

## ADRENAL HISTO-TOXICOLOGICAL IMPACTS OF BIFENTHRIN (A CHLORO-FLUORIDATED-PYRETHROID) ARE REVERSED ON *NIGELLA SATIVA* SEED OIL TREATMENT IN MICE

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**ABSTRACT:** The adrenal histopathologies resulting from exposure to the fluoridated insecticide bifenthrin (Bif) and their amelioration upon post-treatment with *Nigella sativa* (NS) seed oil were explored in mice. Six groups of five animals each, namely (i) Vehicle Control (VC)—0.1 mL corn oil daily for 14 days; (ii) Positive Control (PC)—0.1 mL corn oil for 7 days followed by 0.1 mL of 10% NS seed oil in corn oil (v/v) for the next 7 days; (iii) and (iv) Bif 2.5 mg/kg (Bif2.5) and Bif 5mg/kg (Bif5)—2.5 and 5 mg Bif/kg, respectively, in 0.1 mL corn oil for 7 days + 0.1 mL corn oil only for the next 7 days; and (v) and (vi) Bif 2.5mg/kg+NS (Bif2.5N) and Bif 5 mg/kg+NS (Bif5N)—Bif exposures as in the Bif2.5 and Bif5 groups, respectively, for 7 days + 0.1 mL of 10% NS seed oil in corn oil (v/v) for the next 7 days. All the treatments were provided intragastrically by gavages. The adrenal glands from each animal were processed for histological and micrometric studies after euthanasia on day 15. We found in the medullary part of the adrenal a depletion of the storage granules and a significant enlargement ( $p \leq 0.01$ ) in the mean cross-sectional area (CSA) of the chromaffin cells in the Bif2.5 ( $185 \pm 2.6 \mu\text{m}^2$ , mean  $\pm$  SEM) and Bif5 ( $192 \pm 2.6 \mu\text{m}^2$ ) groups compared to the Bif2.5N ( $167 \pm 1.9 \mu\text{m}^2$ ) and Bif5N ( $158 \pm 2.6 \mu\text{m}^2$ ) groups. Simultaneously, the basal  $\alpha$ -zone of the cortical regions was breached at scattered places with blood profusion in the zona fasciculata. The number of cells per fascicle in the Bif2.5, Bif5, Bif2.5N, and Bif5N groups ( $18.6 \pm 0.4$ ,  $19.4 \pm 0.3$ ,  $16.3 \pm 0.5$ , and  $16.6 \pm 0.4$ , respectively) were significantly higher ( $p \leq 0.05$ ) than in the VC and PC groups ( $14.9 \pm 0.3$  and  $15.5 \pm 0.4$ , respectively). In contrast, the mean CSA of the fascicular cells was significantly higher in the PC ( $101 \pm 2 \mu\text{m}^2$ ) and VC ( $103 \pm 2.8 \mu\text{m}^2$ ) groups than in the Bif treated groups ( $75 \pm 1.9 \mu\text{m}^2$ ,  $66 \pm 2.5 \mu\text{m}^2$ ,  $95 \pm 1.8 \mu\text{m}^2$ , and  $91 \pm 2 \mu\text{m}^2$ , respectively). The results indicate that the histopathological and micrometric toxicity induced by Bif exposure in the Bif2.5 and Bif5 groups showed a rapid recovery on post-treatment with NS oil indicating that although Bif is potentially toxic to the adrenals, at a daily exposure dose of 2.5 mg/kg or higher for 7 or more days, the adrenal pathologies induced can be rapidly reversed by NS seed oil.

Keywords: Adrenal; Bifenthrin; Histo-toxicology; *Nigella sativa*.

### INTRODUCTION

In addition to their systemic, reproductive, developmental, and neurotoxic capacities, pyrethroids have also been found to exhibit endocrine disruptive activities.<sup>1,2</sup> Such endocrine disruptions are usually mediated through receptor interactions and endogenous hormone production.<sup>3</sup> Bifenthrin is a 2<sup>nd</sup> generation chloro-fluoridated type 1 synthetic pyrethroid insecticide that has been used frequently in the agricultural sector as well as domestically and in the workplace.<sup>4</sup> The cis-isomer of Bif has been reported to interfere in the biosynthesis of testosterone<sup>5</sup> and a closely related chloro-fluoridated pyrethroid insecticide,  $\lambda$

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cyhalothrin, a type II fluoridated pyrethroid, has been shown to be able to cause dose-dependent necrosis in the adrenal cortex in rats.<sup>6</sup>

