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Evaluation of Role of Glibenclamide and *Aphanizomenon flos-aquae* Extract on Lymph Node and Spleen of Diabetic Rats

Hemmat M. Abdelhafez*, Tamer M. M. Abu-Amara and Sara M. El-debsi[*]**
*Zoology Department, Faculty of Science, Al-Azhar University-**Histology&Cytology Department, Faculty of Medicine, Al-Azhar University

ABSTRACT

Background:diabetes mellitus is a metabolic disorder in the endocrine system with a common biochemical manifestation, thus hyper-glycemia is a disturbed carbohydrate metabolism. This work aimed to evaluate the role of antidiabetic and hypoglycemic drug glibenclamide as a chemical agent and *Aphanizomenon flos- aquae* extract as a natural agent on lymphoid organs such as lymph nodes and spleen in the diabetic (type-2) white male albino rats.

Material and methods –Fifty male albino rats were used and categorized into five groups; group 1: control (**C**), group 2:Alloxan induced diabetic rats (**D**) (150 mg/kg b .wt); group 3:diabetic rats treated with daonil (**D+Do**)(daonil 5 mg/kg b.wt/day); group 4:*Aphanizomenon flos-aquae* extract (**AFA**)(94.5mg/kg b.wt/day) and group 5:diabetic rats treated with *Aphanizomenon flos -aquae* extract(94.5mg/kg b.wt/day) (**AFA+D**). All groups were dissected after 30 days of treatment. Lymph nodes and spleen samples were taken for histological and histochemical studies. Blood samples were taken for measurement of serum glucose and serum insulin level. **Results**- Diabetic male rats showed very highly significant increase in the serum glucose level, while non significant increase was recorded in the other treated groups in comparison with the control group.Diabetic male rats showed highly significant decrease in the serum insulin level as compared to the control group. Conversely, treatment of diabetic rats with daonil showed a significant increase in the levels of serum insulin. On the other hand non significant increase in the serum insulin was observed in **AFA or AFA+D** groups in comparison with the control group. Many histopathological and histochemical changes were observed in the lymph nodes and spleen of the diabetic rats, but using **AFA** extract succeeded to minimize the drastic changes which were observed in the lymph nodes and spleen of the diabetic rats more than that observed with glibenclamide. **Conclusion**-glibenclamide (daonil) as asynthetic drug and *Aphanizomenon flos-aquae* extract as a natural product ameliorated biochemical, histopathological and histochemical changes in the lymph nodes and splenic tissues of the diabetic rats.*Aphanizomenon flos-aquae* extract proved to be antidiabetic agent better than daonil drug and its antidiabetic action may be due to its anti-inflammatory, antioxidant and hypoglycemic action.

Keywords: Diabetic rats, *Aphanizomenon flos-aquae* extract, lymph node, spleen and hyperglycemia.

INTRODUCTION

About 347 million people worldwide have diabetes according to the world health organization.[1]. Diabetes Mellitus (DM) consists of a group of syndromes characterized by hyperglycemia, altered metabolism of lipids, carbohydrates and proteins and an increased risk of complications of the vascular diseases. Clinically diabetic patients can be classified clinically as having either type-1(insulin dependent diabetes mellitus) or type-2 DM (Non –insulin dependent diabetes mellitus). [2]

This disease remains incurable and can only be controlled with drugs. Several animal models have been used for studying diabetes mellitus or testing antidiabetic agents. Manipulations were done in several animal speciesto induce diabetes mellitus by several agents such as:alloxan monohydrate, streptozotocin with or without nicotinamide, ferric nitilotriacetate, ditizona and anti-insulin serum.[3]Diabetes mellitus remains a major global health problem impacted

by genetic risk propensity. However this makes it largely unpreventable, there exist preventive measures which reduce the onset of the disease and the extent of progression was associated with complications in patients with Type -2 diabetes and this was accompanied with elevation of blood glucose level, abnormal abdominal fat deposition, insulin resistance and number of complications including embryopathy, cardiovascular diseases, nephropathy, neuropathy, microangiopathy and retinopathy. [4] Aging, obesity, insufficient energy consumption, alcohol drinking, smoking, *etc.*, are independent risk factors of pathogenesis of Type- 2 diabetes. Obesity (particularly visceral fat obesity) due to a lack of exercise is accompanied by a decrease in muscle mass, insulin resistance and is closely associated with

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the rapid increase in the number of middle and high aged patients. [5]

Defect in β-cells associated with insulin resistance leads to progressive loss of β-cell mass and function and subsequently onset of diabetes. It is crucial to study the mechanisms by which glucotoxicity induces β-cell failure to develop therapeutic strategies for protecting and recovering a functional β-cell mass. Several mechanisms might explain the glucotoxicity due to prolonged hyperglycemia, such as β-cell exhaustion, oxidative stress induced by free radical oxygen species, endoplasmic reticulum (ER) stress, inflammation caused by proinflammatory cytokines and chemokines, loss of neogenesis and proliferation of β-cells. However, the precise mechanisms of glucotoxicity and its contribution to the pathology of Type-2 diabetes mellitus (T2DM) are still not fully understood. [6] Chronic hyperglycemia and acute glycemic fluctuations from peaks to nadirs lead to diabetes complications through 2 major mechanisms: activation of oxidative stress and increased activity of the innate immune system. [7]Alloxan induced diabetes model appears to be the most reliable and easily reproducible method of inducing diabetes mellitus in the experimental animals [8]**.**They added that alloxan is a hydrophilic and unstable chemical compound which has similar shape as that of glucose, which is responsible for its selective uptake and accumulation by the pancreatic beta cell. Similarity in its shape allows it to transport into the cytosol by the glucose transporter (GLUT2) in the plasma membrane of beta cell.[9]Glibenclamide is an oral hypoglycemic agent of sulphonylureas group which is indicated in patients with type-2 diabetes mellitus as adjunct to diet to lower blood glucose.[10]Glibenclamide is one of drugs from sulphonylureas category. It acts by inhibiting ATP-sensitive potassium channels in pancreatic beta cells which results in cell membrane depolarization opening voltage dependent calcium channel. So the level of intracellular calcium in the beta cell increases and results in stimulation of insulin release. [11]

In spite of its hypoglycemic effect, glibenclamide did not ameliorate oxidative stress in pancreas of the diabetic rats. However, normalization of hyperglycemia is known to be ineffective in preventing the development of macrovascular but only microvascular complications**,** which are linked to oxidative stress. [12]

Blue-green algae in general contain a significant amount of carotenoids, namely beta carotene, lycopene and lutein which provide it with good antioxidant properties. By their quenching action on reactive oxygen species, antioxidants carry intrinsic anti-inflammatory properties. However, blue-green algae also contain specific anti-inflammatory properties as a result of their high phycocyanin content. Phycocyanin is a photo harvesting pigment that provides the intense blue color in blue-green algae. It can constitute up to 15% of the dry weight of a blue-green algae harvest. C-phycocyanin is a free radical scavenger.[13] .

AFA has also been found to increase the production and release of NK (Natural Killer) cells which are the body's first line of defense against rogue cancer cells and viruses. Consumption of AFA leads to rapid changes in immune cell trafficking thus increases the immune surveillance without directly stimulating the immune system. Phycocyanin has been found to have anti-inflammatory and antioxidant properties.[14]Several pharmaceuticals and natural substances can mobilize Adult Stem Cells(ASCs) from human bone marrow deposits. Mobilized ASCs can home into damaged tissue and produce the desired tissue repair or regeneration. However, diseased or damaged tissues provide complex signals which attract migrating regenerative stem cells. [15]

One of the natural products that can mobilize Adult Stem Cells (ASCs) from human bone marrow deposits is Stem Enhance. It is extracted from *Aphanizomenon flos-aquae* (AFA) algae. Stem Enhance capsules contain a blend of the cytoplasmic and cell wall fractions of AFA algae which is enriched by L-selectin ligand (LSL). L-selectin ligand supports the release of stem cells (CD34+ cells) from the bone marrow. Its effect was detected on BM stem cell mobilization. [16]

MATERIAL AND METHODS

The present work was carried out on fifty male albino rats [130 ± 20 gm). Alloxan was purchased from Sigma, St .Louis, MO, USA. Diabetes mellitus was induced in 12hoursfasted animals of **D, D+Do** and **AFA+D** groups by a single intraperitoneal injection of alloxan**(150 mg/kg b.wt.)** [17]. It was dissolved in normal saline. On the 3rd day post-induction of alloxan injection, blood glucose levels were measured by glucometer. Rats with fasting blood glucose level more than 300 mg /dl are considered

diabetic. Tablets (**5mg)** of glibenclamide (Daonil) were soaked in 10 ml water. The diabetic rats treated with glibenclamide at dose level 5mg/kg b. wt/day.[18]AFA Klamath capsules **(350mg)** (German Egyptian Pharmaceutical Company) were opened and then dissolved in distilled water. The drug was administrated orally by gastric tube at a dose of 94.5 mg/kg body weight. The dose for the rat was calculated according to the Paget's formula on the basis of the human dose. [20]After 4 weeks of treatments all animals were anesthetized by ether, blood was collected from the heart puncture by plastic syringes and left to coagulate and the serum was separated by centrifugation at 3000 rpm for 15 min. for histochemical analysis, then sacrificed and specimens of the lymph nodes and spleen were taken from rats of all groups. The specimens were fixed in 10%neutral buffer formol for the histological and histochemical studies. Specimens were washed and dehydrated in ascending grades of alcohol, cleared in xylene and embedded in paraffin wax. Sections were then cut (5µm)and stained by hematoxylin and eosin according to the method of **Drury and Wallington** [21], Mallory's trichrome stain for demonstrating collagen fibers [22] and mercuric bromophenol blue method for demonstrating total protein .[23]

Fasting blood sugar was determined using enzymatic colorimetric method according to the method of **Trinder.**[24]serum insulin level was estimated by ELISA using BioSoure INS-EASIA Kit according to the method of **Yalow and Bauman.**[25] The optical density of mercuric bromophenol blue stained sections of the lymph nodes and spleen of the control and treated groups was recorded using image proplus 4.5.1.22 software analysis. The mean optical density was used to compare the total protein content of the different groups.

