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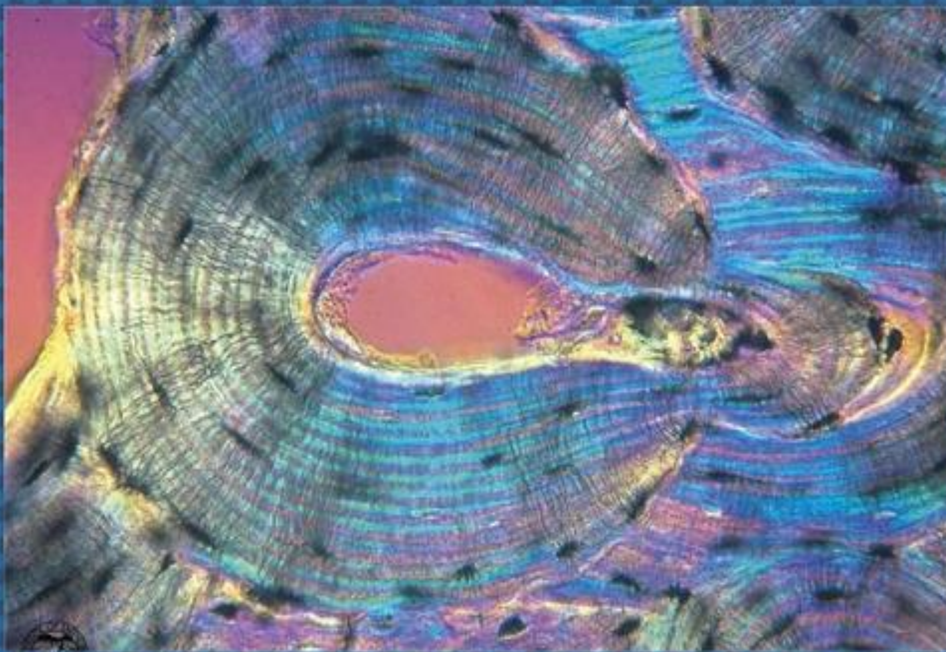

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Treatment Role of *Lagenaria siceraria* and Melatonin on Tramadol-Induced Changes in Spleen of Male Mice

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ABSTRACT

Tramadol is a utilized medication globally that is included in numerous medical guidelines for the treatment of both short-term and long-term pain. There is an increasing body of research indicating the prevalence of tramadol abuse in certain Middle East nations. Tramadol exhibits certain adverse effects.

The purpose of the present investigation is examination and treatment of the histopathological reaction that may develop in the spleen following tramadol administration.

The mice were treated via a dose of tramadol by daily injection for 20 days and 40 days. Additionally, groups of mice were administered tramadol for 40 days, followed by daily treatment with either distilled water, *Lagenaria siceraria* (L.), or melatonin (MT) for another 40 days.The administration of tramadol significantly impacted the quantity of collagen materials present in the spleen cells. As a result, modifications were observed in the typical histological structure of the spleen.Ultimately, we propose that while tramadol has various adverse effects on the spleen tissues of male mice, the use of L. and MT treatments exhibits therapeutic benefits in alleviating these tramadol-induced side effects.

INTRODUCTION

Pain is a significant health issue in the field of medicine. Although different analgesics have been introduced, opioid medications remain the preferred option for alleviating moderate to severe pain disorders that necessitate prolonged treatment (Ceccarelli *et al.,* 2006).

Opioids are the preferred initial treatment for patients who are admitted to the hospital and experiencing moderate to intense pain (Holden *et al.,* 2001). Currently, they are the most efficacious medications for pain relief. Opioids are highly strong and efficacious analgesics that have been widely recognized as suitable therapy options for severe, cancerous, and non-cancerous severe pain (Collet, 2001). Nevertheless, they can also be gratifying, and their frequent utilization can result in reliance and addiction. The rising number of deaths caused by opioid overdoses is more evidence that this addiction is a growing social, economic, and health concern (Hall *et al.,* 2008).

Both in human and veterinary medicine, the use of tramadol has been demonstrated to be effective in the treatment of mild to moderate pain in clinical settings (Pypendop & Ilkiw, 2008).

In addition, it is administered peri-operatively in veterinary anaesthesia to effectively decrease the need for volatile anaesthetics and opioid medications (Seddighi *et al.,* 2009).

Chronic usage of tramadol or morphine in rats leads to significant histological and biochemical alterations in the liver and kidney, resulting in liver and kidney dysfunctions (Atici *et al.,* 2005). *L. siceraria* was employed as a remedy for specific toxins. It exhibits antioxidant, immunomodulatory, antibacterial, anthelmintic, antifungal, anti-allergic, analgesic, cytotoxic, antihyperlipidemic, antidiabetic, anti-inflammatory, hepatoprotective, scavenger for free radicals, memory-enhancing activities and anxiolytic (Aslam, and Najam, 2013).

The pineal gland is the organ that is responsible for producing Melatonin which is a highly effective remover of ROS (Reiter, 2000). Thus, MT possesses the capacity to impede cellular oxidative harm induced by free radicals and can bolster the proper operation of the body's antioxidant defense mechanism (Kumar *et al.,* 1999). Additionally, it is able to activate many antioxidant pathways, hence increasing its functionality and enhancing the stability of cell membranes. Furthermore, it regulates the gene production of many defensive enzymes and decreases programmed cell death and the oxidation of lipids (Reiter & Rubin, 1998). The delivery of MT increased the proliferation of reactive oxygen species inside various tissues, as demonstrated by Cao *et al.* (2013).MT and its metabolite compounds possess anti-inflammation characteristics and have demonstrated significant efficacy in treating many illnesses associated with oxidative stress or inflammation (Carrillo-Vico *et al.,* 2005). Supplementing with MT may reduce the amount of oxidative damage that occurs in the tissues of the kidneys and help minimize renal scarring.

Pretreatment with MT in a cyclosporine-induced nephropathy model resulted in a reduction of kidney damage (Kumar *et al.,* 1999).

Tramadol possesses negative impacts on the male mice ileum at the cellular, chemical, and tissue levels. However, the use of *L. siceraria* and MT as therapies is more successful in alleviating these side effects caused by tramadol (Aref *et al.,* 2018). The objective of this study was to ascertain the detrimental impact of tramadol on the spleen tissues of mice. Furthermore, to authenticate the therapeutic function *of L. siceraria* and MT in mitigating these detrimental consequences.

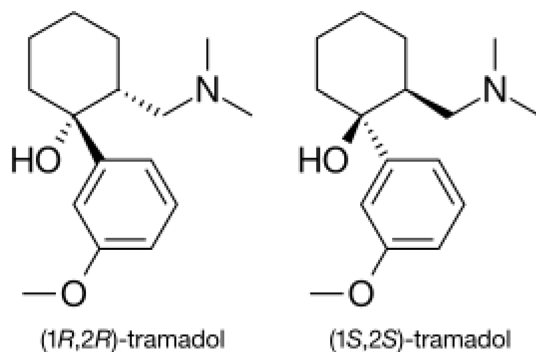
MATERIALS AND METHODS

Experimental Animals:

Total adult male Swiss albino mice were obtained from the Autoradiographic Lab. of Cell Biology and Immunology Studies, Faculty of Science, South Valley University. A group of adult male Swiss albino mice were maintained under identical circumstances of an “artificial light-dark cycle” (12 hours light, 12 hours dark), temperature (23 ± 2 degrees Celsius), and humidity. They were provided with unlimited access to normal food and water. The mice used in the trials had an average age of 90 ± 5 days and weighed approximately 30 ± 2 g each.

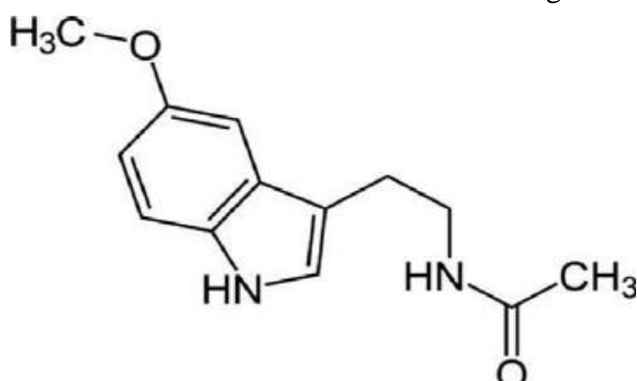
Chemicals and Drugs:

1- The ampoules of tramadol Hcl were purchased from “October Pharma S.A.E.”, located in October City, Egypt, in October 2014. The substances were diluted with distilled water until the concentration reached 150 ug/1ml.



Structural Formula of Tramadol

2- MT from Sigma Co. was solubilized in a small amount of 100% ethanol and then added to distilled water to be diluted to a concentration of 500 mg/ml.



Structural Formula of MT

3- *L. siceraria,* (Aref 1)

L. siceraria

Experimental Design:

The mice that were being tested were split into three groups ten of each.

First group: Group T1 consisted of mice that received subcutaneous daily injections of tramadol Hcl (125ug / 100g b.w.) for a duration of 10 days.

Second group: Group T2 consisted of mice that received a daily subcutaneous injection of tramadol Hcl at a consistent dose for a duration of 20 days.

Third group: Designated as group T_3: mice received the same daily dose of tramadol Hcl subcutaneously for 40 days.

Control group: Mice were categorized into three groups, with each group consisting of 10 male mice. These groups were labeled as C1, C2, and C3. The mice in these groups received a daily intravenous infusion of 25ml of distilled water/ 30g weight. The animals functioned as the control group.

The euthanization of all animals

occurred on the day subsequent to the final dose. Then experiments treated mice were categorized into 3 categories, designated as categories S, M and L, according to the treatment of animals. Mice of each group were categorized into 2 groups each has 10 males.

All treated mice of categories injected with (125ug / 100g b.w.) of tramadol Hcl daily throughout 40 days and mice were treated as follows:

Category S:

The first group: Referred to as Group T3+S1, was administered tramadol and received (0.25ml / 30g b.w.) distilled water daily for a period of 20 days.

The second group: Referred to as Group T3+S2, consisted of mice that were treated with tramadol. These mice were injected daily with a dose of distilled water, measuring 0.25ml per 30g of body weight, for a period of 40 days.

Category M:

The first group: Referred to as Group T3+M1, consisted of animals treated with tramadol. These animals received a daily subcutaneous injection of MT at a dose of 100 mg per 100 g body weight. The injection was administered at 16h, which is 2 hours prior to the end of the illumination period and continued for a duration of 20 days.

The second group: Referred to as Group T3+M2, consisted of animals treated with tramadol. These animals received a daily subcutaneous injection of MT at a dose of 100mg per 100g of body weight. The injection was administered at 16h, which is 2 hours prior to the end of the illumination period and continued for a duration of 40 days.

Category L:

First group: The animals in Group T3+L1, which were given tramadol, received an oral dose of a specialized formulation of "*L. siceraria"* (500mg/1k. b.w.) daily for a duration of 20 days.

Second group: Designated as Group T_3+L_2: tramadol-treated animals were treated with a daily oral dose of Special formulation of *L. siceraria* for (500 mg/ 1k. b.w.) 40 days.The euthanization of all animals occurred on the day subsequent to the final dose.Experiments of this research were conducted in the lab. achieve stability of environmental conditions, separation between treated animals and control ones and IACUC targets" IACUC Approval Project Number:(001/4/2016PN)" and All steps of this experiment were reviewed and approved by the Research Ethics Committee of the Faculty of Science at South Valley University with the approval number (No. 006/2/24).

Tissues Preparation:

Tissue samples of the spleen were collected for the following:

Histological and Histochemical Workup:

Throughout the entire trial length, a series of daily steps were performed to conduct the histological examination.

1- Fixation:

A 10% buffered formalin solution was used as the fixative reagent for the standard hematoxylin and eosin and histochemical stain. (Lillie, 1954).

