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Comparative Histopathological Abnormalities Induced by *Berthellina Citrina* Acid Secretion in Some Organs of Toad and Mice and the Role of Vitamin E

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Abstract: Many marine mollusca secrete sulfuric and hydrochloric acids as a defensive mechanism. Herein, we investigated the histological and histochemical abnormalities of skin acid secretion (SAS) extract toxicity of the sea slug *Berthellina citrina* after oral administration in mice and toads comparatively, including the spleen, liver, stomach, ileum, and kidney. This study also investigated whether vitamin E (Vit. E) could reduce the toxic effects of SAS. The treated spleens of mice and toads with SAS showed atrophy in the white pulp, a decrease in the splenocyte density, megakaryocytes cytoplasmic degeneration, inflammatory cell infiltration and the number of white and red pulp splenocytes decreased. Additionally, the megakaryocyte size increased compared to the control. The treated liver sections of mice and toads also exhibited general inflammation, fibrosis, vasodilation, and angiogenesis. The treated stomachs of mice and toads showed degeneration in the mucosal layer. The treated ileum of mice and toads showed lamina propria detattachment, leukocyte infiltration, and enlargement of villi size. Furthermore, the kidneys of mice treated with SAS showed shrinkage in glomeruli size while toads exhibited enlargement. This demonstrates the potential toxicity of SAS. Vit. E was induced as a protective dose along with SAS administration. Vit. E was able to reduce the adverse effects of SAS extract in both mice and toads. Additionally, this study showed that, Vit. E can protect against SAS-induced toxicity. Generally, Vit. E improved hemoglobin, leukocytes, and lymphocytes number. This study showed important data about the toxicity of SAS and the ameliorative role of Vit. E in reducing SAS side effects.
Keywords: Sea slug, toxicity, antioxidant, histochemistry, acidosis.

1. Introduction

Molluscs are characterized by their unique defensive strategies, like inking [1], sequestration of cnidocysts to protect themselves from the predation of fish and crabs [2] and secretion of inorganic acids [3-5]. These inorganic acids (sulfuric and hydrochloric) are secreted as defensive skin secretion in a wide range of molluscs e.g., many Heterobranchia, as well as prosobranch (Caenogastropoda) [3, 6]. Interestingly, this phenomena are widely presented in sponges [7], echinoderms [8], polychaetes [9] and tunicates [10]. In addition, it was also reported in some marine algae [11].

Marine toxins are a natural part of marine ecosystems. They can be used by organisms as defense mechanisms or as tools for communication. They can also have harmful effects on human health. Marine toxins are an important source of economic activity [12], with potential applications in pharmaceuticals, cosmetics, and other industries. Research is needed to better understand the potential impacts of marine toxins on human health and the environment [13].

Till now, few researchers [14] have been studied the toxicity and biological activity of skin acid secretion extract (SAS) from the sea slug *Berthellina citrina*. It mainly consists of sulfuric and hydrochloric acids and traces of amino acids, 52% of those amino acids is taurine[14]. They showed that it caused toxic effects against Artemia salina and exhibited antimicrobial activity. In addition, it caused significant hemolysis for human RBCs and showed cytotoxicity against prostate carcinoma cells (PC-3), colorectal carcinoma (HCT 116) and lung carcinoma (A549) [14]. On the other hand, studies about the histopathological and toxic abnormalities of SAS on higher tetrapods such as mammalians are rare. Awaad and Moustafa [15] reported that skin acid secretions (SAS) from the sea slug *Berthellina citrina* produced many histopathological and immunotoxicity in the mice's spleen. Acidosis increases the inflammation of blood vessels in vitro and in vivo studies [16, 17]. Moreover, acidosis enhances vascular endothelial growth factor which contributes to tumor angiogenesis [18].

Vitamin E (Vit. E) is a potent antioxidant with anti-inflammatory properties [18]. Vit. E has an opposite effect on 2 rate-limiting steps in the biosynthesis of the vasodilator prostanoids PGI2 and PGE2. The net effect was increased production of both prostanoids PGI2 and PGE2, indicating that substrate availability is the predominant factor through which Vit. E increases prostanoid production [19]. Vit. E, and Tocopherols, exhibited anti-angiogenic effects [20]. Thus, this study aims to determine the biochemical toxicities of the skin acid secretion of the sea slug *Berthelina citrina* (SAS) for

mammalian and amphibian representatives and evaluate the histological and histochemical effects produced by SAS in the spleen, liver, stomach, intestine, and kidney of mammalian and amphibian representatives as well as the ability of Vit. E to ameliorate the abnormalities generated by SAS extract in both mammals and amphibians.

2. Materials And Methods

2.1. Materials

Skin acid secretion extracted from *Berthellina citrina*, Soluble Vit. E (drug store) from Pure Vet Pharma, Alexandria, Egypt. Hematoxylin stain, sodium metabisulfite, basic fuchsin, and periodic acid were purchased from Sigma-Aldrich (Ontario, Canada). The rodent chow diet was obtained from Feedmix-Egypt.co, Al Qalyubia City, El Gharbia, Egypt.

2.2. *Berthellina citrina* collection and skin acid secretion preparation

Berthellina citrina (Mollusca: Heterobranchia: Nudipleura) specimens were collected from from seven sites located along the Red Sea coast of Egypt, ranging from the south of Al-Qusair to the south of Hurghada. These specimens were collected during low tide during the daytime. They were collected by hand from the lower surfaces of stones and dead coral blocks and kept in seawater-filled plastic containers. Animals were identified according to the World register of Marine Species (WORMS) [21]. To obtain acid secretion, the specimens were washed three times using 3.2% NaCl, and then animals gently were dried with absorbent paper to remove sea salt traces. In a vial, animals' skin was rubbed with a glass rod with a smooth end, then animals were left for 3 minutes [5]. Afterward, the mucus secretions were collected and lyophilized using a freeze-dryer and stored at -20°C for further testing.

2.3. Experimental design

The experiment was performed on two types of vertebrates (mammals and amphibians), the Albino mice (*Mus musculus*) was chosen from mammals and the common African toad, *(Bufo regularis*) was chosen from amphibians. During all experimental procedures, animals were used in accordance with ethical guidelines CSRE-1-23. Under optimal living conditions (25 ± 2 ◦C; 12 h light/dark cycle), mice and toads were kept in stainless steel cages in the animal room. During a period of 45 days preceding the experiment, the animals were fed and provided with water ad libitum and acclimated to the new cages. A total number of 10 animals from each representative were divided into three groups as follows: 4 animals in the control group, 3 in SAS treated group and 3 in SAS + Vit. E treated group, some mammalian adult males mice were bred and raised in an artificial cage and received a standard mice chow diet and water ad libitum only and considered as a control group. For the amphibian toads, they were collected from water canals in the Sohag Governorate near agricultural fields. In an artificial habitat, this was made to mimic the natural habitat of toads. The toads had free access to water, houseflies (Musca domestica) and earthworms (Allolobophora caliginosa) as standard food which were provided by the Department of Zoology, Faculty of Science, Sohag University.

2.4. Experimental animals

Using an animal feeding needle a single dose of 25 mg/kg from SAS was orally administered daily to mice as well as toads for 3 weeks, this group termed as SAS-treated group. While animals were administrated with SAS extract (25 mg/kg) [15] for 3 weeks and simultaneously administrated with a preventive dose of Vit. E (100 mg/kg) for 5 weeks [22] termed as Vit. E+SAS treated group. The treatment with Vit. E started a week before SAS administration and ended a week after SAS administration.

2.5. Hematological examination

Samples of mice blood only were collected in tubes containing EDTA and test results were obtained using an automatic hematology analyzer (HA-Vet clindiag system BVBA, Belgium). The following blood parameters were evaluated: hemoglobin concentration (HGB), red blood corpuscles (RBCs) count, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC), total leukocytic count, neutrophils% and lymphocytes%.

2.6. Histopathological investigation

In each group, the liver, spleen, stomach, ileum, and kidney were fixed in 10% formalin and then dehydrated with ethyl alcohol. They were cleared in methyl benzoate and toluene and finally embedded in paraffin. From the paraffin blocks, 7 m-thick paraffin sections of each sample were cut and mounted on glass slides. After being deparaffinized in xylene, sections were hydrated using ethanol alcohol and then washed with distilled water. Following hydration, sections were stained with hematoxylin and eosin, mounted in DPX, covered with cover glass, and viewed under an AxioCamERc5 equipped light microscope (Axio Lab.A1, Carl ZEISS, Germany).

2.7. Histochemical investigation

To illustrate collagen fibers, some sections of the liver, ileum, and stomach were stained with Masson's trichrome stain [23] and periodic acid-Schiff stain (PAS) [24] was used to demonstrate carbohydrates.

2.8. Quantitative analysis

For image analysis, ImageJ software (National Institutes of Health, Bethesda, Maryland) was used to count the blood vessels in liver sections of mice and toads (n=20), megakaryocytes in spleens of mice (n=10), and melanomacrophages in liver sections and spleens of toads (n=30) and (n=10) respectively in an area of 0.2 mm^2 in different representative sections from each tissue. A brief overview of the method used to select the images is as follows: the selected images were opened by the software, the scale bar was standardized, the regions of interest were determined and analyzed, and prospective cells were manually counted. For measuring the color intensity of PAS reaction in mice' and toads' liver sections and to count goblet cells in intestines and mucus-producing cells in stomachs ImageJ software was used

as follows: Using the software, the selected images were opened, the scale bar was standardized, the regions of interest were determined and analyzed in grayscale, and prospective analysis was conducted area color intensity was measured and compared to section background, then the mean color intensity was determined using Microsoft Excel.

Villi size in mice' and toads' ileums also was measured by ImageJ software as follows: Using the software, selected images were opened, the scale bar was standardized, regions of interest were identified and analyzed, and prospective analysis was undertaken, villi widest diameter was measured, then the mean size was determined using Microsoft Excel.

2.9. Statistical analysis

All values are expressed as means with standard deviations. Statistical differences between the groups or biochemical data were determined using a student *t-test* with a significant level at $p \leq 0.05$ and a highly significant level at $p \leq 0.01$.

3. Results

3.1. Histopathological examinations

3.1.1. Histopathological examination of the spleen of albino mice

The normal control spleen showed normal histological architectures of splenic follicles with normal white and red pulp. The splenocytes in both red pulp and white pulp are normal with basophilic appearance (Figure **1A** and **1B**). On the other hand, mice's spleen after treatment with SAS exhibited distortion in the white pulp follicle. Additionally, the density of splenocytes in the red pulp was relatively lower in comparison with that in the control (Figure **1C** and **1D**). The megakaryocyte number was also higher as compared with that in the control and showed cytoplasmic degeneration (Figure 1D). The white pulp follicles were atrophied and also distorted compared to control pulps (Figure **1C**). Moreover, the density of splenocytes in the white and red pulps decreased, creating wide spaces between the splenic cords. Compared to the SAS treated group a slight difference was observed in the third group when mice treated with SAS simultaneously with a protective dose of Vit. E. Additionally, the splenic follicles were much more distinct, and slight atrophy in the case of SAS and Vit. E treated group than that in the SAS-only treated group (Figure **1E**). Compared to the SAS group, however, the number of inflammatory cells aggregated in the red pulp and megakaryocytes were similar, the megakaryocytes were larger but not distorted in the case of SAS and Vit. E treated group (Figure **1F**). Quantitatively, as shown in Figure **2**, the number of megakaryocytes in the spleen of mice treated with SAS only or with SAS and Vit. E was significantly increased for SAS treated group by 3 times higher than the control group with significancy $8.3 \times 10^{-8}$and for SAS and Vit. E treated group by 2.7 times higher with significancy P value $\leq 1.29941 \times 10^{-6}$ compared with those of control mice.

3.1.1. Histopathological examination of the spleen of toad

The normal control spleen was histologically distinguished with normal white and red pulp and a normal number of Melanomacrophages (MMs). White pulp splenocytes and red pulp splenocytes both contain basophilic splenocytes (Figures **3A**, **3B** and **3C**).

