Protective effect of Pentoxifylline versus Nigella sativa against Cyclophosphamide induced splenic damage in adult male albino rats.

Ereny Fekry [1], Mona M. Awny [2]*, Samy Makary [3], Rahman A. A.[1]

KEYWORDS
Toxicity,
Histopathology,
Immunohistochemistry,
Cyclophosphamide,
Pentoxifylline,
Nigella sativa,
Spleen.

ABSTRACT

Cyclophosphamide (CTX) is an effective anticancer therapy. It is known to cause oxidative stress and cytotoxic effects, especially on the immune system. Pentoxifylline and Nigella sativa seeds both possess antioxidant and anti-inflammatory properties. This study explores the ameliorative impact of both pentoxifylline and nigella sativa over the splenic damage induced by cyclophosphamide. Thirty-six adult male albino rats were separated into six groups from six each and received treatment for 10 days. Group I (control) received saline 0.9% solution orally via intragastric tube, group II received pentoxifylline (150 mg/kg) orally, group III received nigella sativa oil (20 mg/day) orally, group IV received a single intraperitoneal injection (IP) of CTX (200 mg/kg), group V received pentoxifylline (150 mg/day) orally for 10 days and at the 8th-day animals received single IP injection of CTX (200 mg/kg), group VI received nigella sativa oil (20 mg/day) orally for 10 days and similarly at the 8th-day animals received single IP injection of CTX (200 mg/kg). Light microscopic and ultrastructure changes in the spleen of the different experimental groups were examined. CTX induced splenic damage in the form of a disrupted and detached capsule of the spleen, lymphocyte loss with marked thickening and dilatation of the central artery in the white pulp, and cellular loss and congested blood sinusoids in the red pulp. The use of pentoxifylline and nigella sativa resulted in the restoration of splenic tissue. Pentoxifylline showed a more protective effect than nigella sativa on the splenic tissue against CTX-induced damage.

Introduction

Cyclophosphamide, also known as Cytophosphane or Cytoxan (CTX), is an alkylating agent that has an effective anticancer activity. The United States licensed it for medicinal use in 1959 (Brayfield, 2017). The mode of action of CTX was reported to occur through introducing alkyl radicals into DNA strands that interfere with DNA replication, stopping cancer cells from division & growing (Huyan et al., 2011). CTX may potentially have immunosuppressive properties due to its cytotoxic action on lymphocytes. So, it can be used in the treatment of some autoimmune diseases (Xu and Zhang, 2015).

It is widely used as a chemo-therapeutic drug to treat hematological malignancies and solid cancers such as lymphoma, multiple myeloma, leukemia, ovarian cancer, breast cancer, small cell lung cancer, neuroblastoma, and sarcoma. It is used as an immunosuppressant in nephrotic syndrome and after organ transplantation (Brayfield, 2017).

(1) *Histology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.*
(2) *Forensic Medicine & Clinical Toxicology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.*
(3) *Physiology Department, Faculty of Medicine, Suez Canal University, Ismailia, Egypt.*
***Corresponding author:** Mona M. Awny
E-mail address: mona_awny@med.suez.edu.eg
Phone 0097336121613

Most people develop side effects after receiving CTX. Leukopenia, particularly lymphocytopenia, anorexia, vomiting, hair loss, and hemorrhagic cystitis are all typical adverse consequences. Infertility and lung fibrosis are two more serious toxic effects (Cengiz, 2018). Cyclophosphamide toxicity is caused by the generation of extremely reactive oxygen free radicals, which causes oxidative stress (Elshater et al., 2018).

Pentoxifylline, a synthetic dimethyl-xanthine derivative, is used to treat peripheral vascular diseases, cerebrovascular insufficiency, and diabetic neuropathy. The mode of action of pentoxifylline is through inhibiting erythrocyte phosphodiesterase, causing an increment in erythrocyte cyclic adenosine monophosphate (cAMP) activity; as a result, the erythrocyte membrane becomes more deformation resistant. It also increases microcirculatory flow and tissue oxygen perfusion by decreasing blood viscosity through decreasing plasma fibrinogen levels, boosting fibrinolytic activity, and inhibiting platelets aggregation (McCarty et al., 2016)

Additionally, pentoxifylline has immunomodulating properties. It reduces the pro-inflammatory response in patients as denoted through the reduction of pro-inflammatory markers. It also increases anti-inflammatory activity in the form of improving leukocyte deformability and chemotaxis (McCarty et al., 2016). Many studies confirmed its potential effects as antioxidants *in vitro* & *in vivo* through its function in lowering the creation of free radicals (Horvath et al., 2002; Vircheva et al., 2010). Another study confirmed the efficacy of pentoxifylline in preventing hemorrhagic cystitis induced by CTX in an experimental model, through its antioxidant and anti-inflammatory effects (Abo-Salem, 2013).

Nigella sativa seeds are also known as black seeds or black cumin. They have been utilized as a spice and in traditional medicine for treating various conditions (Khader and Eckl, 2014). Nigella sativa *s*eed extract (oil) demonstrated a wide range of beneficial biological actions including antioxidant, anti-inflammatory (Entok et al., 2014), antibacterial (Habib and Choudhry, 2021), and anti-cancer activities (Ulasli et al., 2013). Studies confirmed its role in reducing various chemotherapeutic toxicities such as CTX-induced lung injury, cisplatin-induced liver toxicity, and renal damage (Suddek et al., 2013; Al-Malki and Sayed, 2014).

This study was conducted to examine and compare the preventive effects of pentoxifylline and nigella sativa against CTX-induced splenic damage.

Materials and Methods

The research was conducted in the Histology Department of the Faculty of Medicine at Suez Canal University in Ismailia, Egypt. Animals were managed following institutional animal care ethical norms established by the Scientific Research Ethics Committee of the Faculty of Medicine, Suez Canal University, with permission code: 4037＃.

Animals

The experiment was conducted on 36 adult male albino rats of comparable age (3 months) and body weight (150-200 g). The animals utilized in this study were collected from the Veterinary Medicine animal house at Suez Canal University's Faculty of Veterinary Medicine. Before the trial began, they were acclimatized and housed in plastic cages with unrestricted access to water and a typical animal pellet diet. The animal habitat was

kept at a constant room temperature (22-24°C), with a 12-hour day and 12-hour night cycle.

Preparation of drugs

Cyclophosphamide: Endoxan, vials containing 1000 mg dry powder (produced by Baxter Oncology GmbH). Each vial was dissolved in 10 ml of saline (100 mg in each ml).

Pentoxifylline: Trental-coated tablets 400 mg (produced by SANOFI). Each pill was dissolved in 8 ml of saline solution (50 mg in each ml).

Nigella sativa oil: Baraka, 450 mg soft gelatin capsules (manufactured by Pharco pharmaceuticals). The oil was emptied from each capsule using a syringe after each capsule was opened with a sharp knife.

Experimental design

Six groups of animals were formed at random (six animals each).

Group I (Control group): Saline 0.9% solution was administered orally via an intragastric tube for 10 days.

Group II (Pentoxifylline group): pentoxifylline (150 mg/kg) was administered orally via an intragastric tube for 10 days (Abo-Salem, 2013).

Group III (Nigella sativa group): nigella sativa (20 mg/day) was administered orally via an intragastric tube for 10 days (Mohammed et al., 2010; Gore et al., 2016).

Group IV (CTX group): received saline orally daily for 10 days via intragastric tube. On the eighth day CTX (200 mg/kg) was given as a single IP injection (Gore et al., 2016).

Group V (Pentoxifylline + CTX group): received pentoxifylline (150 mg/day) orally via an intragastric tube for 10 days and on the 8th day the animals of this group received a single IP injection of CTX (200 mg/kg).

Group VI (Nigella sativa + CTX group): received nigella sativa oil (20 mg/day) orally via an intragastric tube for 10 days and similarly on the 8th day received a single IP injection of CTX (200 mg/kg).

Treating the animals with a single IP injection of CTX on the eighth day was selected based on earlier reports (Dantas et al., 2010; Khedr, 2015). By the end of the 10th day, all animals were anesthetized by ether and decapitated. The spleen was removed through an abdominal incision and freed of adherent connective tissue. Each spleen was divided into two halves; one half was taken for light microscopic (LM) examination and the other half for transmission electron microscopic **(**TEM) examination.