As well as disrupting the functions of the thyroid, testes, ovaries, and adrenals, the direct oxidative pathologies resulting from exposure to various pesticides may also involve hypothalamic and pituitary regulatory mechanisms.<sup>7-10</sup>

The adrenals are endocrine organs with multifarious functions that regulate diverse physiological processes including those involving the stress response, immune-inflammatory responses, homeostasis, metabolism, and cardiovascular function.<sup>11</sup> Thus the adrenal hormones directly affect several vital bodily organs, including the heart, liver, kidney, bones, muscles, and brain, through their regulatory effects on the metabolism of carbohydrates, proteins, and lipids.<sup>12</sup> Stress-related actions such as exposure to an insecticide trigger the adrenal medulla and cortex resulting in enhanced secretions of catecholamines and glucocorticoids, respectively.<sup>13</sup> Various insecticides, diazinon, malathion, cypermethrin, and  $\lambda$ -cyhalothrin, have been reported to cause adrenal pathologies.<sup>6,14-17</sup> In the present research work we report on the adrenal pathologies induced in mice by exposure to Bif, a fluoridated insecticide. Additionally, we also discuss the protective neutraceutical capacity on these pathological manifestations of *Nigella sativa* seed oil, which contains a huge variety of bioactive compounds (polyphenols and tocopherols, campesterol, stigmasterol,  $\beta$ -sitosterol,  $\alpha$ -spinasterol, citronellol, limonene, p-cymene, citronellyl acetate, carvone, nigellone, thymoquinone, dithymoquinone, thymohydroquinone, thymol) and fatty acids (arachidic, linolenic, linoleic, myristic, oleic, palmitic, palmitoleic, and stearic acids).<sup>18-21</sup>

## MATERIALS AND METHODS

*Animal maintenance and care:* The present study was carried out on thirty 56 $\pm$ 2 day-old-male mice, weighing 30 $\pm$ 1g. The animals were reared and maintained under the set protocols of our lab (12 hr dark-light cycles, 23 $\pm$ 2°C temperature with free access to food and water).<sup>22</sup>

*Preparation of Bif solutions:* The required dilutions (2.5 mg/kg and 5 mg/kg) of Technical (98%) Grade Bif (Batch No# Auc/20130611, manufactured by Be Star China) were prepared in corn oil.

*Preparation of the Nigella sativa oil dilutions:* *Nigella sativa* seed oil (a product of Marhaba Laboratories Limited, Lahore Pakistan) was diluted in corn oil (10% v/v) for the intra-gastric animal treatments in the PC, Bif2.5N, and Bif5N groups.

*Treatment profiles and the animal groups:* The names and treatment profile of six study groups (n=5) were as follows.

(i) Vehicle Control (VC): Each animal was force fed 0.1 mL corn oil daily for 14 days by gavages.

(ii) Positive Control (PC): Each animal was force fed 0.1 mL of pure corn oil daily for 7 days and 0.1 mL 10% (v/v) NS oil/corn oil for the next 7 days.

(iii) 2.5 mg/kg Bif (Bif2.5): Each animal was force fed 2.5 mg/kg Bif dissolved in 0.1 mL corn oil daily for 7 days and 0.1 mL pure corn oil for the next 7 days.

(iv) 5 mg/kg Bif (Bif5): Each animal was force fed 5 mg/kg Bif dissolved in 0.1 mL corn oil daily for 7 days and 0.1 mL pure corn oil for the next 7 days.

(v) 2.5 mg/kg Bif + NS (Bif2.5N): Each animal was force fed 2.5 mg/kg Bif dissolved in 0.1 mL corn oil daily for 7 days and 0.1 mL 10% (v/v) NS oil/corn oil for the next 7 days.

(vi) 5 mg/kg Bif + N.S (Bif5N): Each animal was force fed 5mg/kg Bif dissolved in 0.1mL corn oil daily for 7 days and 0.1 mL 10% (v/v) NS oil/corn oil for the next 7 days.

*Excision of the adrenals and the histological preparations:* The animals were sacrificed on day 15 to obtain the adrenals. The entire adrenal glands of each animal were fixed in 10% formaldehyde buffered solution for 48 hr. They were finally processed for dehydration through various grades (50, 70, 90, and 100%) of absolute ethanol and finally treated with xylol before wax embedding at 57°C. Serial sections (3 µm thick) of the adrenal glands were obtained on a rotary microtome (ERMA, TOKYO JAPAN) and finally stained using hematoxylin and eosin stains to produce permanent slides for the histopathological and micrometric studies.