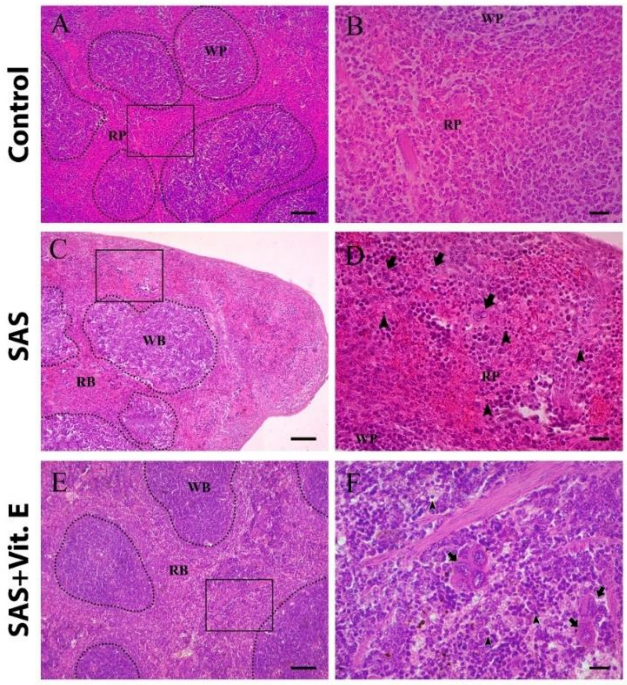

Figure 1: Light microscope photographs showing mice spleen stained with general staining H& E. (A and B), control spleen exhibited intact splenic cords and distinct splenic follicles with normal red and white pulps and splenocyte density. (C and D), spleen treated with SAS showed distortion in splenic follicle shapes, cytoplasmic degeneration, shrinkage of megakaryocytes (bold arrows), and several aggregations of inflammatory cells within the splenic red bulb (arrowheads). (E and F), spleen treated with Vit. E simultaneously with SAS showed distinct splenic follicles, however, there is an increase in the size of megakaryocytes (bold arrows) and an aggregation of inflammatory cells (arrowheads). Scale bare: A, C, and E = 50 µm; B, D, and F = 10 µm.

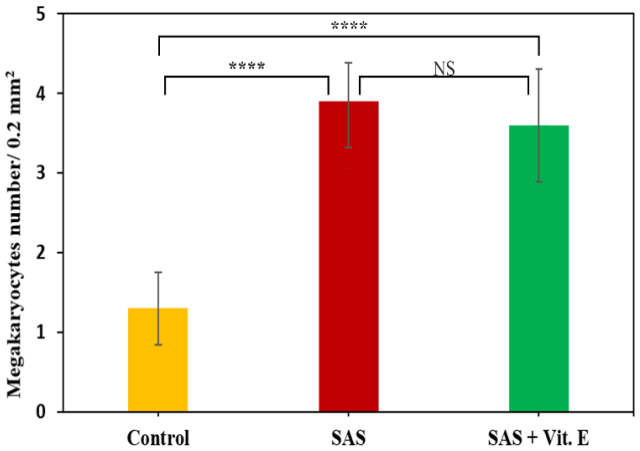

Figure 2: Quantitative anlaysis of megakaryocytes number in mice spleen red pulp after treatment with SAS only or with SAS and Vit. E. The number of megakaryocytes significantly increased after treatment with both SAS alone or SAS simultaneously with Vit. E compared with control. Statistical data are presented as bars with standard deviations. Ten representative sections (n = 10) were obtained from each group of mice for counting megakaryocytes. **** and NS indicate p value ≤ 0.0001 and ≥ 0.05 respectively (Student's t-test).

After SAS treatment, the white pulp follicles in toad spleen showed severe distortion. The red pulp had a lower density of splenocytes than the control (Figure 3C and 3D). The number of MMs was also significantly higher than the control, and the splenocytes exhibited cytoplasmic degeneration (Figure 3D). White pulp follicles showed atrophies and distortions as compared to control pulps (Figure 3D). In addition, the number of splenocytes in the white and red pulps decreased, resulting in wide spaces between the splenic cords. As a result of receiving the protective doses of Vit. E along with SAS treatment (Figure 3G, H and I), there was a slight difference in the third group; the splenic follicles were much more distinct than those in the second group, and there was relatively less atrophy than SAS-only treated group (Figure 3H and 3I). Red pulp and megakaryocytes in the third group (Figure 3F) and the second group (Figure 3I) had roughly similar numbers of inflammatory cells, but MMs were larger and not distorted. As shown in (Figure 4), following SAS administration, there was a significant increase by 3.1 times in MMs with significant P value $\leq 6.96741 \times 10^{-11}$ and Following SAS combined with Vit. E there was a significant decrease by 3.4 times in MMs with significant P value $\leq 1.15574 \times 10^{-11}$.

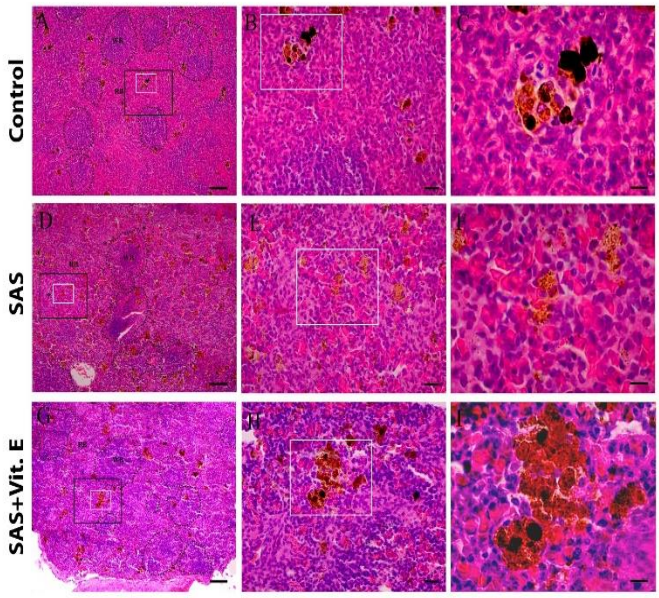

Figure 3: Light microscope photographs showing toad spleen stained with general staining H&E. (A, B and C); Control spleen showing intact splenic cords and distinct splenic follicles with normal white and red pulp and normal splenocytes density. (D, E, and F); Spleen treated with SAS showing distortion in splenic follicle shapes, several aggregations of inflammatory cells within splenic red bulb with numerous amount of MMS. (G, H and I); spleen treated with Vit. E + SAS showing less damaged splenic follicles but with an increase of megakaryocytes size and aggregation of inflammatory cells. Scale bare: A, D and G = 50 µm; B, E and H = 10 µm; C, F and I = 5 µm.

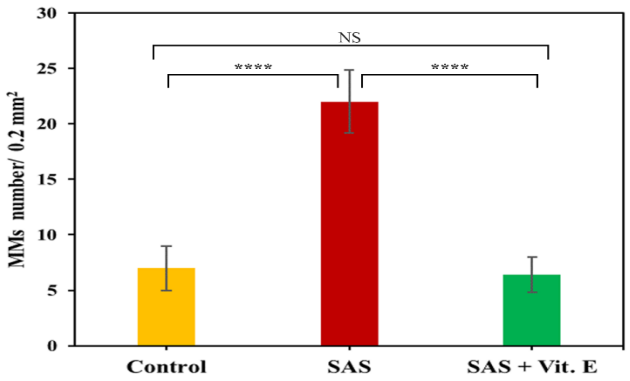

Figure 4: The number of MMs in toad spleen after treatment with SAS or with SAS and Vit. E. The number of MMs significantly increased after treatment with SAS. The treatment with SAS and Vit. E significantly decreased the number of MMs compared to SAS only treated group. Statistical data are presented as bars with standard deviations. Ten representative sections were obtained from each group of mice (n = 10) for counting MMs. **** and NS indicates p value ≤0.0001 and ≥ 0.05 respectively.

3.1.2. Histopathological examination of the liver of albino mice

Control liver sections displayed normal histological architecture with intact strands of hepatocytes (Figures 5A, 5B, and 5C). The liver showed lymphocyte infiltrations into the hepatic parenchyma after 21 days of SAS extract administration (Figures 5D, 5E, and 5F). Additionally, a vasodilatory effect was noted as well as an enlargement and congestion of numerous blood vessels compared with the control group (Figure 5D). Furthermore, there are indications that the blood vessels were initiating angiogenesis as shown in (Figure 5F). Histology of hepatic parenchyma showed clear indications of vascular degeneration (Figure 5D, 5E, and 5F). As shown in Figure 5, despite still being abnormal, the third group had less congested blood vessels and less vasodilation than the second group. Furthermore, the SAS + Vit. E treated group exhibited fewer signs of angiogenesis in comparison to the second group. In general, there is less lymphocyte infiltration in the SAS + Vit. E treated group compared to SAS only treated group (Figure 5G, 5H, and 5I).

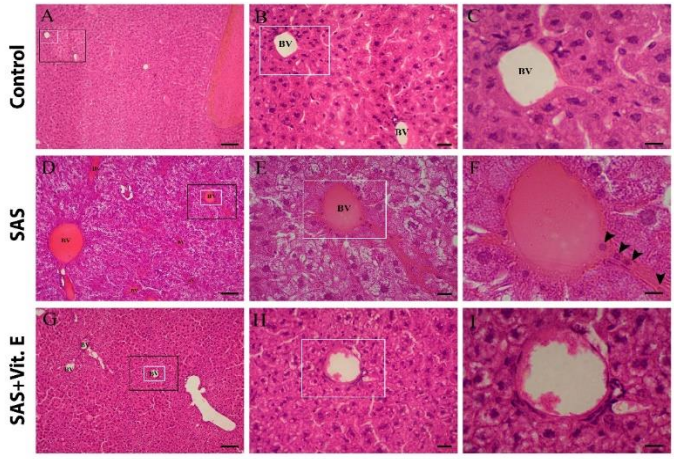

Figure 5: Light microscope photographs showing mice liver stained with general staining H&E. (A, B, and C); the Control liver showed normal histological architecture and normal blood vessel

biodistribution. (D, E, and F); Liver treated with SAS showed blood vessel inflammation with lymphocyte leakage "infiltration" (arrowheads) from a damaged blood vessel and obvious vasodilation. (G, H, and I); Liver treated with Vit. E + SAS showed slight vasodilation with less damaged blood vessels and lower inflammatory response by blood vessels. Scale bare: A, D and G = 50 µm; B, E and H = 10 µm; C, F and 1 = 5 µm

3.1.3.Histopathological examination of the liver of toad

The histological architecture of the control liver sections showed intact and normal strands of hepatocytes as well as a normal number of MMs with normal size (Figures **6A**, **6B**, **6C1,** and **6C2**). As a result of SAS extract administration for 21 days, hepatic parenchyma was infiltrated with lymphocytes (Figure **6F1**). As shown in Figure **6D**, **6E,** and **6F**, a huge vasodilatory effect, as well as initiation of angiogenesis were seen after treatment with SAS (Figure **6F2**). Additionally, the liver parenchyma displayed slight vascular degeneration (Figure **6D**). MMs also displayed enlargement in size (Figure **6 F1**). The treatment of SAS along with Vit. E showed some improvement in the histological architectures of the liver compared with SAS only treated group (Figure **6G**, **6H** and **6I**). There was less vasodilation in the blood vessels of the toad liver in SAS + Vit. E treated group compared with SAS only treated group. Additionally, there were no signs of angiogenesis in the third group compared to the second group (Figure **6G**). Generally, SAS + Vit. E treated group showed lower lymphocyte infiltration compared with SAS only treated group (Figure **6G**, **6H**, and **6I1**). Furthermore, MMs exhibited an increase in size similar compared to SAS only treated group (Figure **6F1**). Quantitatively, Figure **7** displays the number of MMs through the liver parenchyma. There was an increase in the number of MMs in the liver of toads treated with SAS by 1.3 times compared with the control group with significant P value ≤ 0.00000163, indication of some inflammation occurred. While it was observed that the number of MMs in the liver treated with SAS and Vit. E was nearly similar to the number in the SAS-only treated group only higher by 1.1 times with significant P value ≤ 0.01.