Methods

(1) Histological and Immunohistochemical studies

Specimens were fixed in 10% **(v/v)** neutral formalin before being processed into 5 µm thick paraffin slices. The sections were stained using the following methods:

- *H&E staining:* used for regular histopathology examinations.
- *Masson trichrome stain:* for connective tissue examination.
- *CD3 immunohistochemistry:* for detection of T lymphocytes; Anti-CD3 antibody [SP7] is a distinguishing property of T cell lineage cells and may thus be employed as a T cell marker. (Code**:** ab16669, Abcam company,

United Kingdom) (Lee et al., 2017). Negative and positive controls were run.

- *CD20 immunohistochemistry:* for the detection of B lymphocytes; the Anti-CD20 antibody was used to detect the CD20 antigen which is found on the surface of B cells. (Code: ab88247, Abcam company, United Kingdom) (Lee et al., 2017)**.** Negative and positive controls were run.

Qualitative assessment was conducted by examining 5 high power fields (X400) in 10 serial sections from each animal of all analyzed groups. In all groups assessed, the frequency distribution of histopathological abnormalities was established. These histopathological changes included the following:

- *Capsule changes:* Thickening, disorganization, and disruption.
- *White pulp changes:*
  - Mild thickening & dilatation of the central artery.
  - Marked thickening & dilatation of central artery.
  - Homogenized material, and cellular loss.
- *Red pulp changes:* Congested blood sinusoids, and cellular loss.

All Images were taken with a calibrated standard digital microscope camera (Tucsen ISH1000 digital microscope camera) and an Olympus® CX21 microscope at a resolution of 10 MP (megapixels) (3656 ×2740 pixels per image).

Quantitative assessment using the software Image J. was also done to contrast the optical density of CD3 & CD20 in various groups.

(2) Transmission electron microscopy studies

The spleen was split into thin specimens of 1mm thickness and then fixed in a new solution of 2.5% glutaraldehyde and 1.5% paraformaldehyde in 0.1 mol/L phosphate buffer. Specimens were evaluated in the TEM unit at Al-Azhar University.

Statistical analysis:

The Statistical Package for the Social Sciences was used for statistical analysis (SPSS 26 software, IBM Corporation). The differences between groups were determined using one-way analysis of variance (ANOVA) and Tukey's Multiple Comparison Test. The differences between the examined groups were only statistically significant when $P < 0.05$.

Results

The frequency distribution of the various histological alterations of the spleen in different groups is demonstrated in (Table 1). Almost all the animals in group I (the control group) had capsule & septa. White pulp with central artery surrounded by lymphocyte population and red pulp was also seen (Figures 1A, 2A & 3A). Likewise, all animals in groups II & III (Pentoxifylline & Nigella sativa) were nearly normal. Occasionally, hemosiderin pigments were detected (Figures 1B, 2B & 3B) and (Figures. 1C, 2C & 3C).

Almost all the animals of group IV (CTX group) demonstrated disorganized and/or disrupted capsules in 10% of animals while it was detached in 85% of animals of this group. Regarding white pulps, there was lymphocyte loss in 83% of animals, while marked thickened and dilated central artery in 85%. Additionally, homogenized material was noticed in 75% of animals in such a group. The red pulp also showed multiple cellular losses in 90% and congested blood sinusoids in 85% of animals (Figures 1D, 2D & 3D).

Group V (Pentoxifylline + CTX) was similar to control, except that 90 % of animals of this group showed mild thickened and dilated central arteries (Figures 1E, 2E & 3E). Sections of group VI (Nigella sativa + CTX group) showed thickened capsules in 65% of animals. White pulp showed mild thickened and dilated central arteries in 87% of animals. Congested blood sinusoids were observed in 82% of animals (Figures 1F, 2F & 3F).

Table (1): The percentages of different histological changes in the spleen of all different animal groups.

Histopathological changes	Groups (n= 6 in each group)					
	Control %	Pentoxifylline %	Nigella %	CTX %	Pentoxifylline + CTX %	Nigella + CTX %
Capsule						
• Thickened	3	4	4	5	10	65
• Disorganized	2	5	3	10	5	8
• Disrupted	0	0	0	85	4	6
White pulp						
• Mild thickening & dilatation of the central artery	2	3	5	10	90	87
• Marked thickening & dilatation of the central artery	0	0	0	85	10	13
• Homogenized material	0	0	0	75	2	3
• Cellular loss	3	5	7	83	8	10
Red pulp						
• Congested blood sinusoids	2	4	9	85	15	82
• Cellular loss	4	6	8	90	10	12

n: number, CTX: Cyclophosphamide

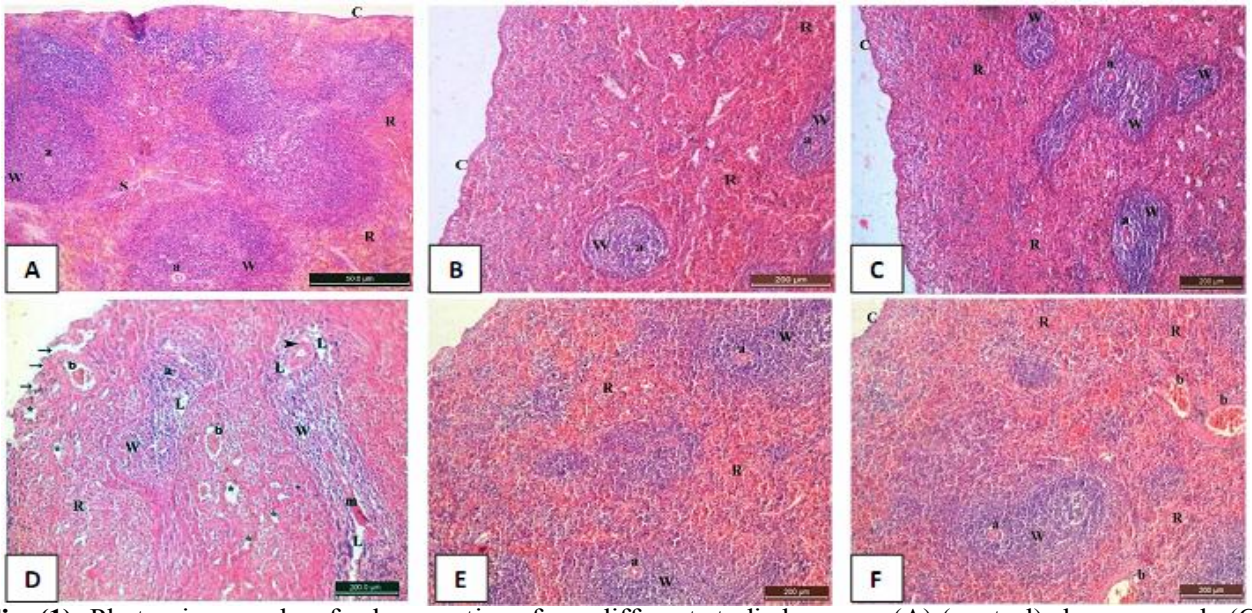


Fig. (1): Photomicrographs of spleen sections from different studied groups. (A) (control) shows capsule (C) & septa (S). White pulp (W) with the central artery (a) and red pulp (R). (B) (Pentoxifylline) and (C) (Nigella sativa) are almost as control. (D) (CTX) shows a disrupted capsule (arrows). The white pulps (W) show lymphocyte loss (L), thickened, and dilated central artery (arrowhead) & homogenized material (m). The red pulp (R) also shows multiple tissue loss (*) and congested blood sinusoids (b). (E) (Pentoxifylline + CTX) is nearly similar to control (F) (Nigella sativa + CTX) Thickened capsule (C), and congested blood sinusoids (b). While white pulp (W) with the central artery (a) is almost as control. (H &E X100).