*Histological observations:* The histological sections were observed and digitally captured at magnifications of 100× and 400× in a 7.2 MP Sony (DSC-W35) camera mechanically affixed on a trinocular research microscope (Labomed CXR<sub>2</sub>).

*Processing and labeling of the digital photomicrographs:* Selected digital camera shots were processed for adjustments in brightness/contrast, cropping, and insertion of various labels to highlight the histological/pathological signs in corelDRAW11 for presentation in the result section (Figures 1–3).

*Digital micrometry and data analysis:* Four randomly selected digital camera shots (2 from each adrenal) were used for the various micrometric measurements of the adrenals with help of a pre-calibrated digital scale in corelDRAW11. The digital images (100× and 400×) of the stage micrometer (ERMA, JAPAN) obtained on the defined camera specs, for histological photography, were used to obtain calibrated scales for the said magnifications. The micrometric measurements were obtained for measuring the cross sectional areas (CSAs) of the cells of the cortical region (zona glomerulosa and zona fasciculata) and the number of endocrine cells per fascicle in the zona fasciculata. Similarly, the number of chromaffin cells per unit area (µm<sup>2</sup>) of the adrenal medulla, the cross-sectional areas of the medullary chromaffin cells, their nuclei, cytoplasts and the collective area of the basophilic cytoplasmic granules per chromaffin cell were measured. The following formulae were employed to obtain the various micrometric values:

$$CSA = \left( \frac{\text{Length} \times \text{Width}}{4} \right) \times \pi$$

where:

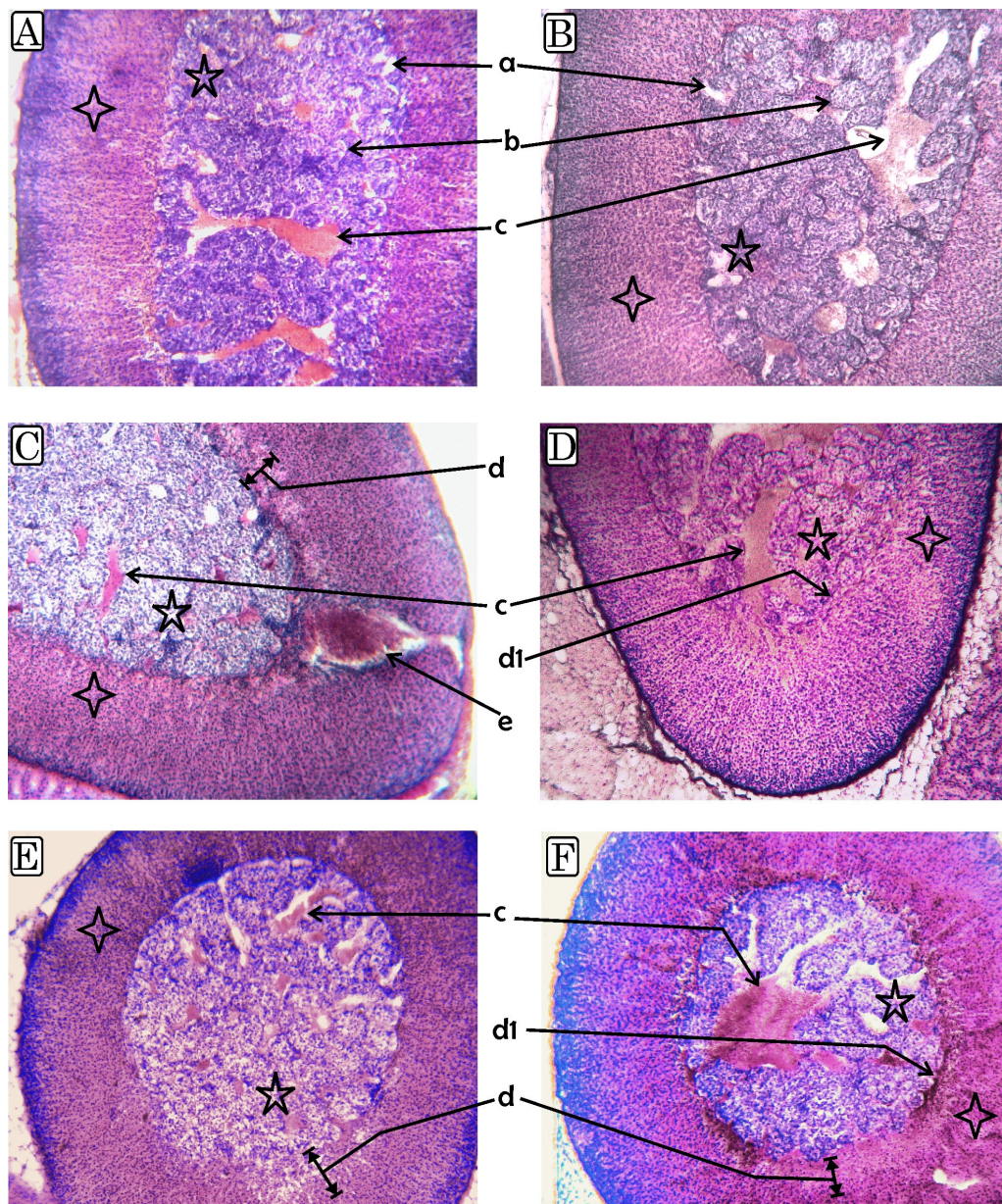
CSA = cross sectional area of the cortical and medullary cells, cell nucleus, cytoplasmic granules, etc in µm<sup>2</sup>, the length is in µm, and the width is in µm.

