AMELIORATION BY BLACK SEED (*NIGELLA SATIVA*) OIL OF HEPATO-HISTOPATHOLOGIES INDUCED IN MICE BY EXPOSURE TO THE TRI-FLUORIDATED PYRETHROID INSECTICIDE BIFENTHRIN

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ABSTRACT: The rescuing potential of the oil of *Nigella sativa* (NS) seeds on the hepato-histopathologies of liver induced with bifenthrin (BF) exposure were explored in 12–15-week-old male Swiss Webster mice. Six groups of five animals (n=5) were studied: (i) VC (vehicle control) group (0.1 mL corn oil once daily for 14 days); (ii) NS group (corn oil treatment as in VC group for 7 days+0.1 mL 10% NS oil in corn oil for the next 7 days); (iii) BF2.5 and (iv) BF5 groups (2.5 and 5 mg/kg BF in 0.1 mL corn oil for 7 days, respectively, +0.1mL pure corn oil for the next 7 days); (v) BF2.5+NS and (vi) BF5+NS groups (2.5 and 5 mg/kg BF as in the respective BF groups and NS as in the NS group). The entire liver from each animal was recovered on the 15th day after cervical dislocation. The characteristic pathologies of the liver observed in the BF groups included damaged hepatic portal veins and profuse fibrosis of the peri-portal vein areas, shrinkage of the sinusoids, misalignment of the cords, especially at the margins, and necrosis of the hepatocytes. The marginal area of the lobules also showed infestation of macrophages followed by the migration of hepatoblasts from the marginal triads to the central lobular vein. The analysis of the data showed a significant decrease ($p \leq 0.05$) in the mean animal body and liver weights in the BF2.5 group (27.3±1.1 and 2.4±0.3, respectively) and in the BF5 group (27±1.3 and 1.62±0.13, respectively) compared to the VC group (33.7±1.1 and 2.42±0.25, respectively) and the NS group (31.4±1.09 and 2.48±0.14, respectively). A convincing recovery in the mean animal body and liver weights was present in the BF2.5+NS group (30±1.1, and 2.5±0.31, respectively) and the BF5+NS group (30.3±1.9 and 2.31±0.3, respectively). A similar pattern of significant ($p \leq 0.05$) alterations was present in the analyses of the micrometric data for the differential counts of the mono-nucleated and bi-nucleated hepatocytes per unit area of the hepatic lobules and in the percent of the hepatolobular areas occupied by the hepatocytes vs the sinusoidal+arterial+venous spaces and the cellular debris+fibrotic mass+non-parenchymal cells. These findings suggest that the sub-chronic exposure of BF may lead to various hepato-histopathologies and micrometric alterations in mice while the oil of NS seeds can convincingly enhance the pace of rehabilitation indicating its hepato-protective and regenerative potentials.

Keywords: Bifenthrin; Hepatotoxicity; *Nigella sativa.*

INTRODUCTION

Background: Bifenthrin (BF) is a mono-chloro, tri-fluoridated compound with the chemical formula of $C_{23}H_{22}ClF_3O_2$) and the IUPAC name of (2-Methyl-3-biphenylyl) methyl (1S,3S)-3-[(1Z)-2-chloro-3,3,3-trifluoro-1-propen-1-yl]-2,2-dimethylcyclopropanecarboxylate.[1] It is a non-systemic third generation type 1

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synthetic pyrethroid insecticide (Figure 1).[2] In last two decades BF has been used extensively in urban and agricultural formulations to control insect pests.[3] Like inorganic fluoride, BF has been found to cause toxic damage to vital body organs like liver.[4,5]

Nigella sativa (NS) is a flowering plant of the ranunculaceae family[6] and, in Islamic history, it is regarded as being one of the best remedial folk medicines[7,8] Recently NS oil has been investigated for its protective capacity against the damaging outcomes of a variety of poisonous substances.[9] Phytochemical investigations have confirmed the presence of a variety of natural antioxidant constituents in NS oil such as fatty acids, trans-anethole, p-cymene, limonene, carvone, thymoquinone, dithymoquinone, thymol, thymohydroquinone, carvacrol, 3-thujene, α-pinene, and γ-terpinene.[10,11] Additionally, it also contains minerals, vitamins, and essential organic compounds like protein, fat, carbohydrates and crude fibers.[12] NS has been shown to provide hepato-protection in conditions of fibrosis and cirrhosis.[13] Thymoquinone, the major component of NS oil, has been found to exhibit antitumor, anti-gastric mucosal ischemia, and anti-pyrethroid induced oxidative stress effects in rodents.[14-15] The aim of the present study was to examine, in mice, the hepato-histopathological potential of BF exposure and its amelioration with NS oil treatment.