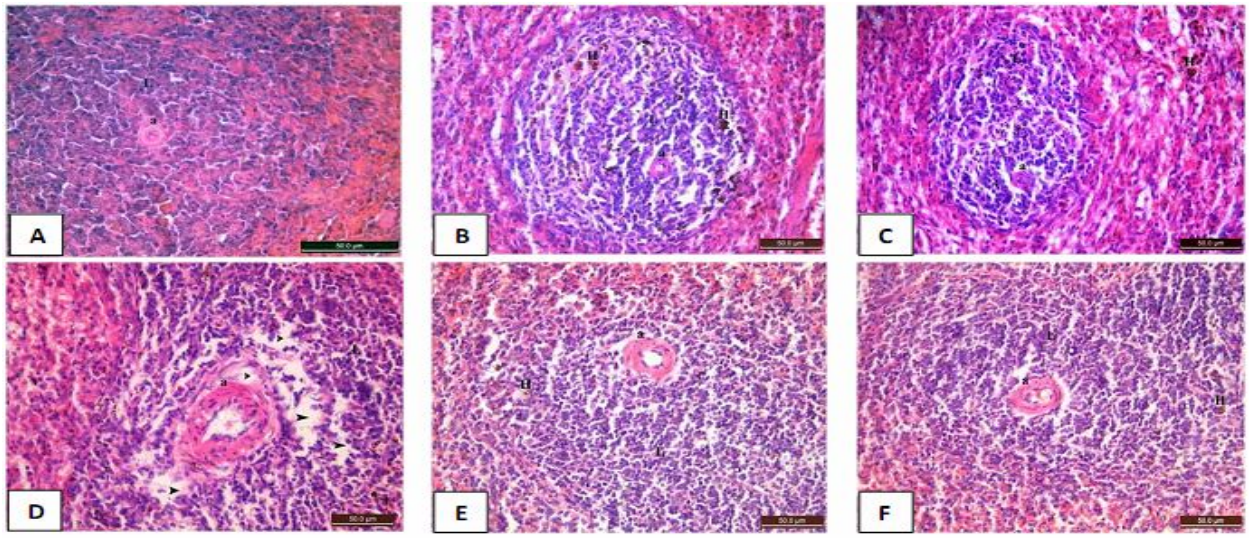


Fig. (2): Photomicrographs of the same preceding spleen sections at higher magnification from different studied groups. (A) (control) shows the central artery of the white pulp (a) surrounded by lymphocyte population (L). (B) (Pentoxifylline) and (C) (Nigella sativa) are nearly similar to control. Hemosiderin pigment (H) is also shown. (D) (CTX) marked thickened and dilated central artery (a) surrounded by lymphocytes (L), which show many losses (arrowheads). (E) (Pentoxifylline + CTX) mild thickened and dilated central artery (a). (F) (Nigella sativa + CTX) mild thickened and dilated central artery (a) surrounded by lymphocyte population (L). (H&E X400)

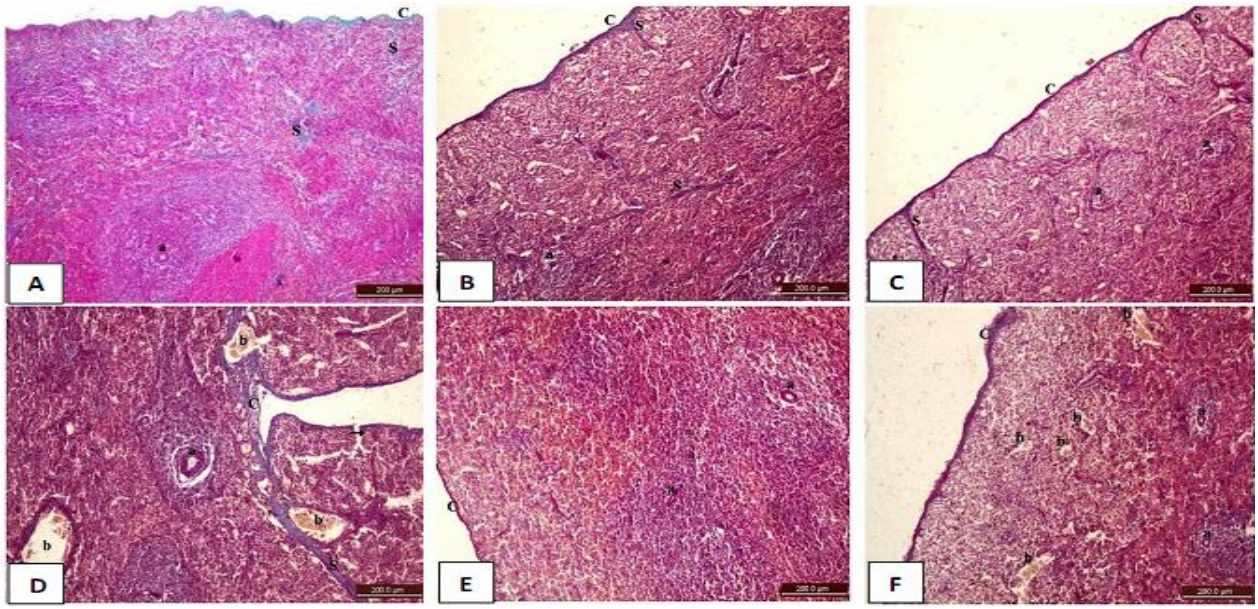


Fig. (3): Photomicrographs of spleen sections stained with Masson trichrome stainX100 from different studied groups. (A) (control) shows capsule (C) & Septa (S) & central artery (a). (B)(Pentoxifylline) & (C) (Nigella sativa) are nearly similar to control. (D) (CTX) Disorganized capsule (C) & disrupted in some areas (arrow), thickened central artery (a), congested blood sinusoids (b). (E) (Pentoxifylline + CTX) nearly similar to control. (F) (Nigella sativa + CTX) thickened capsule (C), congested blood sinusoids (b). Central arteries (a) are almost as control group.

Statistical analysis of the optical density of lymphocyte immunostaining in different groups is shown in (Histograms 1 & 2). Regarding CD3 immunostaining, T lymphocytes surrounding the central artery in a periarterial lymphatic sheath (PALS), in the white pulp, showed a positive cytoplasmic reaction in control, Pentoxifylline& Nigella sativa groups (Figure. 4 A, B & C). There was no significant difference between these three groups ($P > 0.05$). CTX group showed a significant decrease in CD3 reaction in all animals of group IV compared to previous groups ($P < 0.05$) (Figure. 4D). Compared to the CTX group, the reaction was significantly increased in both (Pentoxifylline + CTX) and (Nigella sativa + CTX) ($P < 0.05$) (Histogram 1 & Figure. 4 E & F). Regarding the cytoplasmic reaction of B lymphocytes, comparable results were found on examination of sections stained with CD20 immunostaining (Histogram 2 & Figure. 5 A-F).

