Available online at www.derpharmachemica.com

ISSN 0975-413X
CODEN (USA): PCHHAX

Der Pharma Chemica, 2017, 9(9):24-30
(http://www.derpharmachemica.com/archive.html)

A Cytoprotectant Effect of *Morus alba* against Streptozotocin-Induced Diabetic Damage in Rat Brains

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ABSTRACT

Morus alba is being used in the prevention of diabetes mellitus. Our goals are to evaluate the precise mechanisms underlying neuroprotective effects of Egyptian M. alba against brain damage induced by diabetes in rats. Diabetes was induced by streptozotocin (STZ; 52 mg/kg; i.p). Rats were divided into five groups: normal group received saline, diabetic group, Glimepiride (0.5 mg/kg) and M. alba (250 & 500 mg/kg) groups. All treatments daily administered orally for a period of 10 days. STZ group increased serum glucose level, brain nitric oxide and malondialdehyde contents, decreased brain glutathione and B-cell lymphoma-2 contents, produced alteration in seretonine, norepinephrine and dopamine brain contents and showed many degenerating neurons and cell injury with caspase-3 expression. Whereas treatment with Morus alba corrected all biochemical and histopathological changes with inhibition of caspase-3 expression. In conclusion, Morus alba ameliorated neurologic complications induced by diabetes and considered as an anti-apoptotic and neuro-protective plant.

Keywords: *Morus alba*, STZ-diabetes, Norepinephrine, Seretonine, Rat

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INTRODUCTION

The complications in the nervous system were associated with the increased glucose concentration. Glucose-induced oxidative-nitrosative stress play key role in pathogenic mechanism of neurovascular. The world is now exposed to an explosive elevation in the incidence of diabetes mellitus and cost-effective complementary therapies are needed. Diabetes is defined to be a vascular disease. Controlling the macro-vascular component (neuropathy) is very important on health resources perturbations [1]. Brain tissues, especially, are vulnerable to oxidative stress that is involved in development of diabetic mediated disorders through the formation of free radicals [2]. Diabetes, also, provokes a reduction in brain weight and neocortical volume that is accompanied by a decrement in number of cortical neurons, consequantly neuronal activity decreased [3].

Apoptosis could be considered as a possible mechanism for hyperglycemia-induced neurological complications and neuronal cell death. Apoptosis is proposed as a key element in several neurodegenerative disorders like Parkinson's disease and Alzheimer's disease through shrinkage of cell, condensation of chromatin and nucleus and DNA fragmentation. Apoptosis is gene regulated by two main families of proteins: caspase enzymes and Bcl-2 family [4].

Long-term complications of diabetes were not prevented using insulin administration, so an alternative strategy will be needed to attenuate the diabetes-induced complications. A promising plant, *Morus alba*, of the family Moraceae, is widely cultivated in different area in Egypt and has been traditionally used in treatment of several diseases [5]. It has been shown too utilized for blood serum glucose reduction [6] and for lipid level reduction [7].

This investigation was conducted to examine the role of neurotransmitters in STZ induced diabetes. The potential therapeutic effects of *Morus alba* leaves water extract on CNS sensory function of diabetic rats were assessed.

MATERIALS AND METHODS

Animals

Adult male albino Wistar rats, weighing 120-140 g were used in the current study. They were purchased from the National Research Centre (NRC; Giza, Egypt). Animals received human care in compliance with the guidelines of the animal care and use committee of the NRC. The animals were kept in a quiet place and were allowed free access to water and standard food pellets throughout the period of investigation. Experiments were performed according to the National Regulations of Animal Welfare and Institutional Animal Ethical Committee (IAEC) and the national and international ethics guidelines stated by the ethics committee of NRC.

Plant

Crude water extraction of *Morus alba* leaves 150 g dried *Morus alba* leaves were powdered and extracted with 1000 ml (w/v) of hot water (85ºC) for 3 h. The extract was filtered with Whatman No. 1 filter paper and concentrated by heating at a non-boiling temperature near 100ºC, and then dried completely under vacuum at 25ºC. The dried extract (yield=30%) was used during experimentation and administered at dose of 250-500 mg/kg body weight.

Drugs and chemicals

Glimepiride was obtained from Sanofi-Aventis, *Egypt.*
Streptozotocin was obtained from Sigma Aldrich Chemical Co., USA.