Cytoplasmic granular area per cell = cumulative CSA of all the cytoplasmic granules

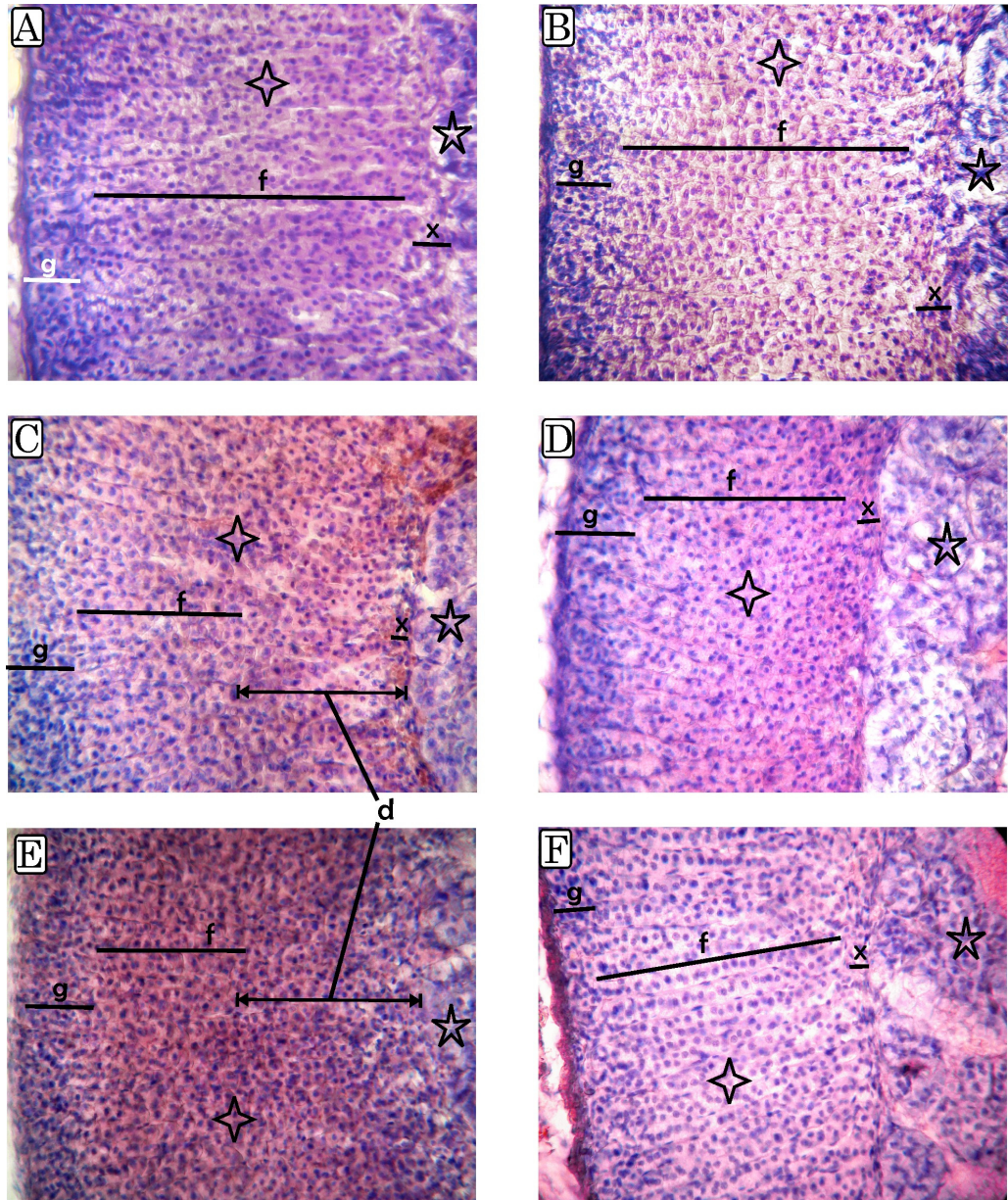
The micrometric data obtained for these parameters was analyzed statistically for “analysis of variance” and “Tukey’s HSD” through SPSS20 software.