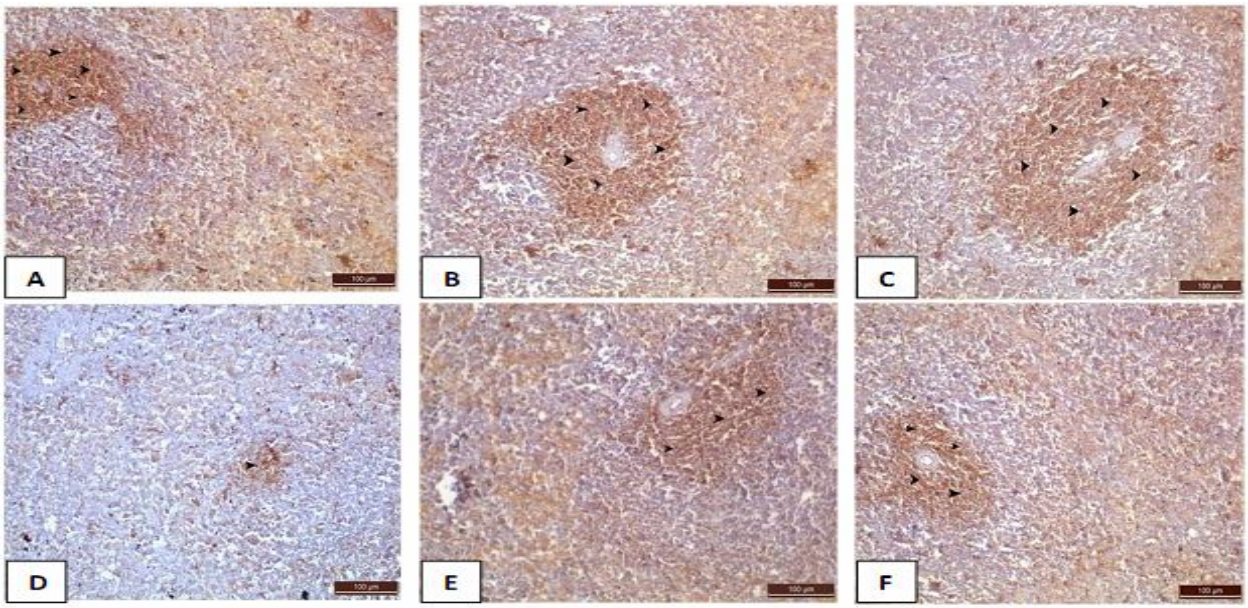


Fig. (4): Photomicrographs of spleen sections from different groups, showing a positive cytoplasmic reaction (arrowheads) of T lymphocytes surrounding the central artery of the white pulp in (A) (control), (B) (Pentoxifylline) &(C) (Nigella sativa). (D) (CTX) shows a weak reaction. (E) (Pentoxifylline + CTX) shows increased reaction compared to D. (F) (Nigella sativa + CTX) shows increased reaction compared to D, but less than the control. (CD3 immuno-staining X200)

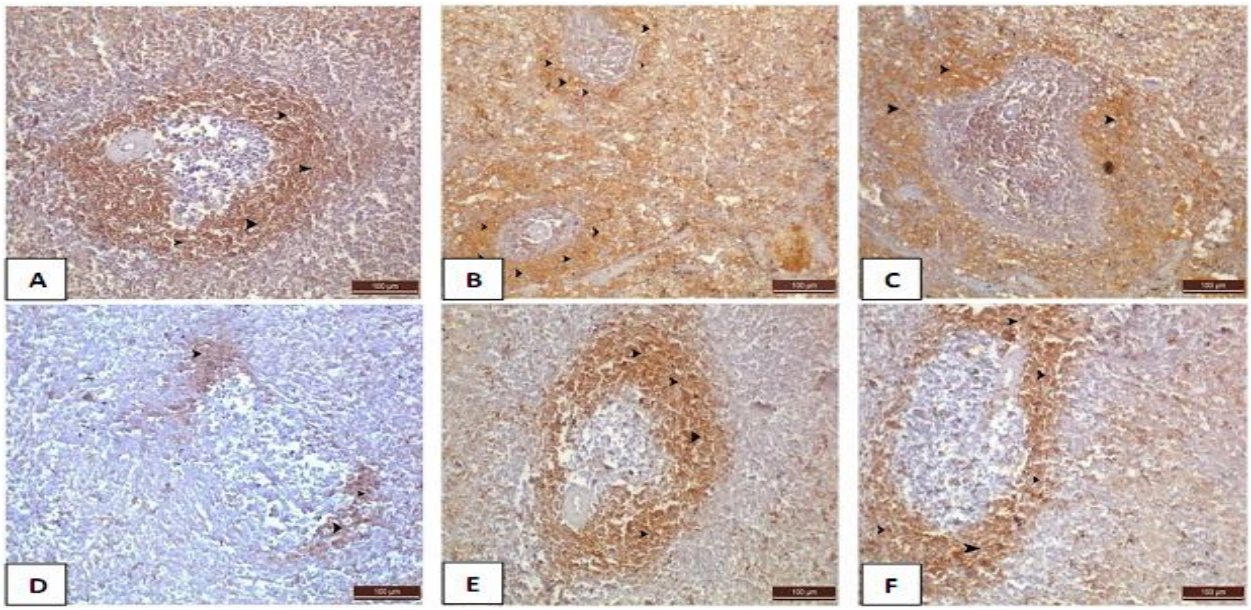
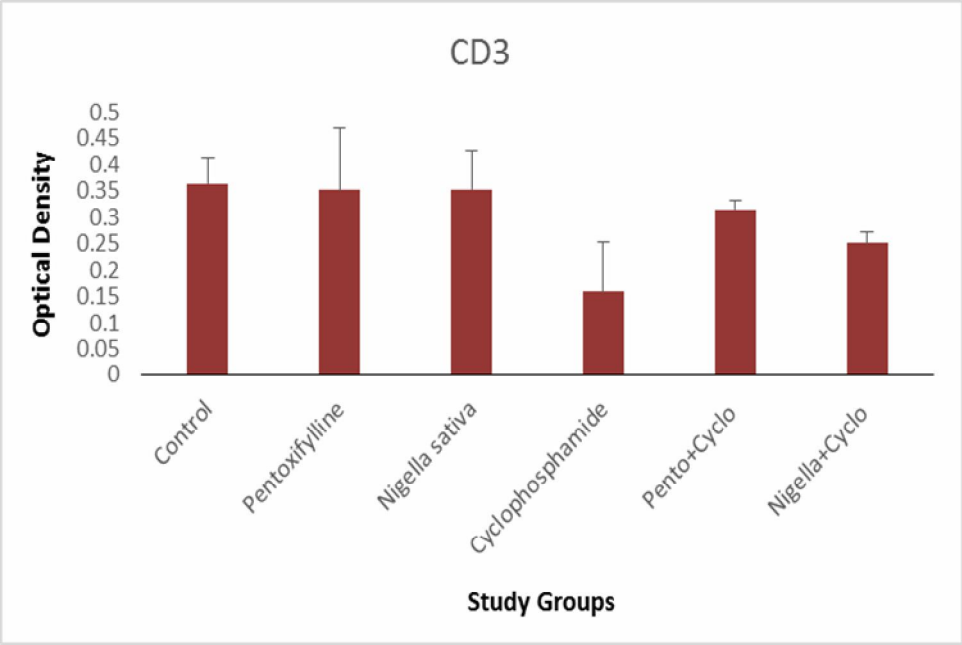
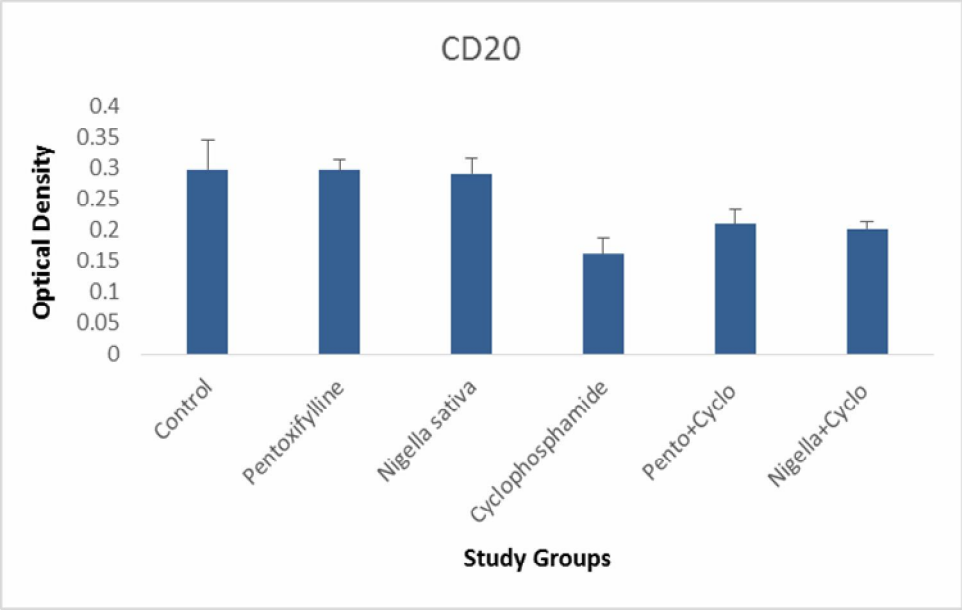


Fig. (5): Photomicrographs of spleen sections stained from various groups, displaying a positive cytoplasmic reaction (arrowheads) of B lymphocytes spread through the rest of the white pulp in (A) (control), (B) (Pentoxifylline) &(C) (Nigella sativa). (D) (CTX) shows a weak reaction. (E) (Pentoxifylline + CTX) shows increased reaction compared to D. (F) (Nigella sativa + CTX) shows increased reaction compared to D, but less than the control. (CD20 immuno-staining X200)