2- Processing:

The preparation involved a first fixation process that included immersing the tissue in a 10% buffered formalin solution for two hours, followed by removing the fixative using distilled water for 30 minutes. The dehydration process involved sequentially immersing the tissues in alcohol solutions with varying concentrations (70%, 90%, and 100%). The tissues were first subjected to 70% alcohol for a duration of 30 minutes, then exposed to 90% alcohol for 1 hour, and then treated with two rounds of pure alcohol, each lasting for one hour. After dehydration, the samples were then cleared by immersing them in several solutions of xylene. The procedure involved immersing the tissue in a solution consisting of equal parts alcohol and xylene for one hour, followed by pure xylene for an additional one and a half hours. The samples were subsequently saturated with melted paraffin wax, followed by embedding and blocking. The paraffin sections, with a thickness of 4-5 micrometers, were

attached to slides (Underwood and Egan, 1985).

3- Items Of Staining:

A-Routine Histological Stain:

Sections were stained using the standard Harris's alum hematoxylin and eosin method (Davenport, 1960). The sections were initially de-paraffinized and hydrated with water, followed by staining with Harris's hematoxylin for a duration of 10 minutes. The sections were distinguished by immersing them 3 to 4 times in a solution of 1% acid alcohol after a brief washing in water. Subsequently, they were rinsed with flowing tap water for a duration of 20 minutes afterward, the sample was treated with eosin dye for a period ranging from 15 seconds to 2 minutes, based on the condition of the eosin and the desired level of counterstaining. For the final step, immerse the sample in 95% and absolute alcohols, repeating the process twice for 2 minutes every time or until the elimination of excess eosin. Rinse the sample in xylene twice, with each rinse lasting 2 minutes, and then mount it. The nuclei appear blue, whereas the cytoplasm appears pink.

2-Collagen Fibers:

Collagen fibers were visualized by Mallory trichrome technique. All methods were designed according to Carleton *et al*. (1980); Lewiński & Sewerynek (1984); Pearse (1980).

RESULTS

Spleen (Figs. 1-12):

The spleen of the control mice showed normal splenic pulps with normal lymphoid distribution in the white pulps and normal red blood corpuscles in the red pulps (Fig. 1). Spleen of the second stage of tramadol injection showed pronounced lymphoid depletion with protrusion of splenic trabeculae (Figs. 2 & 3) and Table (1). During third stage of tramadol injection, spleen exhibited severe necrosis and depletion of the splenic parenchyma (Fig. 4) and Table (1), besides marked protrusion of splenic trabeculae (Fig. 5) and Table (1). Also, there was extensive and severe vascular hypertrophy and congestion (Figs.6) and Table (1). The mice's spleen exhibited a moderate presence of collagen fibers and infiltration of fibrinous secretions following the cessation of tramadol injection. (Fig. 7), in addition to subcapsular lymphoid depletion and necrosis (Fig. 8). The mice were administered a combination of tramadol and *L. siceraria* detected a slight degree of lymphoid depletion of the white pulp (Fig. 9) with slight necrosis of splenic parenchyma (Fig. 10). Some of the mice that were administered tramadol in combination with MT exhibited normal white and red pulps in their splenic tissues. (Fig. 11) with a mild degree of lymphoid depletion in other mice (Fig. 12).

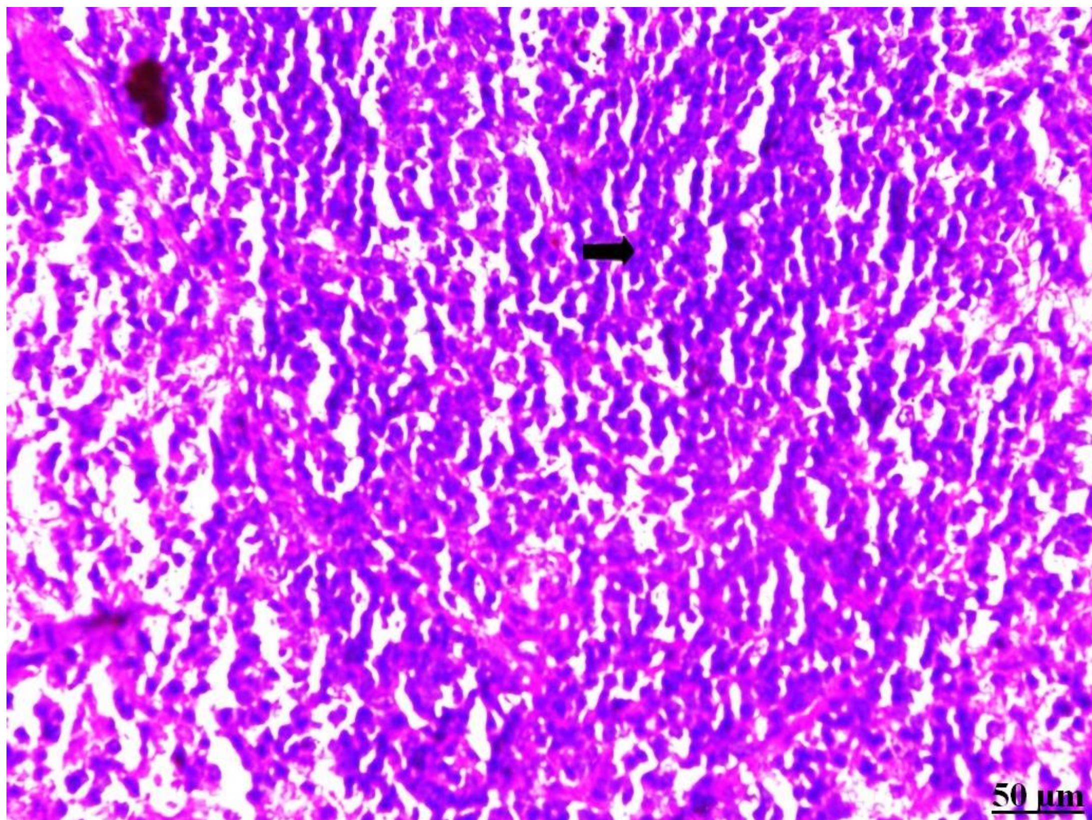


Fig. 1: Microscopy photograph of the spleen from the control mice displaying typical splenic pulps.

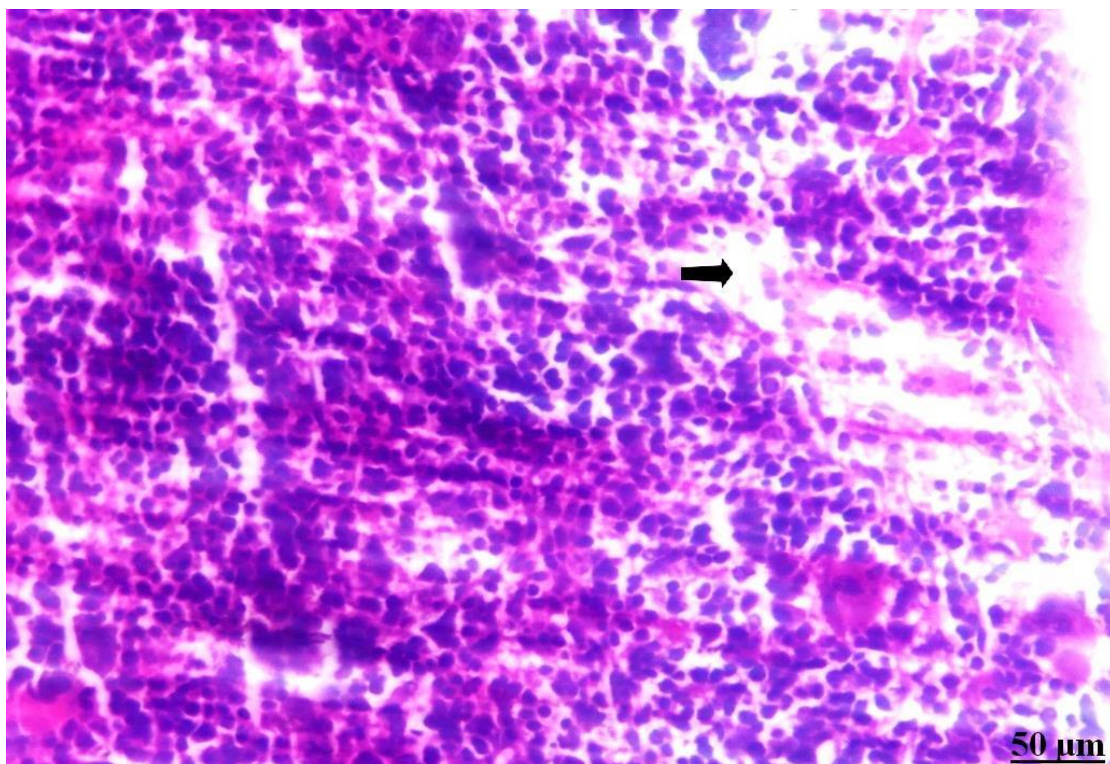


Fig. 2: Microscopy photograph of the spleen from mice who were given tramadol during the second stage showing significant depletion of lymphoid cells.

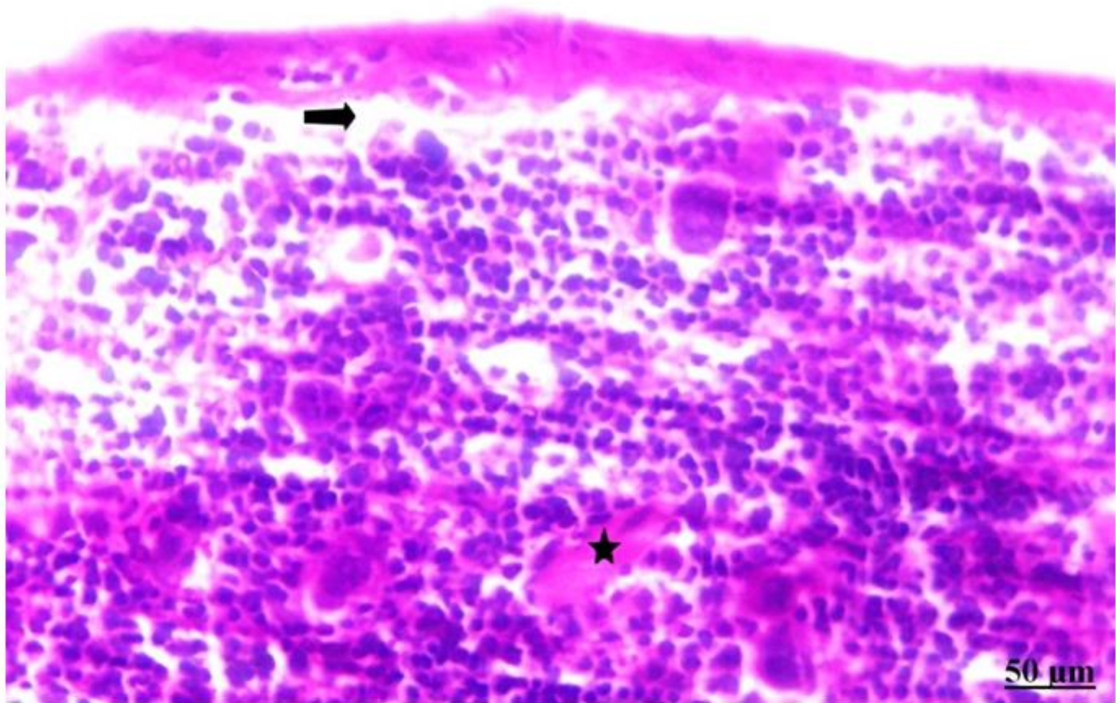


Fig. 3: Microscopy photograph of spleen of the mice administered tramadol (second stage) showing subcapsular lymphoid depletion (arrow) with protrusion of splenic trabeculae (star).