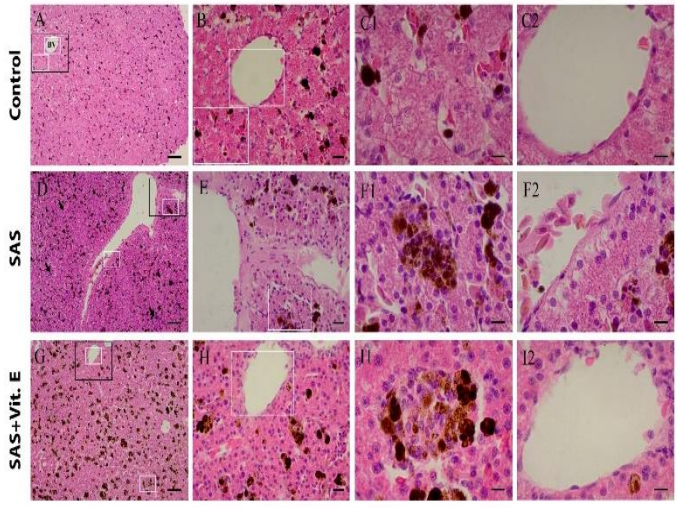

Figure 6: Light microscope photographs showing toad liver of toad stained with general staining H&E. (A, B and C); Control liver showing normal histological architecture, normal blood vessel biodistribution, and normal biodistribution of melanomacrophages MMs. (D, E, and F); Liver treated with SAS showing blood vessel inflammation with lymphocytes aggregations and enlarged MMs, blood vessel showed blood clotting. (G, H, and I); Liver treated with Vit.E+SAS showing blood vessels with normal archterctures but with numerous amount of MMs with aggregation of lymphocytes in the liver paranchyma (G, H, I1 and I2). Scale bare: A, D and G = 50 µm; B, E and H = 10 µm; C1,C2, F1,F2,I1 and I2 = 50 µm.

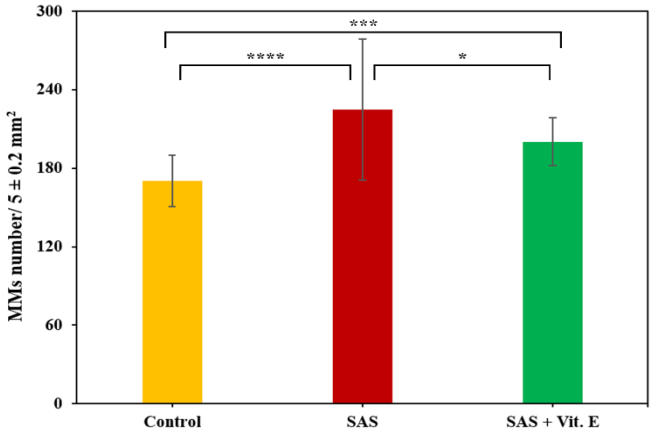

Figure 7: Quantitative analysis of MMs numbers in liver of toad. In SAS only treated group SAS administration increased the number of MMs more than in the control group. After administration of the protective dose of Vit. E along with SAS treatment, the number of MMs decreased and was very close to the control group. Statistical data are presented as bars with standard deviations. Thirty representative sections were obtained from each group of toads (n = 30) for counting MMs.

3.1.4.Quantitative analysis of Liver of toad blood vessels

Figure **8A** showed the number of blood vessels through the liver parenchyma. The liver treated with SAS illustrated an increase in number of blood vessels which indicates initiating angiogenesis as mentioned before. While the liver treated with SAS and Vit. E showed a similar number of blood vessels compared to the control liver. The number of blood vessels changes after treatment with SAS extract through the liver parenchyma of toad is illustrated in Figure **8B**. As previously stated, the liver of toad treated with SAS only showed an increase in the number of blood vessels by 1.6 times higher with significant P value ≤ 0.0014, indicating the onset of angiogenesis. The number of blood vessels in the liver treated with SAS and Vit. E was similar to those in the control liver.

3.1.5.Histopathological examination of the stomach of albino mice

The control stomach revealed intact and normal mucosa with normal epithelium, as well as normal submucosa and lamina propria, and normal muscles (Figure **9A** and **9B**). On the other hand, it was apparent that the mucosal layer as well as the epithelium were severely damaged in the second group (Figure **9C** and **9D**). A more intact mucosal layer was observed in SAS and Vit. E treated group, although some degree of degeneration was still observed and a slight detachment of muscularis mucosal layer (Figure **9E** and **9F**).

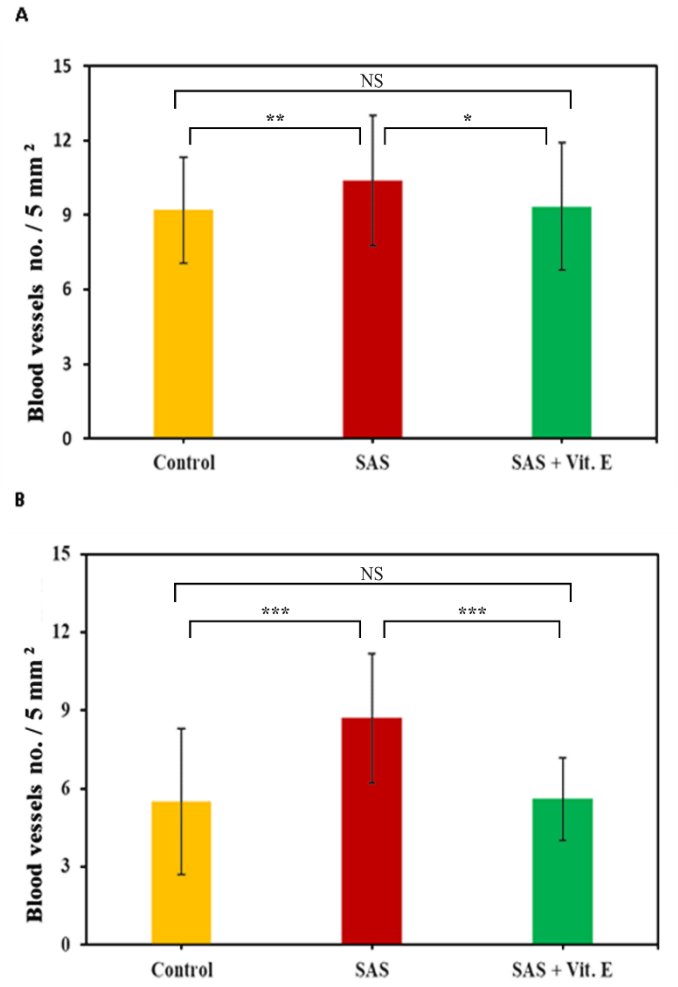

Figure 8: Quantitative analysis of blood vessel numbers in liver tissues. (A) Blood vessels number in albino mice's liver after treatment with SAS or with SAS +Vit. E. Treatment with SAS extract increased the number of blood vessels more than those in the control group. While the number of blood vessels decreased significantly after co-administration of Vit. E simultaneously with SAS. (B) Blood vessels number in toad liver after treatment with SAS or with SAS and vit E. Compared with the control group, SAS extract treatment significantly increased the number of blood vessels compared with the control group. While the number of blood vessels returned to normal following the co-administration of Vit E and SAS. Statistical data are presented as bars with standard deviations. Twenty representative sections were obtained from each group of mice (n = 20) for counting blood vessels. *** and NS indicate p value ≤0.001 and ≥ 0.05 respectively (Student's t-test).

3.1.6.Histopathological examination of the stomach of toad

Mucosa, epithelium, and lamina propria in the control stomach were intact, as were the submucosa and muscles (Figure **10A** and **10B**). Meanwhile, the mucosal layer as well as the epithelium in the SAS only treated group exhibited a slight degree of damage and light degeneration in the mucosal epithelial layer (Figure **10C** and **10D**). Despite less damage to the epithelium in the SAS along with Vit. E treated group, the mucosal layer appeared to be more intact (Figure **10E** and **10F**).

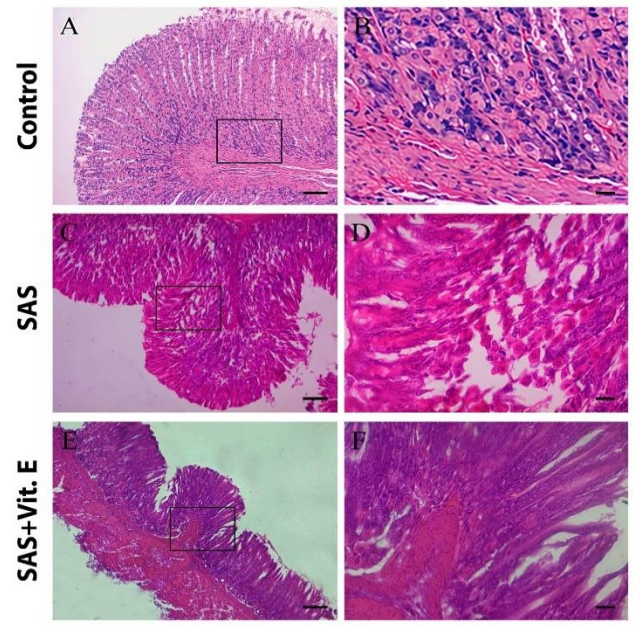

Figure 9: Light microscope photographs showing mice stomach stained with general staining H&E. Control stomach showed normal architectures in most of mucosal layer (A and B). The stomach treated with SAS extract only showed distortion and abnormal mucosal lay architectures and degeneration in mucosal layer and epithelium (C and D). Stomach treated with Vit. E+SAS showed a slight detachment of muscularis mucosa and low inflammation and degeneration in the mucosal epithelium (E and F). Scale bare: : A, C and E = 50 µm; B, D and F = 10 µm.

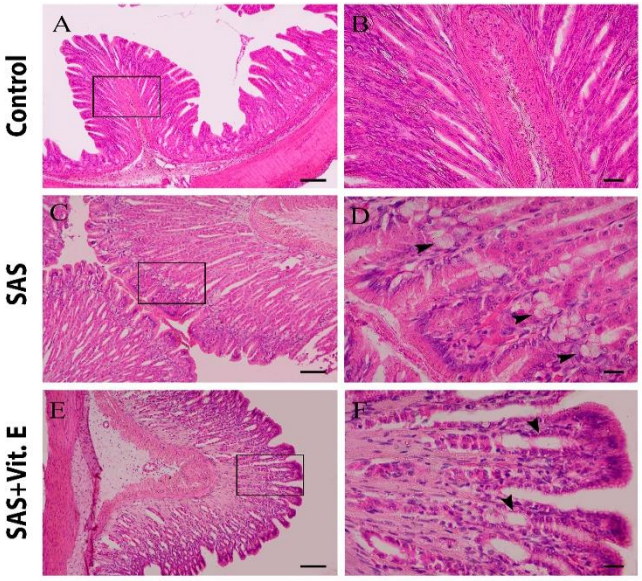

Figure 10: Light microscope photographs showing toad stomach stained with general staining H&E. Control stomach showed normal architecture in all layers especially mucosal epithelial layer (A and B). The stomach treated with SAS extract only showing slight degeneration in the gastric glands with slight damage in the lining epithelial cells (arrow heads) (C and D). While stomach treated with Vit. E+SAS showed a normal mucosal architecture especially related to epithelial cells (E and F). Scale bare: A, C and E = 50 µm; B, D and F = 10 µm.

3.1.7.Histopathological examination of the ileum of albino mice

The ileum in the control group showed intact and normal appearance of villi, brush border, and lamina propria (Figure 11A and 11B). In the case of SAS treated group, hyperplasia of the villi in the ileum was observed, along with slight degeneration and detachment of the lamina propria area, as well as lymphocyte infiltration (Figure 11C and 11D). It should be noted that villi in the SAS extract and Vit. E treated group did not show hyperplasia or lymphocyte infiltration. However, there was a slight detachment of the lamina propria in some areas (Figure 11E and 11F).

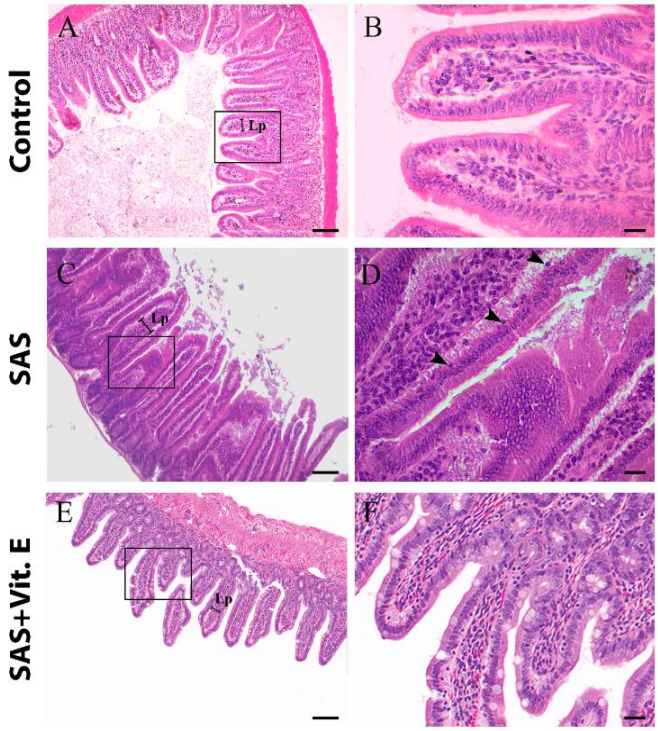

Figure 11: Light microscope photographs showing mice ileum stained with general staining H&E. Control ileum showed normal architectures and normal villi with intact mucosal epithelium (A and B). Ileum treated with SAS showed villi hyperplasia, detachment of lamina propria an increased number of lymphocytes (arrowheads), and some lamina propria degeneration (C and D). While ileum treated with Vit. E+SAS showed slight detachments of lamina propria and less lamina propria degeneration (E and F). Scale bare: A, C and E = 50 µm; B, D and F = 10 µm.