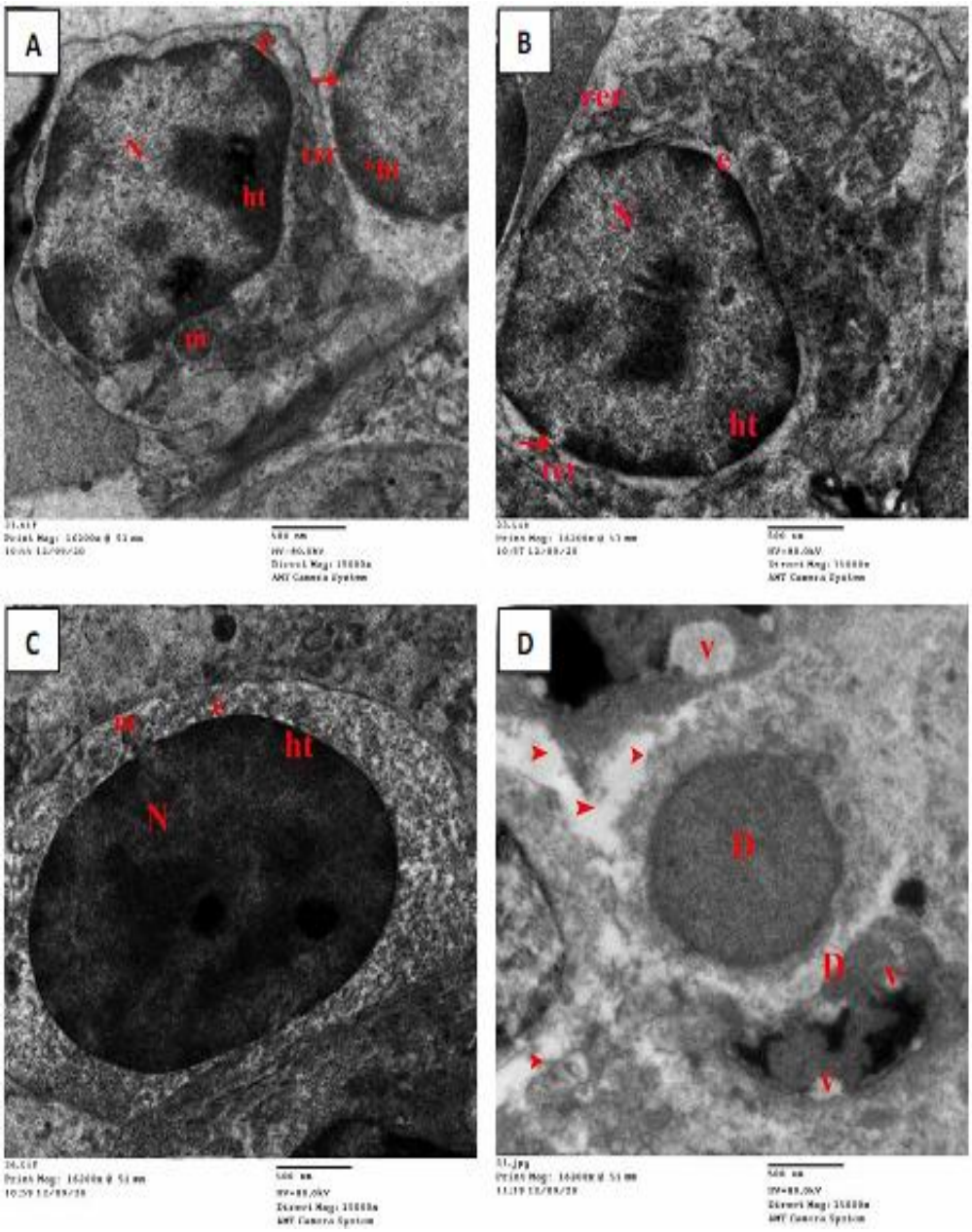


Histograms 1: The mean ± SD of optical density of CD3 immunostaining in different groups (n = 6).



Histograms 2: The mean ± SD of optical density of CD20 immunostaining in different groups (n = 6).

Group I (control group) showed a lymphocyte with a central nucleus, nuclear envelope, and electron-dense nuclear heterochromatin. The cytoplasm showed normal mitochondria and rough endoplasmic reticulum (Figure. 6A). Groups II & III (Pentoxifylline & Nigella sativa were similar to the control group (Figure. 6B & 6C). Group IV (CTX group) showed vacuoles in the nuclear sap and tissue loss. No cytoplasmic organelles were seen (Figure. 6D). Group V (Pentoxifylline + CTX) showed a lymphocyte nucleus, similar to the control group. Cytoplasmic/ tissue loss and vacuolated mitochondria were also noticed (Figure. 6E). Group VI (Nigella sativa + CTX) showed a lymphocyte which was more or less similar to that of the control group. However, many cytoplasmic vacuoles and tissue loss were also shown (Figure. 6F).



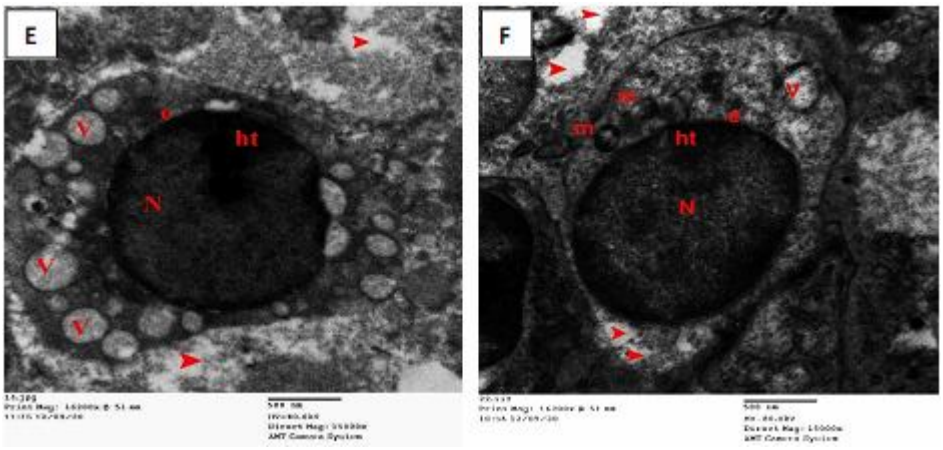


Fig. (6): Electron photomicrograph of splenic white pulp lymphocytes from groups A, B, C, D, E & F (TEMX 15000). (A) (control) shows a lymphocyte with a central nucleus (N), nuclear envelope (e), and electron-dense nuclear heterochromatin (ht). Mitochondria (m) and rough endoplasmic reticulum (rer) are shown. Heterochromatin of adjacent lymphocyte nucleus (ht*) and nuclear pore (arrow) is also demonstrated. (B) (Pentoxifylline) and (C) (Nigella sativa) are nearly similar to the control. (D) (CTX) displaying nuclei of some adjacent lymphocytes (D). Vacuoles (V) in the nuclear sap and tissue loss can be seen (arrowheads). No cytoplasmic organelles were seen. (E) Pentoxifylline + Cyclophosphamide showing lymphocyte nucleus (N), nearly similar to the control group, with nuclear envelop (e) and nuclear heterochromatin (ht). Cytoplasmic/tissue loss (arrowheads) and vacuolated mitochondria (m). (F) Nigella sativa + cyclophosphamide group showing a lymphocyte with nucleus (N), nuclear envelop (e) and nuclear heterochromatin (ht) more or less similar to the control group. Many cytoplasmic vacuoles (V) and tissue loss can be seen (arrowheads).