EXPERIMENTAL DESIGN

Induction of diabetes

STZ was dissolved in in 0.1 M citrate buffer (pH 4.5) 50 mM sodium citrate solution in a dose 50 mg/kg [8]. This solution was intraperitoneally injected in rats; fasting blood sugar was estimated after 2 days to confirm the development of diabetes mellitus.

Experimental design

Fifty male albino rats divided into five groups (ten rats in each group) as follow: Group I: healthy rats received buffer citrate orally. Group II: diabetic rats. Group III: diabetic rats received Glimepiride (0.5 mg/kg; p.o.) [9]. Group IV & V: diabetic rats received *Morus alba* (250 and 500 mg/kg; p.o.). After 10 days, animals were kept fasting for 12 h, blood glucose level was estimated then brains were removed for preparation of homogenate, histopathological studies and immunohistochemistry for Caspase- 3.

METHODS

Determination of blood glucose level

Diabetes was confirmed at 48 h after STZ injection by measuring the glucose concentrations of peripheral blood obtained from the tail vein (One Touch SureStep Meter, LifeScan, Calif, USA).

Preparation of brain homogenate

After 10 days, animals were kept fasting for 12 h then Brains were removed quickly and placed in iced normal saline, perfused with the same solution to remove blood cells, plotted on filter paper and frozen at -80ºC until used for estimation of other biochemical parameters.

Biochemical measurements

Brain Nitric Oxide (NO), Malonedialdehyde (MDA) and reduced Glutathione (GSH) were measured using Biodiagnostic kits, Egypt. Brain B-cell lymphoma 2 (Bcl-2) was measured using Eliza kit (LifeSpan BioSciences, Inc.).

Determination of brain monoamines

Determination of brain serotonin, norepinephrine and dopamine were measured by HPLC method modified from previous method [10]. It was carried out using HPLC system, Agilent technologies 1100 series, equipped with a quaternary pump. Separation was achieved on ODS-reversed phase column (C18, 25 × 0.46 cm i.d. 5 µm). The mobile phase consisted of potassium phosphate buffer/methanol 97/3 (v/v) and was delivered at a flow rate of 1.5 ml/min. UV detection was performed at 270 nm, and the injection volume was 20 µl. The concentration of both catecholamines and seretonine were determined by external standard method using peak areas. Serial dilutions of standards were injected, and their peak areas were determined. A linear standard curve was constructed by plotting peak areas versus the corresponding concentrations.

Histopathological studies

Brains tissues of all animals were dissected immediately after death. The specimens were fixed in 10% neutral-buffered formalin saline for 72 h at least. All the specimens were washed in tap water for half an hour then dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin.

Staining for general morphology

Serial sections of 5 µm thick were cut and stained with haematoxylin and eosin for histopathological investigation. Images were examined and photographed under a digital camera (Microscope Digital Camera DP70, Tokyo) and processed using Adobe Photoshop version 8.0.

Immunohistochemistry for Caspase-3

Paraffin-embedded Brain tissue sections were deparaffinized, and hydrated. Immunohistochemistry was performed with a mouse monoclonal caspase-3 for detection of the caspase cleavage. The paraffin sections were heated in a microwave oven (25 min at 720 W) for antigen retrieval and incubated with either anti-caspase (1:50 dilution) overnight at 4ºC.

After washing with PBS, followed by incubation with biotinylated goat-anti-rabbit immunoglobulin G secondary antibodies (1:200 dilution; Dako Corp.) and streptavidin/alkaline phosphatase complex (1:200 dilution; Dako) for 30 min at room temperature, the binding sites of antibody were visualized with DAB (Sigma). After washing with PBS, the samples were counterstained with H&E for 2-3 min, and dehydrated by transferring them through increasing ethanol solutions (30%, 50%, 70%, 80%, 95%, and 100% ethanol). Following dehydration, the slices were soaked twice in xylen at room temperature for 5 min mounted, examined and evaluated by high power light microscope [11].

Immunomorphometric analysis

The word morphometry means measurement of any form. The reasons for increasing interest for application of morphometry in diagnostic pathology are its advantage of objectivity, reproducibility and possibility of detecting minor differences or variations in a specimen that may escape by subjective evaluation. Quantitative analysis of histologic images attempts to enhance diagnostic pathology in three ways; by isolating the application of individual criteria, thus contributing to their objective use; by increasing precision in the evaluation of quantitative criteria; and by evaluating features that are simply not appreciated by the human.