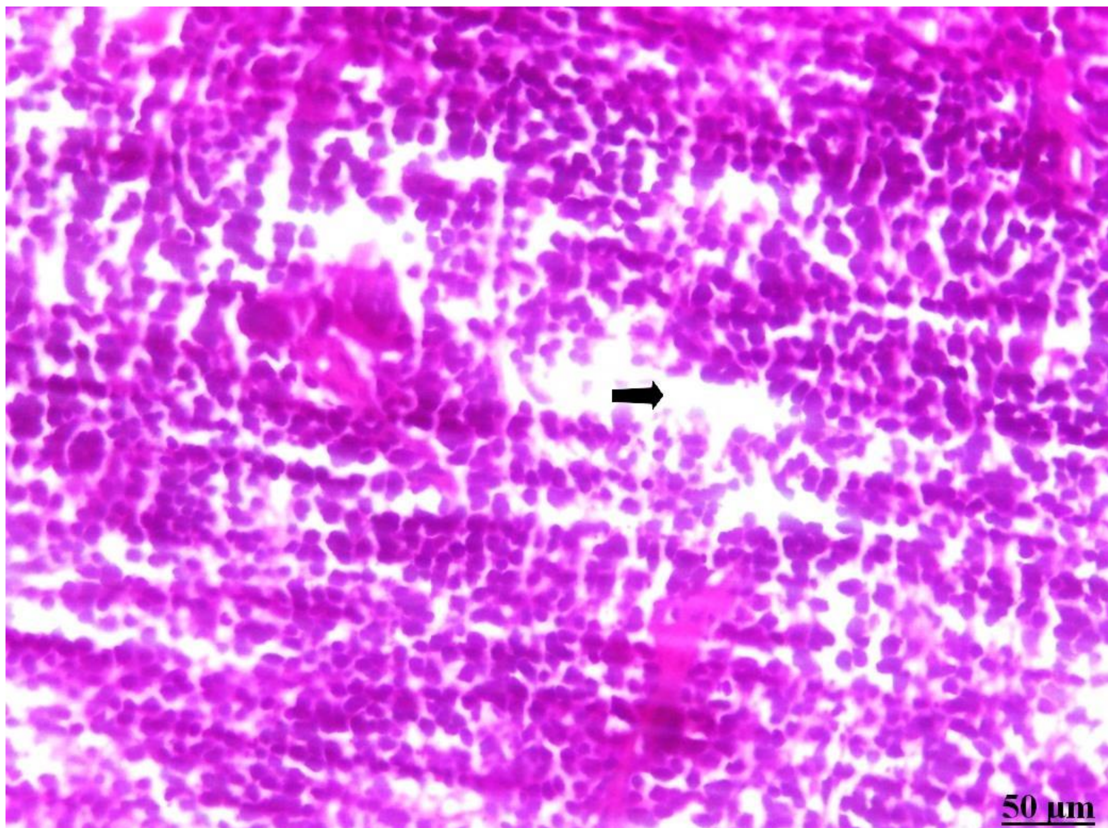


Fig. 4: Microscopy photograph of the spleen of the mice administered tramadol (third stage) possessed severe necrosis and depletion of the splenic parenchyma.

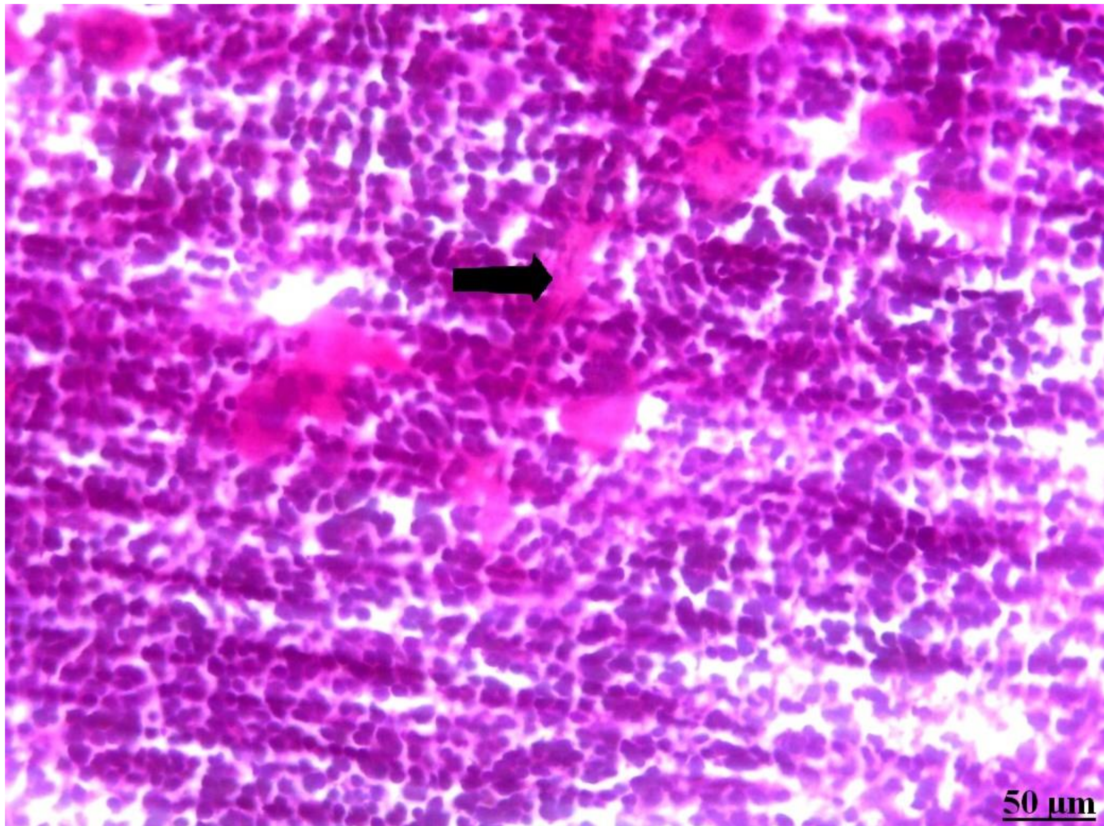


Fig. 5: Microscopy photograph of spleen of mice received tramadol (third stage) showing marked protrusion of splenic trabeculae.

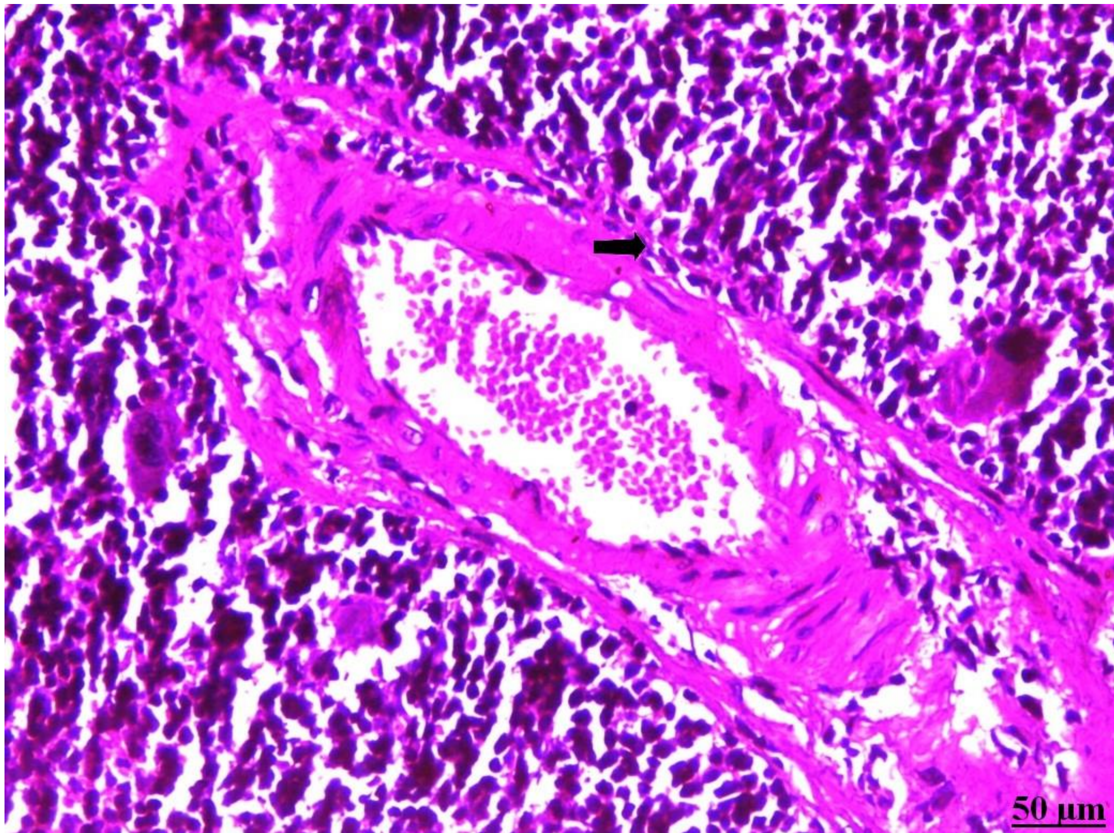


Fig. 6: Microscopy photograph of mice spleen administered tramadol (third stage) showing severe thickening and congestion of the blood vessels.

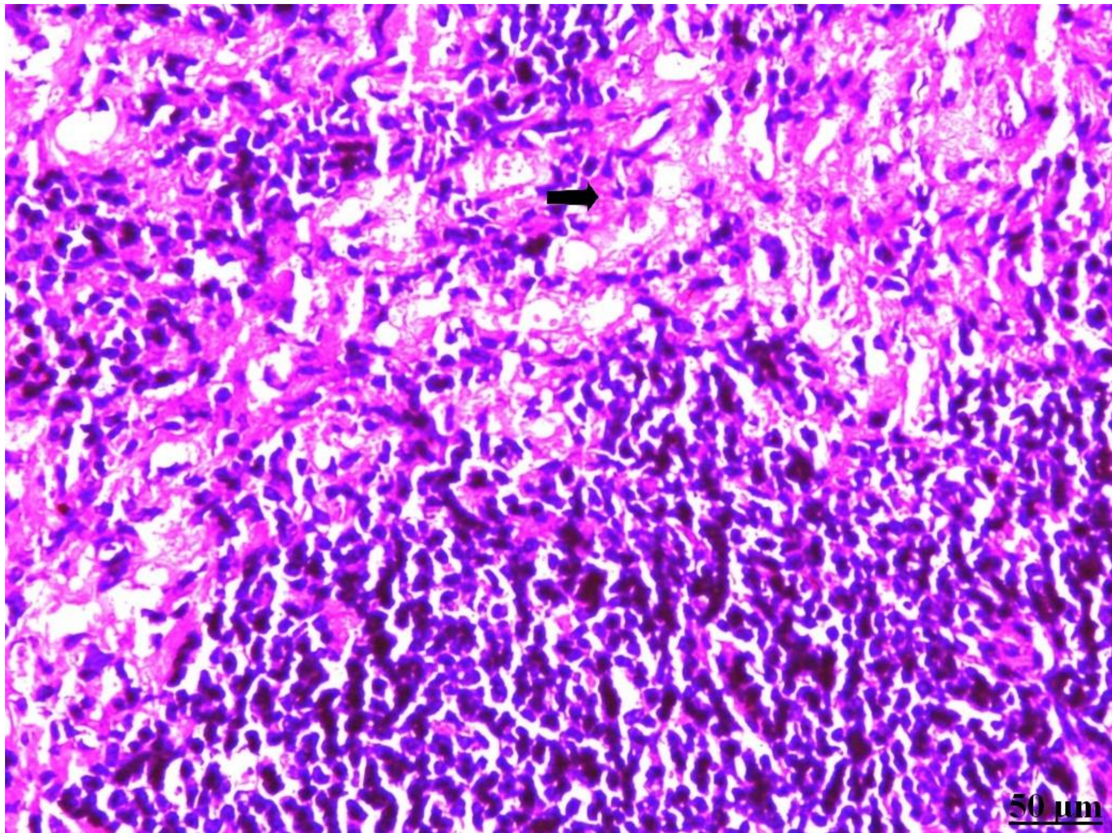


Fig. 7: Microscopy photograph of mice spleen after stopping tramadol injection showing collagen fibers precipitation and fibrinous fluids infiltration.

