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SUB-ACUTE TOXICITY OF BLACK SEED (*Nigella sativa*) AND HONEY MIXTURE

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ABSTRACT

Consumption of black seed (*Nigella sativa*) and honey mixture (BSH) has been reported to provide multiple health advantages. However, the toxicity effect of black seed and honey mixture (BSH) consumption has not been reported, although it has been consumed for centuries. Thus, this toxicity study was conducted, including determination of 50% lethal dose (LD_{50}), changes in body and relative organ weights, differential leukocytes count, liver function test and histopathology analysis of liver and kidney. Thirty male Sprague Dawley rats (120-300 g) were used in the study and treated with varying dosages of BSH (100, 500, 1000 and 2000 mg/kg, respectively) for 14 days. Half of the rats from each group (n=3) were euthanized on day 14 for a sub-acute toxicity study to obtain relative organ weight, haematology, liver function test and histopathology analysis. Another half of animals from each group (n=3) were kept for another 14 days without any treatment for delayed toxicity study. No lethality was observed in all dosage groups, while the LD_{50} value was evaluated to be more than 2000 mg/kg. No significant alterations ($p>0.05$) were observed in animal body weight, differential leukocytes count and relative organ weight in all treatment groups as compared to control for both sub-acute and delayed toxicity studies. However, AST enzymes drop significantly at dosage 500 and 2000 mg/kg in recovery period, which suggested delayed hepatoprotective effect of the mixture. Histopathology analysis of the liver and kidney confirmed no abnormalities in cell morphology. This study clearly demonstrates that consumption of BSH is safe and do not provide any adverse or delayed toxicity effect.

Key words: Black seed and honey mixture, toxicity, haematology, histopatology, liver enzymes

INTRODUCTION

Black seed (*Nigella sativa*) is also referred to as black cumin seeds, that are used as a natural remedy to cure numerous diseases (Bhat, 2011), particularly in reducing blood glucose level (Alimohammadi *et al*., 2013; Kaleem *et al.,* 2006), lowering blood pressure (Sahebkar *et al.*, 2016), and controlling blood cholesterol level (Kaatabi *et al.*, 2012; Chavez-santoscoy *et al*., 2014). Honey is known as another health beneficial food and has a very long history of human consumption. While honey has varying compositions based on its origins (Chan *et al*., 2017; Chua & Adnan, 2014; Isopescu *et al*., 2012; Kek *et al*., 2016; Khalil *et al*., 2010; Oddo *et al*., 2008; Oroian, 2012), it has been reported for its wound healing, antioxidant properties, and reduction of fasting blood glucose level (Al-Waili, 2003a; Al-Waili, 2003b; Afroz *et al.*, 2014; Goharshenasan *et al.*, 2016; Tan *et al.*, 2012). Recently, black seed has been consumed together with honey to reduce the viability of cancer cells and protect against inflammatory response and carcinogenesis c. Consumption of black seed alone is considered as safe (Assayed, 2010; Dollah *et al*., 2013; Zaoui *et al.*, 2002), and honey has a wide margin of safety (Al-Waili, 2003a; Al-Waili, 2003b; Wilson *et al.*, 2011). However, there is no study documented that the black seed & honey mixture are safe. Toxic effects may occur at any concen-

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tration level, and to date, there is no published data on range of BSH concentration safe for human consumption.

Toxicology studies are essential in order to establish the safety and efficacy of new drugs or natural substances which will be used later in human as a health supplement or medicine. A toxicology study covers pharmacological aspects which deal with the adverse effects of bioactive substance on living organisms and acts as a guide for the researchers to make evaluation on the suitability of a new drug to be adopted or applied for clinical use (Anadón, 2016; Gosslau, 2016; Parasuraman, 2011). By identifying the safe range of dosage to consume, BSH could provide maximum advantages to human health with minimum side effects. Therefore, the current study was conducted to evaluate the safety and toxicity effects of the consumption of BSH in an animal model.