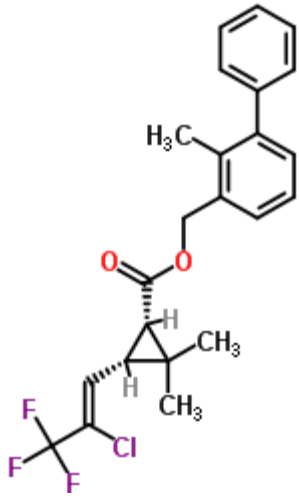

Figure 1. Structural formula of bifenthrin (BF).

MATERIALS AND METHODS

Experimental animals and their housing: The study was carried out on thirty adult male Swiss Webster albino mice weighing 29–32 g. They were accommodated comfortably under similar conditions to those reported in our previous work, strictly following the guidelines of University of Sargodha regarding animal care and experimentation.[5]

Preparation of BF solutions: The desired doses (2.5 mg/kg and 5 mg/kg) of technical grade BF (Batch No# Auc/20130611, manufactured by Be Star China and donated by Auriga Chemical Enterprises, Lahore, Pakistan) were prepared in corn oil. The single dose volume for each animal remained constant at 0.1 mL with the concentration of BF being adjusted by appropriate dilutions of the standard solution in accordance with the body weight.

Preparation of NS oil solution: Nigella sativa oil (extracted and marketed by Marhaba Laboratories Ltd, Lahore, Pakistan) was purchased from the local market and 0.1 mL of its 10% (v/v) dilution in corn oil was provided to each experimental animal in the NS, BF2.5+NS, and BF5+NS groups.

Animal groups and treatment profile: The names and treatment profile of six study groups (n=5) were as follows:

(i) VC (vehicle control) group: Received an intragastric once daily dose of 0.1 mL of pure corn oil for 14 days.

(ii) BF2.5 group (2.5 mg/kg bifenthrin treatment group)**:** Received an intragastric once daily dose of 2.5 mg/kg of BF in 0.1 mL of corn oil for 7 days followed by an intragastric once daily dose of 0.1mL pure corn oil for the next 7 days.

(iii) BF5 group (5 mg/kg bifenthrin treatment group)**:** Received an intragastric once daily dose of 5 mg/kg of BF in 0.1 mL of corn oil for 7 days followed by an intragastric once daily dose of 0.1mL pure corn oil for the next 7 days.

(iv) NS group (*Nigella sativa* oil treatment) group)**:** Received an intragastric once daily dose of 0.1 mL of corn oil for 7 days followed by an intragastric once daily dose 0.1 mL of 10% NS seed oil in corn oil (v/v) for the next 7 days.

(v) BF2.5+NS group (2.5 mg/kg bifenthrin+*Nigella sativa* oil treatment) group**:** Received 2.5mg/kg BF as in BF2.5 group followed by 10% NS seed oil as in the NS group.

(vi) BF5+NS group (5 mg/kg bifenthrin+*Nigella sativa* oil treatment) group**:** Received 5mg/kg BF as in BF5 group followed by 10% NS seed oil as in NS group.

Daily observations: Each animal was weighed on a 0.1 g precision digital balance daily prior to the feeding and dose treatment to record the variations in the group body weight profile during the entire period of the study.

Organ Recovery: The animals were euthanized by cervical dislocation on the 15^{th} day of the study to recover the liver (entire organ). The body weight of each animal was recorded just prior to dissection. In each case, the liver was washed, after exteriorization, in normal saline, surface dried with a paper towel, weighed, and fixed in 10% formalin for 24 hr.