Discussion

Cyclophosphamide is a commonly used chemotherapeutic drug involved in treating many adult and pediatric malignancies, as well as an immunosuppressive agent used to treat refractory autoimmune disorders (Hughes et al., 2018). It is associated with several organ toxicities including nephrotoxicity, gonadal toxicity in the form of oligospermia or azoospermia, and ovarian failure. Neurotoxicity, cardiotoxicity, bladder toxicity, teratogenic effects, increased risk of late malignancy & immunotoxicity were also reported (Barnes et al., 2017). Cyclophosphamide immunotoxicity was reported to occur in the form of serious functional and structural damage to both central and peripheral components of lymphoid tissues (Huyan et al., 2011). For this reason, patients on cyclophosphamide chemotherapy are more susceptible to infection with reduced quality of life and compliance with treatment (Zimecki et al., 2014). Therefore, much research was performed on cyclophosphamide with other adjuvant treatments that could stimulate the immune system and overcome cyclo-phosphamide immunotoxicity, without interfering with its antineoplastic activity. Currently, great attention was paid to natural plants searching for their immunomodulatory properties (Song et al., 2014). As a result, this work intended to investigate and compare the possible protective effect of pentoxifylline and nigella sativa on CTX-induced splenic toxic effects, using light microscopy, immunohistochemical, and electron microscopic techniques.

In the current study, CTX caused significant splenic damage as evidenced by capsule disruption/disorganization, depletion of lymphocytes in both white and red pulp, and congested blood vessels. Our results are in accordance with other studies that showed a reduction in lymphoid cellularity of the spleen in CTX-treated rats (Senin et al., 2020). Moreover, the greatest cyclophosphamide cytotoxicity against cells of the immune and hematopoietic systems was due to the active and continuous replication of their DNA resulting in serious cytotoxic damage (Abd Elhalim et al., 2017).

Light microscopic findings of this study were confirmed ultra-structurally, as EM results showed degenerated lymphocytes with cytoplasmic vacuoles and tissue loss, in the CTX group. This was in accordance with other several studies (Gu et al., 2017; Anan et al., 2017; Li et al., 2018; Ekiz et al., 2020). These findings can be attributed to the oxidative stress generated by cyclophosphamide toxic metabolites and reactive oxygen species (ROS) which resulted in the disruption of the antioxidant intracellular mechanisms. This disruption occurred via DNA crosslinking, lipid peroxidation, and oxidative damage of protein causing eventually cellular injury followed by apoptosis (Guneli et al., 2008; Johnson et al., 2011).

Regarding immunohistochemical results, in the present study, there was a weak CD3 reaction in the spleens of CTX-treated animals due to the massive depletion of T lymphocytes surrounding the central artery in a periarterial lymphatic sheath (PALS) in the white pulp. Cyclophosphamide has apoptotic, necrotic, and anti-proliferative direct effects on many types of cells, especially lymphocytes. Zimecki et al. (2014) reported the deterioration of levels of circulating and splenic lymphocytes in mice treated with CTX. Ustunsoy et al. (2016) also proved CTX's damaging effects on human lymphocytes. Moreover, Qi et al. (2018) reported that treatment with CTX rapidly declined the activity of all lymphoid tissue. Additionally, Mackall et al. (1994) observed that CTX decreased the amount of $CD3^+$ T cells in the patients' peripheral blood during day one of the institution therapy and persistence of that reduction throughout the whole therapy duration.

Concerning the current work, using CD20, a marker of the B lymphocyte population, it revealed a weak reaction in the spleen of animals treated with CTX due to the severe damage of B lymphocytes in the white pulp. This can be attributed to the profound depletion of B cells because of dose-intensive cytotoxic antineoplastic therapy (Abd Elhalim et al., 2017). Moreover, Al-Joufi et al. (2022) findings suggested that CTX suppresses the lymphocyte count and function in the spleen and Peyer's patches of the ileum in a time-dependent fashion, especially B cells which could lead eventually to bacteraemia.

Current study results revealed that pre-treatment of animals with pentoxifylline reversed almost all the histopathological changes caused by CTX apart from mild thickening and dilatation of the central artery. The protective activity of pentoxifylline was through regulating the secretion of proinflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and IL-6, together with nitric oxide and preserving the cellular antioxidants (Abo-Salem, 2013). Furthermore, the protective effect of pentoxifylline against other chemotherapeutic agents was also investigated. Al-Saedi, et al. (2022) showed its efficacy in ameliorating Adriamycin-induced cardiotoxicity in rabbits. Pavitrakar et

al. (2022) studied pentoxifylline's protective effect against cisplatin-induced nephrotoxicity, they concluded that PTX restored renal functions, and decreased inflammation and oxidative stress of renal tissue.

Additionally, the regenerative effect of pentoxifylline was confirmed ultra-structurally in Pentoxifylline and CTX group. Transmission electron microscopy studies showed the ability of pentoxifylline in the restoration of the lymphocyte with its nucleus, nuclear heterochromatin, and nuclear envelope. These findings were consistent with the conclusions provided by Queiroz et al. (2013) who observed that treatment of adult rats with pentoxifylline inhibited the apoptotic cell mechanisms and decreased the pathological lesions induced by testicular heat shock, improving spermatogenesis regeneration. However, some vacuolated mitochondria were still observed which may be attributed to the potent degenerative effect of cyclophosphamide (Ekiz et al., 2020).

Pentoxifylline administration remarkably restored T and B lymphocyte populations of the spleen which was confirmed by the marked increased reaction of both CD3 and CD20, respectively. In contrast to our results, González-Amaro et al. (1998) showed that PTX reduced both T lymphocyte cell cycle progression and proliferation mediated by CD3. Wang et al. (1997) described the same inhibitory effect of PTX on T cells by blocking the expression of the c-Rel transcription factor, which participates in T lymphocyte activation. However, his results confirmed that PTX did not suppress B cells. The difference in T lymphocyte outcomes between the current research and prior studies might be related to the use of various PTX dosages and durations. González-Amaro et al. (1998) discovered that the inhibitory action of PTX on T cells was dosage dependent and appeared early, between 12 and 24 hours. In our work, the stimulatory impact of PTX on T cells occurred after 10 days of administration. A variety of study designs were also employed. Our work used mature splenic cells in vivo, whereas their investigation used normal human lymphocytes or a mouse cell line in vitro. This might explain why PTX has such a distinct effect on T cell proliferation in vitro and in vivo. Pentoxifylline appears to limit T-cell growth in vitro but not in vivo (Wang et al., 1997).

In the current study, examination of H & E and Masson trichrome stained sections of nigella sativa and cyclophosphamide group revealed that nigella sativa could restore some histopathological changes caused by CTX. It was suggested that the underlying protective mechanism of nigella sativa was due to its antitoxic effects which occur through the induction of antioxidant mediators (Mekhemar et al., 2020). The administration of nigella sativa inhibited the generation of reactive oxygen species (ROS) and increased the level of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione (GSH) (Zaki, 2019). Besides improving the endogenous antioxidant enzymes, the protective effect of nigella sativa against CTX-induced haemorrhagic cystitis occurred via lowering inflammatory cytokine levels such as TNF-α, IL-6, and IL-1β. Moreover, DNA fragmentation and lipid peroxidation were also reduced. It was also noticed that nigella sativa was able to restore urinary bladder functions by maintaining the structure and morphology of its tissues (Gore et al., 2016). However, in our study, we found that the blood sinusoids were still obviously congested. Therefore, it is concluded that PTX has a better resolving effect on vascular

congestion, caused by cyclophosphamide, more than nigella sativa. Our results confirm the results of other researchers who observed the decongestant effect of pentoxifylline on blood vessels against both amikacin and amphotericin B-induced nephrotoxicity, which was due to reducing erythrocyte entrapment, platelet aggregation, neutrophil sequestration, and retrieving blood perfusion of the kidney (Vadiei et al., 1996; Ozer et al., 2009). Furthermore, by reducing medullary hyperaemia and renal dysfunction, pentoxifylline alleviated vascular congestion in ischemic acute renal failure. It has been shown that pentoxifylline reduces vascular congestion by increasing prostaglandin synthesis and altering adenine nucleotide metabolism (Nasiri-Toosi et al., 2012).