MATERIALS AND METHODS

Sampling

Black seed origin from Syria was purchased from a local market in Kuala Terengganu. Honey was purchased directly from the local collector of honey bees at Batu Rakit, Terengganu. Only honey collected from respective honey bees collector was used in the sample preparation. The composition of honey used in this study is shown in Table 1 below.

Table 1. Honey composition

Component	Results
Reducing sugar, g/100g:	
Fructose	40.46
Glucose	30.46
Maltose	0.47
Sucrose, g/100g	0.47
Ash, g/100g	0.17
Moisture, g/100g	25.80
Free acidity, meg/kg	67.30
Hidroxymethyl furfural, mg/kg	0.52
Diastase activity, DN	4.51

(Source: Yusof *et al.*, 2017).

Sample preparation

Black seed was cleaned by tap water before dried in oven for 1 hour. The black seed was then finely ground by using dry blender before mixed with honey to form a uniform and thick black seed and honey paste. BSH was prepared in a ratio of 1:1 (Yusof *et al.*, 2017). The prepared black seed and honey mixture was stored in an air-tight container at a room temperature without direct sunlight before used. Treatment dosage was prepared by diluting mixture paste with distilled water for 1-2 ml.

Animal selection

Thirty (30) clinically healthy Sprague Dawley's male rats weighing between 120-300 g (age between 5-7 weeks) were randomly selected for the sub-acute oral toxicity study. All animals were housed in standard environmental conditions at a temperature of 25 ± 1°C with 12 hours light and 12 hours dark cycle. The animals were acclimatized to hygienic laboratory conditions for at least 7 days prior to the experiment. Animals were fed a standard commercial pellet diet and tap water *ad libitum*. This study was approved by the Universiti Malaysia Terengganu Committee of Research following the university's ethical standards with reference number UMT/JKEPHT/2017/11 dated 22nd November 2017.

Sub-acute oral toxicity study (Repeated dose 14 days)

The applied method was modified from Dollah *et al.* (2013). The experiment was divided into Phase I and Phase II. Fixed dose procedure was followed as described in OECD guidelines 420 (2001) for oral toxicity study in the aim to determine the Lethal Dose (LD_{50}). In Phase I, thirty Sprague Dawley rats (Takrif Bistari Enterprise, Seri Kembangan) were divided into five groups which consisted of six animals per group (n=6). Group A served as negative control (untreated group), Group B (orally treated with diluted black seed and honey paste at 100 mg/kg/day), Group C (500 mg/kg/day), Group D (1000 mg/kg/day), and Group E (with the highest tested dose at 2000 mg/kg/day). Treatment rats were treated once daily with diluted BSH using a sterile size 14 ball-tipped oral gavage needle (Harvard Apparatus, US) for 14 days. Close observation was conducted for the first four hours to examine any toxic symptoms such as abnormal behaviour, abnormal posture, evidence of diarrhoea, blood in urine, and increase of heart beat potentially caused by the black seed and honey mixture. Body weight was recorded on days 0, 4, 7, 11 and 14 during the experimental period. Blood was withdrawn from the tail vein using a sterile needle on day 7 and 14 respectively and subject to differential white blood cells counting via Wright's staining. Whole blood samples were collected on day 14 and key hepatic enzyme assays were performed by using Spotchem EZ SP-4430 analyser. Half of the survived rats (n=3) from each group were euthanized on day 14 of the sub-acute oral toxicity study to obtain liver, kidney, spleen, lung and heart for organ relative weight measurements. Toxicity effects in the liver and kidney were further analysed by histopathological analysis for any abnormalities.

