leaves of berries including chokeberry, cranberry, blackcurrant, and bilberry contain high amount of phenolics [8]. Jeszka-Skowron et al. [1] reported that TPC in MLE was higher in ethanol extract (11.9 g GAE/100 g) than in acetone extract (9.26 g GAE/100 g). Total flavonoids content in MLE was higher in ethanol extract (4.8 g QUE/100 g) than in acetone extract (3.97 g QUE/100 g). Phenolic acids such as chlorogenic, caffeic, and vanillic acids were two-fold higher in ethanol extract than in acetone extract. In addition, flavonols such as quercetin 3-(6-malonylglucoside), rutin, isoquercetrin, kaempferol 3-(6-malonylglucoside), and astragalin were measured in higher levels in ethanol extract than in acetone extract. Sánchez-Salcedo et al. [16,17] measured four caffeoylquinic acids, and ten flavonols in *M. alba* leaves. The main phenolic groups were benzoic acid derivatives, cinnamic acid derivatives, flavonols, and anthocyanins. Phenolics possess the ability to sequester metal ions, and could scavenge O_2, and nitrogen radicals, thereby protecting lipid membranes and proteins from oxidative damage.

Vitamin C (ascorbic acid), and β-carotene levels in MLAE were 144 and 0.1 mg/100 g extract, respectively. Vitamin C is an important diet-derived antioxidant, which readily scavenges free radicals and reactive nitrogen species (RNS), such as superoxides and hydroperoxyl radicals, peroxyl radicals, singlet oxygen, nitrogen dioxide and nitro-oxide radicals [48,49]. Thus, it and effectively protects other substrates from oxidative damage. β-Carotene is a well-known plant pigment with the ability to quench free radicals. The levels of TPC, and vitamin C in

Table 1
Biochemical profile in serum of non-diabetic and diabetic rats treated or not with a *M. alba* aqueous extract.

	FBG (mg/dL)	LDLc (mg/dL)	HDLc (mg/dL)	TG (mg/dL)	TC (mg/dL)	CK (U/L)
Control pregnant (C)	248 ± 12.4	50 ± 2.45	40 ± 4.2	34 ± 1.5	28 ± 1.3	82 ± 2.8
MLAE-treated group (M)	255 ± 15.7	59 ± 3.7	43 ± 4.5	36 ± 2.5	34 ± 3.7	78 ± 3.2
Experimental diabetic group (D)	830 ± 32.6*	114 ± 8.3*	159 ± 13.7*	42 ± 3.2*	47 ± 1.4*	348 ± 26.9*
Combined diabetic and MLAE-treated group (DM)	387 ± 11.5*	67 ± 2.9*	67.3 ± 3.9*	37 ± 2.2*	40 ± 3.8*	143 ± 7.4*

Data shown as mean ± standard deviation (SD) (N = 5).
* $p < 0.05$ compared to non-diabetic group.

Table 2
Antioxidant oxidative markers and some enzymes levels in serum of non-diabetic and diabetic rats treated or not with a *M. alba* aqueous extract.

	Control pregnant (C)	MLAE-treated group (M)	Experimental diabetic group (D)	Combined diabetic and MLAE-treated group (DM)
MDA (nmol/mL)	1.02 ± 0.01	1.01 ± 0.01	1.4 ± 0.01*	1.31 ± 0.04
H_2O_2 (mmol/L)	25 ± 0.01	24 ± 0.11	40 ± 0.02*1	31 ± ±0.01
SOD (U/mL)	16.22 ± 1.60	16.87 ± 0.6	15.31 ± *1.26	16.20 ± 1.26
CAT (U/L)	29.79 ± 0. 6	28 ± 0.72	55.76 ± *0.90	32.09 ± 0.9
GST (U/mL)	1.02 ± 0.05	1.5 ± 0.1	1.31 ± 0.5*	1.40 ± 0.09
GSPase (U/L)	17.23 ± 1.2	18.23 ± 2.59	16.4 ± 2.82*	15.21 ± 1.5
GSH (mg/dl)	12.43 ± 0.	11.5 ± 0.58	19.07 ± *1.64	15.3 ± 0.75
GOT (U/mL)	1.25 ± 0.01	1.24 ± 0.02	2.11 ± 0.5*	1.11 ± 0.01
GPT (U/mL)	29.79 ± 1.62	30.5 ± 1.02	45.85 ± 2.82*	33.99 ± 1.3
Albumin (mg/dl)	12.43 ± 1.31	13.3 ± 1.34	23.56 ± 3.67*	15.29 ± 3.73
Bilirubin (mg/dl)	1.02 ± 0.01	1.05 ± 0.03	1.4 ± 0.01*	1.31 ± 0.09*
Arginase (U/L)	4.08 ± 1.22	4.0 ± 1.5	50.6 ± 11.21*	10.75 ± 1.31
α-L-Fucosidase (U/L)	21.81 ± 5.39	20.5 ± 4.39	45.09 ± 0.66*	25.5 ± 5.15*