**RESULTS**

*Histology of the adrenal cortex and medulla:* The histological sections of the VC group animals showed two distinct cortical zones, the zona glomerulosa and zona fasciculata). The basophilic chromaffin cells of the adrenal medulla were arranged in a follicular pattern embedded in the fibro-vascular stroma containing scattered blood filled spaces (sinusoids) and blood vessels (Figures 1A, 2A, and 3A).



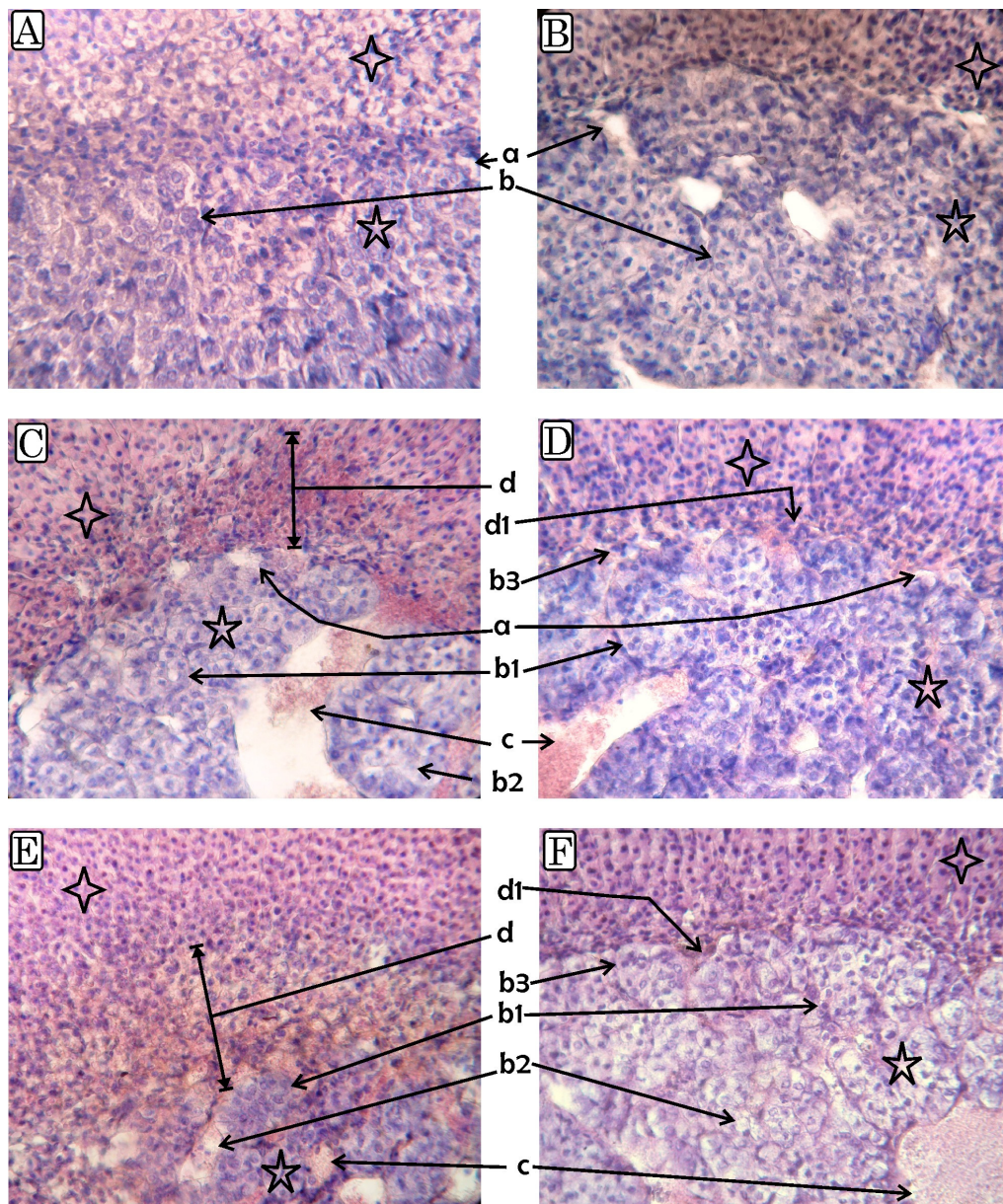
**Figure 1.** Histological sections of adrenals (100×); A: (VC), B: (PC), C: (Bif2.5), D: (Bif2.5N), E: (Bif5), F: (Bif5N); four-pointed star: adrenal cortex; five-pointed star: adrenal medulla; a: medullary marginal spaces; b: medullary follicle of the chromaffin cells; c: medullary blood sinusoids; d: cortical blood infiltration due to breached xzone barrier; d1: xzone repaired; and e: cortical blood vessel.