3.1.8.Histopathological examination of the ileum of toad

A normal lamina propria and intact villi were seen in the ileum of the control group (Figures 12A and 12B). As a result of SAS treatment, hyperplasia of the ileum villi was observed, as well as lamina propria degeneration and detachment, and lymphocyte infiltration (Figure 12C and 12D). When SAS extract was administrated with Vit. E villi hyperplasia or lymphocyte infiltration decreased compared with SAS treated group. However, slight inflammation and degeneration were seen in the villi mucosal epithelium and lamina propria in the SAS and Vit. E treated group (Figure 12E and 12F).

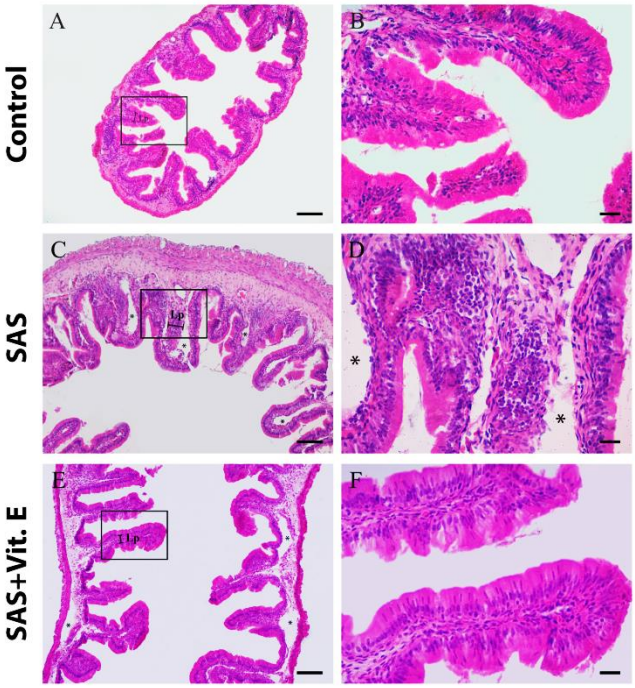

Figure 12: Light microscope photographs showing toad ileum with general staining H&E. Control ileum with normal architectures and normal villi (A and B). Ileum treated with SAS showing villi hyperplasia, detachment of lamina propria (asterisks) and some lamina propria degeneration including a sever lymphocytes infiltration. (C and D). Ileum treated with Vit. E+SAS showing less detachments of sub mucosa but normal structured villi with lower lymphocyte infiltration in the lamina propria area (E and F). Scale bare: A, C and E = 50 µm; B, D and F = 10 µm

3.1.9.Quantitative analysis of the villi size

As shown in (Figure 13A) the size of villi in ileum of albino mice treated with SAS increased by 1.5 times higher with significant P value ≤ 0.0000015 which indicates hyperplasia as mentioned above. While ileum treated with SAS and Vit. E showed normal sized villi similar to those in the control group. Additionally, As shown in (Figure 13B), the size of villi in ileum of toad treated with SAS increased significantly by 2 times higher with significant P value ≤ 0.0018 which indicates hyperplasia in the villi tissues. Furthermore, ileum of toad treated with SAS and Vit. E showed normal sized villi similar to that in the control animals.

3.1.10.Histopathological examination of the kidney of albino mice

In the control kidney, the cortex was intact and normal, as well as normal distal and proximal tubules, and the glomerulus of renal capsules was of normal size (Figure 14A and 14B). Compared to the control group, the glomerulus in the renal capsules of SAS treated group showed shrinkage as well as general degeneration in the cortex (Figure 14C and 14D). However, in the SAS and Vit. E treated group, the glomerulus of the renal capsules showed normal size and the cortex was more intact than in those in the SAS only treated group (Figure 14E and 14F).

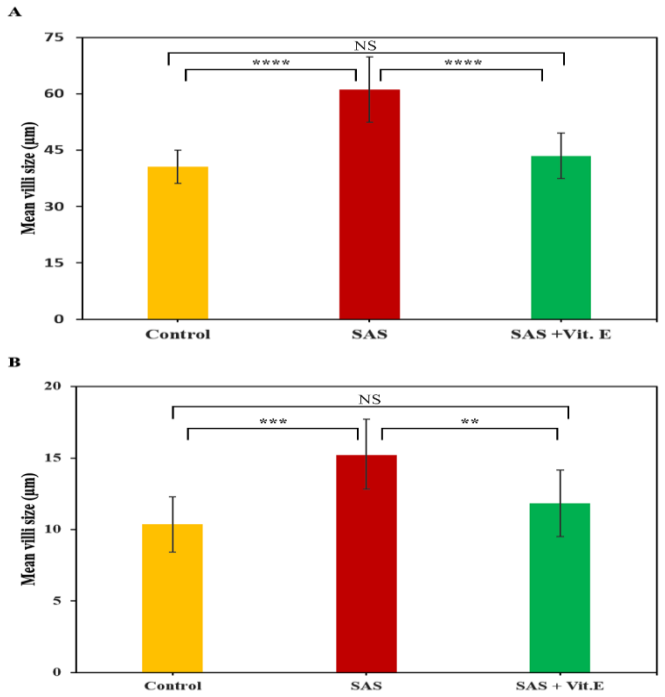

Figure 13: Measurement of the change in the size of villi after treatment with SAS only or with SAS and Vit. E. SAS. (A) The size of villi in albino mice's ileum after treatment with SAS administration slightly increased compared to that in the control group. Additionally, the size of villi decreased significantly after co-administration of Vit. E simultaneously with SAS. (B) The size of villi number in toad ileum after treatment with SAS increased compared with that in the control group, while those sizes were significantly decreased compared with SAS only treatment when Vit. E and SAS co-administrated together. Statistical data are presented as bars with standard deviations. Twenty representative sections were obtained from each group of mice (n = 20) for measuring villi size. ***, ** and NS indicate p value ≤0.001, ≤ 0.01 and ≥ 0.05 respectively (Student's t-test).

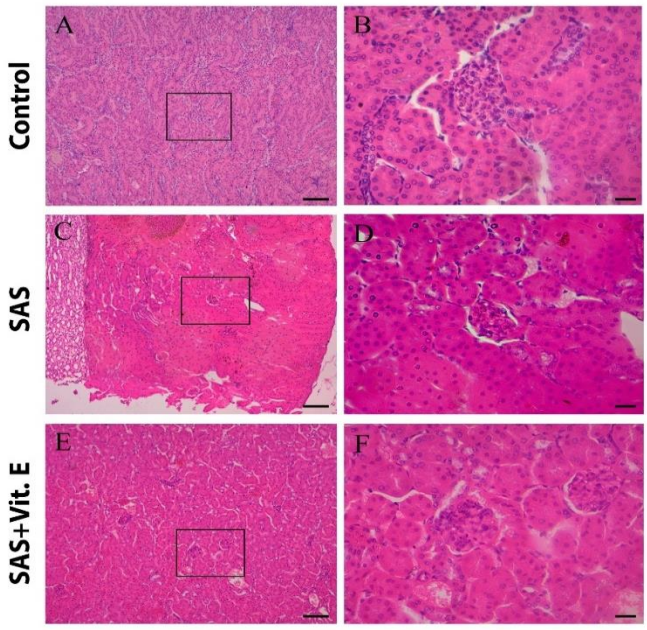

Figure 14: Light microscope photographs showing mice kidney stained with general staining H&E. Control kidney with normal architecture (A and B). Kidney treated with SAS showed shrinkage of glomerulus and some cellular degeneration (C and D). Kidney treated with Vit. E+SAS showed relatively normal glomerulus with slight cellular degenerations (E and F). Scale bare: A, C and E = 50 µm; B, D and F = 10 µm.

3.1.11. Histopathological examination of the kidney of toad

The cortex as well as the distal and proximal tubules of the control kidney were intact and normal, and the renal capsule glomerulus was of normal size (Figure **15A** and **15B**). There was enlargement of the glomeruli in the renal capsules of the SAS only treated group, as well as general degeneration in the cortex and some glomeruli (Figure **15C** and **15D**). While the glomerulus of the renal capsules in the SAS and Vit. E treated group showed normal architecture and appearance and the cortex appeared to be more intact than in the SAS treated group, in addition to lower cellular degeneration was observed (Figure **15E** and **15F**).

3.1.1. Quantitative analysis of glomerulus size in the kidney

The size of the glomerulus in the kidney of mice treated with SAS shrunk by 1.2 times lower with significant P value ≤ 0.000162 when compared to the control group, as demonstrated in (Figure **16A**). The kidney treated with SAS and Vit. E exhibited normal sized glomeruli similar to control group. As indicated in (Figure **16B**), the size of the glomerulus in kidneys of toad treated with SAS enlarged by 1.3 times higher with significant P value ≤ 0.00053 when compared to the control group. The kidney treated with SAS and Vit. E has a normal sized glomerulus

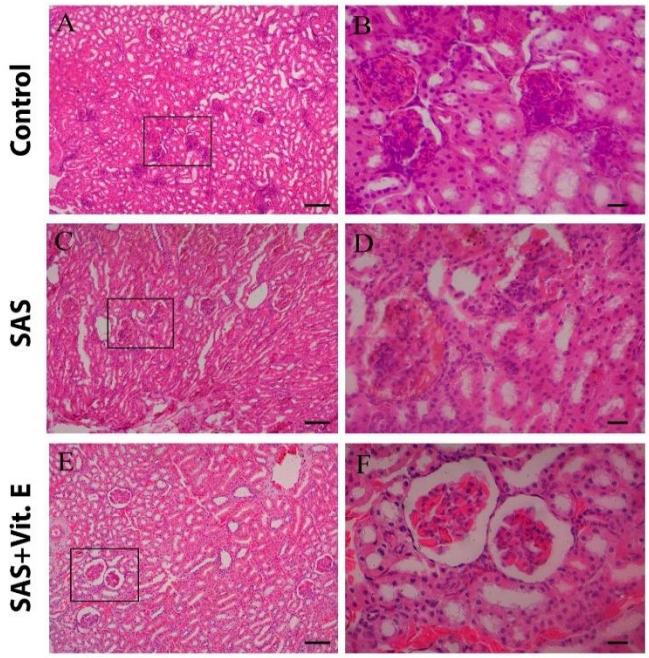

Figure 15: Light microscope photographs showing toad kidney stained with general staining H&E Control kidney with normal architecture (A and B). Kidney treated with SAS showing enlargement and degeneration of glomerulus and some cellular degeneration and swelling (C and D). Kidney treated with Vit. E+SAS showed glomerulus with normal size similar to control (E and F). Scale bare: A, C and E = 50 µm; B, D and F = 10 µm.

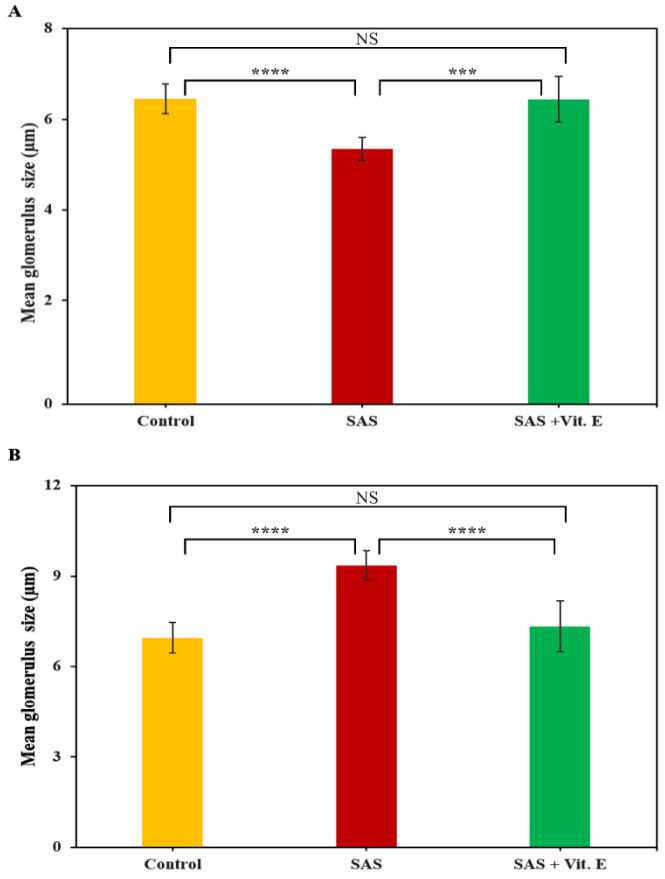

Figure 16: Quantative analysis of the size of glomerulus in the kidney after treatment with SAS or with SAS and Vit. E (A) The size of glomerulus in albino mice's kidney after treatment with SAS shranked slightly compared to those in the control group. While after co-administration of Vit. E and SAS, the glomerulus size turned back to normal similar to those in the control. (B) The size of glomerulus number in toad kidney after treatment with SAS significantly increased size as compared to those in the control group. Following Vit. E and SAS co-administration, glomerulus size almost restored to normal to the normal size. Statistical data are presented as bars with standard deviations. Ten representative sections were obtained from each group of mice (n = 10) for counting glomerulus. ****, *** and NS indicate p value ≤0.0001, ≤ 0.001 and ≥ 0.05 respectively (Student's t-test).