Each result represents the mean ± SD of 10 replicates.
MDA, malondialdhyde concentration; **SOD**, superoxide dismutase activity; **H_2O_2**, hydrogen peroxide; **CAT**, catalase activity; **GST**, Glutathione-S-transferase activity; **GSPase**, Glutathione peroxidase activity; **GSH**, Glutathione reduced concentration; **GOT**, Glutamic-oxaloacetic transaminase; **GPT**, Glutamic–pyruvic transaminase.
* Significant at $p < 0.05$ after studying paired sample *t*-test statically analysis and # non significant at $p > 0.05$.

Table 3
Pregnancy characteristics, fetuses and pups as affected by different treatments.

	C	M	D	DM
Number of mothers	25 (100%)	25 (100%)	25 (100%)	25 (100%)
Number and percentage of aborted mothers	0 (0%)	0 (0%)	5 (20%)	5 (20%)
Number and percentage of pregnant	25 (100%)	25 (100%)	20 (80%)	20 (80%)
Number of fetuses and newborn	120 (100%)	130 (100%)	75 (100%)	60 (100%)
Percentage of numerical reduction of fetuses and newly born from control	0 (0%)	0 (0%)	47 (62.5%)	30 (50%)
Total number (% of fetal mortality)	0 (0%)	0 (0%)	21 (28%)	17 (28.4%)
Number and percentage of alive fetuses and newly born	120 (100%)	130 (100%)	54 (72%)	43 (71.6%)

MLAE may support the key role of these phytochemicals in the health-promoting features related to the supplementation of *M. alba* leaves.

The antioxidant capacity of extracts is dictated by the different mechanisms of action of their constituents. Therefore, this capacity should be evaluated by a variety of methods dealing with different mechanisms [50]. In our study, MLAE exhibited DPPH·, NO·, and $O\cdot_2$ radical scavenging activities (Fig. 1). Bioactive compounds present in MLAE reduce DPPH· radicals (Fig. 1). DPPH· radicals react with reducing agents; the electrons became paired off wherein the solution loses its color upon the number of electrons taken up [51]. Jeszka-Skowron et al. [1] reported that ethanol extract of *M. alba* leaves had higher capacity to reduce $ABTS\cdot^+$ radical cation, and ability to reduce the DPPH· than acetone extract.

The bioactive compounds found in MLAE compete with nitrogen to form NO·, thereby inhibiting the generation of nitrites and this was indicated by less color development with Greiss reagent (Fig. 1). As scavengers of NO· radicals, MLAE can protect humans wherein excess NO· is associated with many diseases.

MLAF inhibited the generation of $O\cdot_2$ *in vitro* (Fig. 1). A material might act as an antioxidant upon its ability to reduce ROS by donating hydrogen [52]. Phenolic compounds found in MLAE may contribute to antioxidant activity, because they have direct antioxidant properties due to the presence of hydroxyl groups, which can act as hydrogen donors [53]. Phenolic compounds present in the leaves donate electrons, and react with radicals to convert them to stable products, and terminate the radical chain reactions [52].

3.2. Biochemical analysis

Data that shown in Table 1 presents the levels of serum biochemical markers including FBG, TC, TG, HDL, LDL, and creatine phosphokinase (CK) in diabetic mothers, and animal groups treated with MLAF. FBG level reached the highest level (830 mg/dL) in DM mothers (group D). Treatment of the experimental-diseased group with MLAE resoluted serum FBG levels (378 mg/dL) but the value was still above the normal level. FBG concentrations in control (C) group, and MLAE-treated group (M) group did not differ significantly. D group recorded the highest level of FBG, TC, TG, HDL, LDL, and CK, while MLAE treatment reduced significantly those levels. There were no significant differences between C group, and M group in the levels of FBG, TC, TG, HDL, LDL, and CK. It could be said that treatment with MLAF led to marked reduction of abnormal changes.