Histological preparations: The organs, fixed in formalin, were processed for wax embedding, serial microtomy, and hematoxylin and eosin staining to produce permanent slides for histopathological and micrometric studies.

Histological studies: The stained histological sections of liver were keenly observed at 100× and 400× to record all the pathological anomalies as digital photographs with the help of a 7.2MP "Sony DSC-W35" digital camera mounted on a "Labomed CXR_2" trinocular research microscope. For the lucid presentation of the histo-pathological and histo-ameliorative signs in the resulting sections, the digital photographs of the selected liver sections were processed in CorelDRAW11® for color, contrast, cropping, and snap on labeling.

Micrometry: The micrometric data was generated, from digital photo micrographs (100× and 400×) of 5 randomly selected histological sections of liver from each animal of all the six groups, in CorelDRAW11.® From each of the 400× photographs, the percent area occupied by (i) hepatocytes, (ii) sinusoids+hepatic arteries+hepatic portal veins, and (iii) cellular debris+fibrotic mass+non-parenchymal cells was calculated by using the grid tool application on the snapshots. The number of mono-nucleated and bi-nucleated cells was counted from the 100× photographs of the above mentioned sections with the help of a 200

μ × 200 μ (40,000 μ^2) quadrate. The differential count of the cells was obtained from 4 different areas of each photograph. Thus, the means of the 20 separate counts, for both categories of cells, from each animal of a group were used as a unit reading to obtain the group mean±SEM values. For the calibration of the quadrate size, the grid squares digital snapshots of the stage micrometer obtained on 100× and 400×, respectively, were used while keeping the camera specifications unchanged

Data analysis and statistical application: The micrometric data obtained were subjected to Analysis of Variance (ANOVA) and the Tukey Multiple Range Test. The body weight and organ (liver) weight data were subjected to Analysis of Covariance (ANCOVA) and the Least Significant Difference Test as a post hoc multiple comparison, by setting the initial and final weights of the animals as covariates, respectively. These statistical analyses were carried out using Softonic SPSS-20 software.

RESULTS

HISTOLOGICAL RESULTS: The typical histological signs of healthy mouse liver were evident in the form of easily identifiable central lobular veins surrounded by one cell thick hepatic cords interjected by narrow sinusoidal spaces. The peri-lobular hepatic portal veins were also easily identifiable. However, the complete hepatic triad structure with all four components (hepatic portal vein, hepatic artery, bile duct, and lymphatic vessel) was not easily recognizable. Most of the hepatocytes were mono-nucleated but some bi-nucleated hepatocytes were also present in various hepatic cords (Figures 2A and 3A).

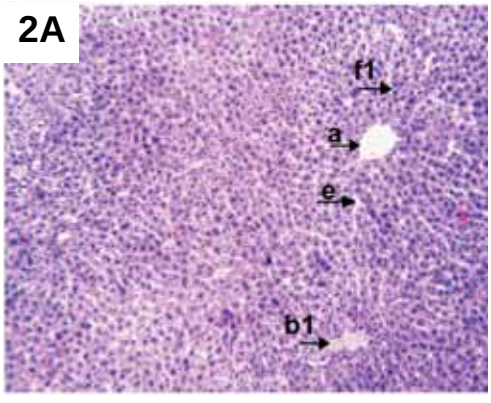

Figure 2A. Selected hepatolobular section of mice liver from the vehicle control (VC) group (100×).

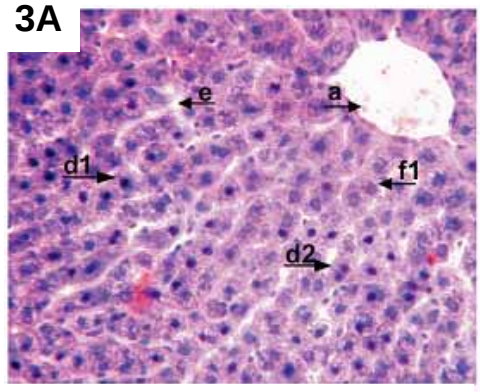

Figure 3A. Selected hepatolobular section of mice liver from the vehicle control (VC) group (400×).

a: central vein, b1: normal hepatic portal vein, d1: mono-nucleated hepatocytes, d2: bi-nucleated hepatocytes, e: sinusoidal spaces, f1: normal hepatic cord.