The morphometric analysis was performed at the Pathology Department, National Research Centre using the Leica Qwin 500 Image Analyzer (LEICA Imaging Systems Ltd, Cambridge, England,) which consists of Leica DM-LB microscope with JVC color video camera attached to a computer system Leica Q 500IW.

The morphometric analysis is carried out on immunohistochemical stained slides. We place the slide to be examined on the stage of the microscope, and focus it at high power magnification (400X). Ten randomly sections were examined for each slide. We start by detection of the marker color, then the software form a binary image for the area of stained by the marker. This area is determined as an area per field in micrometer square, area fraction and area percentage by using the interactive measurement software of the system. The results appear automatically on the monitor in the form of a table with the total, mean, standard deviation, standard error, the minimum area and the maximum area measured.

Statistical analysis

Data are expressed as mean ± SE. Data analysis was done using one way analysis of variance (ANOVA) followed by Tukey test for multiple comparisons. Difference was considered significant when *p* is less than 0.05. SPSS (version 11) program was used to carry out these statistical tests.

All results from pathology are expressed as mean ± SE. Comparison of the values before and after the treatments were made by paired Student's t-test. P<0.05 was considered to be statistically significant, P< 0.001 considered to be statistically highly significant

RESULTS

Effect of *Morus alba* on blood glucose level

STZ injection caused a significant elevation in plasma level of glucose by 2.6 fold of the normal values. Both doses of *Morus alba* treatments produced a significant decrease in plasma glucose level by 66% and 68% respectively of the diabetic values (Table 1).

Table 1: Effect of *Morus alba* on blood glucose level

	Normal control	STZ control	Glimepiride (0.5 mg/kg)	Morus alba (250 mg/kg)	Morus alba (500 mg/kg)
Blood glucose level (mg/dl)	134.60 ± 3.83	484.25 ± 2.86[a]	144.02 ± 7.86[b]	163.16 ± 23.84[b]	155.98 ± 7.26[b]

Data were expressed as mean ± SE. Statistical analysis was carried out by one-way ANOVA followed by Tukey HSD test for multiple comparisons. [a]Significantly different from normal control (Saline) at *P<0.05*. [b]Significantly different from STZ control at *P<0.05*.

Effect of *Morus alba* on brain contents of NO, MDA and GSH

Injection of STZ produced a significant increase in brain NO and MDA contents by 1.4 fold and 0.1 fold respectively as well as exhibited a decrease in brain GSH content by 35% of the normal values, respectively. Treatment with *Morus alba* low dose decreased NO brain content and normalized MDA brain content and had no effect on GSH brain content while high dose normalized the brain contents of 3 parameters (Table 2).

	Normal control	STZ control	Glimepiride (0.5 mg/kg)	Morus alba (250 mg/kg)	Morus alba (500 mg/kg)
NO (umol/g tissue)	22.31 ± 0.11	53.98 ± 0.04[a]	50.19 ± 0.11[a]	31.63 ± 1.26[ab]	25.16 ± 2.00[b]
MDA (nmol/g tissue)	7.70 ± 0.05	8.65 ± 0.06 [a]	7.88 ± 0.04 [b]	7.90 ± 0.15 [b]	7.53 ± 0.16 [b]
GSH (mg/g tissue)	3.74 ± 0.12	2.45 ± 0.02 [a]	3.40 ± 0.18 [b]	2.61 ± 0.01 [a]	3.44 ± 0.16 [b]

Table 2: Effect of *Morus alba* on brain contents of NO, MDA and GSH

Data were expressed as mean ± SE. Statistical analysis was carried out by one-way ANOVA followed by Tukey HSD test for multiple comparisons. [a] Significantly different from normal control (Saline) at *P<0.05*. [b] Significantly different from STZ control at *P<0.05*.

Effect of *Morus alba* on brain contents of Seretonine, Norepinephrine and Dopamine

STZ injection exhibited a decrement in brain seretonine and norepinephrine contents by 52% and 20% respectively as well as produced an elevation in brain dopamine content by 17% of the normal values.

Treatment with *M. alba* high dose increased brain seretonine and norepinephrine contents by 1 fold and 0.2 fold respectively as well as decreased brain dopamine content by 13%, also, treatment with glimibride increased brain seretonine content by 0.9 fold as well as decreased brain dopamine content by 17% of STZ group values (Figure 1).