Forteen days recovery period

In Phase II of toxicity, the method modified from Takahashi *et al.* (2012) and as described in OECD

(2001) was followed. Half of the survived rats (n=3) for each group were returned to their own cage and kept for another 14 days of observation period. During the recovery period, all groups of the rats were left untreated (without treatment of any BSH) and had access to food pellets and water *ad libitum*. The occurrence of delayed toxicity symptoms was observed twice daily, including abnormal behaviour, increase of heart beat and abnormal posture. Body weight changes were recorded on days 0, 4, 7, 11 and 14. All rats were euthanized at the end of day 14 of the recovery period to obtain blood and organ samples for differential white blood cell counting, hepatic enzyme assays and relative organ weight. Liver and kidney samples were subjected to histopathological analysis.

Histopathological analysis

All liver and kidney samples were fixed in 10% buffered formalin for 48 hours and subjected to tissue processor (LEICA^{TM}, Germany). Processed tissue samples were embedded in paraffin wax, sectioned at approximately 5 µm using Rotary Microtome Machine (LEICA^{TM}, Germany), and stained with haematoxylin and eosin (H&E) (Sigma, US). All stained tissues were examined under light microscope to observe any abnormalities in the liver and kidney tissue samples (modified from Takahashi *et al.*, 2012).

Statistical analysis

All data were analysed using IBM Statistical Package for the Social Sciences (SPSS) Statistics software (Version 20). The differences of all toxicological parameters between treatment and control (untreated) groups were compared using one-way ANOVA followed by multiple comparison Tukey post-hoc tests. All data are presented as mean ± S.D. In all analysis, $p < 0.05$ was taken to indicate significant difference.

RESULTS AND DISCUSSION

Results indicated that the lethal dose (LD_{50}) of BSH could not be determined in this study, as no lethality observed in any animal during the 28 days of the experiment (Phase I: 14 days of sub-acute study followed by Phase II: 14 days recovery period). The LD_{50} of BSH is thus more than 2000 mg/kg body weight and may be ranked to Globally Harmonised System (GHS) Category 5 (LD_{50} = 2000-5000 mg/kg body weight) (OECD, 2001a). Gross observations revealed that the oral administration of BSH at all dosages tested did not produce any sign of distress and significant change in behaviour, breathing and nervous responses in tested male rats upon the first 4 hours of the treatment. This indicates that the oral feeding of BSH did not cause any acute toxicity effect (Lippmann *et al*., 2007; Pinault, 2008). Furthermore, no significant body weight increment of all animals compared to untreated group during the 28 days of experiment indicating normal body metabolism and no occurrence of toxic effect even after administration has been stopped. This result is in alignment with a study by Dollah *et al.* (2013), which found that oral administration of grounded black seed for 28 days continuously shown insignificant change in body weight.

Assessment of organ weight and ratios to its body weight is important as alteration in organ-to-body weight ratio may be an indicator as a result of organ damage and precede morphological changes (Olaniyan *et al.*, 2016). The present findings indicated no significant difference ($p > 0.05$) in the relative organ weights of all treatment groups as compared to normal untreated group which demonstrated that the consumption of BSH may not elicit any deleterious effects to the host and was not toxic to the organs in both 14 days of sub-acute toxicity study and 14 days of recovery period (Table 2).

Differential white blood cells count

Blood plays an important role in regulating normal body physiological functions and homeostasis (Doctor & Spinella, 2012). Differential white blood cells counts of circulating peripheral blood was performed in order to investigate if continuous consumption of BSH for 14 days during sub-acute toxicity study could cause any inflammation or any delayed allergic reactions for the next 14 days. Results demonstrated no significant alteration in percentage of leukocytes subtypes between treatment groups and normal untreated group on day 7 and 14 for both Phase I and Phase II toxicity studies (Table 3 & 4). However, there was a significant increase ($p < 0.05$) in basophil counts in dosage group of 100 mg/kg compared to normal untreated group at day 14 of Phase I, indicating the possible occurrence of inflammation reactions (Miyake & Karasuyama, 2017). However, this result can be neglected as the basophil counts is in the normal proportion as proposed by Voehringer (2016), that basophil contribute 1-2%, eosinophil contribute around 5% and monocyte contribute for 2-8% to the circulating blood. This indicates that no inflammation or delayed allergic reactions had occurred upon administration of BSH as all the leukocytes subtypes is in normal proportion as compared to normal untreated group. This contradicts the results of studies by Abel-salam (2012) and Kamil (2013), which reported inflammation due to an increase of