Table 2 shows the changes of antioxidant, oxidative markers and enzymes levels in the serum of different treated mothers. In diabetic group (D) the highest levels of MDA, H_2O_2, SOD, CAT, GST, GSPase, GSH, GOT, GPT, albumin, bilirubin, arginase, and α-L-fucosidase were

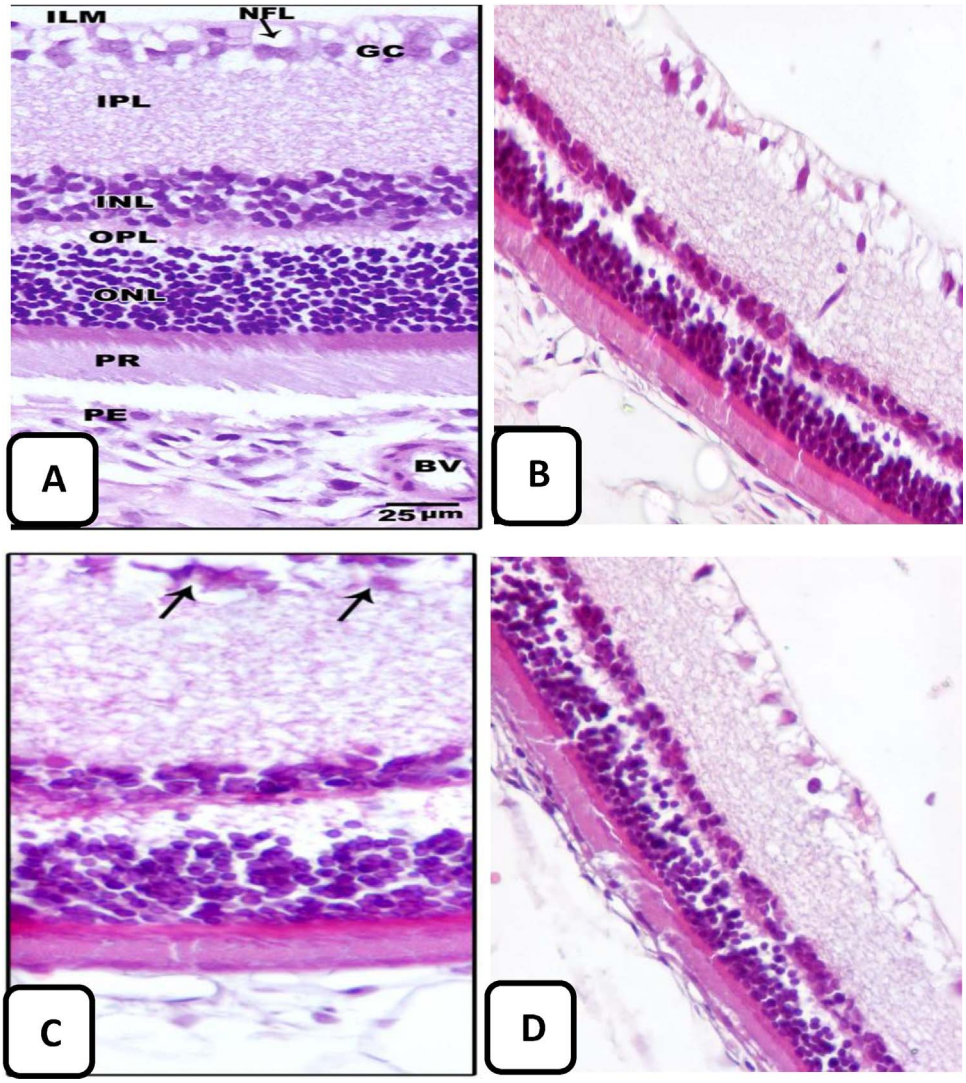

Fig. 2. Photomicrographs of histological sections from the maternal retina.
A: Control rats showing transverse histological sections of control maternal retina with pattern arrangement of retinal layers
B: MLAF-treated rats showing normal pattern arrangement of retinal layers
C: Experimental diabetic rats showing degenerated ganglionic cells and neuvascularization of neve fiber layer with edematous inner plexiform layer and partial loss of outer plexiform layer
D: Experimental D-MLAE rats showing amelioration Sections were stained with hematoxylin and eosin (H & E ×400)

recorded, while MLAE reduced significantly those levels wherein no significant differences between the values for C group, and M group was noted.