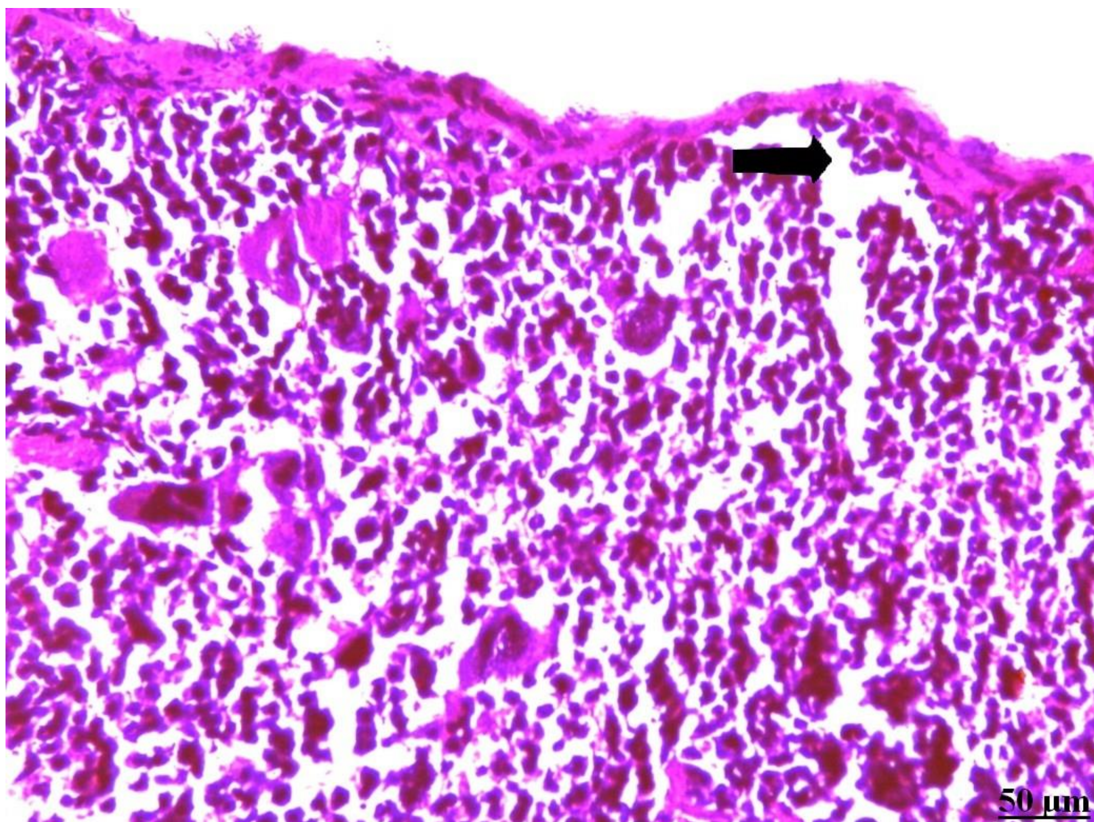


Fig. 8: Microscopy photograph of spleen of the mice following the cessation of tramadol injection showing subcapsular lymphoid depletion and necrosis.

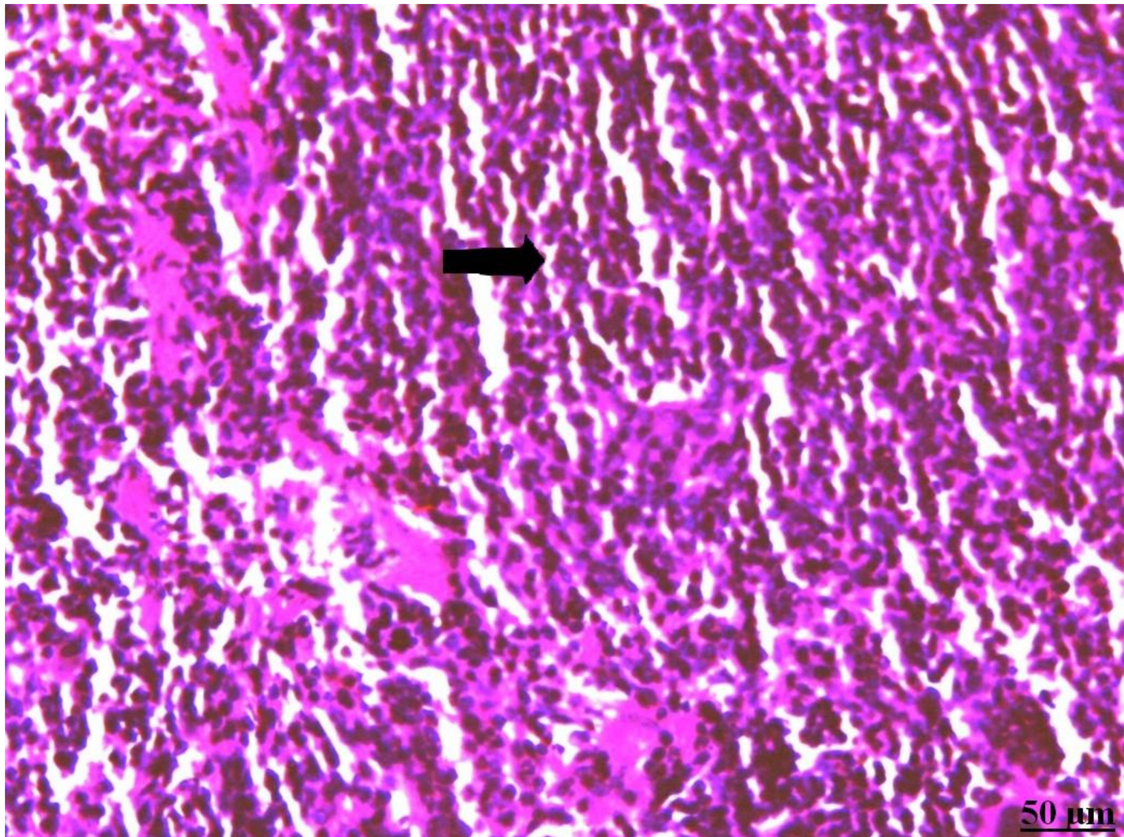


Fig. 9: Microscopy photograph of spleen of the mice were administered tramadol and *L. siceraria*, resulting in a minor effect. degree of lymphoid depletion of the white pulp.

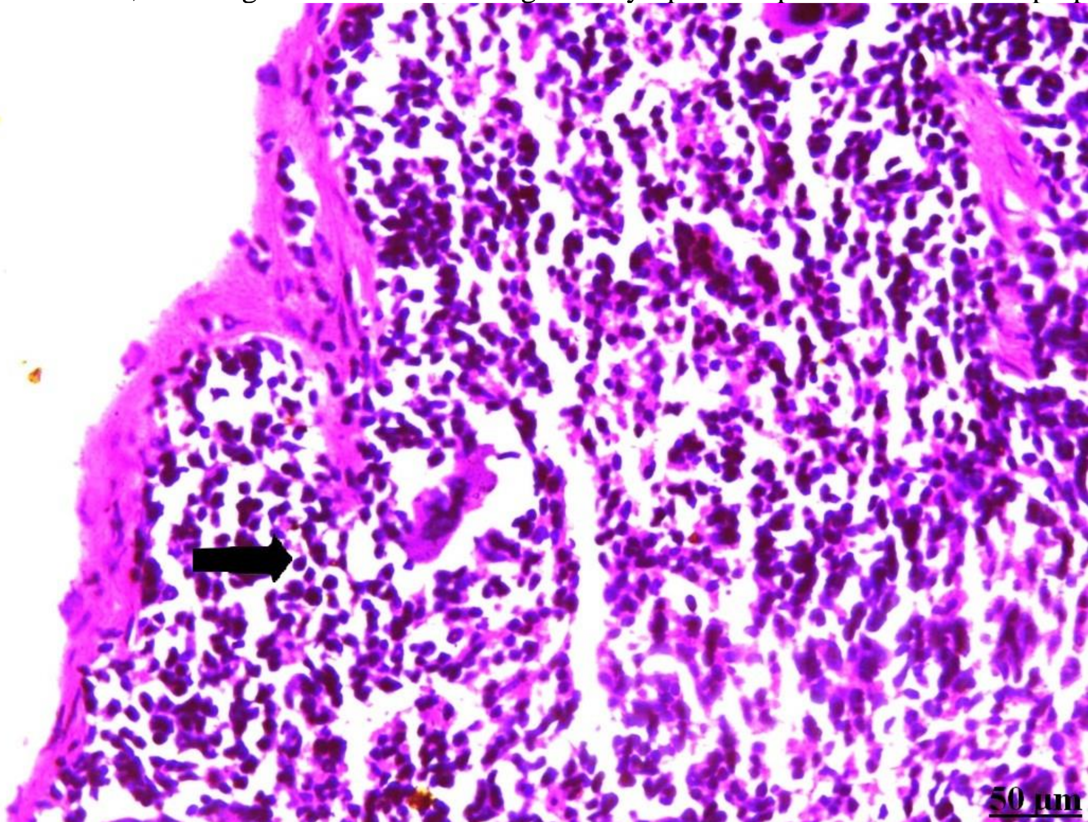


Fig. 10: A microscopy photograph of the spleen from mice that were administered tramadol in combination with *L. siceraria* reveals a little necrosis of the splenic parenchyma.

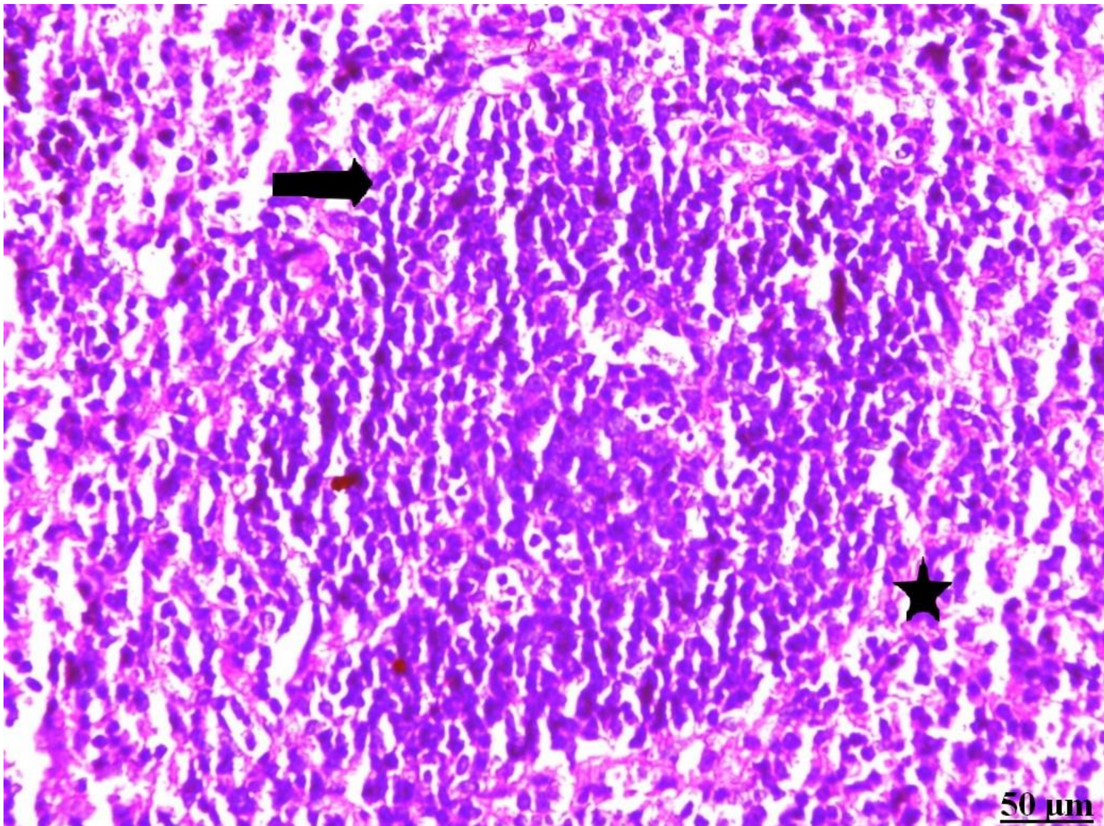


Fig.11: microscopy photograph of the mice that were administered tramadol and MT exhibited normal splenic tissues of white (arrow) and red pulps (star).