Moreover, ultrastructural changes of the spleen of animals that received nigella sativa concomitant with CTX, revealed that nigella sativa could also ameliorate the degenerative effect of cyclophosphamide but also with persistent cytoplasmic vacuoles and tissue loss. These findings corroborated previous research on other chemotherapeutic agents which reported that nigella sativa could partially decrease vacuolar degeneration after intraperitoneal injection of carboplatin (Erisgin et al., 2019). It was also found that administration of nigella sativa resulted in the preservation of hepatic histoarchitecture after hepatotoxicity induced by tramadol but with residual cytoplasmic vacuolations and darkly stained nuclei of hepatocytes (Omar and Mohammed, 2017).

In the current work, results demonstrated that nigella sativa administration had a protective role against CTX and could restore both splenic T & B lymphocytes. This agreed with Abuharfeil et al. (2001) who observed that oral administration of aqueous extracts of nigella sativa seeds for 1 week resulted in a two-fold rise in the number and function of splenic non-killer (NK) cells as compared to control NK cells, targeting YAC-1 tumour targets. Furthermore, the boosting effects of nigella sativa on the B lymphocytes were concluded by Ebaid et al. (2011) who reported that oral administration of nigella sativa oil for 30–60 days can boost humoral immunological responses. This was evidenced by antibody hemagglutination titre that almost returned to normal levels in a time-dependent way, after treatment with chloramphenicol in albino rats. Moreover, it was reported that intraperitoneal injection of nigella sativa in a dose of 1 mg/kg once daily for 7 days, in male albino rats, reversed the pesticide - stimulated immune-toxicological effects resulting in higher total immunoglobulins (Ig) levels (especially IgGs) and antibody hemagglutination (Mohany et al., 2012).

In conclusion, both pentoxifylline and nigella sativa resulted in the protection of splenic tissue against CTX -induced cytotoxicity based on light and electron microscopic results. However, Pentoxifylline was more effective than nigella sativa. Accordingly, authors recommend PTX administration for cancer patients before and during CTX therapy, to prevent or at least minimize its immunosuppressive effect.

Conflicts of interest

The authors state that they have no conflicts of interest.

Funding sources

This study was not funded by any funding agency.

References

Abd Elhalim, S., Sharada, H., Abulyazid, I., et al. (2017). "Ameliorative effect of Carob Pods extract (Ceratonia siliqua L.) against cyclophosphamide-induced alterations in bone marrow and spleen of rats," *Journal of Applied Pharmaceutical Science*, 7(10), pp.168–181. Doi:10.7324/japs.2017.71025.

Abo-Salem, O.M. (2013). "Uroprotective effect of pentoxifylline in cyclophosphamide-induced hemorrhagic cystitis in rats," *Journal of Biochemical and Molecular Toxicology*, 27(7), pp.343–350. Doi:10.1002/jbt.21494.

Abuharfeil, N.M., Salim, M. and Von Kleist, S. (2001). "Augmentation of natural killer cell activity in vivo against tumor cells by some wild plants from Jordan," *Phytotherapy Research*, 15(2), pp. 109–113. Doi:10.1002/ptr.692.

Al-Joufi, F.A., Senin, M.M., Matloub, G.I., et al. (2022). "Histological and immunohistochemical study of the protective effect of virgin coconut oil on cyclophosphamide-induced immunotoxicity of the spleen and Peyer's patches," *International Journal of Health Sciences*, 6(S2), pp.1092–1107. Doi:10.53730/ijhs.v6ns2.5151.

Al-Malki, A.L. and Sayed, A.A.R. (2014). "Thymoquinone attenuates cisplatin-induced hepatotoxicity via nuclear factor kappa- β," *BMC Complementary and Alternative Medicine*, 14(1). Doi:10.1186/1472-6882-14-282.

Al-Saedi, H., Hussein, U., and Ramadhan, M. (2022). "Protective effects of pentoxifylline against adriamycin-induced cardiotoxicity in rabbits," *Journal of Pharmaceutical Negative Results*, 13(2), pp. 51–56. Doi: 10.47750/pnr.2022.13.02.008.

Anan, H.H., Wahba, N.S., Abdallah, M.A., et al. (2017). "Histological and immunohistochemical study of cyclophosphamide effect on adult rat testis," *International Journal of Scientific Reports*, 3(2), pp.39. doi:10.18203/issn.2454-2156.intjscirep20170356.

Barnes, H., Holland, A., Westall, G., et al. (2017). "Cyclophosphamide for connective tissue disease-associated interstitial lung disease," *Chest*, 152(4), p.A442. Doi: 10.1016/j.chest.2017.08.469.

Brayfield, A., ed. "Cyclophosphamide: Martindale: The complete drug reference". Medicines Complete. London, UK: *Pharmaceutical Press*. Retrieved 12 August 2017.

Cengiz, M. (2018). "Hematoprotective effect of boron on cyclophosphamide toxicity in rats," *Cellular and Molecular Biology*, 64 (5), pp. 62. Doi:10.14715/cmb/2018.64.5.10.

Dantas, A.C.B., Batista-Júnior, F.F. de A., Macedo, L.F., et al. (2010). "Protective effect of Simvastatin in the cyclophosphamide-induced hemorrhagic cystitis in rats," *Acta Cirurgica Brasileira*, 25(1), pp.43–46. Doi:10.1590/s0102-86502010000100011.

Ebaid, H., Dkhil, M., Zahran, W., et al. (2011). "Role of Nigella sativa in ameliorating chloramphenicol induced tissue damage in rats," *J. Med. Plant Res*, 5(2), pp.280–288.

Ekiz Y., Taşdemir, M., Kaya, M., et al. (2020). "The effects of magnesium sulfate on cyclophosphamide-induced ovarian damage: Folliculogenesis," *Acta Histochemica*, 122(2), pp.151470.

Doi: 10.1016/j.acthis.2019.151470.

Elshater, A.-E.A., Haridy, M.A.M., Salman, M.M.A., et al. (2018). "Fullerene C60 nanoparticles ameliorated cyclophosphamide-induced acute hepatotoxicity in rats", *Biomedicine & Pharmacotherapy*, 97, pp. 53–59.

Doi:10.1016/j.biopha.2017.10.134.

Entok, E., Ustuner, M.C., Ozbayer, C., et al. (2014). "Anti-inflammatory and anti-oxidative effects of Nigella sativa L.: 18FDG-PET imaging of inflammation", *Molecular Biology Reports*, 41(5), pp.2827–2834.

Doi:10.1007/s11033-014-3137-2.

Erisgin, Z., Atasever, M., Cetinkaya, K., et al. (2019). "Protective effects of Nigella sativa oil against carboplatin-induced liver damage in rats," *Biomedicine & Pharmacotherapy*, 110, pp.742–747. Doi: 10.1016/j.biopha.2018.12.037.

González-Amaro, R., Portales-Perez, D., Baranda, L., et al. (1998). "Pentoxifylline inhibits adhesion and activation of human T lymphocytes," *J Immunol,* 161, pp.65-72.

Gore, P.R., Prajapati, C.P., Mahajan, U.B., et al. (2016). "Protective effect of Thymoquinone against cyclophosphamide -induced hemorrhagic cystitis through inhibiting DNA damage and upregulation of Nrf2 expression", *International Journal of Biological Sciences*, 12(8), pp.944–953.

Doi:10.7150/ijbs.15781.

Gu, Y.P., Yang, X.M., Duan, Z.H., et al. (2017). "Squid ink polysaccharide prevents autophagy and oxidative stress affected by cyclophosphamide in Leydig cells of mice: A pilot study," *Iran J Basic Med Sci*, 20, pp.1194-1199.

Guneli, E., Tugyan, K., Ozturk, H., et al. (2008). "Effect of melatonin on testicular damage in streptozotocin-induced diabetes rats," *European Surgical Research*, 40(4), pp.354–360.

Doi:10.1159/000118032.

Habib, N. and Choudhry, S. (2021). "HPLC Quantification of thymoquinone extracted from Nigella sativa L. (Ranunculaceae) seeds and antibacterial activity of its extracts against Bacillus Species," *Evidence -Based Complementary and Alternative Medicine,* pp.1–11.

Doi:10.1155/2021/6645680.

Horvath, B., Marton, Z., Halmosi, et al. (2002). "In vitro antioxidant properties of pentoxifylline, piracetam, and vinpocetine," *Clinical Neuro-pharmacology*, 25(1), pp.37–42.

Doi:10.1097/00002826-200201000-00007.