As shown in Table 3, the effect of DM, and MLAE treatments on some pregnancy characteristics, fetuses, and pups were listed. The most important changes were noted for D, and D-MLAE groups. Total number of aborted mothers for both D, and D-MLAE groups was five. The percentage of pregnant for both D, and D-MLAE groups was 80%. The percentage of numerical reduction of fetuses, and newly born from control was 62.5% for D group while the percentage was 50% for D-MLAE group. The percentage of alive fetuses, and newly born was 72% for D group and 71.6% for D-MLAE group.

3.3. Histological examination

Fig. 2 showed histological photomicrographs of the maternal retina. DM rats in group D showed degenerated ganglionic cells, and neovascularization of nerve fiber layer with edematous inner plexiform layer as well as partial loss of outer plexiform layer. Histological photomicrographs of the retina from pups are shown in Fig. 3. DM rats (group D) showed degeneration of ganglionic cell layers, and nuclear cell layers while DM rats treated with MLAE (group D-MLAE) showed amelioration histological traits.

3.4. Comet assay results

Figs. 4 and 5 showed photomicrographs of Comet assay of the eye from mothers (Fig. 4) and pups (Fig. 5). DM mothers and pups (group D) showed increased stretching of apoptotic cells while mothers and pups form D-MLAE group showed normal cell content.

4. Discussion

The present study was undertaken to investigate the antioxidant activity of MLAE and antidiabetic properties of MLAE in STZ-induced diabetic rats focusing on the ameliorative effects against embryogenesis defects. DM is a severe health problem with increasing rates of incidence [54,14]. DM is characterized by a high blood glucose levels resulting from insufficient insulin leading to metabolic abnormalities in lipids, carbohydrates, and proteins. Free radicals play an important role in the DM pathogenesis. Free radicals are well known for their role as beneficial and toxic components, wherein high levels of free radicals resulted in damage to membrane lipids, cellular proteins, and nucleic acids that leads to cell death. By donating their own electrons, antioxidants are effective against free radicals [55,13]. The importance of using aromatic plants as a source of natural antioxidants is being investigated, and the information about the plant effects was recently reported. Rahimi-Madiseh et al. [3] investigated the impact of *Allium ampeloprasum* extract on the oxidative stress and dyslipidemia in