**Figure 2.** Histological sections of adrenal cortex (400×); A: (VC), B: (PC), C: (Bif2.5), D: (Bif2.5N), E: (Bif5), F: (Bif5N); four-pointed star: adrenal cortex; five-pointed star: adrenal medulla; g: zona glomerulosa; f: zona fasciculata; x: x-zone; and d: cortical blood infiltration due to breached x-zone barrier.

The cortical and medullary regions were clearly separated by a thin basal cortical region the so called “x-zone”. The cells in the zona glomerulosa were basophilic and smaller in size as compared to the cells in the zona fasciculata (Figure 2A). The PC group adrenal sections showed a more or less similar histological distribution to that present in the VC adrenal (Figures 1B, 2B, and 3B). The histopathological signs appearing in the Bif-treated groups (Bif2.5 and Bif5) include randomly placed enlarged follicles with hypertrophy of the chromaffin cells. However, generally the

the chromaffin cells were less densely stained, possibly due to the depletion of the stored catecholamines. The signs of apoptosis in individual medullary endocrine cells were also identifiable in the Bif2.5, Bif5, and Bif5N groups (Figures 3C, 3E, and 3F). The zona glomerulosa remained almost completely undisturbed. However, the zona fasciculata showed cellular hypertrophy and, in addition, the breaches of the  $\times$ -zone which allowed a profusion of blood in the zona fasciculata and caused disruption of the cortical fascicles at various places (Figures 1C, 1E, 2C, 2E, 3C, and 3E).



**Figure 3.** Histological sections of adrenal medulla (400 $\times$ ); A: (VC), B: (PC), C: (Bif2.5), D: (Bif2.5N), E: (Bif5), F: (Bif5N); four-pointed star: adrenal cortex; five-pointed star: adrenal medulla; a: medullary marginal spaces; b: medullary follicle of the chromaffin cells; b1: medullary follicles containing chromaffin cells with granular depletion; b2: probable apoptosis of the chromaffin cells; b3: medullary follicle regeneration; c: medullary blood sinusoids; d: cortical blood infiltration due to breached  $\times$ zone barrier; and d1:  $\times$ zone repaired.

Like the Bif2.5 and Bif5 groups, the histological sections in the Bif2.5N and Bif5N groups also showed chromaffin cell apoptosis at scattered places. However, regeneration of the medullary follicle, with prominent signs of chromaffin cells mitosis, was especially visible below the  $\times$ -zone (Figures 1D, 1F, 3D, and 3F). Blood profusions were observed at scattered places in the cortical portion of the adrenal in the Bif5N group (Figure 1F) while the cortical region in the Bif2.5N group showed a completely repaired  $\times$ -zone and, like that of the VC and PC groups, the zona glomerulosa and zona fasciculata showed fairly normal cell distributions (Figures 1D and 2D).