RESULTS

The biochemical results:

Diabetic male rats showed very highly significant increase in the serum glucose level which reached 341.4 with percent of change 201.0% as compared to the control group (113.40). While non significant increase was recorded for the other treated groups in comparison with the control group. They reached 117.80; 116.80 and 119.6 with percent of change 3.88**%**;3.0**%** and 5.47**%** in groups **D+Do, AFA and AFA+D** respectively**(Fig.1** and **table 1)**.

Diabetic male rats showed a very highly significant decrease in the serum insulin level which amounted 6.38 with percent of change -64.86% as compared to the control group (18.16).

Conversely, treatment of diabetic rats with daonil showed a significant increase in the levels of serum insulin which amounted 20.50 with percent of change12.89%. On the other hand non significant increase in the serum insulin was observed in **AFA or AFA+D** groups in comparison with the control group and they reached 19.88&20.22respectively with percent of change9.4**%**&11.34 **%**.**(Fig. 2** and **table1)**.

Lymph node: Normal histological structure of the lymph node of the control rats was noticed in **fig.3.** Slightly normal appearance of lymph node tissue of groups **D+Do, AFA and AFA+D** was observed in **figs.5,6&7.**

The diabetic group showed highly dilated and congested blood sinuses with numerous degenerated areas in the cortex, medulla and hilum; degenerated areas contained degenerated cells and the blood sinuses contained hemolysed RBCs with numerous hemorrhagic areas**.** Hypocellularity in some cortical follicles was detected with highly distorted cortical follicles **(Figs.4-A,B,C,D&E)**.

Normal distribution of collagen fibres was detected in the lymph node tissue of the control rat. Thin collagen bundles are supporting the capsule, cortex, medulla and paracortex (**Fig.8)**. Highly increased collagen fibres were detected in the cortical region and they were scattered in the degenerated areas of the medulla of lymph node of the diabetic group (**Figs.9 A&B)**.Slightly reduced collagen fibres were detected in the capsule, cortex and medulla of lymph node of group **D+Do(Figs.10-A&B).**

Nearly normal distribution of collagen fibres was realized in the lymph node of **AFA** group but some collagen fibres were scattered in the cortex and medulla specially in walls of the blood vessels **(Fig.11)**.Somewhat normal distribution of collagen fibres was demonstrated in the cortical and medullary regions of the lymph node of **AFA+D** group, but thick collagen bundles were detected in the capsule (**Fig.12).**

Moderate staining affinity of total protein was detected in the cortex of lymph node of the control group with less stained medulla **(Fig.13).** Highly increased staining affinity of total protein was detected in lymph node of the diabetic group, degenerated areas were

negatively stained **(Figs.14-A&B).Table 2** showed a significant increase in the mean(MOD)optical density of total protein content of lymph node tissue of the diabetic group(0.54) compared to the control value (0.37). The percentage of increase was 45.95%.

The diabetic groups treated with daonil or AFA resulted in a significant increase in total protein content of lymph node.MOD reached 0.41 and 0.44 in **D+Do and AFA+D** groups with percent of change10.8% and 18.92% respectively .These data reflect the serious effect of diabetes on total protein content in the lymph node. While non significant increase in total protein content was observed in AFA group.

Spleen:

Normal histological structure of spleen of the control rat has been observed in **fig.18.**In spleen of the diabetic group (D) there were thickened arterial walls with narrow lumens ,necrotic trabeculae, numerous hemosidrin granules with hemolysed RBCs in blood sinuses of the red pulps , necrotic areas in the white pulps, thickened trabeculae which contained highly dilated trabecular vein ,lots of hemolysed RBCs ,highly reduced lymphocytes in the white pulps with numerous degenerated areas **(Figs. 19-A,B&C).**

Slightly normal appearance of white pulps of splenic tissue of **D+Do** group was observed, but the central arteries had thickened arterial walls with numerous degenerated areas in the red pulps and dilated blood sinuses **(Fig. 20)**.

Similary, somewhat normal appearance of splenic tissue of AFA group was detected **in fig.21**. Nearly normal appearance was also observed in the splenic tissue of **AFA+D** group, but thickened arterial walls were still detected **(Fig.22)**.

Splenic tissue of the control group has collagen fibres which are supporting the capsule and trabeculae with scattered fibres in the red and white pulps **(Fig.23).** Highly increased collagen fibres with common fibrosis were detected in the splenic tissue of the diabetic group specially under the capsule, in the red and white pulps, in the thickened trabecular walls and around the highly dilated trabecular vein **(Figs.24-A,B&C).**Normal distribution of collagen fibres was observed in the white pulps of the splenic tissue of **D+Do** group **(Fig. 25).**Somewhat normal appearance of collagen fibres was realized in the splenic tissues of **AFA** and **AFA+D** group**s (Figs. 26 & 27)** . Sections of the splenic tissue of the control group showed moderately stained total protein in the capsule, trabeculae and red pulps with less stained white pulps **(Fig.28).** In the spleen of the diabetic group pools of deeply stained red blood cells were realized in the red pulps with less stained white pulps, but degenerated areas showed negatively stained total protein **(Figs. 29-A&B**and **table 3)**.

Sections of spleen of **D+Do** group showed deeply stained walls of the central arteries with nearly normal content of total protein in the white and red pulps (**Fig.30** and **table 3)**.

Distribution of total protein was somewhat normal in the red and white pulps of the splenic tissue of **AFA** group as shown in **fig. 31;** while a significant decrease in total protein content was observed in the spleen tissue of **AFA+D** and **D+Do** groups where MOD reached 0.35 with percent of change -5.41% in the 2 groups. Such decrease was lower in the diabetic group which reached 0.31with percent of change -16.22% (**Fig**.**29-32** and **table 3)**.

DISCUSSION

Diabetes mellitus is one of the most common metabolic diseases of human beings. Type-2 diabetes is a more common kind of diabetes found in 90% of the diabetic population.[26] Diabetes mellitus (DM) is a heterogeneous clinical syndrome featured by high levels of glucose. Whether it is type 1 DM (T1DM) or type 2 DM (T2DM) the immune system is involved in development and progression of both of them. [27] In type -2 diabetes there is a cytokine associated acute phase reaction, part of the innate immune response .[28]The acute phase proteins are synthesized in the liver, stimulated by cytokines, mainly interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF) ,which are produced in macrophages, monocytes, endothelium and many other cells in the body.[29]Glibenclamide enhances insulin secretion by residual beta cell function, which declines progressively with duration of type -2 diabetes; therefore, the best role for sulfonylureas would be early in the disease spectrum, where physicians may be thought to offer the maximum benefit. [30] .In some of the societies there is a strong desire to use herbs or plants for treatment, due to less side effects, easier consumption or availability. However, very few of the traditional treatments for diabetes have received scientific or medical scrutiny and several have been shown to assist glycemic control in non-insulin dependent form of diabetes.[31] Blue green algae have a high concentration of vitamins, minerals and enzymes with a complete spectrum of essential and non-essential amino acids that are all easily absorbed

by the body. Due to these properties, a large number of researchers were interested in employment of blue green algae as food supplementation .**Mani *et al*.** [32] mentioned the lipid lowering effect of blue green algae in healthy and diabetic patients. It has been shown that blue green algae increases the stem cells trafficking or homing in animals through induction of a transient boosting in the population of stem cells in animal's circulatory systems .[33]

Results of this study showed highly significant increase in the serum glucose level of the diabetic group, while non significant increase was recorded in the other treated groups in comparison with the control group. These results are in agreement with those of **Mostafa *et al*.** [34] They reported that severe hyperglycemia in the diabetic rats can be considered as a direct reflex to the marked hypoinsulinemia caused by the selective destructive cytotoxic effect of alloxan on the β-cells of the pancreas. It has a direct effect on the cell membrane permeability by causing failure of ionic pumps and increased cells size.

Ahmadi *et al*.[35] reported that decreased insulin level and sensitivity cause an increase in hepatic glucose production. As well as a decrease in peripheral glucose uptake and a significant decrease in the conversion of glucose to glycogen in the liver. Finally this produces an increase in blood glucose level and decreased its intercellular level.

In the present study serum insulin level recorded a very highly significant decrease in the diabetic group. In contrast a significant increase in the serum insulin level was observed in **D+Do** group compared to the control group. While non significant increase in the serum insulin level was recorded in **AFA** and **AFA+ D** groups.

Zheng *et al*.[36]found that glibenclamide is a lowering blood glucose level agent that has the mechanism of action to stimulate β-cell of pancreatic islet to release insulin; decrease the leading out of glycogen; enhance the use of glucose in the tissues and organs and to improve microcirculation in the body.

In agreement with the present results improvement in the diabetic rats treated by **AFA** extract may be due to stimulation of β-cells of Langerhans islets to increase the production of insulin or due to enhancement of transport of blood glucose to the peripheral tissue. This may possibly be due to the high fibre content of blue green algae that interferes with the glucose absorption or probable action of producing polypeptides after digestion of blue green algae. The mobilization, migration and differentiation of bone marrow stem cells in the target tissue constitute a natural phenomenon of healing in the human body. [37]

The histopathological and histochemical changes in the lymph nodes and spleen of treated rats:

Lymph node:

Histological results of this study showed nearlynormal structure of the lymph node of **D+Do, AFA and AFA+D** groups

The microscopic appearance of lymph node of the diabetic rats showed severe histopathological changes. These changes include: highly dilated and congested blood sinuses with numerous degenerated areas in the cortex,debris of degenerated cells and congested blood sinuses which contain hemolysed RBCs with numerous hemorrhagic areas**.** Hypocellularity in some cortical follicles was detected with degenerated areas in medulla and hilum of the lymph node of the diabetic group**.**

In agreement with the present study **Guttmann *et al.***[38]found that lymph node T-cell zones showed hypocellularity in the diabetic patients.