Table 2. Effects of administration of black seed and honey mixture on experimental rat's relative organ weight during 14 days of sub-acute toxicity study and 14 days of recovery period

Dose of black seed and Honey Mixture (mg/kg body weight)	14 days of sub-acute toxicity study				
	Liver (%)	Kidney (%)	Heart (%)	Lung (%)	Spleen (%)
Control (untreated)	3.26 ± 0.32	0.69 ± 0.08	0.31 ± 0.03	0.52 ± 0.08	0.19 ± 0.03
100	3.23 ± 0.09	0.68 ± 0.06	0.37 ± 0.08	0.51 ± 0.08	0.18 ± 0.03
500	3.40 ± 0.16	0.71 ± 0.06	0.34 ± 0.07	0.57 ± 0.11	0.18 ± 0.05
1000	3.29 ± 0.50	0.69 ± 0.08	0.36 ± 0.05	0.49 ± 0.05	0.18 ± 0.04
2000	3.14 ± 0.28	0.65 ± 0.04	0.34 ± 0.03	0.52 ± 0.06	0.16 ± 0.03
	14 days of recovery period				
Control (untreated)	3.12 ± 0.20	0.69 ± 0.06	0.34 ± 0.03	0.50 ± 0.08	0.17 ± 0.03
100	3.24 ± 0.11	0.66 ± 0.04	0.37 ± 0.04	0.52 ± 0.13	0.17 ± 0.05
500	3.13 ± 0.52	0.61 ± 0.03	0.34 ± 0.03	0.46 ± 0.06	0.15 ± 0.03
1000	3.18 ± 0.56	0.68 ± 0.04	0.38 ± 0.03	0.50 ± 0.02	0.19 ± 0.02
2000	2.87 ± 0.08	0.69 ± 0.07	0.38 ± 0.01	0.49 ± 0.05	0.18 ± 0.01

All data are presented as mean ± S.D (n=3) and analysed by using One-Way ANOVA followed by multiple comparisons Tukey Post Hoc Test. No significant difference ($p > 0.05$) between all treatment groups.

Table 3. Effects of administration of black seed and honey mixture on experimental rat's differential leukocytes counts on day 7 and 14 of 14 days of sub-acute toxicity study

Dose of black seed and honey mixture (mg/kg body weight)	Day 7				
	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)	Basophil (%)	Eosinophil (%)
Control(untreated)	45.00 ± 18.52^{A}	36.33 ± 20.01^{A}	18.33 ± 2.31^{A}	0.00 ± 0.00^{A}	0.00 ± 0.00^{A}
100	46.00 ± 27.88^{A}	41.67 ± 30.67^{A}	11.67 ± 5.51^{A}	0.00 ± 0.00^{A}	0.67 ± 0.58^{A}
500	66.00 ± 7.55^{A}	16.33 ± 1.16^{A}	14.00 ± 5.29^{A}	0.00 ± 0.00^{A}	4.00 ± 3.46^{A}
1000	54.33 ± 14.74^{A}	25.67 ± 17.93^{A}	17.33 ± 1.53^{A}	0.33 ± 0.58^{A}	3.33 ± 1.53^{A}
2000	54.33 ± 4.04^{A}	30.00 ± 4.58^{A}	13.33 ± 3.06^{A}	0.00 ± 0.00^{A}	2.33 ± 1.53^{A}
	Day 14				
Control(untreated)	64.00 ± 17.44^{a}	27.33 ± 8.39^{a}	5.33 ± 4.51^{a}	0.00 ± 0.00^{a}	3.33 ± 4.93^{a}
100	73.33 ± 6.51^{a}	15.67 ± 9.87^{a}	5.33 ± 6.81^{a}	2.00 ± 0.00^{b*}	3.67 ± 0.58^{a}
500	62.67 ± 4.04^{a}	27.67 ± 5.51^{a}	8.33 ± 7.23^{a}	0.00 ± 0.00^{a}	1.67 ± 1.53^{a}
1000	62.00 ± 5.29^{a}	20.33 ± 1.53^{a}	14.33 ± 9.02^{a}	1.33 ± 1.16^{a}	2.00 ± 1.73^{a}
2000	61.00 ± 3.46^{a}	25.33 ± 4.51^{a}	12.67 ± 3.22^{a}	0.33 ± 0.58^{a}	1.33 ± 1.16^{a}