3.2. Histochemical examinations

3.2.1.Histochemical examination of liver

3.2.1.1. Collagen fibers in the liver of mice

Using Masson's trichrome stain, hepatic collagen fibers accumulation was investigated. Compared with those of the control (Figure **17A** and **17B**), the treated liver with SAS extract showed higher expression of collagens in the blood vessels wall as well as interstitial areas collagen (Figure **17C** and **17D**). As a result of the Vit. E treatment as a protective dose along with SAS extract, collagen expression on the wall of blood vessels as well as on the peri-cellular levels significantly decreased (Figure **17E** and **17F**).

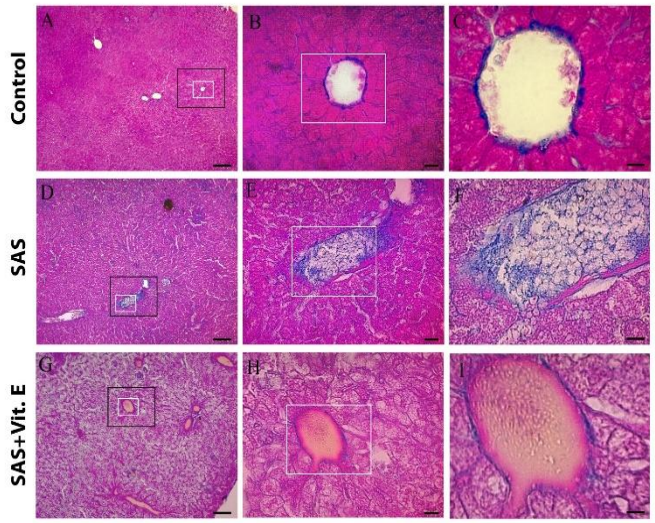

Figure 17: Light microscope photographs showing mice liver with Masson's triple stain. (A, B and C), Control liver showed normal blood vessel and collagen fibers biodistribution. (D, E and F), Liver treated with SAS showed an increase of collagen fibers in blood vessel walls and in interstitial area. (G, H and I), Liver treated with Vit. E+SAS showed less accumulation of collagen fibers in blood vessel inflammation and interstitial areas. Scale bare: A, D and G = 50 µm; B, E and H = 10 µm; C, F and 1 = 5 µm.

3.2.1.2. Collagen fibers in the liver of toad

Similar to liver in the mice, hepatic fibrosis was investigated using Masson's trichrome stain in the case of toad liver. As compared to the control (Figure **18A**, **18B**, **18C1** and **18C2**), liver sections treated with SAS only showed higher accumulation of collagen fibers in the blood vessels wall as well as in the interstitial areas between hepatocytes indicates interstitial fibrosis (Figure **18D**, **18E**, **18F1**, and **18F2**). In the Vit. E and SAS treated group, collagen levels slightly decreased at the peri-cellular and blood vessel walls indicating the protective role of Vit. E against changes produced by SAS extract treatment (Figure **18E** and **18F**).

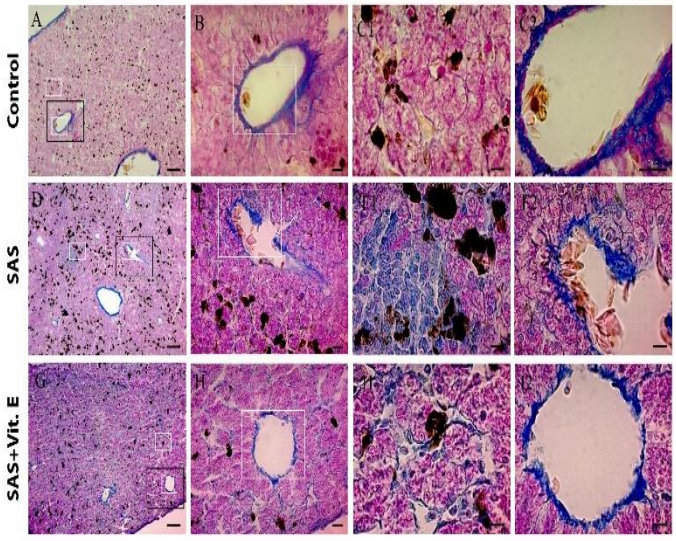

Figure 18: Light microscope photographs showing toad liver stained with Masson's triple stain. (A, B and C), Control liver showed normal blood vessel biodistribution with normal collagen fibers. (D, E and F),

Liver treated with SAS showing higher accumulation of collagen fibers in the walls of blood vessels as well as in the interstitial area between hepatocytes, Liver treated with Vit. E+SAS showed lower accumulation of collagen fibers in all hepatic tissue architectures especially in blood vessels (G, H and I). Scale bare: A, D and G = 50 µm; B, E and H = 10 µm; C1,C2, F1,F2,I1 and I2 = 50 µm.

3.2.1.3. Carbohydrates biodistribution in the liver of mice

The PAS stain showed that in the control liver sections (Figure **19A** and **19B**), carbohydrate levels were normal in the parenchyma and hepatocytes, and staining intensity was homogenous throughout the liver. Compared to the control group, the levels of carbohydrates in the hepatic parenchyma in the group treated with SAS (Figure **19C** and **19D**) were significantly reduced. The general level of carbohydrates in the hepatocytes and parenchyma in the group treated with SAS and Vit. E was similar to that of the control group (Figure **19E** and **19F**).

3.2.1.4. Carbohydrates biodistribution in the liver of toad

Figure **20** showed the biodistribution profiles of carbohydrates in the liver of toad using PAS stain. As shown in (Figure **20 A** and **B**), the parenchyma and hepatocytes of the control liver sections had normal carbohydrate levels and homogenous staining intensity throughout all the liver architecture. In the case of SAS treated group (Figure **20C** and **20D**), carbohydrate levels in the hepatic parenchyma were significantly reduced compared to that in the control group. The SAS and Vit. E treated group had nearly similar levels of carbohydrates in the hepatocytes and parenchyma (Figure **20E** and **20F**), in a comparason to control group.

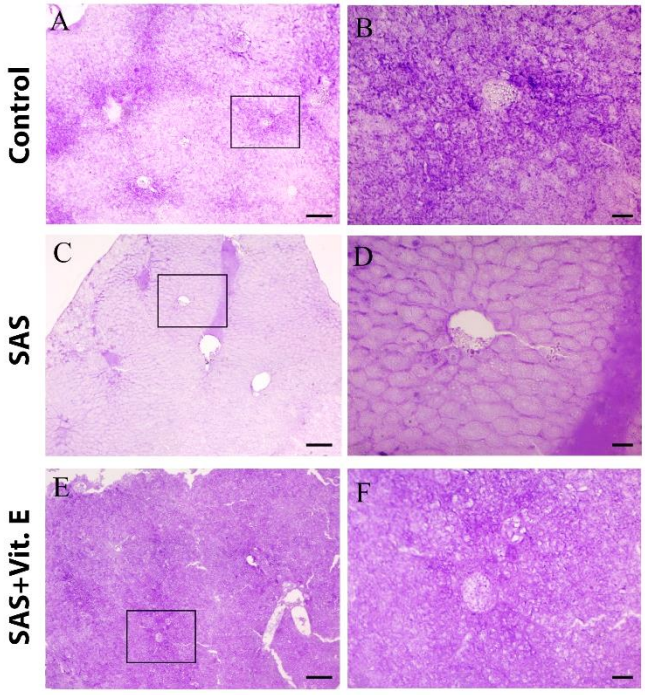

Figure 19: Light microscope photographs showing mice liver stained with PAS to illustrate general carbohydrates biodistribution. (A and B), Control liver showed normal biodistribution of carbohydrates through all liver parenchyma. (C and D), The liver treated with SAS extract showed a significant decrease in carbohydrates. (E and F), Liver treated with Vit. E+SAS showed similar biodistribution of carbohydrates to the control group. Scale bare: A, C and E = 50 µm; B, D and F = 10 µm.

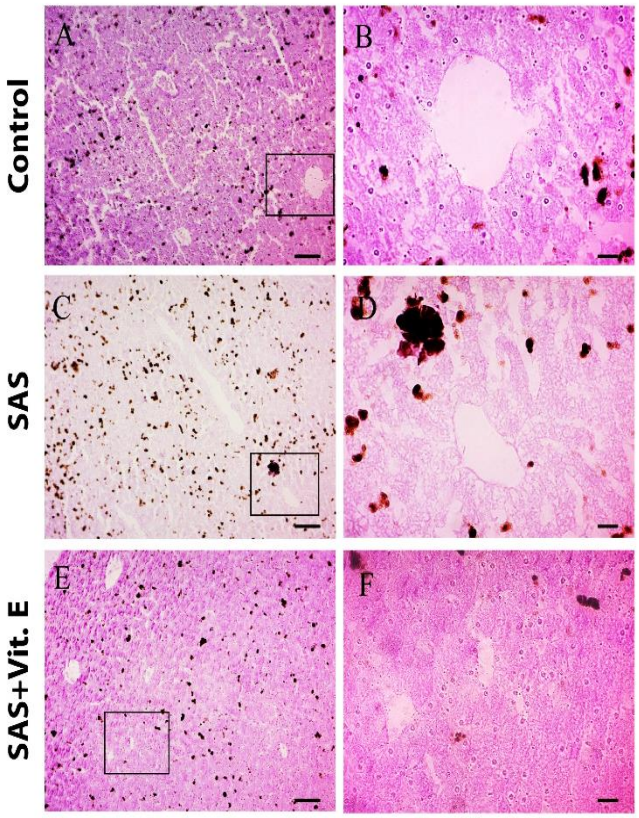

Figure 20: Light microscope photographs showing toad liver stained with PAS to illustrate general carbohydrates biodistribution. (A and B), Control liver showed normal biodistribution of carbohydrates. (C and D), Liver treated with SAS showing significant general decrease in the level of carbohydrates compared to control. (E and F), Liver treated with Vit. E+SAS showed normal and homogenous biodistribution of carbohydrates through all liver parenchyma. Scale bare A, C and E = 50 µm; B, D and F = 10 µm.

3.2.1.5. Quantitative analysis of carbohydrates the liver

As shown in Figure **21A**, the levels of carbohydrates throughout the mice's liver of SAS treated group was compared with that after Vit. E and SAS treated and control group. The SAS treated group showed lower level by 1.3 times with significant P value ≤ 0.0223 of carbohydrate expression compared to the control group, whereas the Vit. E and SAS treated group showed a similar level of carbohydrates to that in the control liver. (Figure **21B**) analyzes the levels of carbohydrates throughout the toad's liver. Compared to the control group, the SAS treated group displayed a lower of carbohydrates by 2.3 times with significant P value ≤ 0.04 level of carbohydrate expression compared to that in the control group, While the Vit. E and SAS treated group exhibited similar levels of carbohydrates to that in the control.