These results are in agreement with **Bellgrau *et al*.**[39] They found that mixed lymphocyte reactions (MLR) and other *invitro* proliferative responses revealed markedly abnormal lymphocyte function and granulomatous lesions in lymph nodes of the diabetic rats**.**

Tae Hu *et al*.[40] revealed the relation between diabetic status and lymphocytes. Diabetes induced apoptosis in lymphocytes of rats and humans with reduced number of blood-circulating lymphocytes.They also reported that diabetes induced impairment of lymphocyte function.

Abnormalities in the defense mechanisms of diabetic rats were studied by **Otton *et al*.** [41].They detected decreased proliferation of lymphocytes which play a vital role for initiating immunity. Decreased proliferative response of lymphocytes owing to high glucose concentration may inhibit DNA synthesis. Therefore, high glycemic in addition to the lack of insulin may participate in the reduced proliferation capacity of lymphocytes in the diabetic rats.

Salil *et al*.[42]observed that the adverse reaction of synthetic medicines in the treatment of diabetes mellitus has restricted in a growing demand for the use of herbal drugs or phytomedicines.

Monostori *et al*. [43] reported that AFA is one of

the natural products that can mobilize Adult Stem Cells (ASCs) from human bone marrow that contain a substance called Stem Enhancer cells.It is extracted from *Aphanizomenon flos-aquae* (AFA),Bone Marrow Cells (BMCs) and Mesenchymal Stem cells (MSCs) and it induces the regeneration of recipient derived pancreatic insulin-secreting cells at first. Second, Mesenchymal Stem Cells (MSCs) inhibit T cell mediated immune responses against the newly formed beta-cells.

In this study highly increased collagen fibres were detected in the cortical regions and they were scattered in the degenerated areas of the medulla of lymph node of the diabetic group, while slightly reduced collagen fibres were demonstrated in the capsule, cortex, paracortex and medulla of the lymph node tissue of daonil diabetic group **(D+Do)**, somewhat normal distribution of collagen fibres in the lymph node of **AFA** group but some collagen fibres were scattered in the cortex and medulla. Nearly normal distribution of collagen fibres was noticed in the cortical and medullary regions of the lymph node of **AFA+D** group, but the capsule contained thick collagen fibres. These results agree with those of **Johnson and Lalande**[44] who reported that diabetes mellitus caused lymphatic infiltration ,disturbance in the lymphatic flow and increased density of collagen fibres in lymph node tissue. Histological examination of diabetic lymph nodes treated with either glibenclamide or captopril showed reduced collagen fibres compared to untreated diabetic rats. [45]

Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions. They do this by being oxidized themselves, so antioxidants are often reducing agents. [46]

Parikh *et al*.[47]estimated the results of blue green algae supplementation on promoting health and controlling a variety of disorders in humans and they reported that this substance can enhance the phagocyte activity in macrophages. The present study showed highly increased staining affinity of total protein in lymph node of the diabetic group**(D)**,degenerated areas were negatively stained .**Nico *et al*.** [48]reported that the reduction in proteins synthesis may be due to the high protein catabolism in the diabetic individuals (breakdown of protein to obtain energy in the absence of carbohydrate).

In the present study the diabetic groups treated with daonil or AFA showed significant increase in total protein content of lymph node, but such increase was lower than that in the diabetic group. While non significant increase in total protein content was noticed in lymph node of AFA group.

. Treatment of diabetic rats with metformin or glibenclamide did not produce any significant effects on antioxidant enzymes activities and total protein content compared to the diabetic control rats.[49]**Bhardwaj *et al.*** [50]realized increased level of total protein in the diabetic rats .and this may be resulted from decreased liver uptake of proteins. The aqueous extract of *Cassia sophera* (AECS) and glibenclamide-treated rats showed a decrease in total protein content compared to the diabetic control. The reduced level of protein may be due to reduced oxidative stress after AECS treatment and hepatoprotection.

In diabetes, oxidative stress has been found to be mainly due to an increased production of oxygen free radicals and a sharp reduction of antioxidant defenses. Hence, compounds with both hypoglycemic and antioxidative properties would be as useful antidiabetic agents.[51]. They added that glibenclamide is often used as an insulin stimulant and also used as a standard antidiabetic drug in STZ induced diabetes to compare the antidiabetic properties of a variety of hypoglycemic compounds. Glibenclamide can either increase the biosynthesis of antioxidant enzymes or reduce the oxidative stress leading to less degradation of antioxidant, or have both effects.

Spleen:

In the present study the microscopic appearance of spleen of the diabetic rats showed severe changes. These changes include: thickened arterial walls with narrow lumens, necrotic trabeculae, numerous hemosidrin granules with hemolysed RBCs in blood sinuses of the red pulps , necrotic areas in the white pulps, thickened trabeculae which contained highly dilated trabecular vein ,highly reduced lymphocytes in the white pulps with numerous degenerated areas.

Selvant *et al.* [52] reported that in the diabetic rats the spleen showed degenerative changes and necrosis of white pulps with inhibition of spleen growth. **Moselhy *et al*.** [53] observed that diffused lymphocytic hyperplasia and hypertrophy lymphoid follicles as well as congested red pulps

with hemosidrosis in spleen of alloxanated diabetic rats.

Treatment of diabetic rats with daonil**(D+Do)** showed slightly improvement in the architecture of white pulps of splenic tissue ,but the central arteries had thickened walls with numerous degenerated areas and dilated blood sinuses in the red pulps.

Kothny *et al*.[54] reported that there was no significant increase in infections and infestations (i.e. no suppression of immune function) with glibenclamide treatment. They also reported that daily administration of wide range doses of glibenclamide for 4 weeks in rats did not affect the development of immunization related splenic and lymph nodes tissues and morphological changes (injection site granuloma formation).

Complications of type-2 diabetes not only include nephropathy, autonomic neuropathy, peripheral neuropathy, retinopathy, patients with T2D also suffer other complications than the microvascular complications. Immuno-deficiency, delayed wound healing, skin ulcer and osteoporosis. [55]They added that treatment with sulphonylureas and some antioxidant herbs led to improvement in complications of type-2 diabetes mellitus in the diabetic rats.

In the present study somewhat normal appearance of splenic tissue of **AFA** and **AFA+D** groups was observed, but thickened arterial walls were still detected in **AFA+D** group.

Ginsberg *et al.* [56]reported that consumption of a moderate amount (1.5 grams) of blue-green algae *Aphanizomenon flos- aquae* resulted in rapid changes in immune cell trafficking. Two hours after AFA consumption, a generalized mobilization of lymphocytes and monocytes, but not polymorph nucleated cells was observed. In addition, the relative proportions and absolute numbers of natural killer (NK) cells were reduced after AFA consumption. A significant reduction in phagocytic activity was observed for polymorph nucleated cells. They added that the changes in immune cell trafficking displayed high degree of cell specificity.

The present results showed highly increased collagen fibres with common fibrosis in the splenic tissue of the diabetic group especially under the capsule, in the red and white pulps, in the thickened trabecular walls and around the highly dilated trabecular vein.

The present results indicated that treatment of diabetic rats with glibenclamide and AFA showed slightly normal distribution of collagen fibres in the splenic tissues of **D+Do,** AFA and AFA+D groups.

In agreement with the present results **Choi *et al*.** [57] demonstrated normal collagen fibres in the spleen of diabetic rats which were treated with sulphonylurea drug (Glyburide) due to improvement in glucose metabolism, while increased collagen fibres were observed in the spleen and liver of streptozotocin-induced diabetic rats due to degenerative changes.

Badary *et al*.[58] realized many degenerated lymphocytes and pyknotic nuclei with increased collagen fibres in the spleen tissue of the diabetic rats.

In the present study quantitative examination of total protein content in the spleen showed a significant decrease in **D, D+Do** and **AFA+D** group, but such decrease was more obvious in the diabetic group and this reflect the improvement in the diabetic groups which were treated with **AFA** or daonil . Non-significant change in total protein content was observed in **AFA** treated group as compared to the control group.

Helal *et al.* [59] recorded that there was a significant decrease in body weight gain and total protein content with severe hyperglycemia in the diabetic untreated rats as a result to severe hypoinsulinemia and increasing insulin resistance. Where, the defect in insulin level or function led to alteration in carbohydrates metabolism causing hyperglycemia and decreasing total protein content. This may be due to the effect of insulin on hepatic cells by stimulating glycogenolysis, gluconeogenesis and inhibition of its effect on peripheral utilization of glucose.

In diabetes, oxidative stress has been found to be mainly due to an increased production of oxygen free radicals and a sharp reduction of antioxidant defense. Hence, compounds with both hypoglycemic and antioxidative properties would be as useful antidiabetic agents.[63]They added that glibenclamide is often used as an insulin stimulant and also used as a standard antidiabetic drug in STZ induced diabetes to compare the antidiabetic properties of a variety of hypoglycemic compounds. Glibenclamide can either increase the biosynthesis of antioxidant enzymes or reduce the oxidative stress leading to less degradation of antioxidantor have both effects.

Several food grade microalgae, including *Spirulina platensis*, *Aphanizomenon flos -aquae* and *Chlorella pyrenoidosa* are also known to contain polysaccharides,potent immune-

stimulators of human monocytes and macrophages.[51]

Effects on the innate immunity and adaptive immunity of oral administrations of three blue green algae(*Spirulina platensis*, *Aphanizomenon flos -aquae* and *Chlorella pyrenoidosa*) function as an immunostimulatory substance of monocytes of the spleen. [60]

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Table 1: The statistical analysis (Mean optical density MOD) of the serum glucose level (mg/dl) and serum insulin values (µIU/dl) and percentage of change in the different experimental groups.