In the NS group the hepatolobular details were similar to the VC group. Additionally, in the marginal triads, hepatic portal veins and hepatic arteries were easily distinguishable. However, the central vein, hepatic portal vein, and

sinusoidal spaces in the various hepatic lobules were apparently more dilated in the NS group than in the VC group (Figures 2B and 3B).

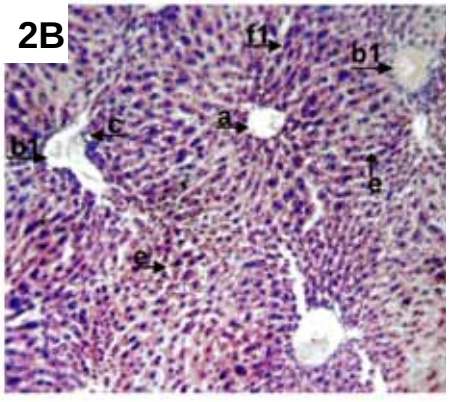

Figure 2B. Selected hepatolobular section of mice liver from the *Nigella sativa* (NS) group (100×).

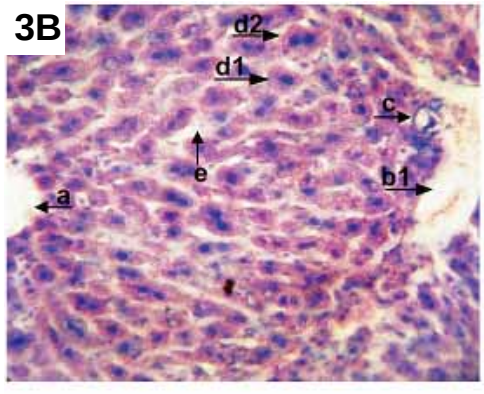

Figure 3B. Selected hepatolobular section of mice liver from the *Nigella sativa* (NS) group (400×).

a: central vein, b1: normal hepatic portal vein, c: hepatic artery, d1: mono-nucleated hepatocytes, d2: bi-nucleated hepatocytes, e: sinusoidal spaces, f1: normal hepatic cord.

The histological sections in the BF2.5 group showed various signs of histopathological damage. These signs include damaged hepatic portal veins with marginal and profuse fibrosis of the peri-portal vein areas, damaged hepatic cords at the marginal area of the lobules, which showed a profuse infestation of macrophages, and the migration of hepatoblasts from the marginal triads to the central vein. These signs represent the natural tendency of hepatolobular repair activity (Figures 2C and 3C).

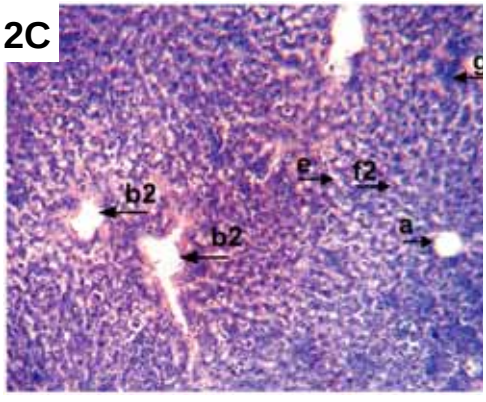

Figure 2C. Selected hepatolobular section of mice liver from the bifenthrin 2.5 mg/kg (BF2.5) group (100×).

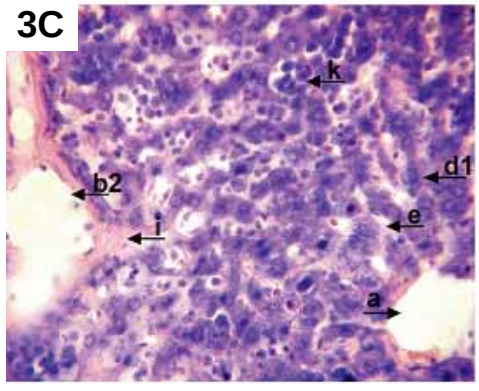

Figure 3C. Selected hepatolobular section of mice liver from the bifenthrin 2.5 mg/kg (BF2.5) group (400×).

a: central vein, b2: damaged hepatic portal vein, c: hepatic artery, d1: mono-nucleated hepatocytes, e: sinusoidal spaces, f2: damaged hepatic cord, g: origin of regenerative cells, i: fibrotic mass, k: macrophage infestation.