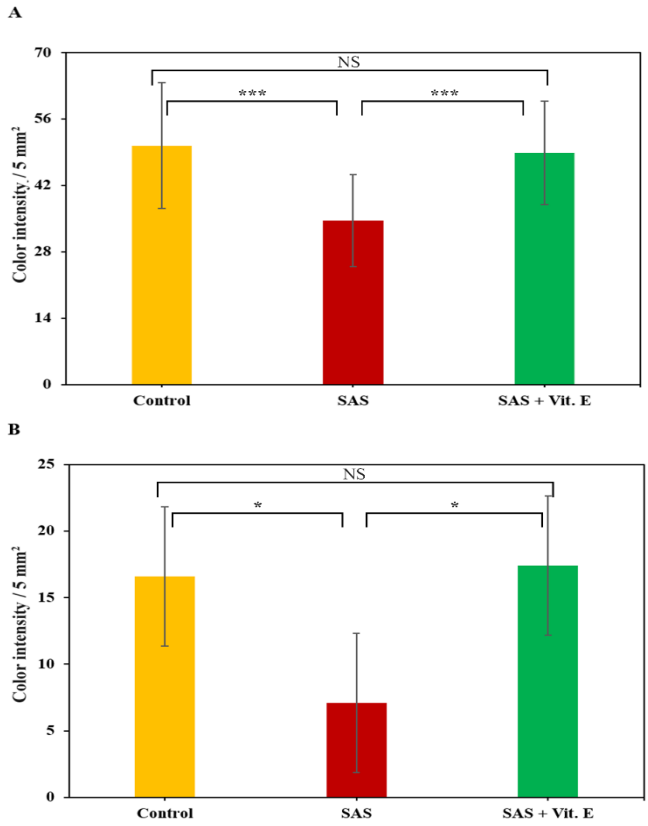

Figure 21: Quantitative analysis of the color intensity of PAS staining indicating the levels of carbohydrates in the hepatocytes. (A) Analysis of carbohydrates levels in albino mice's liver after treatment with SAS or with SAS and Vit. E. The color intensity decreased more after SAS administration than that in the control group. After co-administration of Vit. E and SAS, the color intensity increased to similar color intensity in control group. (B) Analysis of carbohydrates levels in toad liver after treatment with SAS or with SAS and Vit. E. After SAS administration, the color intensity in toad liver decreased more than that in the control group. The color intensity increased to almost similar color intensity in the control group after co-administration of Vit. E and SAS. Statistical data are presented as bars with standard deviations. Ten representative sections were obtained from each group of mice (n = 10) for measuring color intensity. ***, * and NS indicate p value ≤0.001, ≤ 0.01 and ≥ 0.05 respectively (Student's t-test).

3.2.2. Histochemical examination of stomach

3.2.2.1. Carbohydrates distribution in mice's stomach

Using PAS staining, it was observed that the gastric mucosa of the control stomach contained a high level of carbohydrates with homogenous biodistribution (Figure **22 A** and **B**). On the other hand, it was apparent that the level of carbohydrates in the gastric mucosa in the SAS-treated group was reduced compared to that in the control (Figures **22C** and **22D**). While, in the case of Vit. E+SAS treated group showed a similar level of carbohydrates in gastric mucosa as that in the control group (Figure **22E** and **22F**).

3.2.2.2. Carbohydrates distribution in toad stomach

In the control stomach, high levels of carbohydrates were determined in the gastric mucosa using PAS stain (Figures **23A** and **23B**). Meanwhile, there was a reduction in the amount of carbohydrates in the gastric mucosa of SAS-treated toads (Figures **23C** and **23D**). As is shown in (Figures **23E** and **23F**), the Vit. E and SAS treated group showed similar levels of carbohydrates in the gastric mucosa to that in the control group.

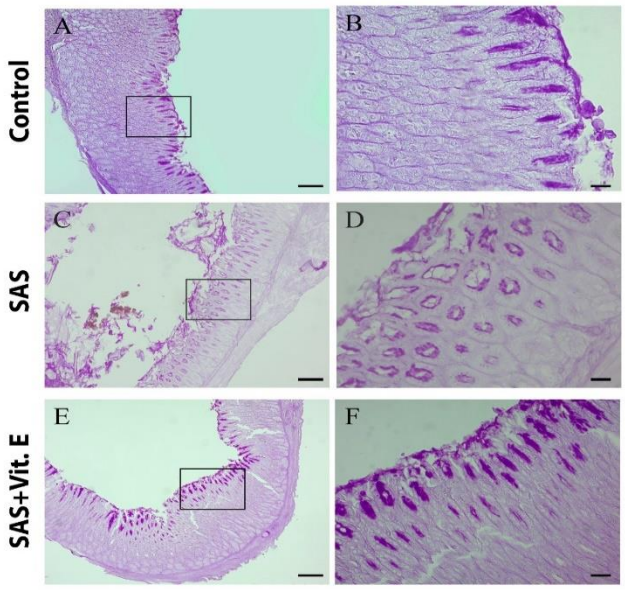

Figure 22: Light microscope photographs showing mice stomach stained with PAS. Control stomach with normal carbohydrate biodistribution (A and B). While the stomach treated with SAS showed a reduction in the level and intensity of carbohydrates (C and D). Stomach treated with Vit. E+SAS showed a higher intensity of carbohydrates similar to that in the control (e and f). Scale bare: A, C and E = 50 µm; B, D and F = 10 µm.

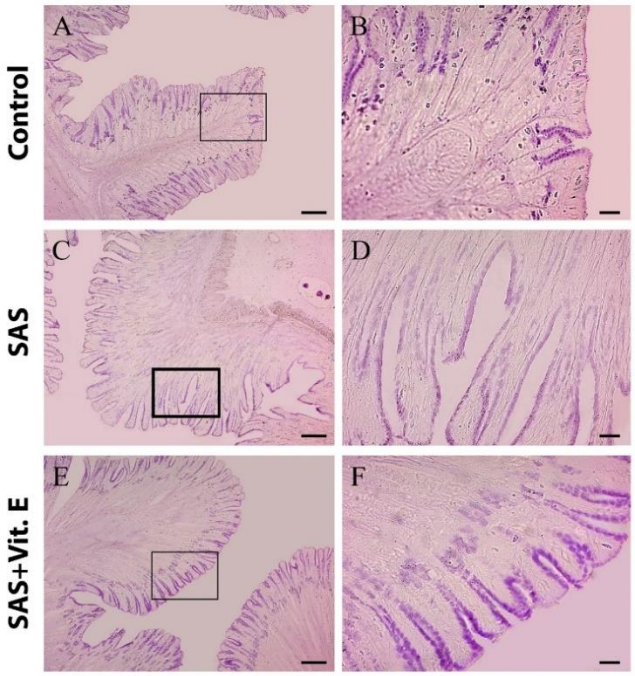

Figure 23: Light microscope photographs showing toad stomach stained with PAS. The control stomach showed normal architecture with normal biodistribution of carbohydrates (A and B). The stomach treated with SAS showing increase in mucus glands secretions which are enriched with carbohydrates (C and D). Stomach treated with Vit. E+SAS showing slightly normal activity of mucus glands similar to control (E and F). Scale bare: A, C, and E = 50 µm; B, D, and F = 10 µm.

3.2.2.3. Quantitative analysis of carbohydrate distribution in the stomach

Figure 24A showed the levels of carbohydrates throughout the stomach of mice in comparison to the control group. The SAS-treated group illustrated a very low level of carbohydrate expression by 2.125 times with significant P value ≤ 1.05795 $x10^{-10}$, less than the control group. Whereas the Vit. E and SAS treated group showed a normal level of carbohydrates, but slightly lower than that in the control group. Figure 24B analyzes the levels of carbohydrates throughout the stomach of toad in comparison to the control group. The SAS-treated group showed a very low level of carbohydrate expression less than the control group, while the Vit. E and SAS treated group showed higher levels of carbohydrates almost similar to that in the control.

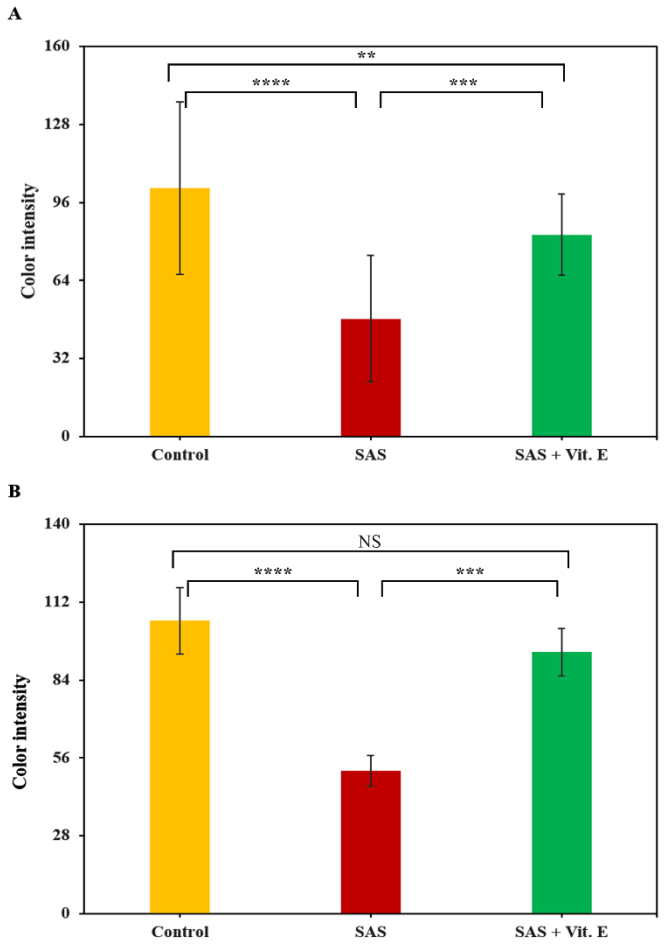

Figure 24: Quantitative analysis of the color intensity of PAS staining indicating the levels of carbohydrates in the gastric mucosa after treatment with SAS or with SAS and Vit. E. (A) Analysis of carbohydrate levels in albino mice's stomach after treatment with SAS or with SAS and Vit. E b. After SAS administration, the color intensity significantly reduced compared to that in the control group. The color intensity increased to a similar level in the control group after co-administration of Vit. E and SAS. (B) Analysis of carbohydrates presence in toad stomach after treatment with SAS or with SAS and Vit. E. The color intensity significantly decreased after SAS administration compared to that in the control group. After the co-administration of Vit. E and SAS, the color intensity elevated again to nearly comparable levels to the control group. Statistical data are presented as bars with standard deviations. Ten representative sections were obtained from each group of mice (n = 10) for measuring color intensity. ****, ***, **, * and NS indicate p value ≤0.0001, ≤ 0.001, ≤ 0.01, ≤ 0.05 and ≥ 0.05 respectively (Student's t-test).

3.2.3. Histochemical examination of stomach

3.2.3.1. Collagen biodistribution in mice's ileum

The level of collagen fibers in the ileum was carried out using Masson's trichrome stain as shown in Figure 25. In the control group, collagen fibers were distributed normally in the submucosal layer as well as in the villus lamina propria (Figure 25A and 25B). In contrast, the SAS-treated group exhibited collagen fibrosis throughout the villi and lamina propria (Figure 25 C and D). Similar to the control group, collagen fibers distribution was normal in the case of Vit. E+SAS treated group (Figure 25E and 25F).

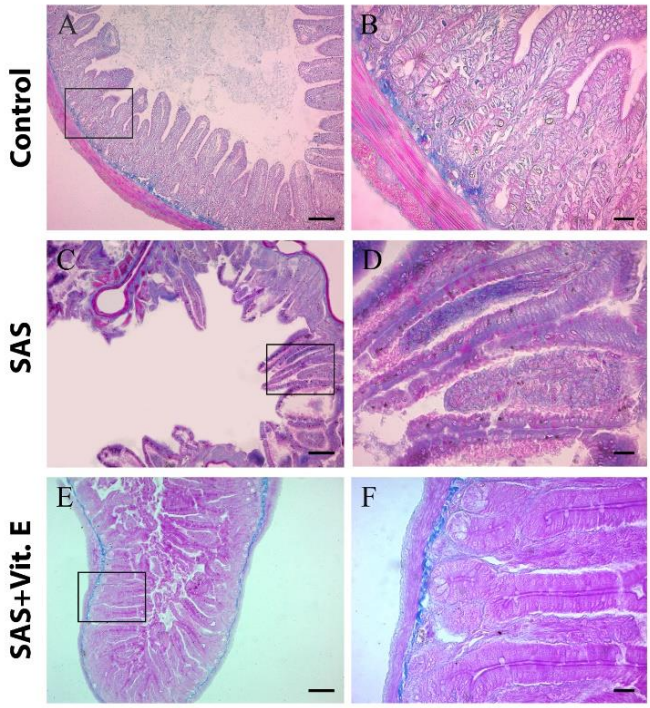

Figure 25: Light microscope photographs showing mice ileum stained with Masson trichrome. Control ileum with normal architectures with normal collagen fibers biodistribution (A and B). The ileum treated with SAS showed higher levels of collagen fibers in the lamina propria compartments (C and D). Ileum treated with Vit. E+SAS showed normal collagen fibers distribution similar to the control group (E and F). Scale bare: A, C and E = 50 µm; B, D and F = 10 µm

3.2.3.2. Collagen biodistribution in toad's ileum

The ileal fibrosis in toad was shown in Figure 26. Collagen fibers were distributed normally in the submucosa as well as lamina propria of the control group ileum (Figure 26A and 26B). Contrary to the first group, the SAS-treated group displayed significant collagen fibers accumulation throughout the villus and lamina propria (Figures 26C and 26D). Slight collagen fibers were observed in the sub-mucosal layer in the Vit. E and SAS treated group (Figure 26E and 26F).