All data are presented as mean ± S.D (n=3) and analysed by using One-Way ANOVA followed by multiple comparisons Tukey Post Hoc Test.
*$p < 0.05$ showed significant difference in differential leukocytes counts between treatment group and normal group.
[A] No significant difference between all treatment groups on day 7 of 14 days of sub-acute toxicity study.
[a] No significant difference between all treatment groups on day 14 of 14 days of sub-acute toxicity study.
[b] Significantly different compared to normal untreated group on day 14 of 14 days of sub-acute toxicity study.

Table 4. Effects of administration of black seed and honey mixture on experimental rat's differential leukocytes counts on day 7 and 14 of 14 days of recovery period

Dose of black seed and honey mixture (mg/kg body weight)	Day 7				
	Lymphocyte (%)	Neutrophil (%)	Monocyte (%)	Basophil (%)	Eosinophil (%)
Control (untreated)	71.33 ± 7.37	15.00 ± 4.00	12.33 ± 9.07	0.00 ± 0.00	1.33 ± 1.53
100	69.67 ± 3.06	18.67 ± 4.93	9.67 ± 3.51	1.00 ± 1.73	1.33 ± 1.53
500	48.67 ± 25.01	37.00 ± 23.39	10.67 ± 4.93	0.00 ± 0.00	3.67 ± 2.08
1000	64.00 ± 8.54	19.33 ± 5.51	15.33 ± 10.69	0.00 ± 0.00	1.67 ± 1.16
2000	53.33 ± 7.64	22.00 ± 2.65	22.33 ± 10.50	0.67 ± 0.58	1.67 ± 0.58
	Day 14				
Control (untreated)	57.00 ± 9.64	24.00 ± 4.36	15.00 ± 7.00	0.33 ± 0.58	3.67 ± 3.79
100	58.00 ± 8.54	24.33 ± 11.02	15.67 ± 4.51	0.00 ± 0.00	2.00 ± 2.00
500	55.67 ± 6.51	28.33 ± 7.57	14.00 ± 2.00	0.67 ± 1.16	1.33 ± 0.58
1000	62.33 ± 10.97	20.67 ± 9.61	14.33 ± 3.22	0.00 ± 0.00	2.33 ± 1.53
2000	59.33 ± 11.85	25.00 ± 10.15	14.67 ± 3.79	0.00 ± 0.00	1.00 ± 0.00

All data are presented as mean ± S.D (n=3) and analysed by using One-Way ANOVA followed by multiple comparisons Tukey Post Hoc Test. No significant difference ($p > 0.05$) between all treatment groups.

granulocytes upon administration of black seed. The difference of outcomes as compared to the current study might be due to higher dosage given, form of black seed used and administration duration. Moreover, this study investigated on mixture of black seed and honey, while previous studies focused on black seed alone.