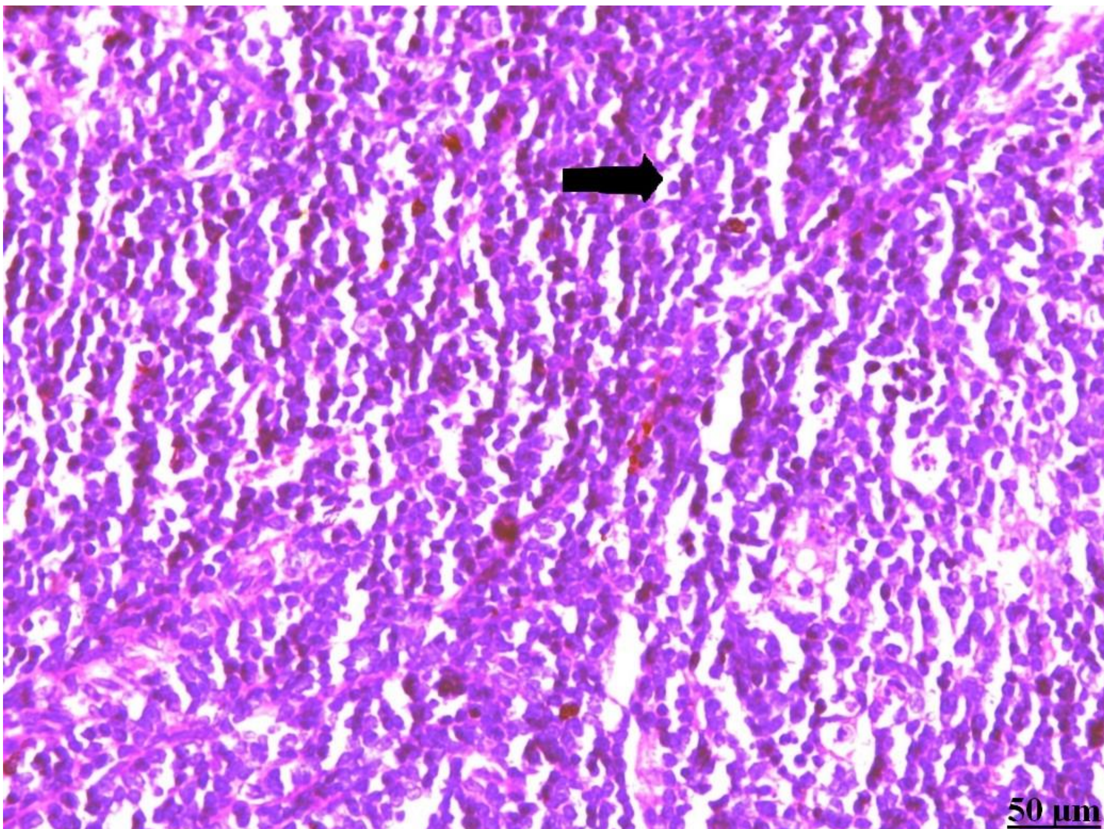


Fig.12: A microscope photograph of the spleen from mice that were administered tramadol and MT reveals a slight reduction in lymphoid cells.

Table (1): The histopathological score of, G. 1 (control), G. 2 (second stage of tramadol), G. 3 (third stage of tramadol), G. 4 (stop tramadol injection), G. 5 (tramadol injection and treated with Lagenaria siceraria) and G. 6 (tramadol injection and treated with melatonin) stained with Hematoxylene and eosin were classified according to severity into severe (+++), moderate (++), mild (+) and absent (-).

Lesions / Groups	G. (1)	G. (2)	G. (3)	G. (4)	G. (5)	G. (6)
Spleen						
Lymphoid depletion and necrosis of splenocytes	-	+++	+++	++	+	+
Collagen fibers infiltration	-	+++	+++	++	+	+
Fibrinous exudates precipitation	-	++	++	+++	+	+
Protrusion of the splenic trabeculae	-	+++	+++	+++	+	+
Hemorrhage of red pulps	-	+++	+++	++	+	+
Thickening and congestion of the blood vessels	+	+++	+++	++	+	+

Spleen (Figs. 13-24):

The control mice's spleen, stained with Mallory trichrome, had a typical arrangement of collagen fibers (Figs. 13 & 14). While spleen of

the mice that received tramadol (second stage) exhibited a pronounced distribution of collagen fibers with a deeply blue color (Figs. 15 & 16) and Table (2). Also, marked perivascular fibrosis with an intense blue color was detected (Fig. 17) and table (2). The mice's spleen, which received tramadol throughout the third stage, exhibited interstitial and capsular fibrosis characterized by a strong blue staining of collagen fibers. (Fig. 18) and Table (2). Furthermore, there was perivascular fibrosis characterized by a pronounced blue staining of collagen fibers (Fig. 19 & 20) and Table (2). After the stop of tramadol injection; mice possessed moderate collagen fibers precipitation (Figs 21). After treatment with *L. siceraria* and MT, the spleen detected minimal and slight collagen fiber precipitation, respectively (Figs. 22, 23, 24).

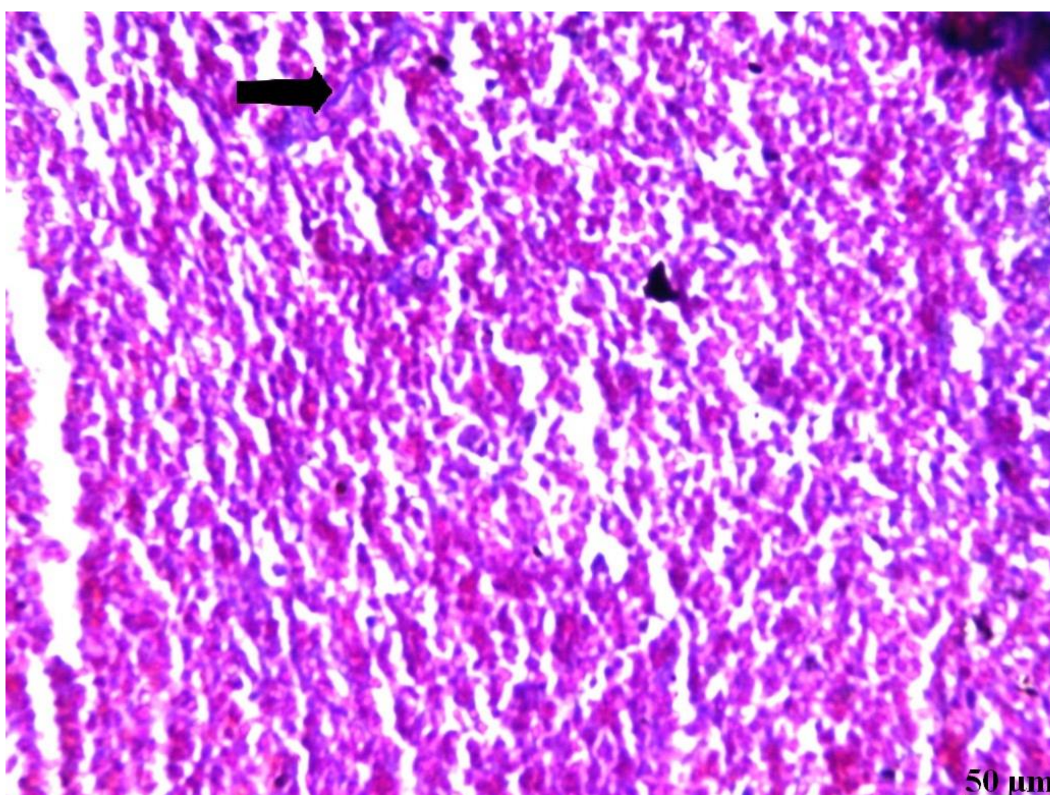


Fig.13: A microscope photograph of the spleen from the control mice, stained with "Mallory trichrome", reveals a typical arrangement of collagen fibers.

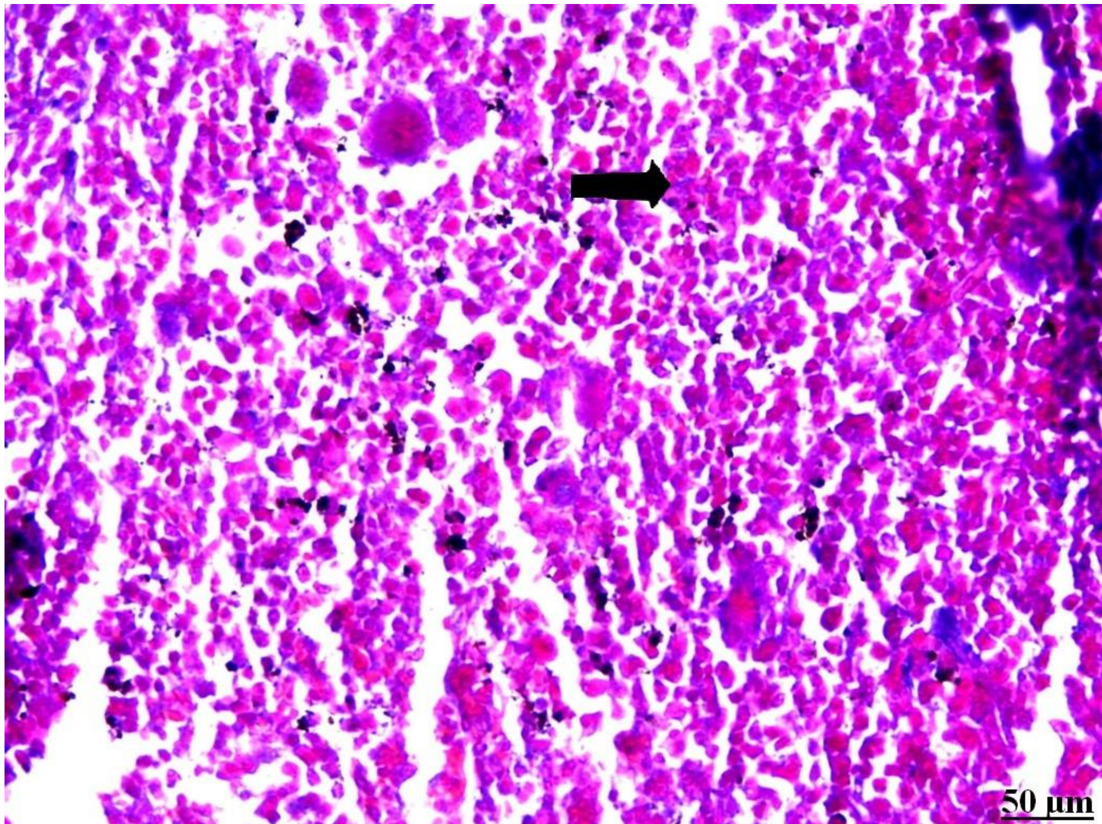


Fig. 14: A photomicrograph of the spleen from mice in the control group, stained with "Mallory trichrome", reveals a typical arrangement of collagen fibers.

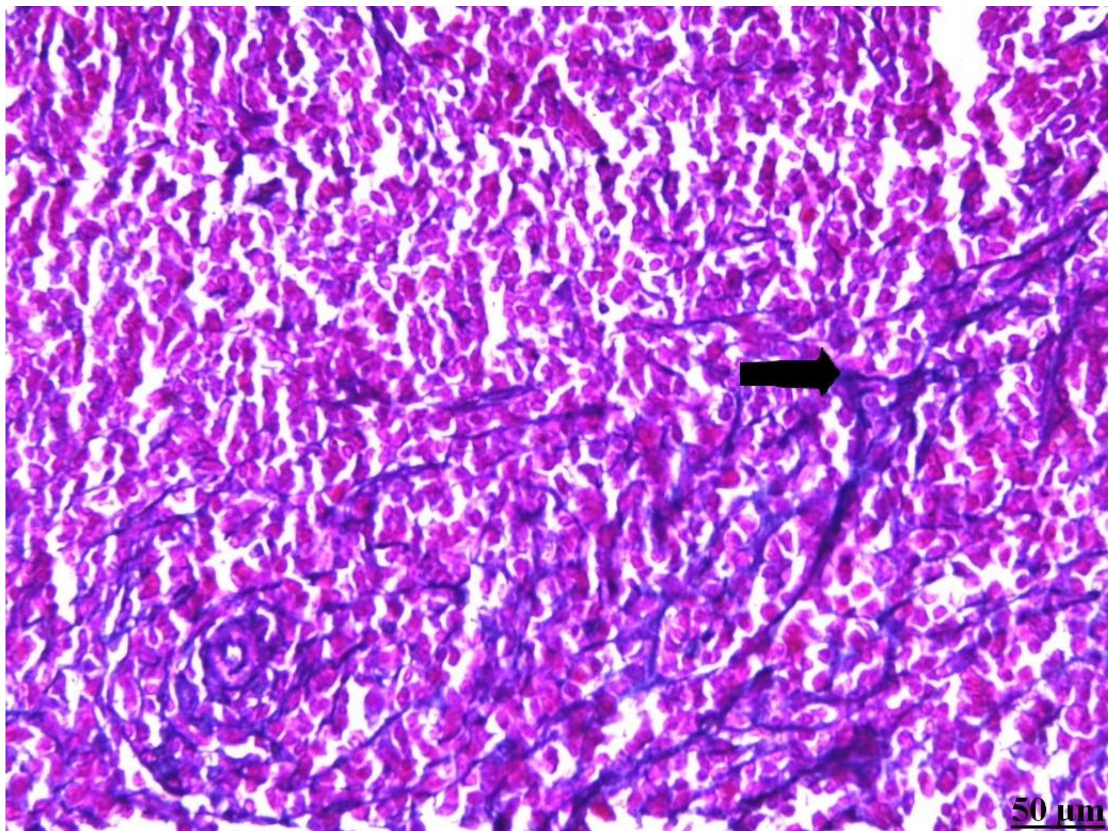


Fig. 15: The photomicrograph depicts the spleen of mice that were administered tramadol during the second stage. The spleen is stained with "Mallory trichrome", revealing a prominent dispersal of collagen fibers that exhibit a deep blue color.