Parameter	Serum glucose level		Serum insulin level	
Time / Groups	30 days		30 days	
	Mean± S.E	% Change	Mean± S.E	% Change
C	113.40±3.08	0.0%	18.16 ± 0.80	0.0%
D	341.4±4.83***	201.0%	6.38 ± 0.47***	-64.86 %
D +Do	117.80±2.48^{ns}	3.88%	20.50± 0.47*	12.89%
AFA	116.80±1.59^{ns}	3.00%	19.88± 0.99^{ns}	9.47%
AFA+D	119.60±3.19^{ns}	5.47%	20.22±0.73^{ns}	11.34%

Each value represented the mean ± standard error (SE).

- **C** control; **D** Diabetic group;**D+Do**diabetic+daonil,**AFA** *Aphanizomenon flos-aquae* extract and **AFA+D,***Aphanizomenon flos-aquae*+ diabetic group .
- The values are considered * significant at $P \leq 0.05$ and *** very highly significant at $P \leq 0.001$ compared to the control group.***ns,* is non significant.**

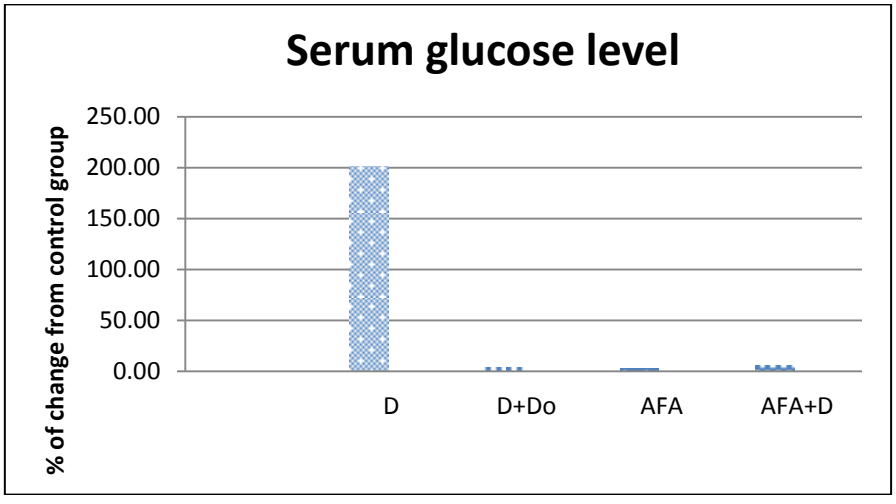


Figure1-The percentage of change of the serum glucose levels in the different experimental groups.

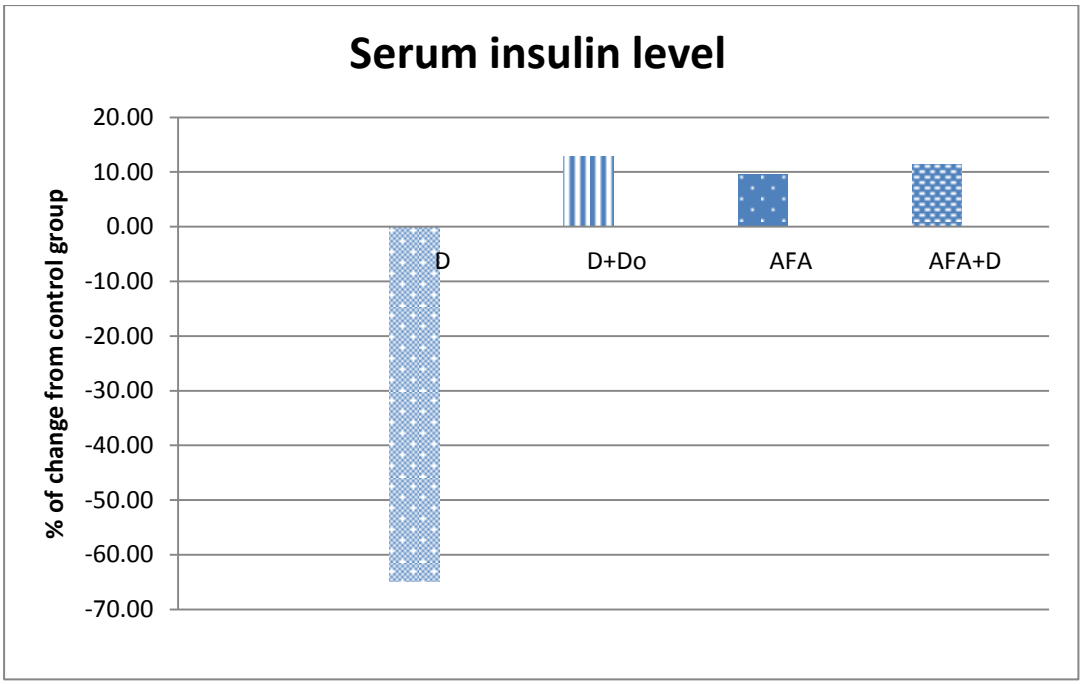


Figure2-The percentage of change of serum insulin levels in the different experimental groups.

Total protein content in the lymph node

Table 2-The mean optical density values (MOD)and percent of change in total protein in the lymph node tissue of the control and the different treated groups

Groups / Parameters	**C**	**D**	**D+Do**	**AFA**	**AFA+D**
Mean ± SE	0.37 ±0.014	0.54* ±0.029	0.41*± 0.028	0.39^{ns} ±0.014	0.44* ± 0.096
% of change	**0%**	45.95%	10.8%	5.41%	18.92%

- Each value represented the mean ± standard deviation (SD).
- The values are considered *significant at $P \leq 0.05$ compared to the control group.
- **C** control; **D** Diabetic group;**D+Do** diabetic+daonil; **AFA** *Aphanizomenon flos-aquae* extract and **AFA+D,** *Aphanizomenon flos -aquae*+ diabetic group .

Total protein content in the spleen

Table 3-The mean optical density values (MOD) and percent of change of total protein in the spleen tissue of the control and the different treated groups.

Groups / Parameters	**C**	**D**	**D+Do**	**AFA**	**AFA+D**
Mean ±SE	0.37 ±0.017	0.31* ±0.014	0.35* ± 0.016	0.371^{ns} ±0.019	0.35* ±0.016
% of change	**0%**	-16.22%	-5.41%	0%	- 5.41%

- Each value represented the mean ± standard deviation (SD).
- The values are considered * significant at $P \leq 0.05$ compared to the control group

- **C** control, **D** Diabetic group, **D+Do** diabetic+daonil,**AFA** extract and **AFA+D,***Aphanizomenon flos-aquae* + diabetic group .

Figures 3-7: photomicrographs of lymph node tissue of the control and treated groups.**(Hx&E X100&200)**

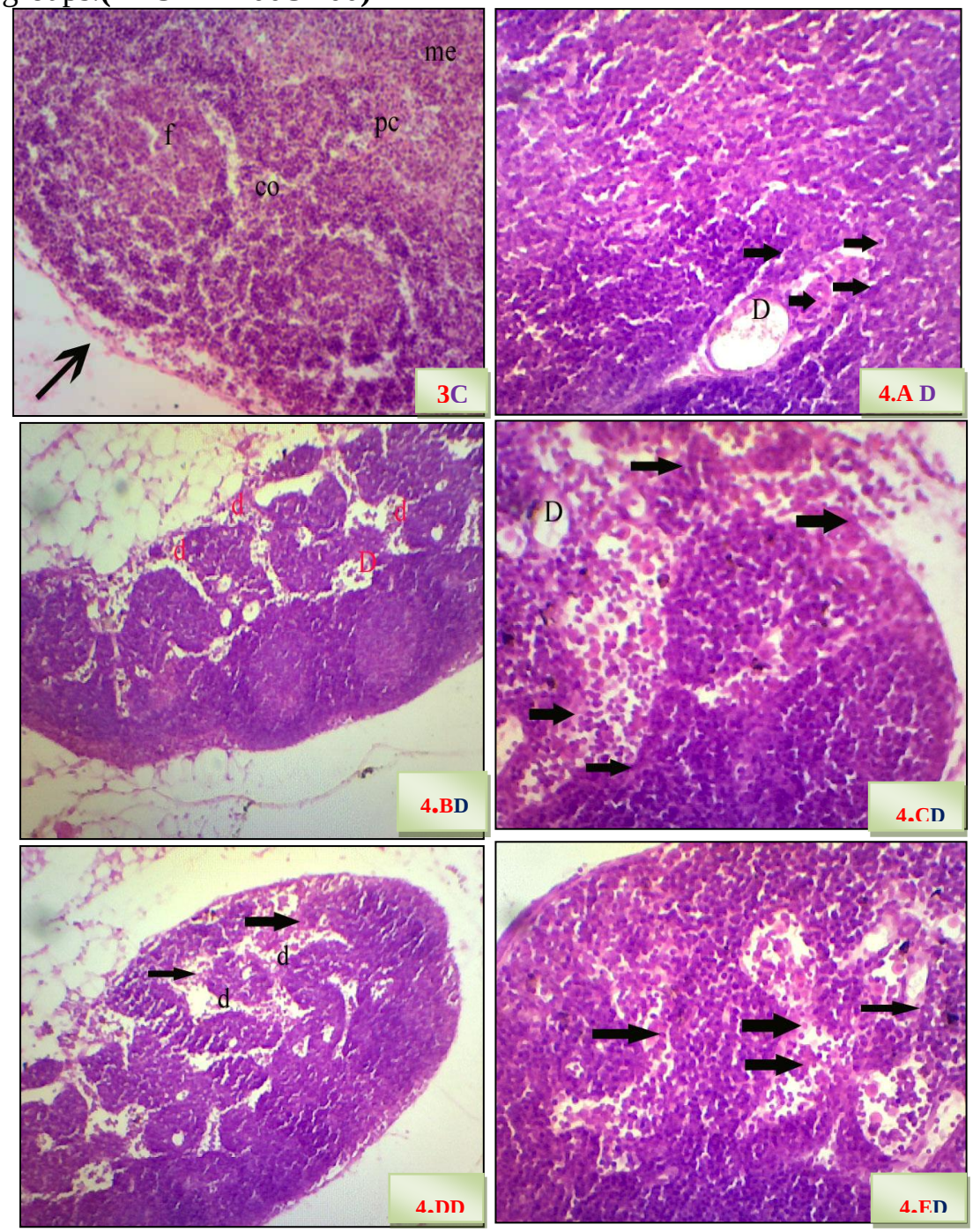


Fig.3:showing normal histological structure of the lymph node which consists of :cortex (co) with cortical follicle (f), paracortex (pc) and medulla(me). They are encircled by the capsule (→).**(X100)**