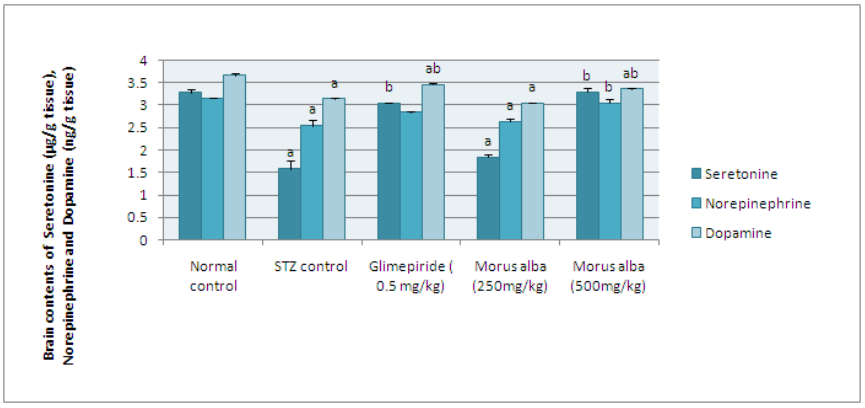

Figure 1: Effect of *Morus alba* on brain contents of Seretonine, Norepinephrine and Dopamine

Data were expressed as mean ± SE. Statistical analysis was carried out by one-way ANOVA followed by Tukey HSD test for multiple comparisons. [a]Significantly different from normal control (Saline) at *P<0.05*. [b]Significantly different from STZ control at *P<0.05*.

Effect of *Morus alba* on brain contents of Bcl2

Anti-apoptotic Bcl-2 protein was down-regulated after STZ injection by 37% as compared to normal control while treatment with *Morus alba* high dose and glimibride up-regulated brain Bcl-2 by 54% and 18% respectively, as compared to STZ group (Figure 2).

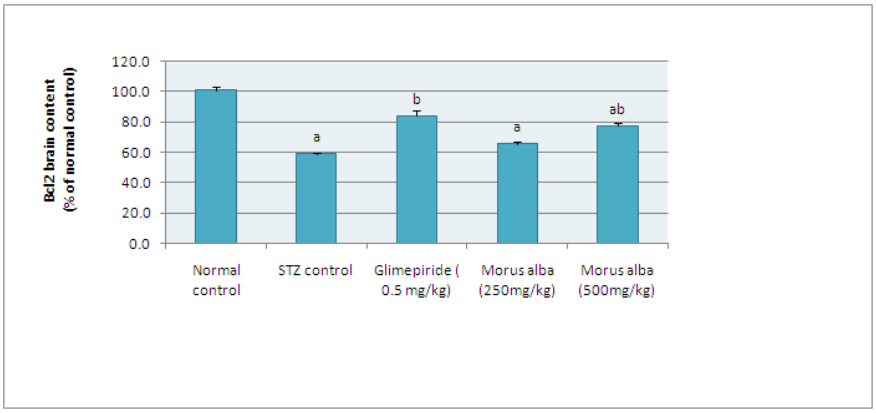

Figure 2: Effect of *Morus alba* on brain contents of Bcl-2

Data were expressed as mean ± SE. Statistical analysis was carried out by one-way ANOVA followed by Tukey HSD test for multiple comparisons. [a]Significantly different from normal control (Saline) at *P<0.05*. [b]Significantly different from STZ control at *P<0.05*.

HISTOPATHOLOGICAL RESULTS

As shown in Figure 3, the normal control group of brain tissue have typical normal neuronal structure of the brain tissue, where it is formed of 3 layers, the innermost one is the Granular layer (G.L) composed of many small cells, the central layer is regularly arranged Purkinje cell layer, formed of large flask-shaped cells with vesicular cytoplasm, prominent nuclei and normal dendrites (yellow arrow) and the outermost molecular layer that contains small nerve cells and many unmyelinated nerve fibers with typically neurofibrillary pattern and vascularity (M.L). The STZ group showing many degenerating neurons with pyknotic nuclei which appear dark and shrunken (blue arrows) and associated with neuropil vacuolation which denoting decreased connectivity (thin black arrows), cerebral edema (C.E.) and congested cerebral blood vessel (thick black arrow) which all points to irritation and cell injury. In the brain tissue from Glimepiride group showing normal neuronal structure with minimally degenerated purkinje cells (red double headed arrow) with congested cerebral blood vessel (thick black arrow). The brain tissue from *Morus alba* (250 mg/kg) group showing occasional degenerating purkinje cells (red double headed arrow) associated with regeneration features of some neurons (green arrows) and mild edema. *Morus alba* (500 mg/kg) group showing the normal structure of the brain except some degenerated purkinje cells (red double headed arrow) and congested blood vessel (thick black arrows).