Hepatic enzyme assays

Results demonstrated that generally both of the concentration of alanine aminotransferase (ALT/GPT) and aspartate aminotransferase (AST/GOT) are insignificant ($p > 0.05$) when compared with all the treatment groups and the control group at the end of sub-acute toxicity study and recovery period (Figure 1). However, there is a significant decrease ($p < 0.05$) in the AST enzyme between dosages of 500 and 2000 mg/kg with the control group (Figure 1). The significant result indicated possible hepatoprotective effects by the administration of BSH (Afroz *et al*., 2014). In the present study, assumption is made that continuous assessments are conducted on the same group of animals, it may thus be concluded that BSH exhibits hepatoprotective effect after consumption.

Histopathological analysis

Histopathological analysis on the livers obtained from sub-acute study and recovery period (Figure 2) confirmed that the consumption of BSH did not give any toxicity effect at any administered dosage. No abnormalities were detected in the central vein, hepatocytes, sinusoids and no fatty change occurred indicating that no lesion has been developed in all the tested groups when compared to control rat's liver. In addition, observations on kidneys obtained from sub-acute study and recovery period revealed non-toxic effects upon the consumption of BSH. Kidney samples from both studies show that the glomerulus encircled with Bowman's capsule in all tested dose did not experience any inflammatory reaction when compared to normal untreated rat kidneys.

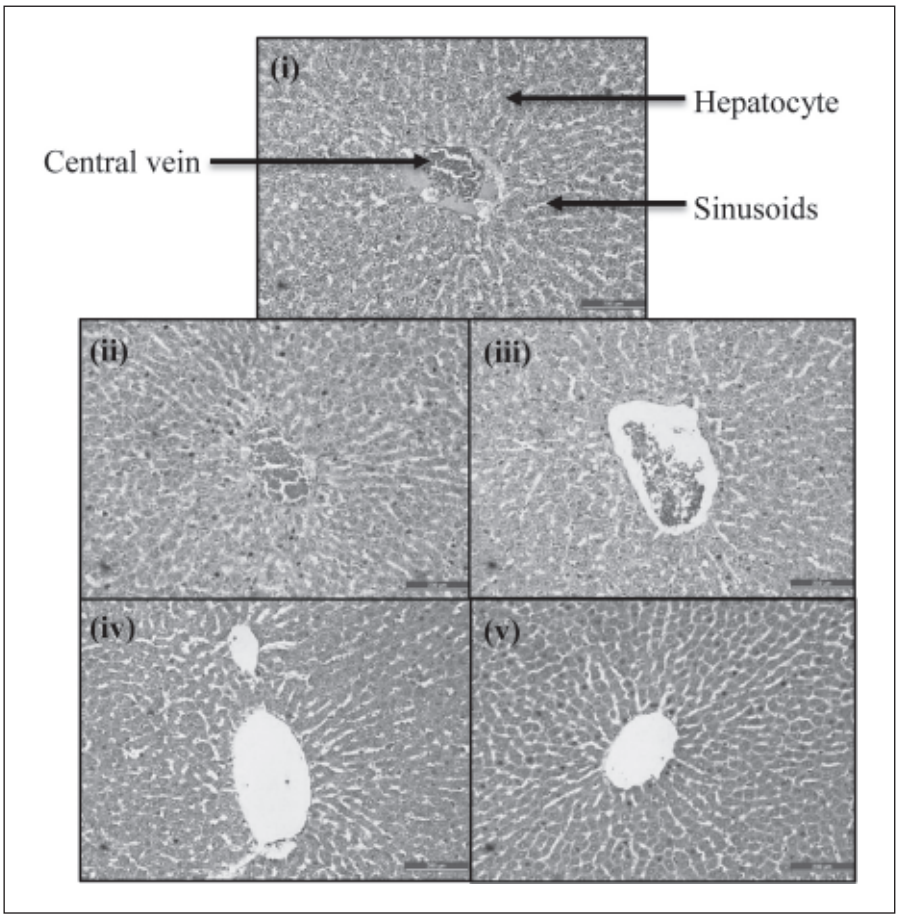

Fig. 1. Photomicrograph of rat liver's cross section from different treatment groups; (i) normal untreated group (ii) 100 mg/kg, (iii) 500 mg/kg, (iv) 1000 mg/kg, (v) 2000 mg/kg during 14 days of sub-acute toxicity study. Noted that the central vein, hepatocytes and sinusoids were in normal condition without any lesions and fat deposition compared to normal untreated group. (Haematoxylin and eosin staining, 200×).