Figs.4:lymph node tissue of diabetic group**(D)**showing highly dilated and congested blood sinuses (→) in the hilum region,they contain haemolysed blood cells with numerous degenerated areas(d) in the cortical and medullary regions which contain debris of degenerated cells with hypocellularity in some cortical follicles.Notice:highly dilated wall of the vein (D).**(AX200,BX100,CX200,DX100,EX100)**

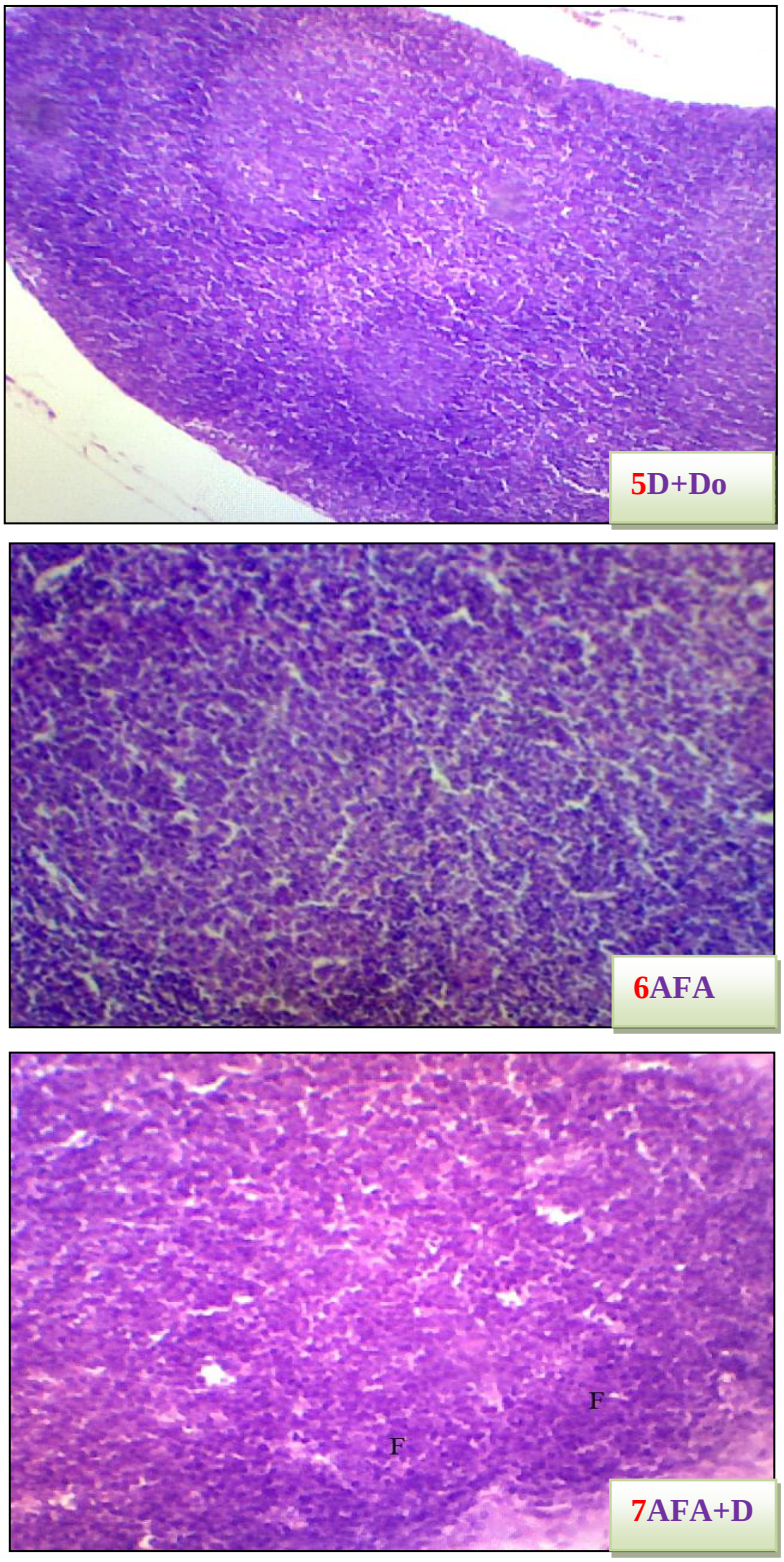


Fig.5: showingslightly normal appearance of lymph node tissue of **D+Do** group.**(X100)**
Fig.6: showing somewhat normal appearance of lymph node tissue of **AFA** group.**(X200)**
Fig.7:- showing nearly normal appearance of lymph node tissue of **AFA+D** group with normal cortical follicles(F). **(X200)**

Figures 8-12: Photomicrographs oflymph node tissueshowing distribution of the collagen fibres in the control and treated groups**(Mallory's trichrome stain X100& 200)**

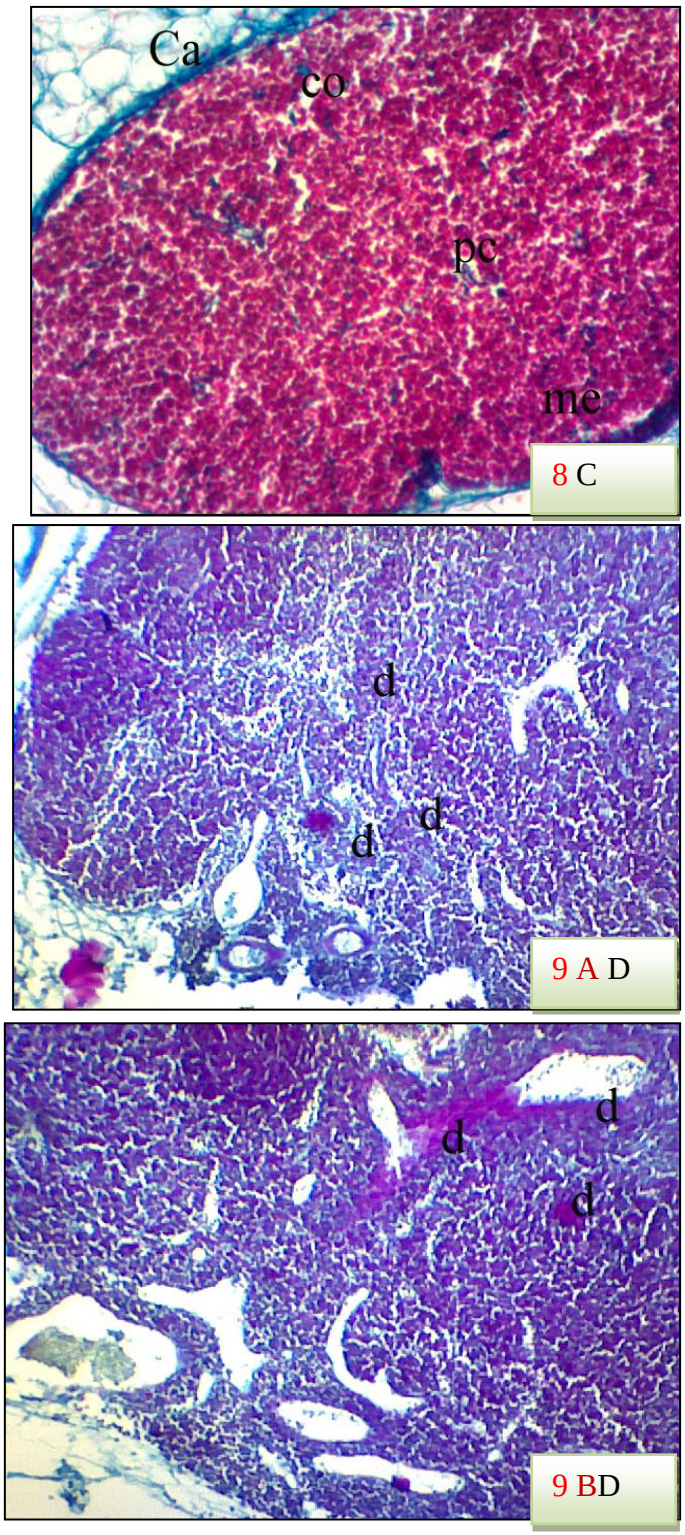
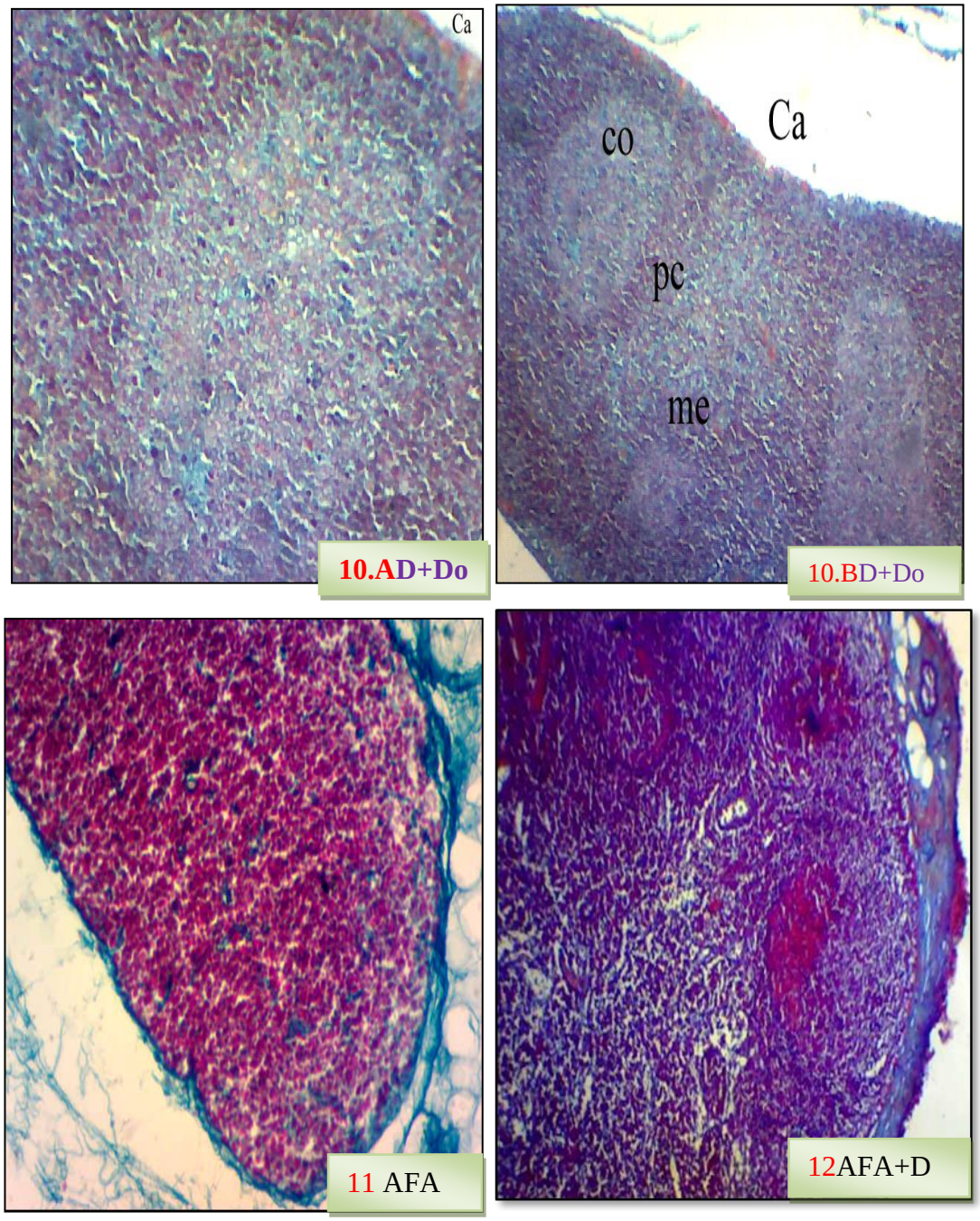