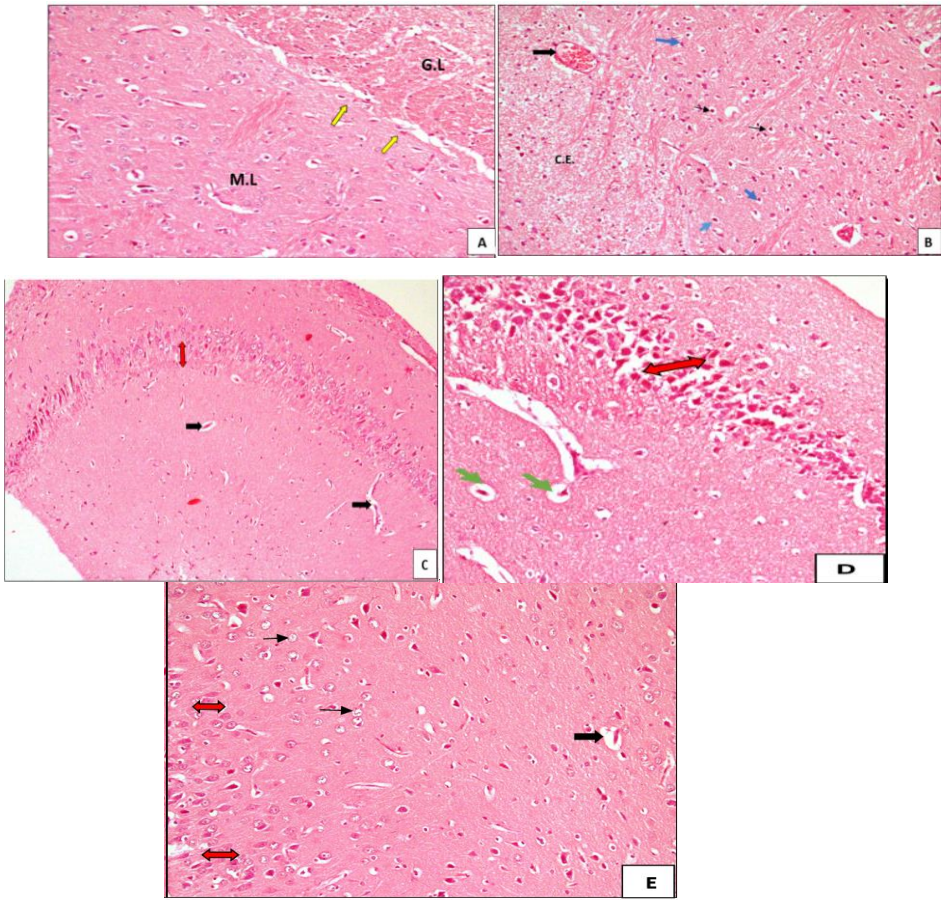

Figure 3: Photomicrography of the brain tissue sections stained by H&E(X200): A ; Normal control, B; STZ control, C; Glimepiride (0.5 mg/kg), D; *Morus alba* (250 mg/kg), E; *Morus alba* (500 mg/kg)

Immunomorphometric results

Activated caspase-3 labeling was specific in the delineating morphologically apoptotic cells, where its expression was localized in the cytoplasm of the neuronal cells which marked by the blue color in image analyzer system to be measured as an area %. There were negligible caspase-3 expressions in the normal control group also in the third group (Glimepiride). On the other hand the maximum expression was in the second group (STZ group) and the lower expressions were in the fourth (*Morus alba;* 250 mg/kg) & fifth (*Morus alba;* 500 mg/kg) examined groups, especially in the fifth group which showed significantly decrease when compared with normal control (saline) and highly significant decline in the apoptotic cells when compared with the diabetic group (STZ), which denote that the *Morus alba can* be a hopeful treatment of the diabetes complication in the brain (Table 3; Figures 4 and 5).