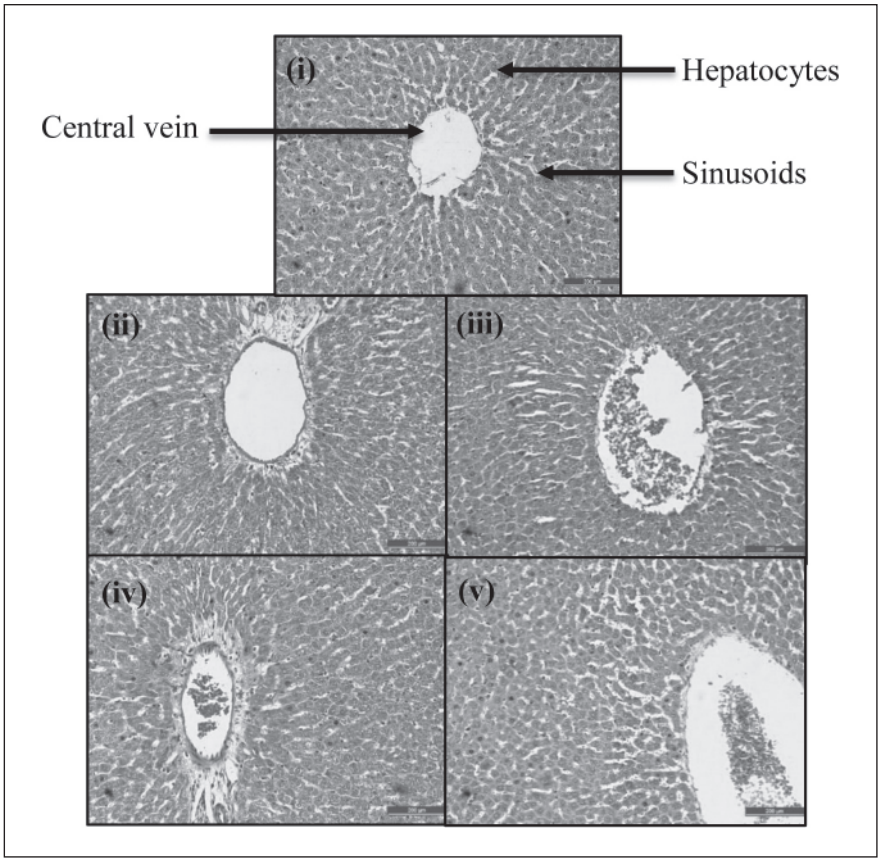

Fig. 2. Photomicrograph of rat liver's cross section from different treatment groups; (i) normal untreated group (ii) 100 mg/kg, (iii) 500 mg/kg, (iv) 1000 mg/kg, (v) 2000 mg/kg during 14 days of recovery period. Noted that the central vein, hepatocytes and sinusoids were in normal condition without any lesions and fat deposition as compared to normal untreated group. (Haematoxylin and eosin staining, 200×).

CONCLUSION

This study confirms that daily administration of 2 g/kg body weight (equivalent to 100 g/day for 50 kg human) of black seed and honey mixture supplementation can be concluded as safe and did not cause any adverse or delayed toxicity effects. All toxicity parameters which were evaluated, such as animal behaviour, body weight, relative organ weight, differential white blood cells count, hepatic enzyme assays and histopathological analysis are unaffected following this administration. It is suggested that a chronic toxicity study should be conducted to increase the duration of administration. Furthermore, blood glucose level, lipid profile and renal function test could be conducted to further examine the effects of BSH consumption on other biochemical measurement.

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