Similar signs of hepato-lobular derangements with a slightly enhanced impact of the peri-lobular damage were seen in the BF5 group (Figures 2D and 3D).

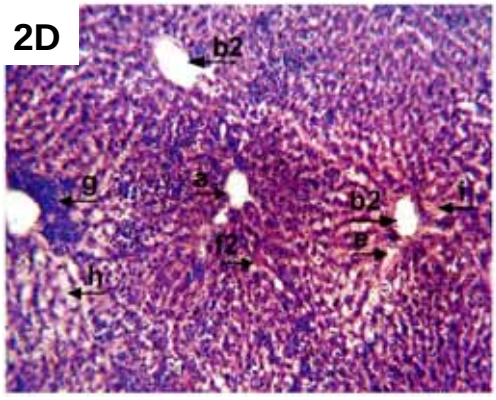

Figure 2D. Selected hepatolobular section of mice liver from the bifenthrin 5 mg/kg (BF5) group (100×).

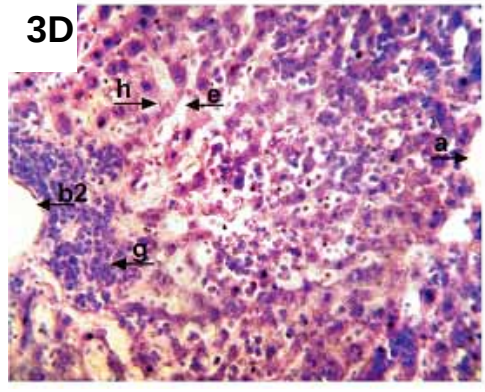

Figure 3D. Selected hepatolobular section of mice liver from the bifenthrin 5 mg/kg (BF5) group (400×).

a: central vein, b2: damaged hepatic portal vein, e: sinusoidal spaces, f2: damaged hepatic cord, g: origin of regenerative cells, h: apoptotic cells, i: fibrotic mass.

The impact of damage on the hepato-lobular architecture was less pronounced in the BF2.5+NS and BF5+NS groups than in the BF2.5 and BF5 groups, respectively. On the other hand, the regenerative signs, as indicated by the presence of numerous hepatoblasts at the margin of hepatic triad and their gradual differentiation and rearrangement into nascent hepatic cords towards the centrilobular area, were obvious (Figures 2E, 3E, 2F, and 3F).

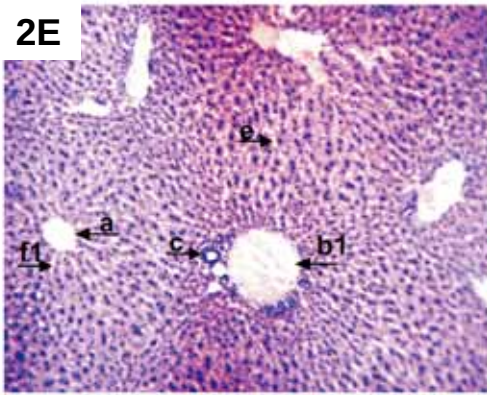

Figure 2E. Selected hepatolobular section of mice liver from the bifenthrin 2.5 mg/kg+*Nigella sativa* (NS) (BF2.5+NS) group (100×).

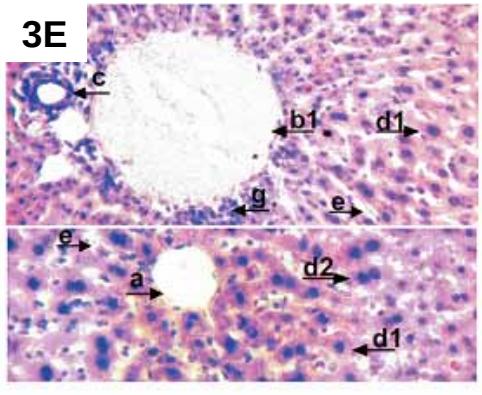

Figure 3E. Selected hepatolobular section of mice liver from the bifenthrin 2.5 mg/kg+*Nigella sativa* (NS) (BF2.5+NS) group (400×).

a: central vein, b1: normal hepatic portal vein, c: hepatic artery, d1: mono-nucleated hepatocytes, d2: bi-nucleated hepatocytes, e: sinusoidal spaces, f1: normal hepatic cord, g: origin of regenerative cells.