Hughes, E., Scurr, M., Campbell, E., et al. (2018). "T-cell modulation by cyclophosphamide for tumor therapy," *Immunology*, 154(1), pp.62–68.

Doi:10.1111/imm.12913.

Huyan, X.-H., Lin, Y.-P., Gao, T., et al. (2011). "Immunosuppressive effect of cyclophosphamide on white blood cells and lymphocyte subpopulations from peripheral blood of Balb/c mice," *International Immunopharmacology*, 11(9), pp.1293–1297.

Doi: 10.1016/j.intimp.2011.04.011.

Johnson, L.A., Malayappan, B., Tretyakova, N., et al. (2011). "Formation of cyclophosphamide specific DNA adducts in hematological

diseases," *Pediatric Blood & Cancer*, 58(5), pp.708–714. Doi:10.1002/pbc.23254.

Khader, M., and Eckl, P.M. (2014). "Thymoquinone: an emerging natural drug with a wide range of medical applications", *Iran J Basic Med Sci*, 17(12), pp. 950–957.

Khedr, N.F. (2015). "Protective effect of mirtazapine and hesperidin on cyclophosphamide-induced oxidative damage and infertility in rat ovaries," *Experimental Biology and Medicine*, 240(12), pp.1682–1689.

Doi:10.1177/1535370215576304.

Lee, E. J., Kim, M., Kim, H. S., et al. (2017). "CD3 and CD20 immunohistochemical staining patterns of bone marrow-infiltrating malignant lymphoma cells," *Annals of Clinical and Laboratory Science*, 47(2), pp136–143.

Li, W., Guo, S., Xu, D., et al. (2018). "Polysaccharide of Atractylodes macrocephala koidz (PAMK) relieves immunosuppression in cyclophosphamide -treated geese by maintaining a humoral and cellular immune balance," *Molecules*, 23(4), pp.932.

Doi:10.3390/molecules23040932.

Mackall, C., Fleisher, T., Brown, M., et al. (1994). "Lymphocyte depletion during treatment with intensive chemotherapy for cancer," *Blood*, 84(7), pp.2221–2228.

Doi:10.1182/blood. v84.7.2221.2221.

McCarty, M.F., O'Keefe, J.H. and DiNicolantonio, J.J. (2016). "Pentoxifylline for vascular health: a brief review of the literature," *Open Heart*, 3(1), pp. e000365.

Doi:10.1136/openhrt-2015-000365.

Mekhemar, M., Hassan, Y. and Dörfer, C. (2020). "Nigella sativa and Thymoquinone: A natural blessing for periodontal therapy," *Antioxidants*, 9(12), pp.1260.

Doi:10.3390/antiox9121260.

Mohammed, S.S.; Mohamed M.N.; Abdel Gawad, S.K.; and Mahmoud, S.H. (2010). "Possible protective effect of Nigella sativa oil against piroxicam-induced gastric mucosal damage in adult male albino rats (Light and scanning electron microscopic study)," *The Egyptian Journal of Histology*, 33(1), pp.127–139.

Doi:10.1097/00767537-201003000-00013.

Mohany, M., El-Feki, M., Refaat, I., et al. (2012). "Thymoquinone ameliorates the immunological and histological changes induced by exposure to imidacloprid insecticide," *The Journal of Toxicological Sciences*, 37(1), pp. 1–11.

Doi: 10.2131/jts.37.1.

Nasiri-Toosi, Z., Dashti-Khavidaki, S., Khalili, H., et al. (2012). "A review of the potential protective effects of pentoxifylline against drug-induced nephrotoxicity," *European Journal of Clinical Pharmacology*, 69 (5), pp.1057–1073.

Doi:10.1007/s00228-012-1452-x.

Omar, N.M. and Mohammed, M.A. (2017). "The impact of black seed oil on tramadol-induced hepatotoxicity: Immunohistochemical and ultra-structural study," *Acta Histochemica*, 119(5), pp.543–554.

Doi: 10.1016/j.acthis.2017.05.008.

Ozer, M.K., Asci, H., Oncu, M., et al. (2009). "Effects of pentoxifylline on amikacin-induced nephrotoxicity in rats," *Ren Fail,* 31, pp.134–139.

Pavitrakar, V.N., Mody, R. and Ravindran, S. (2022). "Protective effects of recombinant human golimumab and pentoxifylline in nephrotoxicity induced by cisplatin," *Journal of Biochemical and Molecular Toxicology*, 36(4). Doi:10.1002/jbt.22990.

Qi, Q., Dong, Z., Sun, Y., et al. (2018). "Protective effect of bergenin against cyclophosphamide-induced immuno-suppression by immuno-modulatory effect and antioxidation in Balb/c mice," *Molecules*, 23 (10), pp.2668. Doi:10.3390/molecules23102668.

Queiroz, G.C.D., Oliveira, V.V.G., Gueiros, O.G., et al. (2013). "Effect of pentoxifylline on the regeneration of rat testicular germ cells after heat shock," *Anim. Reprod,* 10(1), pp. 45-54.

Senin, M.M., Al-Ani, I.M., Mahmud, M.I.A.M., et al. (2020). "Protective effect of virgin Coconut oil on cyclophosphamide-induced histological changes in lymphoid tissues," *IIUM Medical Journal Malaysia*, 17(3). Doi:10.31436/imjm.v17i3.246.

Song, Y.H., Sun, H., Zhang, A.H., et al. (2014). "Plant-derived natural products as leads to anti-cancer drugs," *J. Med. Plant Herb. Ther. Res.*, 2, pp. 6-15.

Suddek, G.M., Ashry, N.A., and Gameil, N.M. (2013). "Thymoquinone attenuates cyclophosphamide-induced pulmonary injury in rats," *Inflammo-pharmacol*, 21, pp 427-435.

Ulasli, S.S., Celik, S., Gunay, E., et al. (2013). "Anticancer effects of Thymoquinone, caffeic acid phenethyl ester, and resveratrol on A549 non-small cell lung cancer cells exposed to benzo(a)pyrene", *Asian Pacific Journal of Cancer Prevention*, 14(10), pp.6159–6164. Doi:10.7314/apjcp.2013.14.10.6159.

Ustunsoy, S., Akal, Z.U., and Alpsoy, L. (2016). "Protective role of Gossypetin against cyclophosphamide toxicity in human lymphocyte culture in vitro*", Medicinal Chemistry*, 6(2), pp. 088-092.

Vadiei, K., Tucker, S.D., Lopcz-Berestein, G., et al. (1996). "Nephroprotective mechanism(s) of pentoxifylline: Reduction of erythrocyte-mediated vascular congestion and inhibition of nitric oxide release" *Pharmacology & Toxicology*, 78(3), pp.174–180. Doi:10.1111/j.1600-0773. 1996.tb00200. x.

Vircheva, S., Alexandrova, A., Georgieva, A., et al. (2010). "In vivo effects of pentoxifylline on enzyme and non-enzyme antioxidant levels in rat liver after carrageenan-induced paw inflammation," *Cell Biochemistry and Function*, 28(8), pp.668–672. Doi:10.1002/cbf.1705.

Wang, W., Tam, W.F., Hughes, C.C.W., et al. (1997). "c-rel is a target of pentoxifylline-mediated inhibition of T lymphocyte activation", *Immunity*, 6(2), pp.165–174. Doi:10.1016/s1074-7613(00)80423-9.

Xu, X. and Zhang, X. (2015). "Effects of cyclophosphamide on immune system and gut microbiota in mice" *Microbiological Research*, 171, pp.97–106. Doi: 10.1016/j.micres.2014.11.002.

Zaki, S.M. (2019). "Evaluation of antioxidant and anti-lipid peroxidation potentials of Nigella sativa and onion extract on nicotine-induced lung damage", *Folia Morphologica*, 78(3), pp.554–563. Doi:10.5603/fm. a2018.0117.

Zimecki, M., Artym, J., Kocięba, M., et al. (2014). "Immune function in cyclophosphamide-treated mice is restored by the T-cell-tropic isoxazole derivative R-13", *Journal of Immunotoxicology*, 12(4), pp. 322–329. Doi:10.3109/1547691x.2014.978486.