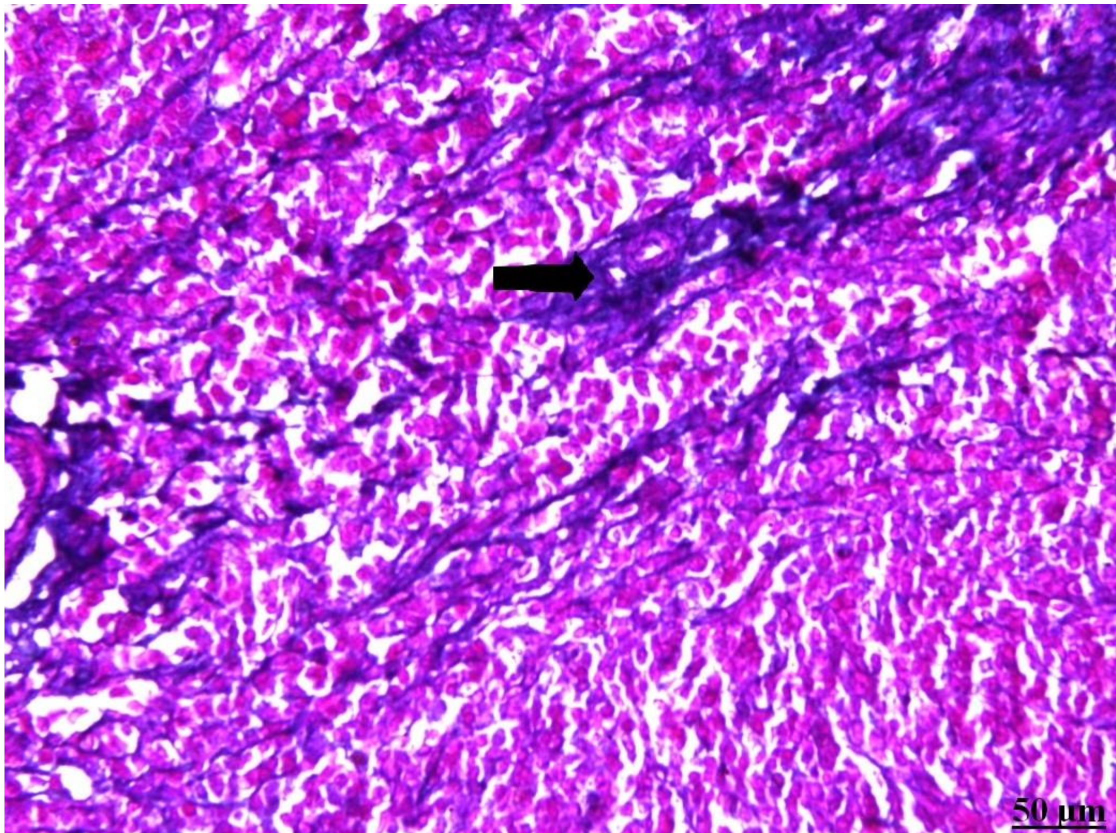


Fig. 16: A photomicrograph of the spleen from mice that were administered tramadol (in the second stage) was stained with Mallory trichrome. The image shows collagen fibers that are heavily stained with a blue tint.

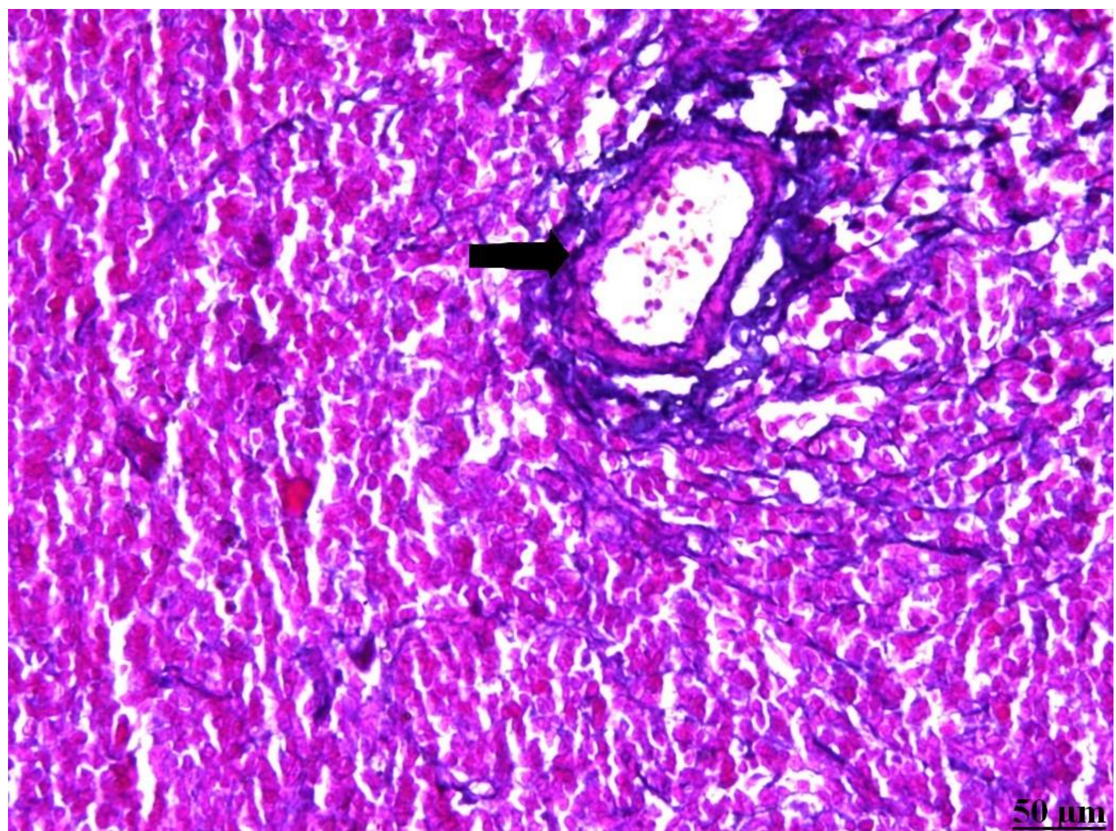


Fig.17: The image displays a microscopic view of the spleen from mice that were administered tramadol during the second stage. The spleen is stained with Mallory trichrome, revealing significant fibrosis surrounding the blood vessels.

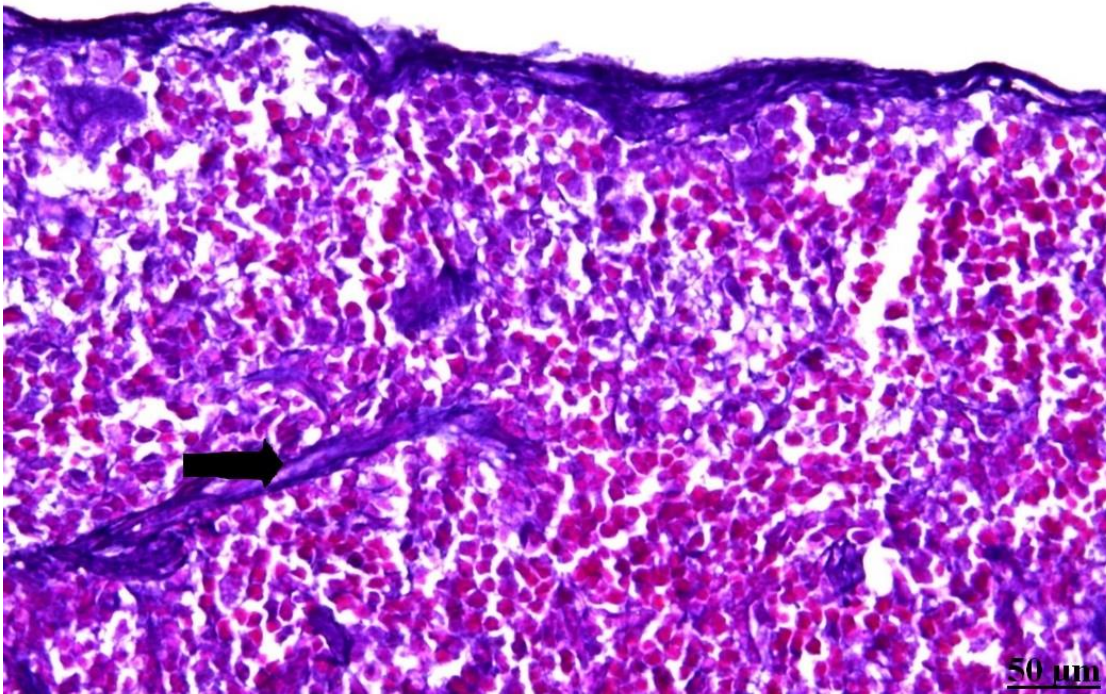


Fig.18: The photomicrograph depicts the spleen of mice that were administered tramadol during the third stage. The image is stained with Mallory trichrome, revealing the presence of interstitial and capsular fibrosis. The collagen fibers appear intensely blue in color.

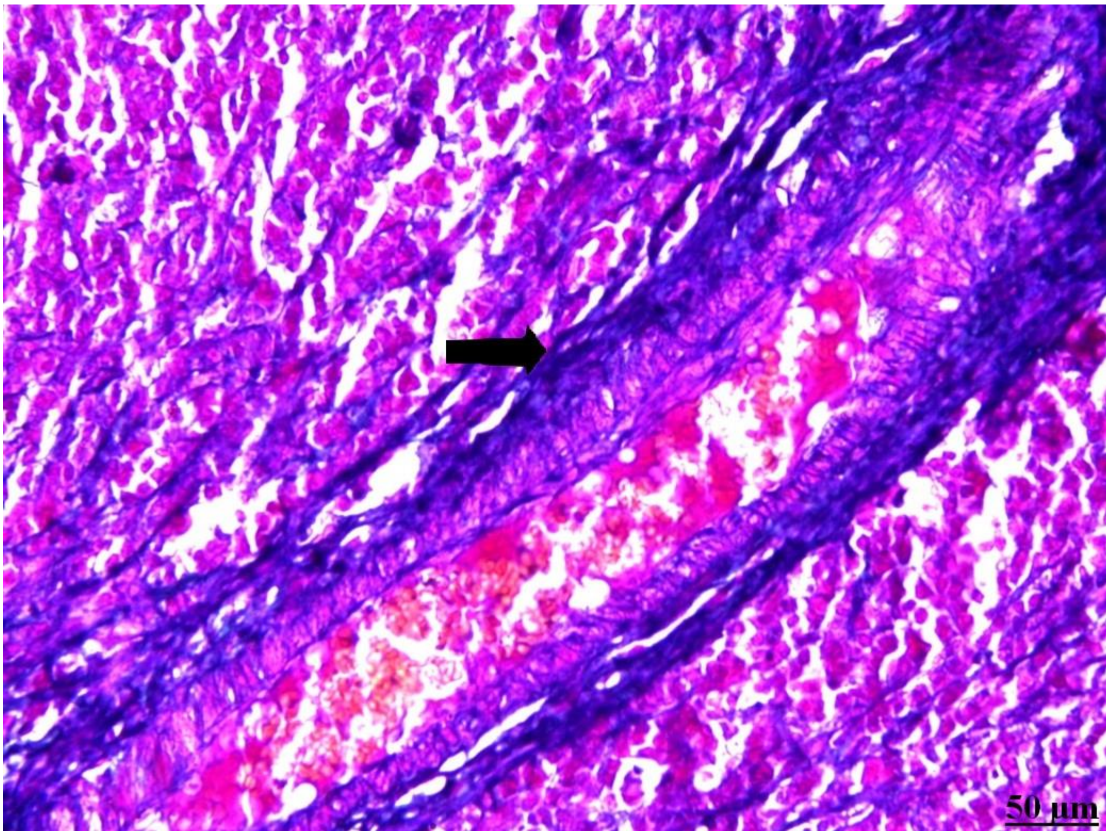


Fig. 19: A photomicrograph of the spleen from mice that were administered tramadol (in the third stage) and stained with Mallory trichrome reveals perivascular fibrosis, characterized by collagen fibers exhibiting a prominent blue hue.