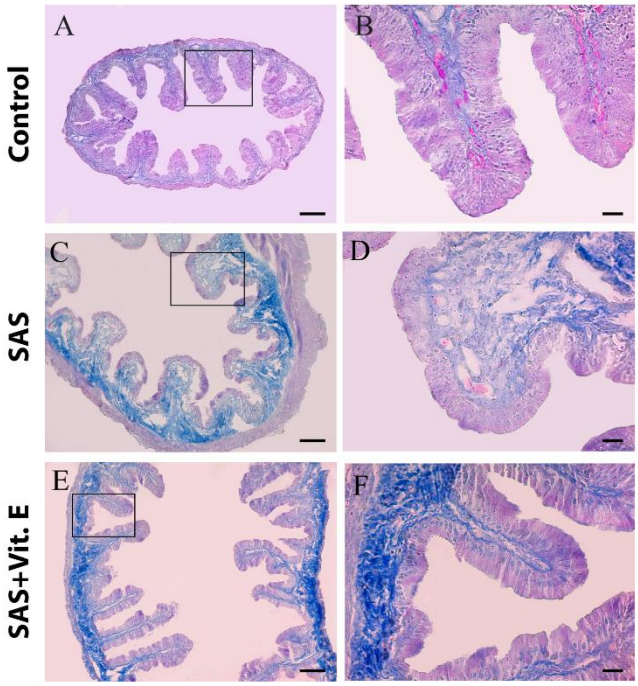

Figure 26: Light microscope photographs showing toad ileum stained with Masson trichrome. Control ileum with normal architecture with normal collagen fibers biodistribution in all compartments of the ileum (A and B). Ileum treated with SAS showing higher accumulation of collagen fibers in the sub-mucosal area and lamina propria compartments (C and D). Ileum treated with Vit. E+SAS showed slightly higher levels of collagen fibers in the submucosa layer and lamina propria compared to that in the control (E and F). Scale bare: A, C and E = 50 µm; B, D and F = 10 µm.

3.2.3.3. Carbohydrates biodistribution in mice's ileum

There was a slight decrease in the expression of carbohydrates in the epithelium and goblet cells of the control ileum (Figures **27A** and **27B**). In contrary with the control group the SAS-treated group showed intense expression of carbohydrates in the epithelium and goblet cells (Figures **27C** and **27D**). As shown in Figures **27E** and **27F**, the Vit. E and SAS treated group showed a decrease in the level of carbohydrate expression as compared to the SAS treated group, but still exhibited a bit higher level of carbohydrates compared to that in the control group.

3.2.3.4. Carbohydrates biodistribution in toads' ileum

Using a PAS stain the control ileum showed normal carbohydrate expression in its epithelium and goblet cells (Figures **28A** and **28B**). The epithelium and goblet cells of the SAS-treated group exhibited low expression of carbohydrates compared to those of the control group (Figures **28C** and **28D**). Compared to the SAS only treated group, the Vit. E and SAS treated group displayed a slightly higher level of carbohydrate expression than the SAS treated group, but still exhibited a bit lower level than the control group (Figure **28E** and **28F**).

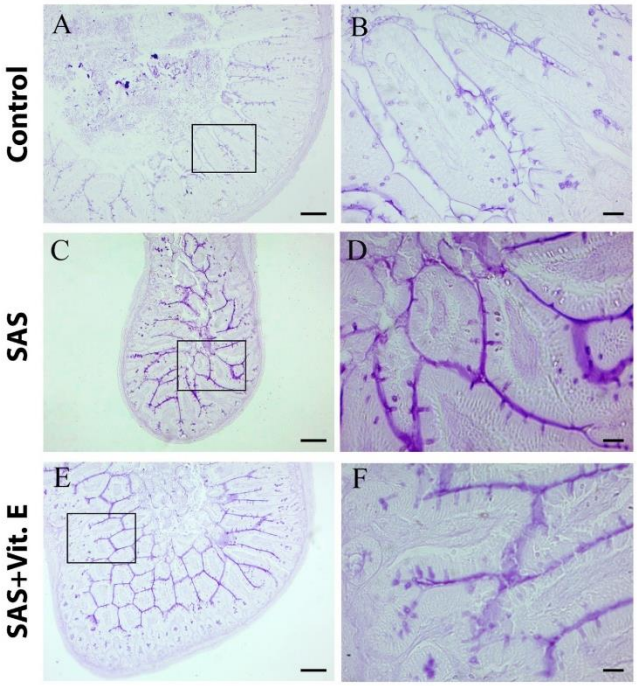

Figure 27: Light microscope photographs showing mice's ileum stained with PAS. Control ileum with normal architecture and normal levels and biodistribution of carbohydrates (A and B). The ileum treated with SAS showed higher sections of carbohydrates produced by goblet cells (C and D). Ileum treated with Vit. E+SAS showed slightly higher activity of goblet cells compared to that in the control group but relatively lower than ileum treated with SAS (E and F). Scale bare: A, C and E = 50 µm; B, D and F = 10 µm.

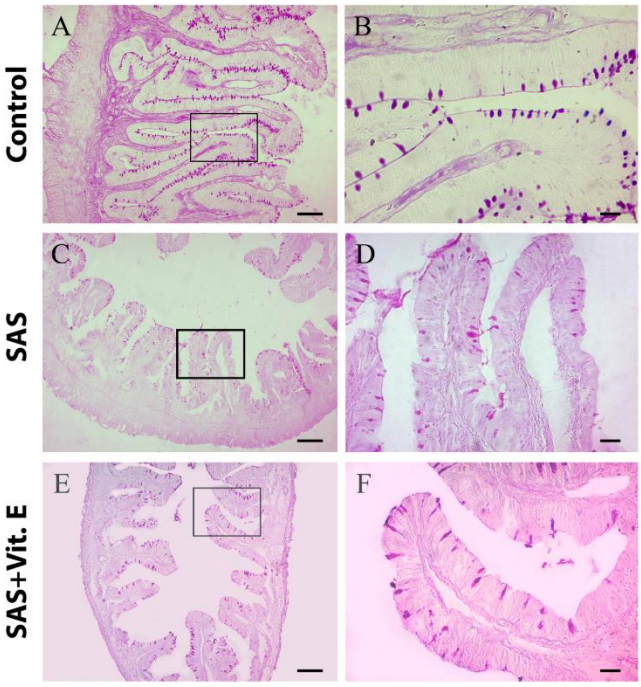

Figure 28: Light microscope photographs showing toad ileum stained with PAS. Control ileum with normal architecture and normal carbohydrate biodistribution (A and B). The ileum treated with SAS showed lower activity of goblet cells and lower production of carbohydrates compared to that in the control (C and D). Ileum treated

with Vit. E+SAS showed relatively lower activity of goblet cells and lower levels of carbohydrates compared with control (e and f). Scale bare: A, C, and E = 50 µm; B, D, and F = 10 µm.

3.2.3.5. Quantitative analysis of carbohydrate distribution in the ileum

As shown in Figure 29A, the levels of carbohydrates throughout the ileum after treatment with SAS or Vit. E and SAS were treated compared to that in the control group. The SAS-treated group had a higher level of carbohydrate expression compared to that in the control group by 1.3 times with significant P value ≤ 0.0004, while the Vit. E and SAS treated group had a normal level of carbohydrates similar to that of the control group. The carbohydrate levels were investigated throughout the ileum of toad (Figure 29B). When compared to the control group, the SAS-treated group showed a significantly lower level of carbohydrate expression compared to the control group by 1.4 times with a significant P value ≤ 0.0002, while the Vit. E and SAS treated group showed a normal level of carbohydrates, which was similar to the control group.

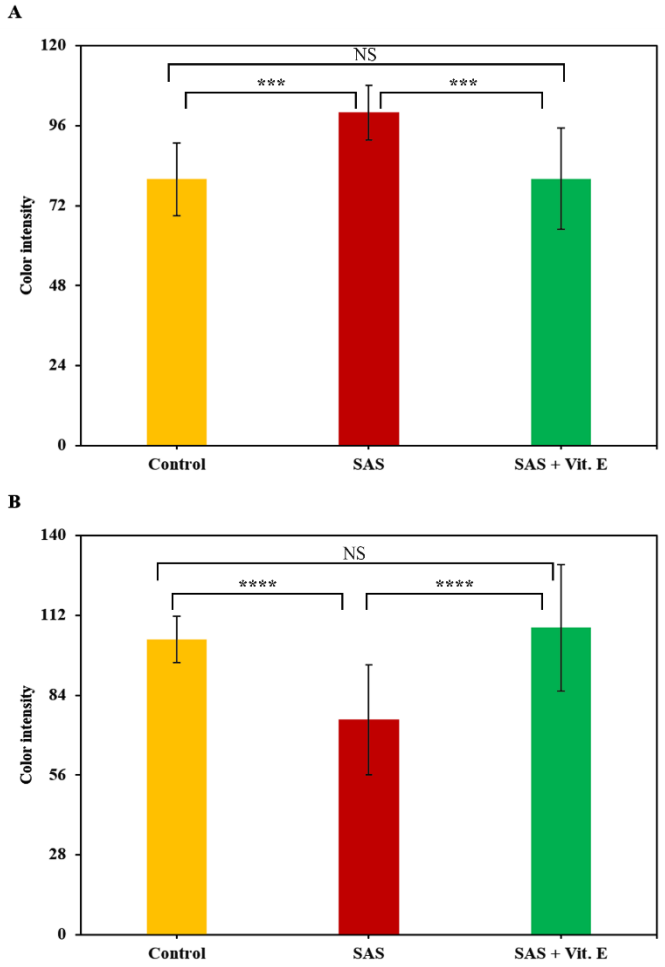

Figure 29: Quantitative analysis of the color intensity of PAS staining indicating the levels of carbohydrates in the ileum after treatment with SAS or with SAS and Vit. E. (A) Analysis of carbohydrates presence in albino mice's ileum after treatment with SAS or with SAS and Vit. E. Following SAS only administration, the color intensity slightly increased to that in the control group. While the color intensity reduced to be similar level in the control group after co-administration of Vit. E and SAS. (B) Analysis of carbohydrates presence in toad ileum after treatment with SAS or with SAS and Vit. E. The color intensity significantly decreased after SAS administration compared with that in the control group. After the co-administration of Vit. E and SAS, the color intensity was similar to the same level in the control group. Statistical data are presented as bars with standard deviations. The color intensity representing the presence of carbohydrates in goblet cells was evaluated in 30 goblet cells obtained from 10 representative sections in each group (n = 30) for measuring color intensity. ****, *** and NS indicate p value ≤0.0001, ≤ 0.001, and ≥ 0.05 respectively (Student's t-test).

3.3. Hematological examinations in mice

Figure 30 illustrated quantitative analysis of RBCs count and its biochemical parameters. Following SAS administration, the hemoglobin and RBCs counts in the SAS-treated group slightly decreased which indicates the onset of anemia. After receiving the protective dose of Vit. E, hemoglobin level reverted to normal, and the mice seemed to be recovering from anemia based on their MCV, MCH, and MCHC levels. Figure 31 shows a quantitative analysis of WBCs. The Total leukocyte count in the SAS-treated group significantly decreased, indicating inflammation induction. While in Vit. E and SAS treated group; the total leukocyte count slightly improved (Figure 31A). As a result of SAS only or Vit. E and SAS treated administration, the percentage of neutrophils significantly decreased, indicating the induction of inflammation (Figure 31B). While the percentage of lymphocytes significantly increased after SAS only treatment but it significantly decreased in the case of Vit. E and SAS treatment, which was consistent with our results and indicated the protective role of Vit. E.