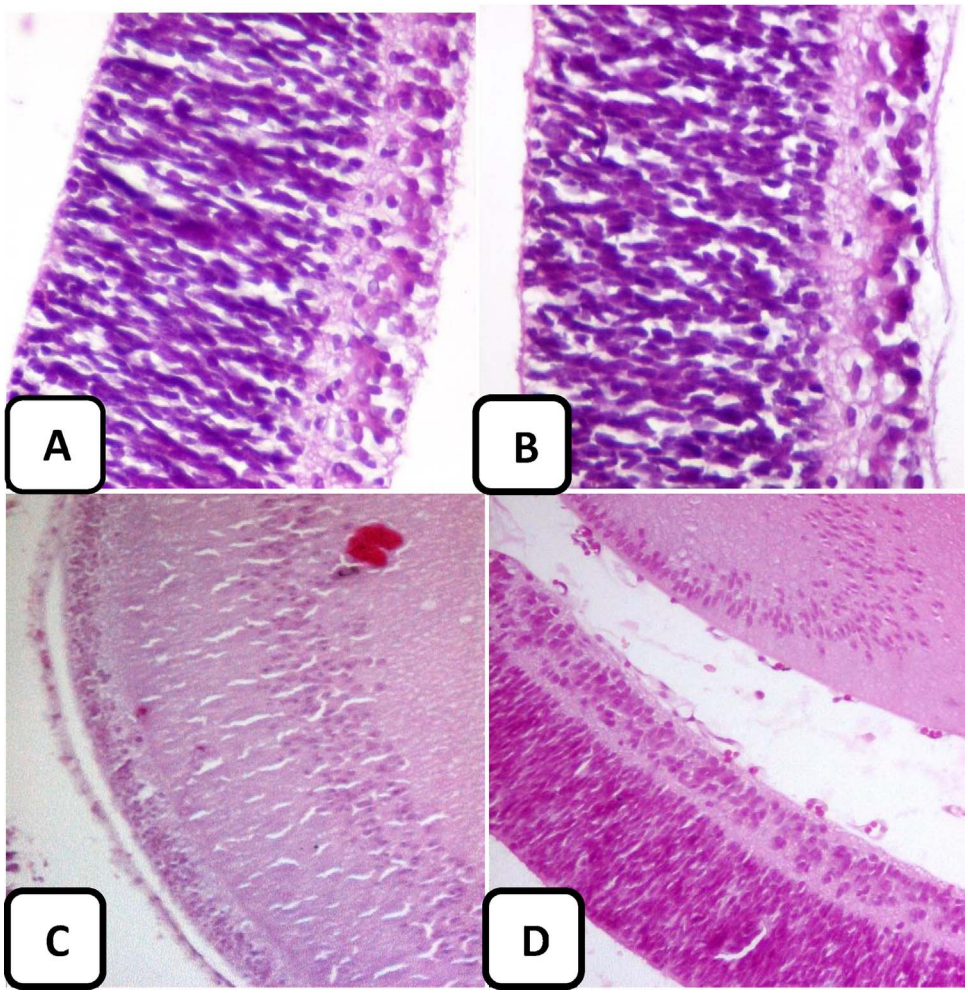

Fig. 3. Photomicrographs of transverse histological sections of retina from pups'.
A: Control group showing normal arrangement of retinal layer
B: MLAE-treated group showing normal arrangement of retinal layer
C: Experimental diabetic group (group D) showing degeneration of ganglionic cell layers and nuclear cell layers of pups
D: Experimental D-MLAE group showing amelioration effect
Sections were stained with hematoxylin and eosin (H & E ×400)

diabetic rats induced by alloxan. The results highlighted the health beneficial impacts of *A. ampeloprasum* extract that exerted hypolipidemic, hypoglycemic, and anti-oxidative stress effects in rats with alloxan-induced diabetes. Adam et al. [11] showed that aqueous *Vitis Vinifera* seed extract could protect the pancreas against inflammation, oxidative stress, and apoptosis-induced damage while preserving pancreatic function near normal in diabetes. Chahdoura et al. [12] studied the hepatoprotective effects of *Opuntia microdasys* flowers against diabetes type 2 induced in rats. The results showed that *Opuntia microdasys* had a protective effect on the protection of liver, thus reducing some of the causes of diabetes in rats. Almuaigel et al. [13] evaluated the reduction potential of aqueous extract of casing of pods of *phaseolus vulgaris* in blood glucose and lipids levels among hyperglycemic STZ-induced rats. Hebi et al. [14] investigated the impact of oral administration of the aerial part aqueous extract of *Tamarix articulata* (5 mg/kg) on blood glucose levels in normal and STZ-induced diabetic rats. The results demonstrated antihyperglycemic, and antioxidant traits of *T. articulata* in severe diabetic state.

M. alba leaves could be used as natural antioxidant source with powerful antioxidant properties [56,57,21]. Our results showed that TPC in MLAE was higher than in *M. alba* variety *alba* or *M. alba* v. *rosa* from Tunisia [18] but lower than in MLE from Taiwan [36]. These results confirm that TPC may depend on a variety, climate and the extraction method. Among the array of bioactive substances in *M. alba* extracts, DJN received special attention as a competitive inhibitor of intestinal α-glucosidase affecting carbohydrate digestion and absorption resulting in suppressed postprandial hyperglycaemia [58]. DNJ has been shown to inhibit $_{D}$-glucose uptake at the intestinal brush border membrane because of its similar size and structure to $_{D}$-glucose [59]. Proposed mechanism is attenuating the expression of proteins participated in the transepithelial glucose transport system, and maintained stable the levels of blood glucose by regulating the expression of enzyme proteins involved in gluconeogenesis and hepatic glycolysis [60].

DM considered the major public health problems especially in pregnant that possess early pregnancy dysfunction, abortion and teratogenesis [61]. Evidence indicates that *M. alba* can exert several health-beneficial effects such as hepatoprotective, gastroprotective, and reproductive effects [62]. Antioxidant properties of *M. alba* on renal and intestinal tissues could enhance the immune system, and enable the reproductive organs to perform its function in a good manner [57].