Fig.8:showing normal distribution of collagen fibres in the lymph node tissue of a control rat .Notice:thin collagen bundles supporting the capsule (Ca),cortex (co),paracortex (pc) and medulla(me). **(X100)**

Figs.9- showing highly increased collagen fibres in the cortical region scattered in the degenerated areas **(d)** of lymph node of the diabetic group **(D).** **(A&BX200)**



Figs.10A,B:showing slightly reduced collagen fibres in the capsule(Ca),Cortex(co),paracortex(pc) and medulla(me) of the lymph node tissue of daonil diabetic group **(D+Do)**. **(AX200& BX100).**

Fig.11: showin gnearly normal distribution of collagen fibres in the cortical and medullary regions of the lymph node of AFA group .Some collagen fibres are scattered in the cortex and medulla. **(X100)**

Fig.12: showing somewhat normal distribution of collagen fibres in the cortical and medullary regions of the lymph node of **AFA+D** group, the capsule contains thick collagen fibres. **(X100)**

Figures 13-17: Photomicrographs of lymph node tissue showing total protein distribution in the control and treated groups

(Mercury bromophenol blue X 100&200)

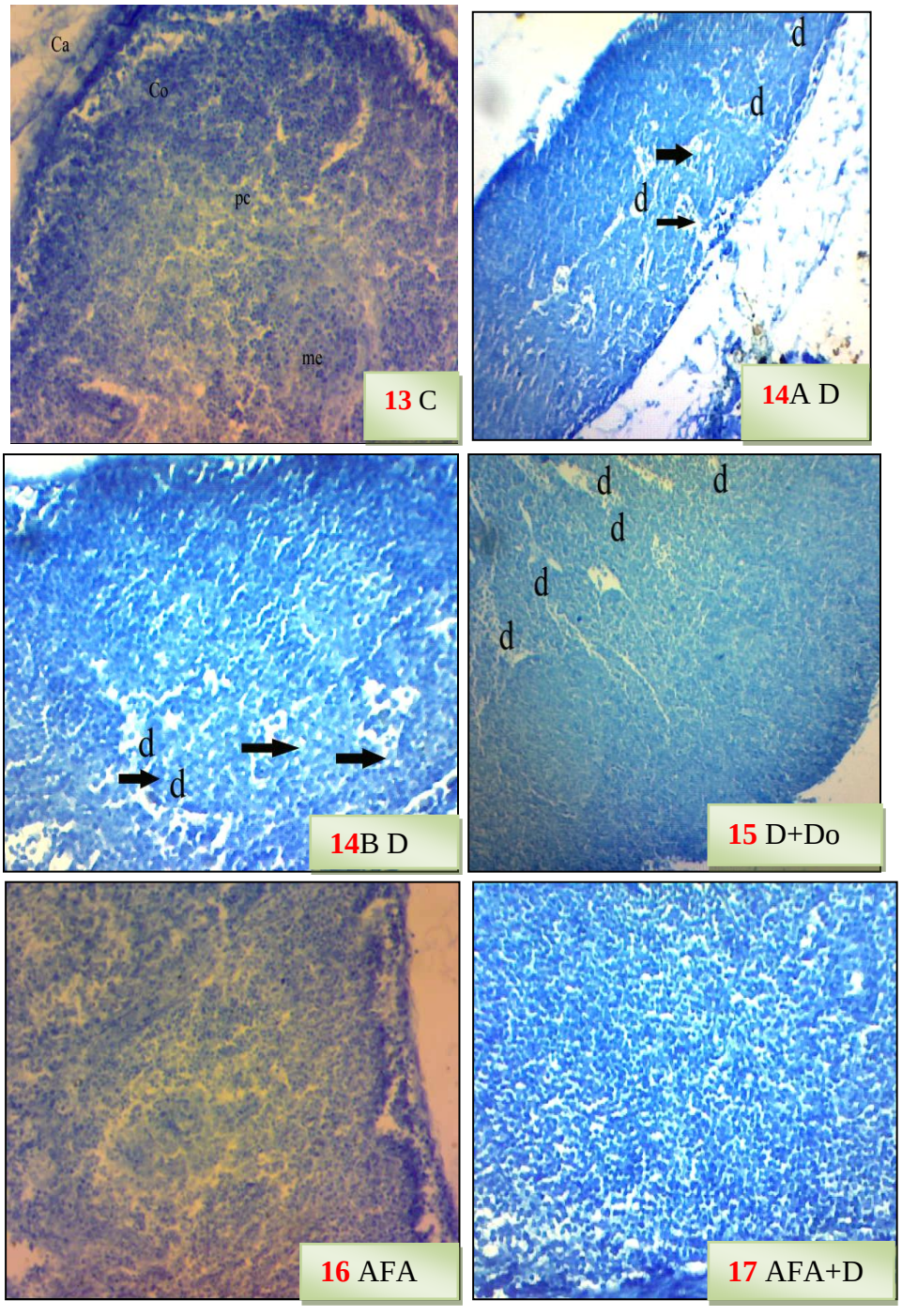


Fig.13: showing normal distribution of total protein in the lymph node tissue of a control rat. Notice: moderate staining affinity in the capsule (ca), cortex (co)with less staining affinity in paracortex (pc) and medulla(me). **(X100)**

Figs.14: Showing increased staining affinity of total protein in lymph node of the diabetic group .Highly dilated blood sinuses containing deeply stained RBCs(→),degenerated areas(d)are negatively stained. **(AX 100&BX200)**

Fig.15 :showing slightly increased total protein in the cortical region of the lymph node of daonil diabetic treated group, some degenerated areas(d) in the medulla are negatively stained and dilated blood sinuses contain moderately stained blood cells**.** **(X100)**

Fig.16: showing somewhat normal appearance of the total protein in the capsule, cortex and medulla of lymph node tissue of the AFA group**.** **(X100)**

Fig.17 Showing nearly increased total protein in lymph node tissue of the AFA diabetic group. **(X200)**

Figures 18-22: photomicrographs of spleen tissue of the control and treated groups.**(Hx&E X100&200)**