*Micrometry of adrenal cortex:* With slight variations, the mean CSA of the endocrine cells in the zona glomerulosa remained almost unchanged. In contrast, the analysis of data for the fascicular endocrine cells indicated a significant decrease ( $p \leq 0.01$ ) in the mean cellular CSA in the Bif5 group compared to the Bif2.5 group. Furthermore, both the Bif2.5 and Bif5 groups showed a significantly smaller cellular CSA of the zona fasciculata to the other four groups. Interestingly, the mean number of cells per fascicle were significantly higher ( $p \leq 0.01$ ) in the two Bif groups compared to the CV, PC, Bif2.5N, and, Bif5N groups (Table 1).

**Table 1.** Fascicular cell count and the cross-sectional area (CSA) of the glomerular and the fascicular cells (Values are mean $\pm$ SEM)

Micrometric parameter	Group					
	VC	PC	Bif2.5	Bif5	Bif2.5N	Bif5N
Mean CSA of cells in the zona glomerulosa ( $\mu\text{m}^2$ )	43 $\pm 1.6$	42 $\pm 1.5$	42 $\pm 2.0$	43 $\pm 2.6$	43 $\pm 1.8$	42 $\pm 1.3$
Mean CSA of cells in the zona fasciculata ( $\mu\text{m}^2$ ) *	101 $\pm 2^a$	103 $\pm 2.8^a$	75 $\pm 1.9^b$	66 $\pm 2.5^c$	95 $\pm 1.8^{ad}$	91 $\pm 2^d$
Mean number of endocrine cells per fascicle*	14.9 $\pm 0.3^a$	15.5 $\pm 0.4^{ab}$	18.6 $\pm 0.4^c$	19.4 $\pm 0.3^c$	16.3 $\pm 0.5^b$	16.6 $\pm 0.4^b$

\*: Any two groups means for a given parameter not sharing a common lower case superscript letter, <sup>a,b,c</sup> and <sup>d</sup>, differ significantly from each other at a significance level of  $p \leq 0.01$ .

*Micrometry of adrenal medulla:* The mean CSA of the chromaffin cells in the Bif2.5 and Bif5 groups remained significantly higher than that of the Bif2.5N and Bif5N groups. However, the smallest mean chromaffin cell CSA was recorded in the VC and PC groups which also differed significantly from all the Bif treated groups, both with and without NS. Almost similar trends were shown in the data for the chromaffin cell nuclear CSA among the groups. An obvious significant decline ( $p \leq 0.05$ ) in the basophilic cytoplasmic granular area per chromaffin cell was found in the Bif2.5 and Bif5 groups as compared to the CV, PC, Bif2.5N, and, Bif5N groups. With slight variations the mean number of chromaffin cells per unit area of the adrenal medulla remained almost unaffected in all the groups except the Bif5 group which showed a significant decrease compared to the other groups (Table 2).

**Table 2.** Micrometric variations in the adrenal medulla (Values are mean±SEM)

Micrometric parameter	Group					
	VC	PC	Bif2.5	Bif5	Bif2.5N	Bif5N
Mean CSA of the chromaffin cells ( $\mu\text{m}^2$ )*	141 ±2.5 <sup>a</sup>	134 ±0.8 <sup>a</sup>	185 ±2.6 <sup>b</sup>	192 ±2.6 <sup>b</sup>	167 ±1.9 <sup>c</sup>	158 ±2.6 <sup>c</sup>
Mean nuclear CSA of the chromaffin cells ( $\mu\text{m}^2$ ) <sup>†</sup>	36 ±1.6 <sup>ab</sup>	34 ±1.1 <sup>a</sup>	44 ±1.9 <sup>cd</sup>	47 ±1.4 <sup>d</sup>	39 ±1.4 <sup>abc</sup>	41 ±2.1 <sup>bcd</sup>
Mean CSA of the cytoplasmic granules per chromaffin cell ( $\mu\text{m}^2$ ) <sup>†</sup>	18 ±1.6 <sup>a</sup>	19 ±2.4 <sup>a</sup>	12 ±1.4 <sup>b</sup>	11 ±1.3 <sup>b</sup>	20 ±1.7 <sup>a</sup>	16 ±2.4 <sup>ab</sup>
Mean non-granular cytoplasmic area of the chromaffin cells ( $\mu\text{m}^2$ ) <sup>†</sup>	89 ±2.6 <sup>a</sup>	82 ±1.8 <sup>a</sup>	133 ±3.1 <sup>b</sup>	142 ±2.9 <sup>b</sup>	111 ±1.8 <sup>c</sup>	103 ±2.7 <sup>c</sup>
Mean no. of cells/unit area in the adrenal medulla	31 ±0.6 <sup>a</sup>	32 ±0.8 <sup>a</sup>	29 ±0.5 <sup>a</sup>	24 ±0.7 <sup>b</sup>	31 ±0.9 <sup>a</sup>	30 ±0.9 <sup>a</sup>

\*: Any two groups means for a given parameter not sharing a common lower case superscript letter, <sup>a,b,c</sup> and <sup>d</sup>, differ significantly from each other at a significance level of  $p \leq 0.01$ .