Normally, the developing embryo utilizes aerobic, and anaerobic metabolic pathways. When embryonic, and fetal circulation is established, the embryo is exposed to high levels of oxygen (Ornoy et al., 1996). At that time, the embryo should develop its antioxidant defense system, slowly increasing the activity of the main antioxidant enzymes. Ornoy [63] reported that the decrease in the potential of endogenous antioxidant enzymes in embryos, and yolk sacs of diabetic mothers results in growth retardation.

In our experimental approach, diabetes is induced by a STZ injection at levels of 60 mg/kg of animal bw. This model has been commonly used to study the pathophysiology of DM [64,55]. We utilized a slightly modified model closer to the etiology of T2DM observed in humans that used previously [65,28]. Our study limitation was that we did not perform morphological examination of the pancreas to further substantiate the beneficial effect of MLAE on pancreatic β-cell function. However, it was reported that STZ causes selective destruction of β-cells

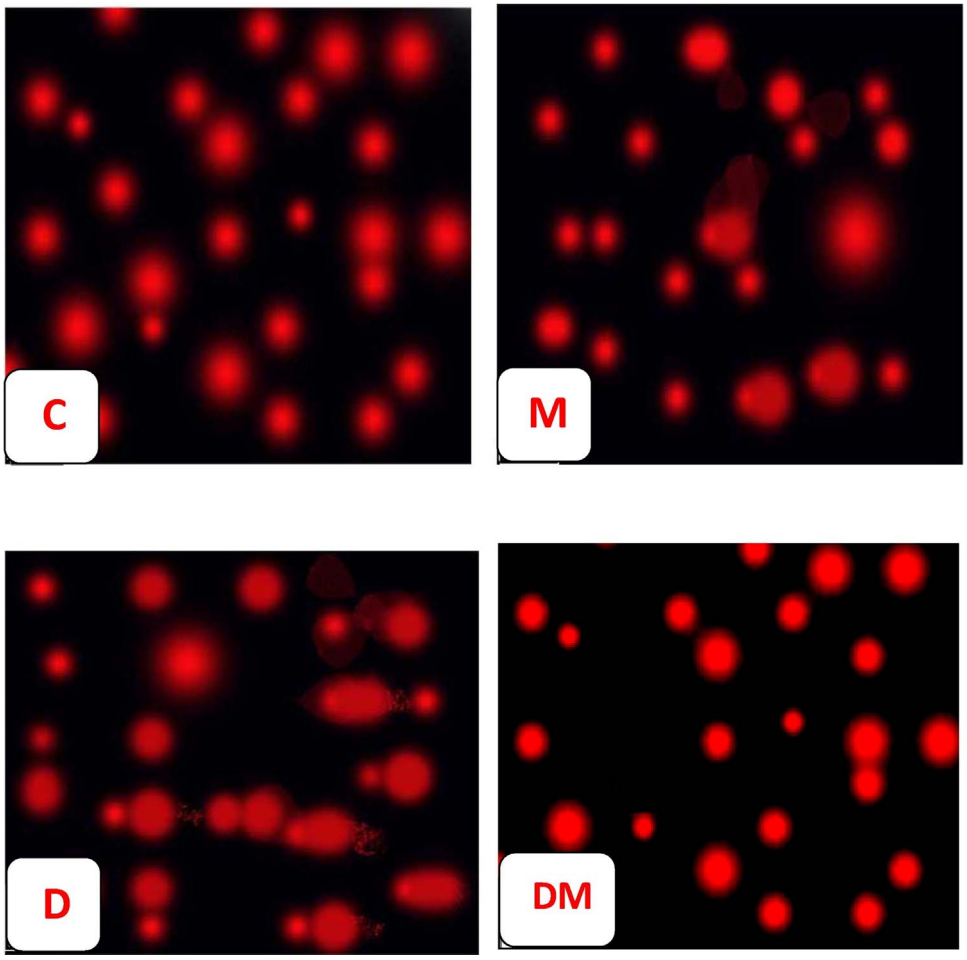

Fig. 4. Photomicrograph of Comet assay of the eye of mothers.
C: Control group showing normal structure of the eye cells
M: MLAE-treated animals showing normal structure of the eye
D: Experimental diabetic group (group D) showing increased stretching of apoptic cells
DM: Experimental D-MLAE group showing amelioration