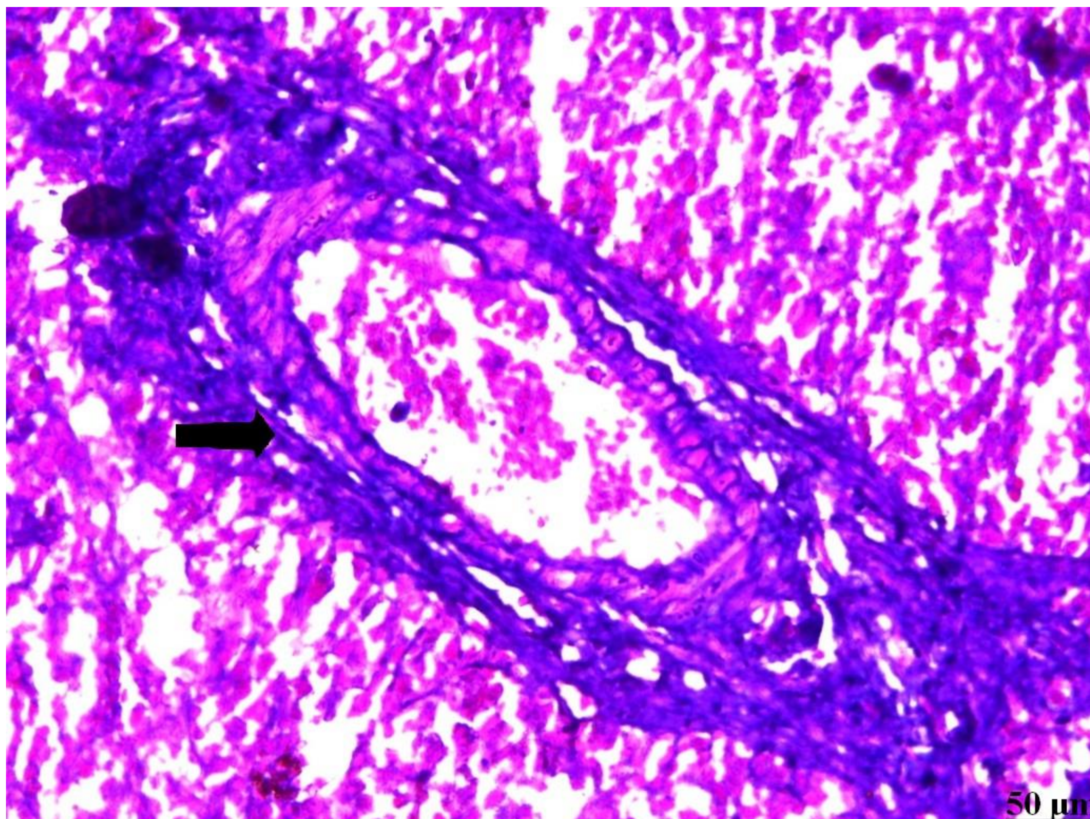


Fig. 20: A photomicrograph of the spleen from mice that were administered tramadol (in the third stage) was stained with Mallory trichrome. The image reveals perivascular fibrosis, characterized by collagen fibers displaying a prominent blue coloration. **(X 400).**

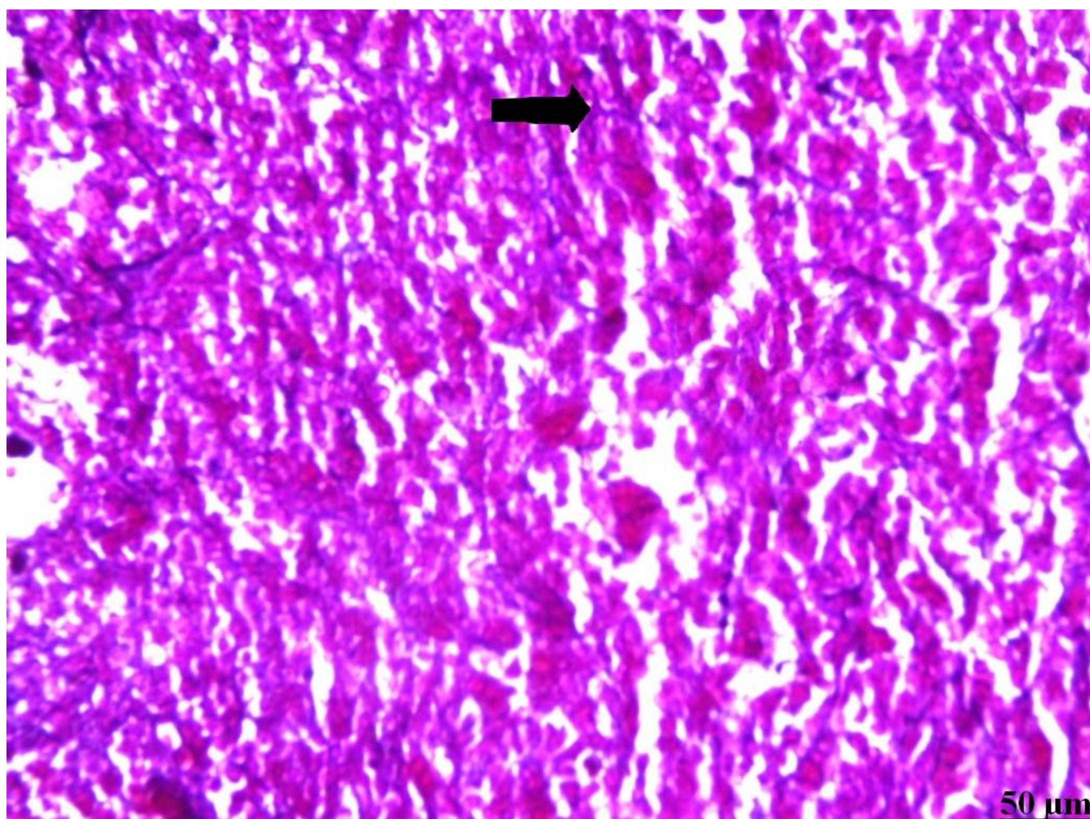


Fig. 21: Photomicrograph of spleen of the mice after stop tramadol injection demonstrating moderately collagen fibers precipitation.

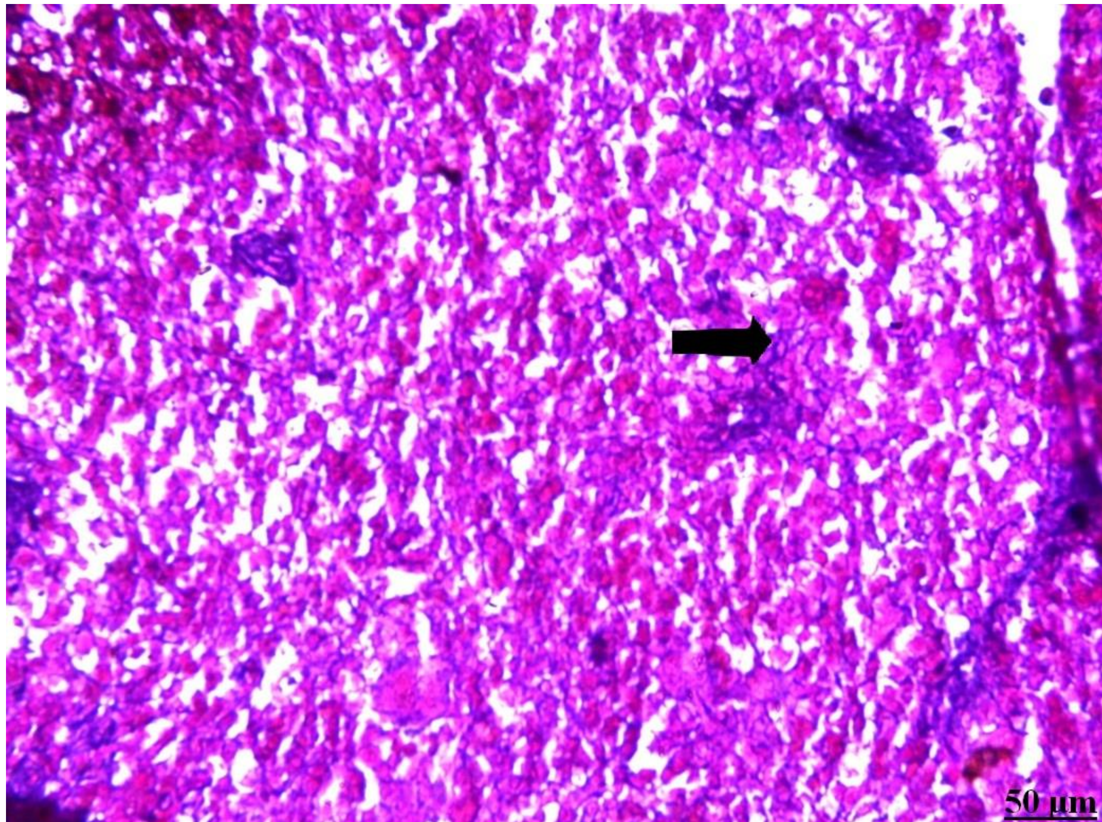


Fig.22: A photomicrograph of the spleen from mice that were administered tramadol in combination with *L. siceraria*, reveals a small amount of collagen fiber precipitation.

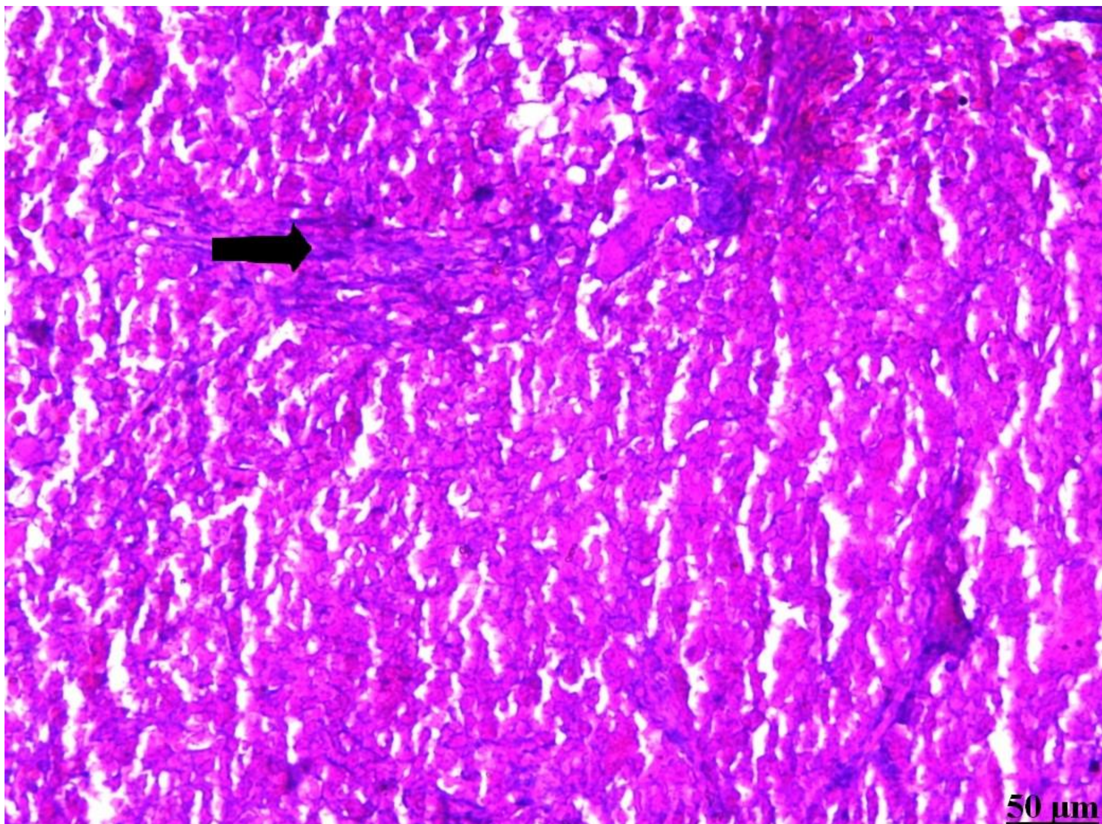


Fig. 23: A photomicrograph of the spleen from mice that were administered tramadol in combination with *L. siceraria*, stained with "Mallory trichrome", reveals a little amount of collagen fibers precipitation.