Table 3: Results of immunomorphometric measurments of Caspase-3

	Mean area of damaged cell ± SE.	P Value from normal control	P values from STZ control
Normal control	1.044 ±0.418	-----	$P = 0.0007^{(HS)}$
STZ control	10.352 ± 6.964	$P = 0.0007^{(HS)}$	-----
Glimepiride (0.5 mg/kg)	1.297 ± 0.673	$P = 0.3259^{(NS)}$	$P=0.0001^{(HS)}$
Morus alba (250 mg/kg)	6.992 ± 5.835	$P = 0.0067^{(S)}$	$P=0.2575^{(NS)}$
Morus alba (500 mg/kg)	3.648 ± 2.914	$P = 0.0230^{(S)}$	$P=0.0116^{(S)}$

Data were expressed as mean ± SE. Statistical analysis was carried out by one-way ANOVA followed by paired Student's t-test. $P<0.05$ was considered to be statistically significant, $P<0.001$ considered to be statistically highly significant.

DISCUSSION

STZ-induced diabetes considered as endogenous chronic oxidative stress linked with hyperglycemia, hyperlipoproteinemia and neurologic complications [12]. Thus, we examined STZ effects on the brain oxidative stress, neurotransmitters changes, apoptosis and anti-apoptotic pathways. In addition we evaluated the potential therapeutic role of *Morus alba* against STZ-induced diabetes and brain complications.

In the current study, there was a significant elevation in blood glucose level in STZ-diabetic group. While this value was decreased by treatment with both doses of *Morus alba* and glimipride. This result was in agreement with previous study [13].

Also, we demonstrated that STZ exhibited a rise in NO and MDA brain contents as well as suppressed the measured brain GSH content. These results were in a line with Modi et al. [14] who found higher lipid peroxidation in STZ group. In this study, both doses of *Morus alba* decreased NO and MDA brain contents while high dose only enhanced GSH brain activity. *Morus alba* during variety of insults can provoke neuronal survival. The mechanism of *Morus alba*-mediated neuroprotection may be in part due to regulating oxidative phosphorylation and scavenging ROS. The treatment with *Morus alba* leaves extract elevated brain antioxidants alleviating the schistosome-induced brain disturbances in mice [15].

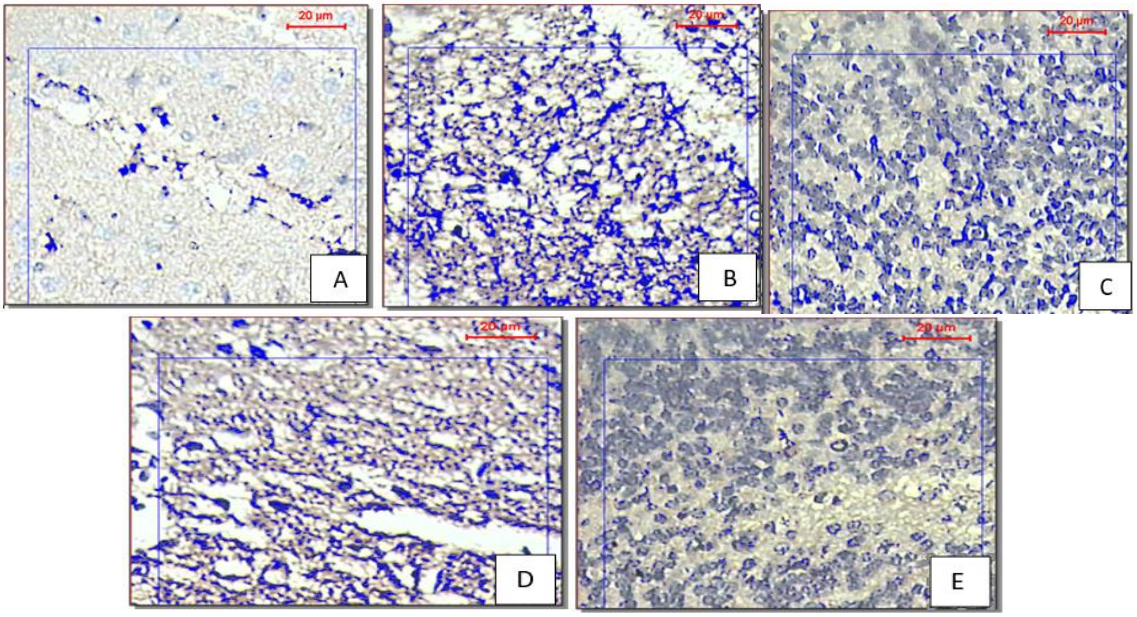

Figure 4 : Captured photos from image analyser system marking the immunoreactivity of brain tissues from the five examined groups to Caspase-3: A: Normal control, B: STZ control, C: Glimepiride (0.5 mg/kg), D: *Morus alba* (250 mg/kg), E: *Morus alba* (500 mg/kg)