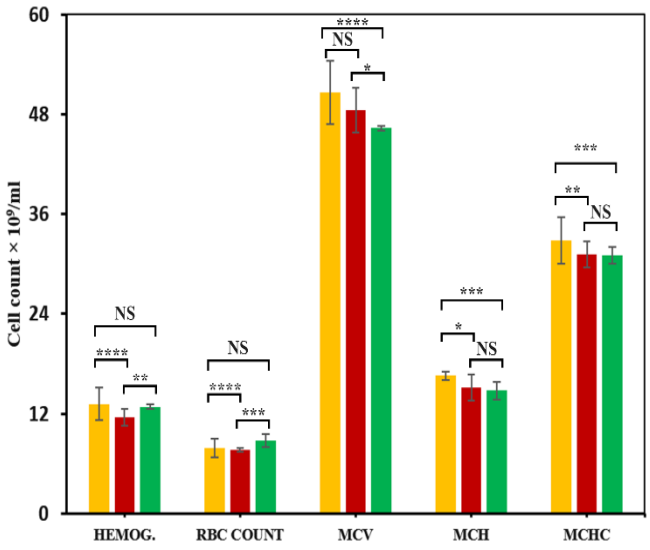

Figure 30: Analysis of RBCs counts and biochemical parameters of mice. The hemoglobin and RBC count in the SAS-treated group slightly decreased. After administration of the protective dose of Vit. E with SAS administration the hemoglobin level returned to normal. Statistical data are presented as bars with standard deviations. 3 representative blood samples were obtained from each group of mice (n = 3). ****, ***, **, * and NS indicate p value ≤0.0001, ≤ 0.001, ≤ 0.01, ≤ 0.05 and ≥ 0.05 respectively (Student's t-test).

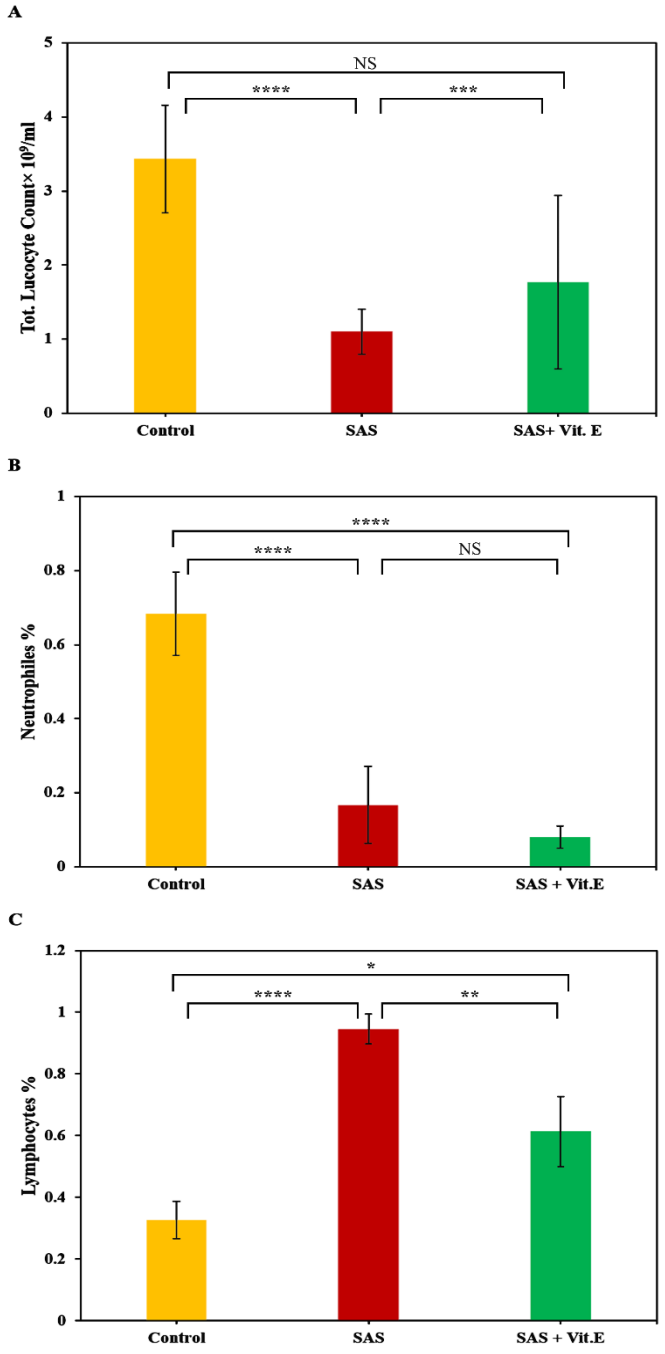

Figure 31: Analysis of WBCs of mice after treatment with SAS only or with Vit. E and SAS treated. (A), As a result of SAS administration, the total leucocyte counts significantly decreased compared to that in the control. After administration of the protective dose of Vit. E, the total leucocyte counts slightly decreased compared with control. (B), As a result of SAS administration, the percentage of neutrophils decreased compared to that in the control. While Vit. E and SAS treatment showed a significant decrease in the percentage of neutrophils compared with the control or SAS-treated group. (C), Lymphocytes percentage decreased significantly increased after SAS treatment compared to those in the control or Vit. E and SAS treated groups. Statistical data is presented as bars with standard deviations. 3 representative blood samples were obtained from each group of mice (n = 3). ****, ***, **, * and NS indicate p value ≤0.0001, ≤ 0.001, ≤ 0.01, ≤ 0.05 and ≥ 0.05 respectively (Student's t-test).

4. Discussion

Different methods can be used by some invertebrate creatures for defense and stealthiness against their predators [25-27]. Among them, gastropods can protect themselves from predators by using secretions from special glands, and skin acid secretions [5]. The gastropod *Berthellina citrina* produces SAS extract as a defense against predators [28]. This extract is composed mainly of acidic contents such as sulfate and chloride ions that characterize SAS secretions with lower pH. In this study the histological and histochemical abnormalities produced by SAS secretion after oral administration into representative of mammalian as well as amphibian representatives, moreover, the role of Vit. E as an antioxidant and anti-inflammatory agent against the toxicities of SAS was investigated.

In the present study, mice spleens treated with SAS exhibited the same histopathological abnormalities such as a high number of megakaryocytes, cytoplasmic degeneration, collagen fibers induction, and white pulp follicles were atrophied. These data are in accordance with the results from the previous study, which showed that SAS induced an increase in megakaryocytes and a decrease in the number of splenocytes in the spleen [15]. Regarding toads, interestingly, the results were much similar considering the histological differences between mice' and toads' spleens. Toad's spleen had severe distortion, the red pulp contained fewer splenocytes, and the spleen exhibited cytoplasmic degeneration and a very high number of melanomacrophages after treatment with SAS. Atrophies and distortions occurred in white pulp follicles, and splenocytes decreased in red and white pulps, resulting in wide spaces between splenic cords as in toad's spleen. This finding finding was consistent with a previous study on the toxicity of SAS extract in spleen [15]. Generally, the function of the spleen of both mammalian and amphibian representative was disrupted by the presence of SAS contents in the blood and splenic compartments after oral administration. The acidic components of SAS might react with splenic immune cells and compartments to produce different inflammatory mediators such as cytokines or chemokines that induced fibrosis and inflammation. A similar effect was observed in the spleen of mice treated with ink fluid of sea hare Aplysia dactylomela which contains a high percent of H_2O_2 [29]. Vit. E has many advantages as antioxidant or anti-inflammatory properties [18]. The simultaneous administration of Vit. E along with SAS extract, reduced inflammation in the spleen of mice and toad, decreased megakaryocytes number in mice spleen, and decreased MMs number in toad spleen. This data suggests that, Vit. E has antioxidant as well as anti-inflammatory effects against spleen inflammation produced by SAS extract administration into higher vertebrates such as amphibians and mammalians.

Herein, SAS extract administration in mice's liver produced several abnormalities including, lymphocyte infiltration, blood vessels dilation and number increase, collagen fibers increase, and carbohydrate levels decreased. While SAS extract administration in toads' liver similar effects to those in the mice's liver such as lymphocyte infiltration, vascular dilation, and degenerations and the number of MMs increased,

indicating severe inflammation. This data indicated that the acidic contents of SAS induced different abnormalities in the liver of mice and toads. These results are in agreement with a report by Pedoto and coworkers [30] who demonstrated that metabolic acidosis led to hepatic inflammation, vasodilation, and shock in healthy rats. In this work Vit. E administration simultaneously with SAS showed improvement induced abnormalities in the liver of toads and mice, such as less congested blood vessels, less vasodilation, less lymphocyte infiltration, improved numbers of blood vessels, and normal levels of collagen and carbohydrates. Importantly Vit. E is an agent for angiogenesis control, reduced blood vessel vasodilation, and angiogenesis. Thus, Vit. E administration appears to show a beneficial effect in reducing SAS's toxic effect on the liver in both mice and toads. The findings are consistent with those reported in [18, 20, 31] which demonstrate the ability of Vit. E in moderating vasodilation, regulation of angiogenesis, and its anti-inflammatory properties.

In the case of the stomach, it was observed that after oral administration of SAS extract, the mucosal epithelium layer in the stomach of mice and toads showed different induced abnormalities. The findings data are similar to previously reported data with Penner who reported that very strong acids such as HCl and H_2SO_4 may produce rapid gastric necrosis with early perforation [32]. The results of this study are consistent with Penner's description as SAS mainly consists of HCl and H_2SO_4. Administration of Vit. E showed improvement in the histological structure of mice and toads' gastric mucosa in this study. Previously, according to a study induced by Kurose and coworkers Vit. E may prevent oxidative damage to gastric mucosa [33].

Some villus lamina propria degeneration and changes in the size of the intestinal villus were seen in the ileum of the mice and toads after administration with SAS. Previously Pedoto and coworkers [30] reported that acidosis caused varying degrees of necrosis in the distal, middle, and proximal small intestines. Inflammatory infiltrations containing neutrophils, eosinophils, and mononuclear cells were associated with superficial necrosis, and crypt distortion, and the lamina propria and mucosa were most affected. The treatment with Vit. E reduced the abnormalities in the lamina propria, improved villi degeneration, and normalized carbohydrate levels in both mice and toads' villi. previous studies reported that, Vit. E has demonstrated its ability to protect the intestinal mucosa from oxidative stress [34]. This data is in agreement with our findings in this study and suggests the ability of Vit. E in ameliorating abnormalities in the villi induced by SAS.

The treated kidney with SAS extract showed shrinkage of the renal glomerulus of mice kidney while enlargement in the renal glomerulus of toads' kidney was observed. Some of these results is in agreement with previous studies, one of them reported that acidosis caused by citric acid in mice's kidneys showed enlargement of renal glomerulus and cortex degeneration [35]. Vit. E administration showed improvement in the kidney cortex structure as well as a normal size of the glomerulus was observed. Previously Vit. E has been reported to possess protective effects against renal degeneration and apoptosis [36], indicating the anti-informatory role of Vit. E against renal degeneration and inflammation.

5. Conclusion

In conclusion according to the findings of this study, oral administration of SAS produced different histological and histochemical abnormalities in the spleen, liver, stomach, intestine, and kidney in both mice and toads. These abnormalities include tissue degeneration, lymphocytes infiltration, immune cells cell number change, blood vessels' structure change, and changes in the levels of collagen and carbohydrates levels. Interestingly, Vit. E as an anti-inflammatory and antioxidant agent had a significant protective role against abnormalities produced by SAS administration in albino mice and toads. Administration of Vit. E reduced the number of inflammatory cells, normalized the collagen and carbohydrate levels and improved the structure of blood vessels in the liver and spleen of treated mice and toads with SAS extract. This data showed the importance of Vit. E against abnormalities produced by SAS extract after SAS administration into higher vertebrates' representatives such as mice and toads. This is a basic study to investigate the toxicity of SAS extract and in the future, this can be used as economical value. Also, understanding the effect of the SAS secretion as a chemical defense can help in understanding its ecological impacts. This study provides new information on the biological activity of a marine extract that could be exploited in the natural products industry, as well as in medical and biological research.

CRediT authorship contribution statement:

The authors confirm their contribution to the paper as follows: study conception and design: Alaa Moustafa & Aziz Awaad; data collection: Alaa Moustafa, Aziz Awaad and Salma El-Saraf; analysis and interpretation of results: Alaa Moustafa, Aziz Awaad & Salma El-Saraf; draft manuscript preparation: Aziz Awaad & Salma El-Saraf; All authors reviewed the results and approved the final version of the manuscript.

Data availability statement

The data used to support the findings of this study are available from the corresponding author upon request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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