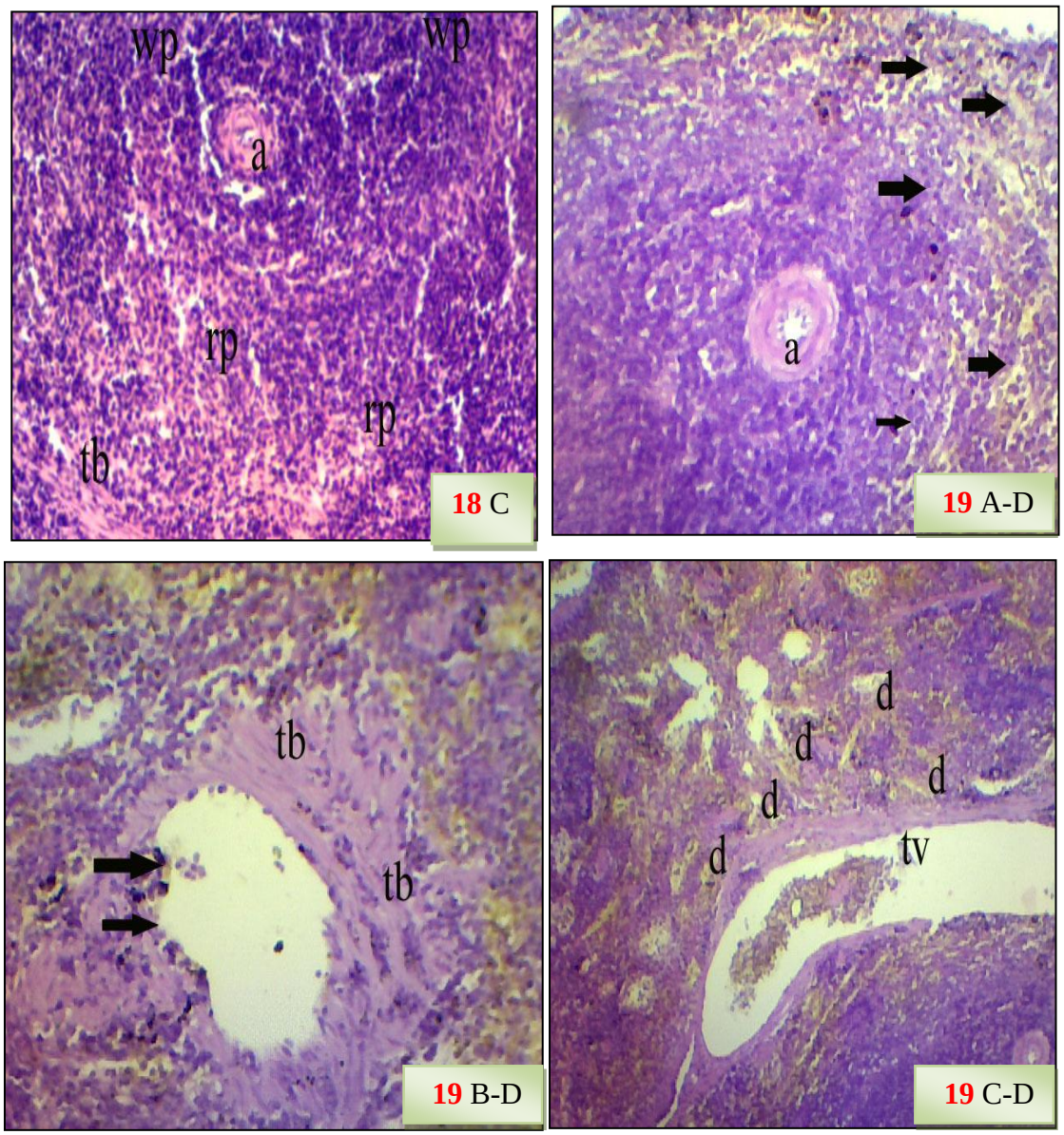
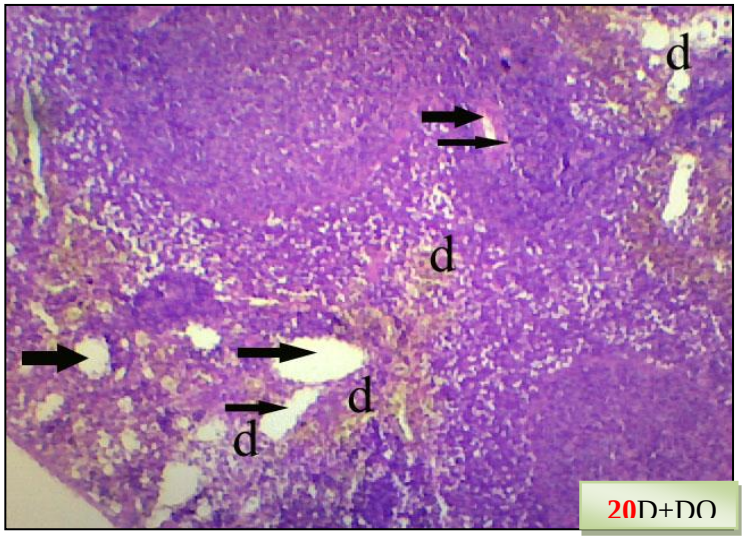
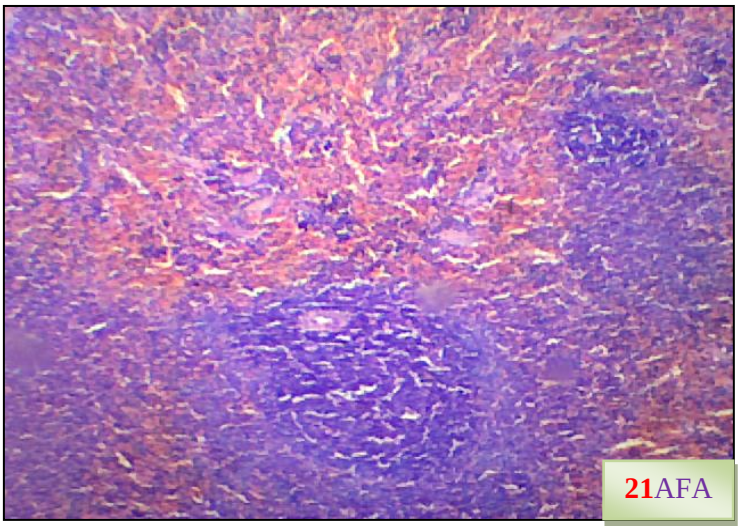


Fig.18: showing normal histological structure of spleen of a control rat which contains red pulps (rp),white pulp (wp) with its central artery(a) and trabeculae (tb)**.** **(X100)**

Figs.19:showing thickened arterial wall (a) with narrow lumen ,necrotic trabeculae (tb) numerous hemosidrin granules(→) with hemolysed blood cells in the dilated blood sinuses of the red pulps and highly dilated trabecular vein (tv) which contains hemolysed blood cells with highly reduced lymphocytes in the white pulps and numerous degenerated areas (d)in the spleen tissue of the diabetic group.

(A,B&C X200)





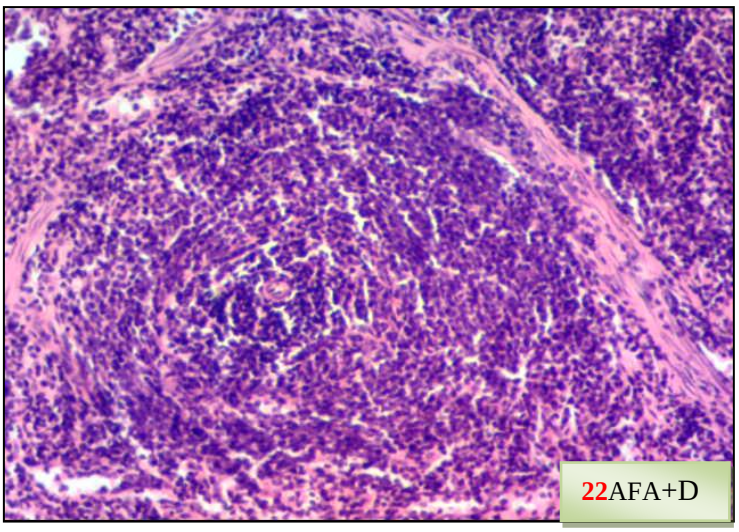


Fig.20 :showing somewhat normal appearance of the white pulps, the central artery has thickened arterial wall with numerous degenerated areas (d) in the red pulps and dilated blood sinuses **(→)** in the spleen tissue of the daonil diabetic treated group. **(X200)**

Fig.21: showing slightly normal appearance of spleen tissue of **AFA** group. **(X100)**

Fig.22 : showing nearly normal appearance of the splenic tissue of **AFA+D** group, thickened arterial wall is still detected. **(X200)**

Figures 23-27: Photomicrographs ofspleen tissueshowing distribution of the collagen fibres in the control and treated groups

(Mallory's trichrome stain X100& 200)