†: Any two groups means for a given parameter not sharing a common lower case superscript letter, <sup>a,b,c</sup> and <sup>d</sup>, differ significantly from each other at a significance level of  $p \leq 0.001$ .

## DISCUSSION

The established neuro-toxic physiology of the pyrethroid insecticides is revealed by their primary action on the sodium channels in the motor neurons causing repeated impulses and the secretion of neurotransmitters on the postsynaptic neurons or the target organs such as muscles and the endocrine and exocrine glands.<sup>23,24</sup> The present study reveals the Bif histopathology of the adrenals in mammals. The results obtained are, on one hand, interesting to unearth as they show, for the first time, the histopathological and micrometric derangements of the adrenals with sub-chronic low dose Bif exposure and, on the other hand, they provide exciting information about the ameliorative potential of NS oil upon these pathological outcomes. Because of an acute dearth of information on adrenal histopathology, the results obtained are not directly comparable to any previous report in the available literature. Nevertheless, in the general perception, the organophosphorus and pyrethroid insecticides are believed to possess neuro-endocrine toxic potentials.<sup>15,25</sup> In this connection  $\lambda$  cyhalothrin, a fluoridated type II pyrethroid, has been reported to possess adrenal toxicity.<sup>6</sup> Similarly, exposure to deltamethrin, a brominated pyrethroid, has been found to increase the circulating epinephrine and nor-epinephrine levels in rats.<sup>26-28</sup>

The secretion from the adrenal medulla under stressful conditions of the catecholamines (adrenaline and noradrenaline) help with the adjustments necessary in the physiology of the animal body for tackling emergency situations.<sup>29,30</sup> The



glucocorticoid secretions from the zona fasciculata of the adrenal cortex, cortisol and corticosterone, have immunosuppressive and anti-inflammatory effects and assist in allowing the body to responses to toxicants and toxoids.<sup>31</sup> The secretion from the zona glomerulosa of mineralocorticoids, such as aldosterone, plays an important role in the regulation of blood pressure and electrolyte balance. The innermost layer of the cortex, the zona reticularis, produces androgens that are converted to fully functional sex hormones in the gonads and other target organs. Taken together, the hormonal responses of the adrenal medulla and cortex to exposure to toxicants such as Bif involve an enhanced secretion of catecholamines and glucocorticoids from the respective regions of the adrenal gland. In the present study, the histopathological and micrometric alterations observed in the medullary adrenal with Bif exposure, such as enlargement of the chromaffin cells, cytoplasmic granular depletion, etc, show that the stress of Bif exposure enhances catecholaminergic secretion. Similarly, the breached sanctity of the  $\times$ -zone, together with a simultaneous significant decrease in the mean cellular CSA and hyperplasia in the zona fasciculata of the adrenal cortex, indicates that Bif exposure stress causes pathologies and micrometric alterations that are the most probably a consequence of the sustained hyperglucocorticoid synthesis and release.

NS seeds have been reported to contain medicinal potential in the Islamic system of medication and treatment for disease and ailments.<sup>32</sup> NS oil contains unique bioactive phytochemicals (thymoquinone, carvacol, t-anethole, 4-terpinol, cholesterol, campesterol, stigmasterol,  $\beta$ -sitosterol,  $\alpha$ -spinasterol, citronellol, limonene, p-cymene, citronellyl acetate, carvone, nigellone, and arachidic, linolenic, linoleic, myristic, oleic, palmitic, palmitoleic, and stearic acids) which have unique anti-oxidative, immunomodulatory, and stress-alleviation potentials.<sup>33,34</sup> In the present study, the NS oil treatment to the Bif-exposed animals normalized the toxic histopathological signs of the Bif exposure on the adrenals.

### CONCLUSION

The present study has shown that Bif exposure at 2.5 mg/kg or more may result in characteristic histopathological and micrometric alterations in mouse adrenal medulla and cortex and that NS seed oil has the potential to rapidly ameliorate these stress-induced changes.

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