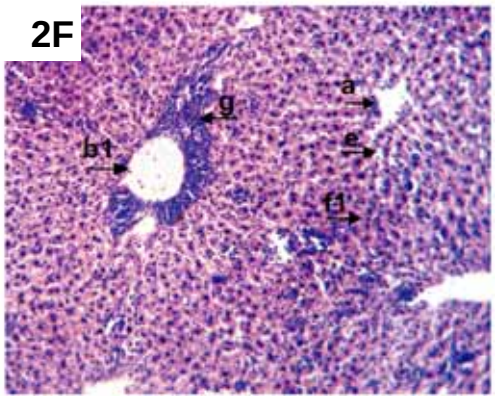

Figure 2 F. Selected hepatolobular section of mice liver from the bifenthrin 5 mg/kg+*Nigella sativa* (NS) (BF5+NS) group (100×).

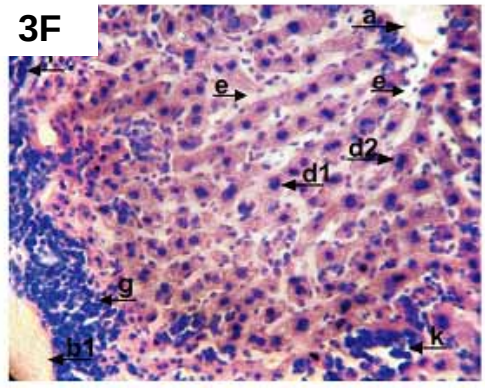

Figure 3 F. Selected hepatolobular section of mice liver from the bifenthrin 5 mg/kg+*Nigella sativa* (NS) (BF5+NS) group (400×).

a: central vein, b1: normal hepatic portal vein, d1: mono-nucleated hepatocytes, d2: bi-nucleated hepatocytes, e: sinusoidal spaces, f1: normal hepatic cord, g: origin of regenerative cells, k: macrophage infestation.

MORPHOMETRIC AND HISTOMETRIC RESULTS:

Mean body weight: Analysis of Co-variance (ANCOVA) for the final body weight, using the initial weight as a covariate, showed a significant difference ($p \leq 0.01$) among the groups. The Least Significant Difference Multiple Comparison (LSDMC) based post hoc analysis indicated that the VC group differed significantly ($p \leq 0.05$) from all the other groups except for the NS group, whereas the NS groups differed significantly ($p \leq 0.05$) only from the BF2.5 and BF5 groups (Table 1).

Mean Liver weight: Analysis of Co-Variance (ANCOVA) for the liver weight, using animal body weight at the time of sacrifice as a covariate, indicated no significant variation among the groups. The LSDMC post hoc analysis indicated a significant ($p \leq 0.05$) difference in the BF5 group from the rest of the 5 groups, whereas the BF2.5+NS group differed significantly ($p \leq 0.05$) from all the other groups except for the NS group (Table 1).

Mean number of mono-nucleated hepatocytes per unit area: Analysis of Variance (ANOVA) showed a significant ($p \leq 0.05$) difference among the groups. The post hoc analysis (Tukey's test) indicated that the NS group differed significantly ($p \leq 0.05$) from the rest of the groups except for the BF2.5+NS group (Table 1).