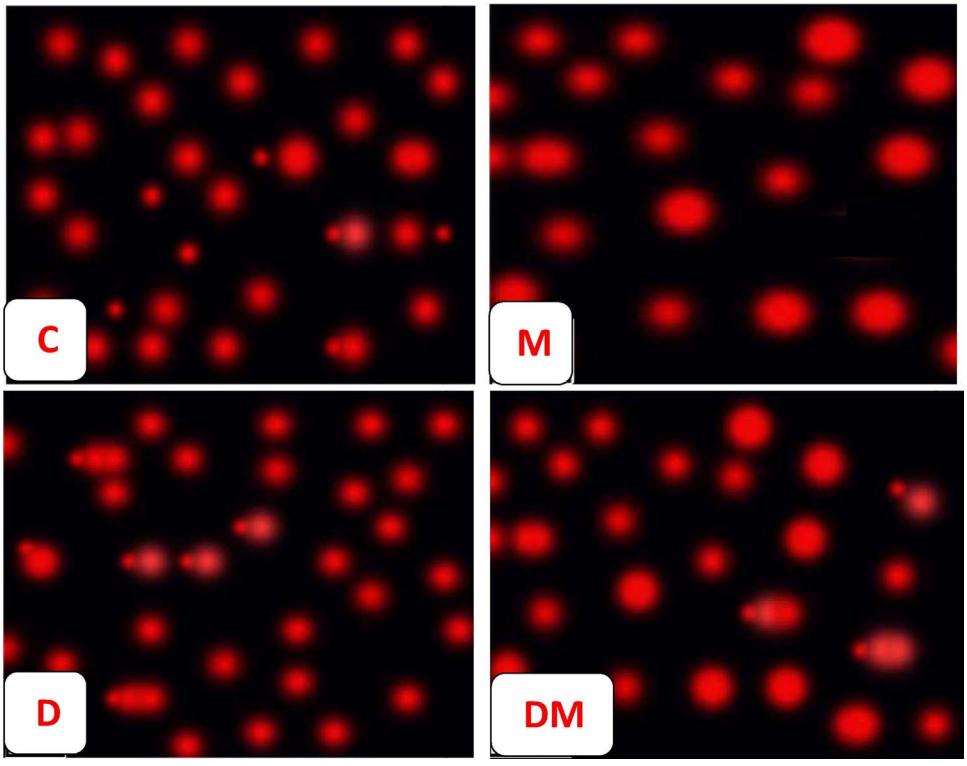

Fig. 5. Photomicrograph of Comet assay of the eye of pups.
C: Control group showing normal structure of the eye cells
M: MLAE-treated group showing normal structure of the eye
D: Experimental diabetic group (group D) showing increased stretching of apoptic cells
DM: Experimental D-MLAE group showing amelioration traits

of islets of Langerhans, resulting in high decrease in insulin [66].

The major finding of our study was that MLAE ingested with food effectively corrected blood glucose levels, and improved oxidative status in STZ-induced diabetic rats. Normalizing glycaemia is a crucial factor in the treatment of DM because chronic exposure of tissues to supraphysiological levels of blood glucose can lead to adverse intracellular outcomes [67,68]. In our study, STZ injection caused significant increase in blood glucose, confirming diabetes. Dietary treatment with MLAE decreased glucose level. These results are in line with other studies reporting a similar decrease in blood glucose in diabetic rats treated with MLE [33].

Insulin deficiency, and resistance is an important feature of DM, and results in deregulation of carbohydrate metabolism, and decreased activity of glycolysis enzymes. These derangements ultimately cause impaired peripheral glucose use, and augmented hepatic glucose production. Our results showed lower insulin level in D group than in control group suggest reduced insulin secretion caused by the STZ-induced pancreas damage. Our results support other studies showed that ameliorating effect of MLE on insulin resistance is most likely caused by both increased sensitivity to insulin receptor, and pancreatic β-cell regeneration [69].