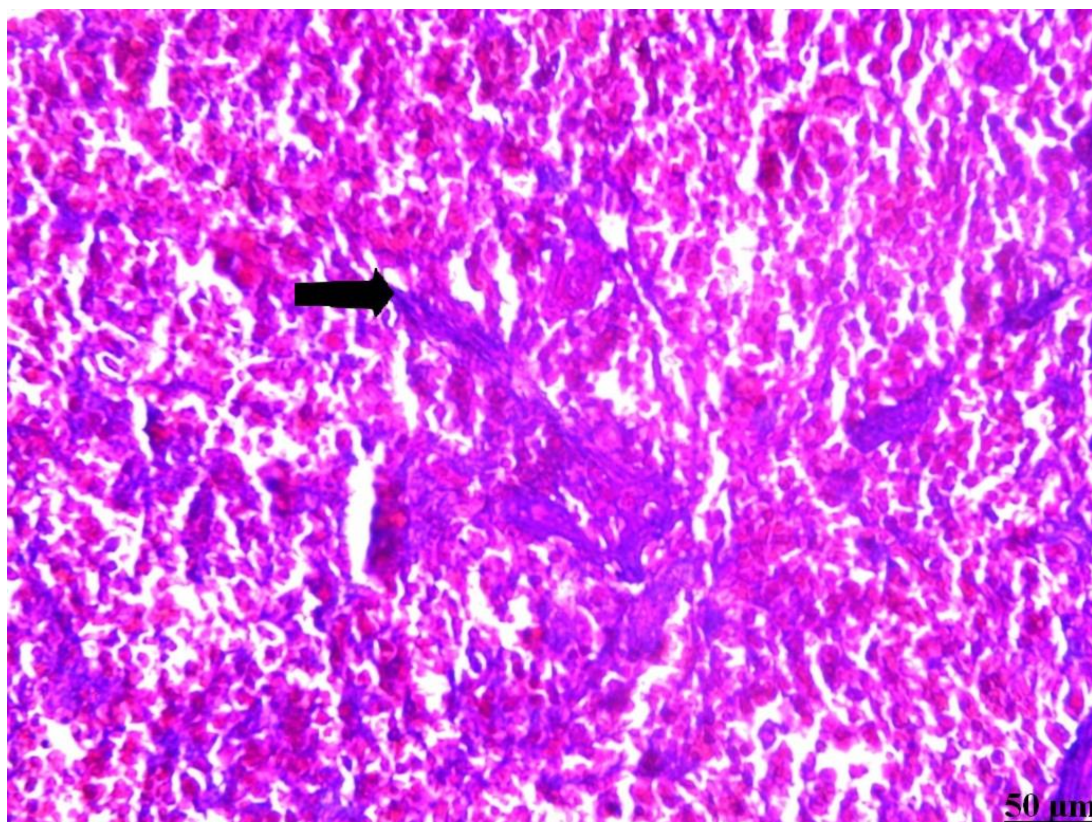


Fig. 24: Photomicrograph of spleen of the mice were administered a combination of tramadol and MT, and their stained samples were analyzed using Mallory trichrome staining, revealing... slight collagen fibers precipitation.

Table 2: The Histochemical score of, G. 1 (control), G. 2 (second stage of tramadol), G. 3 (third stage of tramadol), G. 4 (stop tramadol injection), G. 5 (tramadol injection and treated with Lagenaria siceraria) and G. 6 (tramadol injection and treated with melatonin) stained Trichrome techniques were classified according to severity into severe (+++), moderate (++), mild (+) and absent (-).

Lesions / Groups	G. (1)	G. (2)	G. (3)	G. (4)	G. (5)	G. (6)
Mallory trichrome technique						
Spleen						
Interstitial fibrosis	-	+++	++	++	+	+
Perivascular fibrosis	-	+++	++	++	+	+
Blue stained coloration of dense collagen fibers	-	+++	++	++	+	+
Fibrinous precipitation	-	+++	++	+++	+	+

DISCUSSION

Opioids are highly strong and efficacious analgesics that are widely recognized as suitable therapy options for acute pain, cancer-related pain, and chronic pain unrelated to cancer (Collet, 2001). Nevertheless, they also offer benefits and their frequent utilization can result in reliance and addiction. Undoubtedly, the dependency on opioid analgesics is a growing concern from both a socioeconomic and health standpoint, as evidenced by the rise in overdose-related fatalities (Hall *et al.,* 2008). Clinical applications of tramadol include the treatment of mild to severe pain in both humans and animals (Pypendop and Ilkiw, 2008). Tramadol is additionally employed in veterinary anesthesia pre-operatively due to its substantial reduction in the need for volatile anesthetics and opioid agents (Pearse, 1980). Administering tramadol repeatedly in these patients may result in

the buildup of harmful byproducts in the body, heighten the likelihood of interactions with other drugs, and/or reduce the rate at which tramadol is eliminated, hence raising its potential for causing harm (Seddighi *et al.,* 2009). The current study aimed to evaluate the toxic impact of tramadol on certain tissues in mice, as well as to determine the degree of reversibility and tissue regeneration in the affected organs after discontinuation of the drug.

We obtained a group of adult male Swiss albino mice from the Autoradiographic lab, a research facility focused on Cell Biology and Immunology at the Faculty of Science, South Valley University. Two distinct tests were conducted, differing in the level of exposure to different substances. In the initial round of trials, the animals were categorized into three groups, with each group consisting of 10 male subjects.

The 1st group, referred to as Group T2, received (125ug / 100g b.w.) subcutaneous injection of tramadol Hcl once a day for a duration of 20 days. The 2nd group, referred to as Group T2, received a daily (125ug / 100g b.w.) subcutaneous injection of tramadol Hcl for a duration of 40 days. The control animals were divided into three groups, each consisting of ten males. These groups were labeled as C1, C2, and C3. The animals in these groups were injected with a daily dose of 25ml of distilled water per 30g of body weight. This injection acted as a control for the experiment.The sacrifice of all animals occurred on the day immediately following the final injection. In the second series of studies, the treated animals were classified into three categories: TS, TM, and TL, based on the method of treatment. The mice were categorized and then separated into two groups, each consisting of 10 male mice. All mice in the specified categories received a daily injection of tramadol Hcl at a dosage of 125ug per 100g of body weight for a duration of 40 days. The mice were then subjected to the following treatments. Category S: The 1st group, referred to as Group T2+S, consisted of animals treated with tramadol. The animals were administered a daily intravenous infusion of distilled water at a dosage of 0.25ml per 30g of body weight. for a duration of 40 days. Category M: The 1st group, known as Group T2+M, consisted of animals treated with tramadol. These animals were given a daily subcutaneous injection of MT at a dose of 100mg per 100g body weight, Two hours before the light cycle ends, for a duration of 40 days. Category L: The 1st group, referred to as Group T2+L, consisted of mice administered an oral dose of a unique preparation of *L. siceraria* (500mg /1k. b.w.) for a duration of 40 days. The sacrifice of all animals occurred on the day immediately following the final injection.

Tramadol impacts the body's defense mechanisms and immunological system following administration. The development of a good survival response in animals is attributed to the activation of cell-mediated immune response (De Decker *et al.,* 2008; Kollar and Roan, 1980) provided evidence that tramadol induces changes in the spleen of rats during acute, chronic, and withdrawal phases. The treatment resulted in a modest reduction in the proliferation of T-cells activated by ConA and B-cells stimulated by LPS. Tramadol administration and discontinuation significantly hindered the production of IFN-γ by splenic cells.

The results we obtained could be a consequence of tramadol-induced lipid peroxidation in the liver and kidney tissues. Cell membrane lipid peroxidation results in the reduction of membrane flexibility, modification of membrane electrical potential, and an increased permeability of the membrane. These changes collectively cause a modification in the cellular chemical composition. The heightened presence of collagen fibers is a result of reduced collagen metabolism, which could be

associated with oxidative stress (Mostafa, 2006).

Splenic fibrosis is characterized by the accumulation of excessive collagen resulting from the creation of new fibers, which occurs as a response to chronic spleen injury and the subsequent tissue repair process. The hepatic stellate cell is widely acknowledged as the primary mediator of spleen fibrogenesis, according to their report. Upon experiencing liver injury, hepatocytes undergo an activation phase characterized by increased proliferation and the synthesis of a fibrotic matrix abundant in type I collagen (Altindag *et al.,* 2008).

L. siceraria exhibited a sparse distribution of collagen fibers in the spleen. The immunomodulatory properties of *L. siceraria* were observed in the n-butanol soluble and ethyl acetate soluble fractions of its consecutive methanol extract (Friedman, 1997). *L. siceraria* has the ability to reverse any alterations in the tissues.

In addition, the administration of MT successfully corrected and enhanced the pathogenic changes caused by tramadol. The spleen showed minimal necrosis and degenerative changes, along with mild congestion of the blood vessels. There was a small amount of infiltration by fibrous tissue.

Gangwal *et al.,* (2008); Montilla *et al.,* (2001) said that MT has the ability to inhibit molecular damage produced by harmful reactive substances containing oxygen and nitrogen. MT's role in neutralizing free radicals can be categorized into four primary functions. Firstly, it acts as an antioxidant by directly scavenging reactive oxygen species (ROS) (Reiter, 2000). Secondly, it stimulates the production and activation of antioxidant enzymes (Reiter *et al.,* 2003). Thirdly, it enhances the efficiency of mitochondrial functions by improving the "mitochondrial permeability transition pore" (MPTP), reducing cytochrome c release and enhancing mitochondrial respiratory chain oxidative phosphorylation. As a result, lipid peroxidation in cell membranes is decreased (Rodriguez *et al.,* 2004). Furthermore, Acuna-Castroviejo *et al.,* (2007) demonstrated that MT exerts its anti-neuroinflammatory effects by inhibiting the activation of NF-κB in the cellular signaling pathway. MT, when given before bed, may lower levels of inflammatory cytokines IL-1β and TNF-α and increase levels of the anti-inflammatory cytokine IL-4 in the bloodstream (Permpoonputtana & Govitrapong, 2013). The findings are consistent with prior studies on colitis produced by acetic acid in rats which demonstrated that MT effectively alleviates inflammation in this condition (Carrasco *et al.,* 2013). According to Tahan *et al.,* (2011). MT suppresses the formation of peroxynitrite and the activation of poly ADP ribosesynthetase in rat macrophages that are produced by zymosan-induced shock. MT's anti-inflammatory properties lead to the suppression of peroxynitrite formation, as demonstrated by Maestroni (1993).

Declarations:

Ethical Approval: All steps of this experiment were reviewed and approved by the Research Ethics Committee of the Faculty of Science at South Valley University with the approval number (No. 006/2/24).

Conflict of Interests: No conflicts of interest to declare.

Contributions of the Authors: AA outlined the study design. AA and MJ conducted laboratory experiments and collection of data. AA was responsible for the rats' manipulation. Histological and histochemical study conducted by ZA and MJ.

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