Table 1. Variations in body and liver weights and the differential counts of mono-nucleated and bi-nucleated hepatocytes per unit lobular area (40,000 μ^2) (VC=vehicle control, NS=*Nigella sativa*, BF2.5=bifenthrin 2.5 mg/kg body weight, BF5=bifenthrin 5 mg/kg body weight)

Morphometric and histometric parameters	Groups (values are mean±SEM)					
	VC	NS	BF2.5	BF5	BF2.5+NS	BF5+NS
Mean body weight (g)*†	33.7 ±1.1^{a}	31.4 ±1.09^{ac}	27.3 ±1.1^{b}	27 ±1.3^{b}	30 ±1.1^{bc}	30.3 ±1.9^{bc}
Mean liver weight (g)†	2.42 ±0.25^{a}	2.48 ±0.14^{ac}	2.4 ±0.3^{a}	1.62 ±0.13^{b}	2.5 ±0.31^{c}	2.31 ±0.3^{a}
Mean liver weight (g)†	2.42 ±0.25^{a}	2.48 ±0.14^{ac}	2.4 ±0.3^{a}	1.62 ±0.13^{b}	2.5 ±0.31^{c}	2.31 ±0.3^{a}
Mean number of mono-nucleated hepatocytes per unit lobular area‡§	40.4 ±2.1^{a}	58 ±4.52^{b}	46.4 ±4.4^{a}	37.4 ±1.7^{a}	49 ±5.02^{ab}	45.4 ±3.9^{a}
Mean number of bi-nucleated hepatocytes per unit lobular area‡,§	19.8 ±0.86b^{a}	24 ±2.3^{b}	14.4 ±1.69^{c}	10.6 ±0.81^{d}	21.2 ±2.5^{ab}	18.6 ±1.32^{a}

†analyzed by ANCOVA; §analyzed by ANOVA; ‡$p \leq 0.05$; *$p \leq 0.01$. Any two groups not sharing a common lower case superscript letter differ significantly ($p \leq 0.05$) from each other.

Percent hepatolobular area occupied by i) hepatocytes ii) sinusoids+blood vessels and iii) cellular debris+fibrotic mass+non-parenchymal cells:

Analysis of Variance showed a highly significant ($p \leq 0.001$) variation among the groups for all the three parameters. The post hoc analysis (Tukey's test) for the area occupied by hepatocytes indicated significantly ($p \leq 0.05$) higher mean values in the NS group than in the VC, BF2.5, BF5, and BF5+NS groups while the BF2.5+NS group showed a significantly ($p \leq 0.05$) higher mean value than in the BF2.5 and BF5 groups. For the percent area occupied by arterial, venous, and sinusoidal spaces, the VC group showed a significantly ($p \leq 0.05$) higher mean value than in the other four groups. On the other hand, the mean percent area occupied by cellular debris, fibrotic mass, and non-parenchymal cells in the BF5 group remained significantly ($p \leq 0.05$) higher than in the VC, NS, and BF2.5+NS groups. Although the mean percent values for the area occupied by debris in the BF2.5+NS group was significantly less than in the BF2.5 and BF5+NS groups, it

still remained significantly ($p \leq 0.05$) higher than in the VC and NS groups (Figure 4).