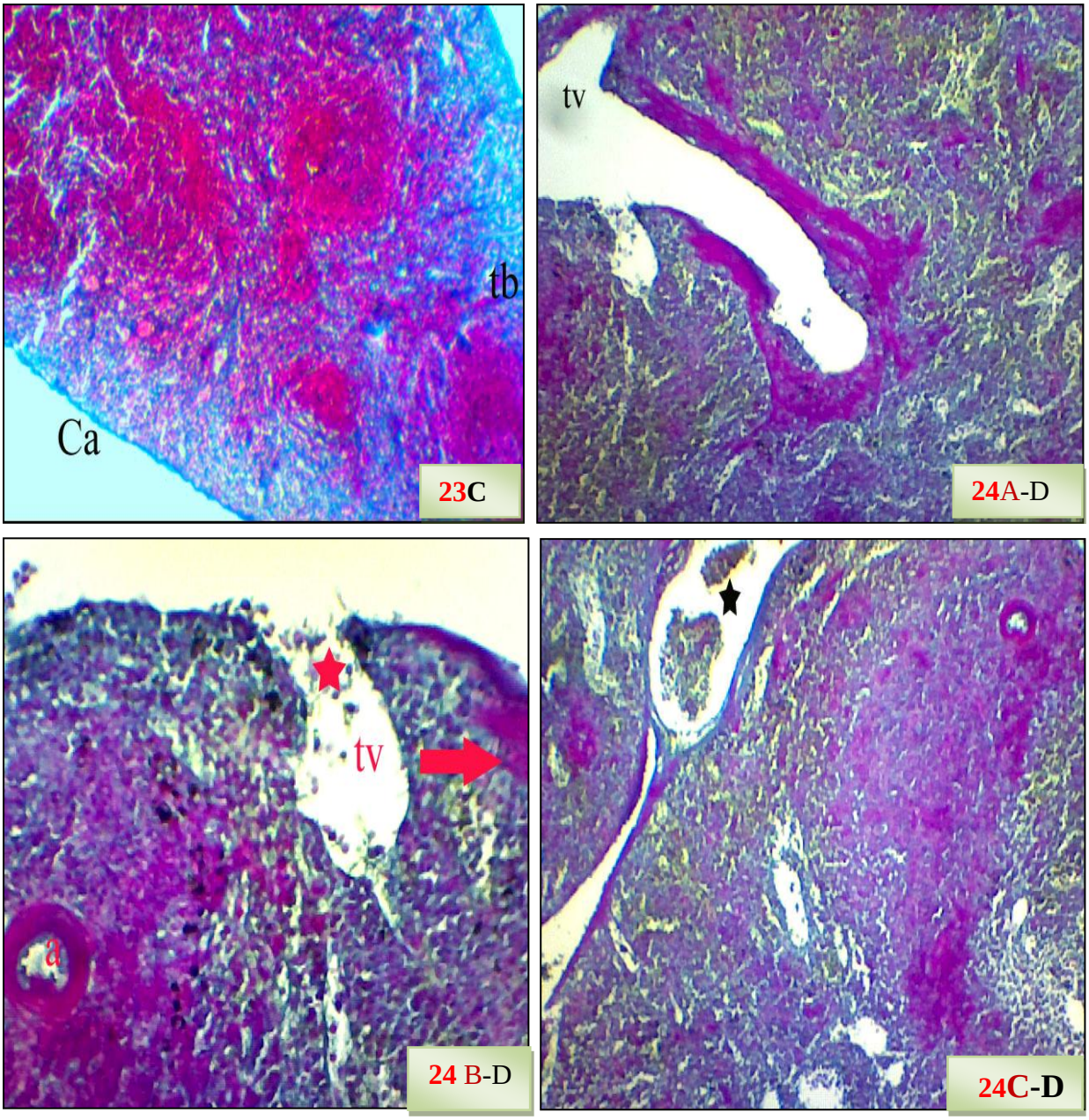


Fig.23 :showing normal distribution of collagen fibres in the splenic tissue of a control rat. Notice: thin collagen fibres support the capsule (Ca), trabeculae (tb) with scattered collagen fibres in the red and white pulps. **(X100)**

Figs.24-:showing highly increased collagen fibres in the red and white pulps of spleen of the diabetic group especially in the thickened trabeculae and walls of their veins(tv),in the thickened walls of the central arteries(a) ,in and under the capsule (→) and inside the trabecular vein **(*).** **(A,B&C X200)**

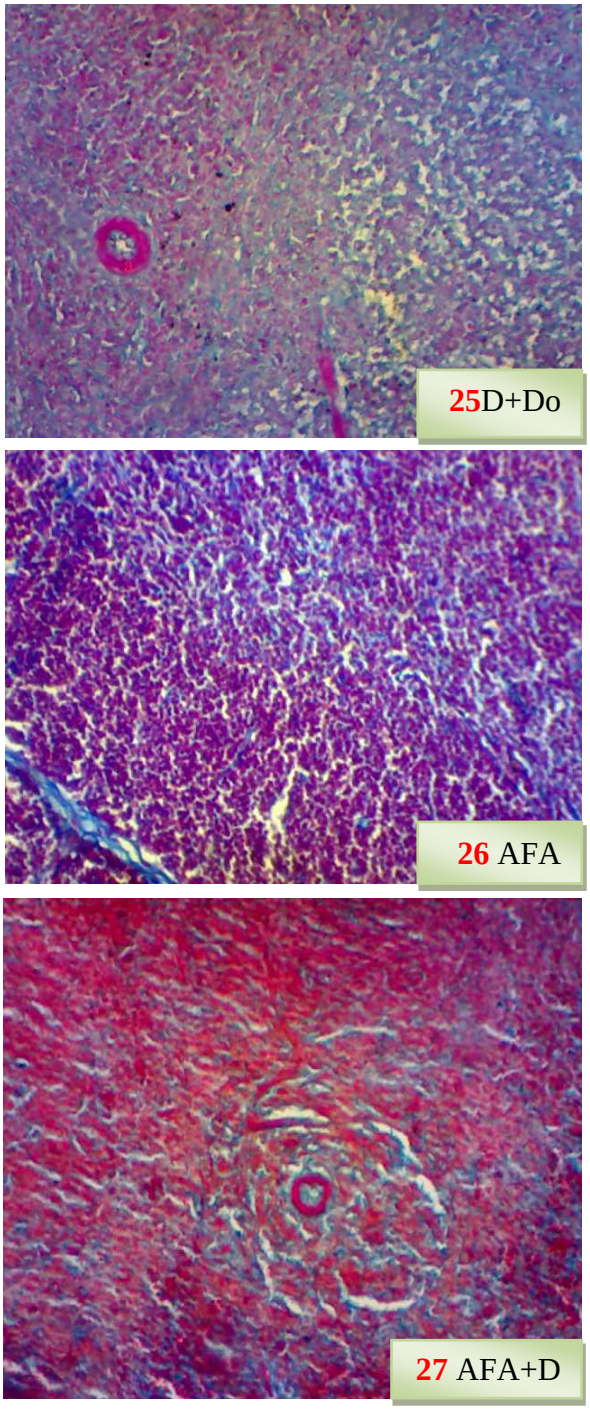


Fig.25: showing somewhat normal distribution of collagen fibres in the white pulps of the splenic tissue of **D+Do** group, the central arteries have thickened arterial walls with numerous degenerated areas in the red pulps. **(X200)**

Fig.26 :showing nearly normal appearance of collagen fibres in the spleen tissue of **AFA** group**.** **(X200)**

Fig.27 :showing slightly normal appearance of collagen fibres in the red and white pulps of spleen of **AFA+D** group**.** **(X200)**

Figures 28-32: Photomicrographs of spleen tissue showing total protein distribution in the control and treated groups

(Mercury bromophenol blue X 100&200)

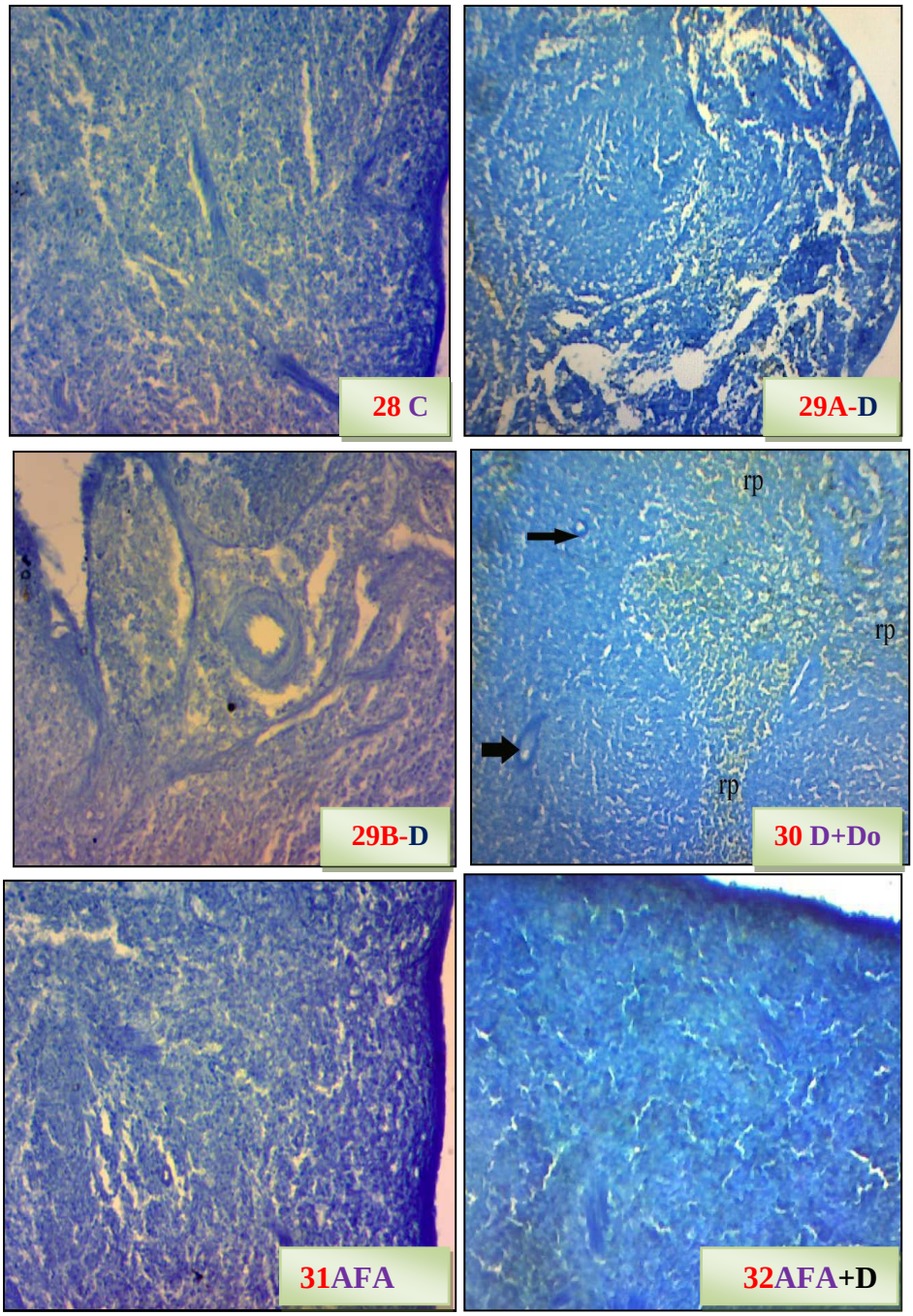


Fig.28: showing moderately stained capsule, trabeculae and red pulps with less stained white pulps in the splenic tissue of a control rat. **(X 100)**

Fig.29:showing deeply stained pools of red blood cells in the red pulps and negatively or poorly stained degenerated areas in splenic tissue of diabetic rats. **(X200)**

Fig.30:showing deeply stained walls of the central arteries (→) with less stained red pulps (rp) and lymphocytes in the white pulps of the splenic tissue of the **D+Do** group**.** **(X200)**

Fig.31: showin gnearly normal appearance of total protein in the splenic tissue of **AFA** group**.(X 100)**

Fig.32:showing slightly decreased the total protein in the red and white pulps of the **AFA+D** group**. (X 100)**