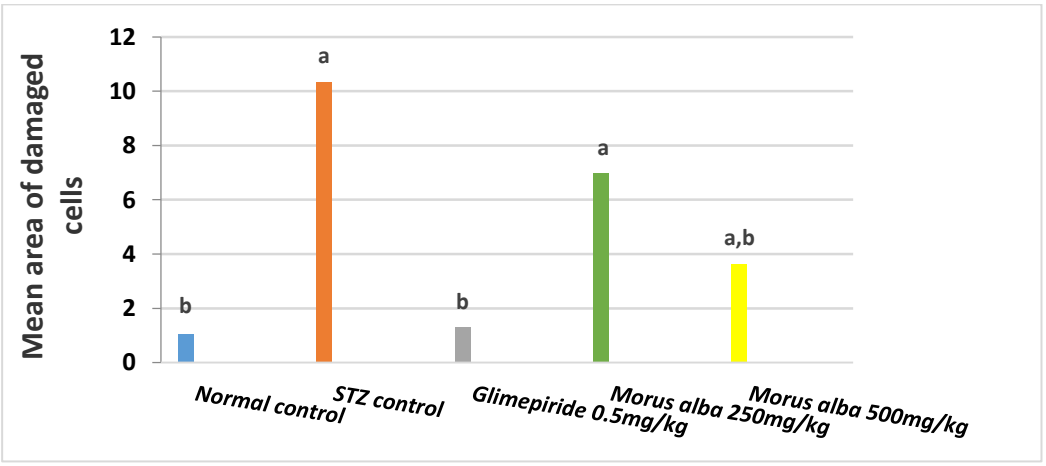

Figure 5: Results of immunomorphometric measurments of Caspase-3. Data were expressed as mean ± SE. Statistical analysis was carried out by one-way ANOVA followed by paired Student's t-test. [a]Significantly different from normal control (Saline). [b]Significantly different from STZ control at P<0.001

The present results concerning dopamine decrease in brain content via reduction of dopamine synthesis and turnover in several brain areas in diabetic group compared to normal values and this agree with previous work [3]. Also, Flseweidy et al. [16] reported similar decrease in diabetic brain neurotransmitters, noradrenaline and serotonin. The reduction in 5-HT levels during diabetes may be through a decrease in amino acids in the brain inducing a chronic anabolic deficit with a decrease in 5-HT synthesis [17]. Motawi et al. [18] found a positive correlation between MDA and neurotransmitters. Treatment with high dose of *Morus alba* provoked brain neurotransmitters improvement that related to its free radicals scavenging effects and restoration of brain antioxidants as GSH. El-Sayyad et al. [19] reported *Morus alba* protective effects on ocular neurotransmitters.

The present histopathological study showed an increased incidence of degenerating neurons, brain cell injury and cell death with and elevation of caspase-3 in brain tissues of diabetic rats. This elevation may in turn disrupt structural components of neuroreceptor inner segment and provoke brain tissues apoptosis incidence which were correlated with the study of Hang et al. [20]. These findings supported by our biochemical study that indicated a marked reduction of anti-apoptotic Bcl-2 protein.

The treatment with *Morus alba* ameliorated the histopathological changes with a reduction of apoptotic cells in diabetic brain tissues and these effects may be due to its phenolics and anthocyanins contents that up regulated Bcl-2 and down regulated caspase-3 as shown in our biochemical and immunohistochemical studies. Song-Tao [21] studied the role of *Morus alba* on diabetic brain tissue as it improved the structural changes of the diabetic peripheral neuropathy and increased the area of myelinated fiber.

In previous study, the protective effects of *Morus alba* against neurotoxicity were examined *in vitro* due to its phenolics and anthocyanins contents [22].

CONCLUSION

We concluded that the ameliorating effect of *Morus alba* on brain oxidative stress, neurotransmitters and apoptosis may be attributed to its flavonoids content, which exhibits potential hypoglycaemic, anti-oxidative and anti-apoptotic effects. So, *Morus alba* seems to be a promising plant in diabetes therapy and its brain complications.

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