In this study, MLAE reduced the indices of lipid peroxidation, suggesting high antioxidant activity of MLAE. We could postulate that the high content of TPC in MLAE might contribute to its high antioxidative potential. Similar results showing antioxidative properties of *M. alba* ethanol extract were reported [70,33]. Quercetin found in *M. alba* exhibited a hypocholesterolemic, and hypotriacylglyceridemic effect due to the stimulatory effect on the β-oxidation of fatty acids [71]. Zafar et al. [62] reported that concomitant administration of hydro alcoholic extract of *M. alba* along with isoniazid significantly reduced the nephrotoxicity as evidenced by marked reduction in blood urea nitrogen and creatinine.

DM increased maternal serum TC, TG, LDLc, HDLc, and CK activity at parturition. In addition, the hepatic tissues of DM rats showed disorganization and apparent pathological alteration ranging from edematous of blood vessel to apparent hepatocyte damage, and distortion of blood sinusoids, widespread of cytoplasmic lipid globules and pyknotic cell death. The observed findings agree with Nadeau et al. [72] who reported an increase of hepatitis in children with T2DM assessed by the prevalence of high alanine aminotransferase. Protection with *M. alba* resulted in significant alterations in the studied groups. This might be due to diabetic oxidative stress causing necrosis of lenticular lens fibers that forming large cysts filled with amorphous material.

Diabetes was found to induce oxidative stress in retinal tissues, resulted in insufficient supply of nutrients to target structures (*i.e.*, optic nerve, retina and head) and raising glutamate levels that initiate the retinal neuronal cell death [73]. Similar lens opacity was detected in DM patients [74,54] and in diabetic animals which showed intracellular accumulation of sugar alcohol and morphological changes including swelling, vacuole formation in peripheral lens epithelial cells of cataract lenses, and rounding of nuclei [75]. Giavini and Prati [76] mentioned congenital diabetic cataract in rat fetuses, and pups of STZ-diabetic mothers. There is a relationship between the development and differentiation of photoreceptors, and retinal pigmented epithelium [77] manifested by secretion of factors that promote photoreceptor survival, and differentiation [78]. Therefore, the increased incidence of apoptotic cell death of retinal pigmented epithelium in pups of diseased groups might disrupt structural components of photoreceptor inner segment.

The present results showed an incidence of retinal cell death, and DNA fragmentation. This increased incidence of apoptosis confirmed the oxidative stress of maternal DM in growth of their pups' retinal tissues. On the other side, amelioration with MLAE in diseased groups was exhibited by attenuating retinal cell death. This may be due to the hypoglycaemic impacts, and the reduction in the activity of LDLc, TC, and CK that have roles in retinal cell damage.

The results showed an increased incidence of retinal cell death and DNA fragmentation in retinal tissues of diabetic mothers and their pups. This increased incidence of apoptosis confirmed the oxidative stress of maternal diabetes in growth of their pups' retinal tissues. Afanasev et al. [79] reported also increased incidence of apoptotic cells in STZ-diabetic rat retina. Amelioration with protected *M. alba* extract was shown by decreasing retinal cell death. This may be due to the reduction of LDL, TC and creatine phosphokinase activity, which have roles in retinal cell damage. Enkhmaa et al. [80] found that quercetin 3-(6-malonylglucoside), the main flavonoids glycoside in mulberry leaves, decrease the incidence of atherosclerotic lesion development in mice.

5. Conclusion

M. alba leaves has excellent radical scavenging properties, antioxidant, anticancer, and antihyperglycemic activities that had long been used as a conventional medicine. In conclusion, our study demonstrates that MLAE improves deranged carbohydrate metabolism in STZ-induced diabetic rats. The mechanism of antidiabetic action of *M. alba* bioactive components most likely involves intracellular pathways involved in insulin signaling or glucose homeostasis. The suppression of oxidative stress might also contribute, at least in part, to the antidiabetic traits of MLAE. The results showed that MLAE can be used as a nutraceutical agent to ameliorate DM. The ameliorating impact of MLAE on retinal neuronal cells activity might be attributed to its TPC, which shows potential antioxidative activity. The mechanisms and sites of this traits and the bioactive constituents of MLAE are still to be determined in addition to toxicological studies in further experiments. In addition, protection with MLAE led to marked preservation of the maternal organs, and their offspring against the oxidative stress of diabetes because of its potential antihyperglycemic, and antihyperlipidemic impacts as well as antioxidative activity.

Disclosures

The authors declare that there is no conflict of interests regarding the publication of this paper.

Ethical approval

All applicable international and institutional guidelines for the care and use of animals were followed.

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