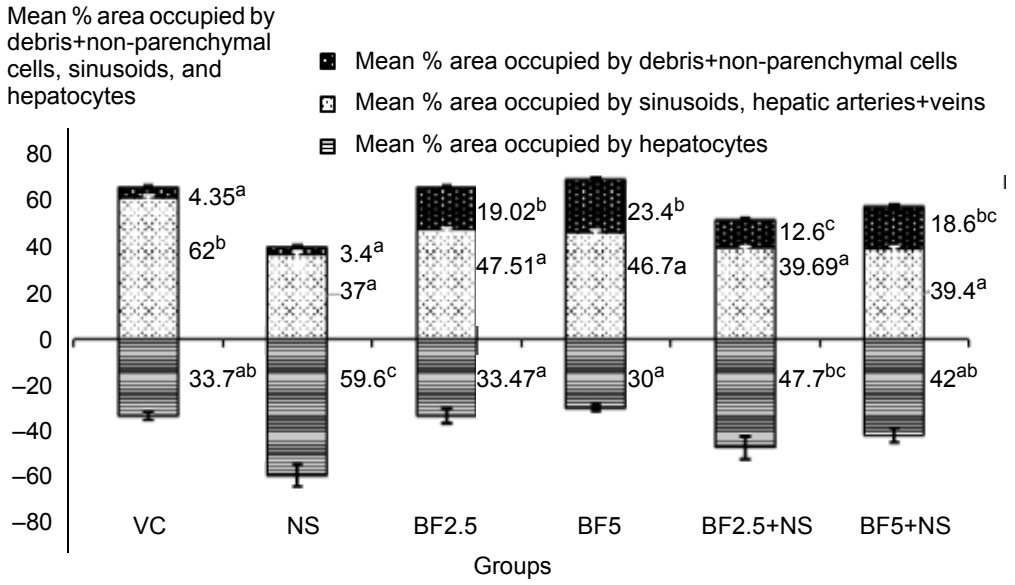

Figure 4. Mean percent liver area occupied in the various groups by: i) cellular debris, fibrotic mass, and non-parenchymal cells, ii) sinusoids, hepatic arteries and hepatic portal veins, and iii) hepatocytes the groups.
± bars indicate SEM.
[abc]indicate a significant difference between the groups not sharing a common lower case letter.
VC=vehicle control, NS=*Nigella sativa*, BF2.5=bifenthrin 2.5 mg/kg body weight, BF5=bifenthrin 5 mg/kg body weight.

DISCUSSION

In general, liver has been found to be the first and foremost target organ for most of the environmental chemicals and toxins, primarily because of its metabolic capability to degrade and detoxify such poisonous materials. Thus, it is the organ of choice for the estimation of the toxic potential of the environmental poisonous chemicals like heavy metals, halogens, and insecticides.[16, 17]

Pyrethroids are generally believed to be safer insecticides as compared to the organophosphate and organochlorine groups, because of their rapid metabolism and removal from the non-target animals (humans and other mammals).[18] Unfortunately, no information exists in available literature that can convincingly address the metabolic fate of 2nd and 3rd generation pyrethroids.[19] Thus the general perception of their rapid metabolism and non-accumulative nature is now being seen with critical eyes. Seemingly, this situation becomes more alarming if the chemical structures of 2nd and 3rd generation pyrethroids are observed closely, particularly because of the presence of toxic halogen ligands such as chlorine, fluorine, and bromine. Unfortunately, BF (the most widely used insecticide in the cotton crop areas of Pakistan) is tetra-halogenated (with mono-chloro, tri-fluoridated ligands) synthetic pyrethroid insecticide.

The hepatotoxicity of insecticides remains an area of choice for investigation by toxicologists worldwide.[20] Unfortunately, there are only few reports that have placed any consideration on bringing forward information on the hepatotoxicity of BF exposure.[4,21] Little is known about its metabolic by-products and their excretion from the animal or human body. Our results show drastic histopathological and micrometric changes in the hepatolobular architecture, such as damaged and punctured hepatic portal veins with marginal and profuse fibrosis of the peri-portal vein areas and damaged hepatic cords, especially at the marginal hepatolobular area. The peri-lobular area may also show an infestation of macrophages that may be followed by a migration of hepatoblasts from the marginal triads to the centric lobular vein during the regenerative phase. Most probably, these hepato-histopathologies are caused by the persistent oxidative stress of BF exposure. The severe histological alterations in the peri-portal areas of the hepatic lobules may be attributable to the higher insecticide exposure in this area as the orally delivered insecticide must pass through the periportal area of the hepatic lobules after its gastrointestinal absorption.[20,21] The rapid rehabilitation of these histological alterations in the liver after treatment with NS oil, compared to the control group, indicates the immense rescuing potential of this heavenly plant seed oil. Its unique phytochemical components (especially “thymoquinone”) must be responsible for the miraculous corrective potentials of the hepato-histopathological manifestations of BF exposure found in this study. The micrometric findings further consolidate this opinion.

CONCLUSION

The results show that the general impression of BF being a non-accumulative and thus safer insecticide for humans and pet mammals needs a thorough revision. There is a genuine need for further investigation of the potential for persistent toxicity from the use of halogenated pyrethroids, such as BF, deltamehrin, cypermethrin, and permethrin. In addition, a thorough study is needed of the potentially immense benefits of NS seed oil for preventing and treating various diseases and ailments, particularly the toxicological manifestations of widely used environmental chemicals such as the pesticides and insecticides.

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