Clinical and Histopathological study of black and red grape seed extracts (*Vitis Vinifera*) effects on the Albino Mice

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Abstract

Grape seed extract (GSE) is a complex mixture of several compounds, mostly represented by polyphenols and phenolic acids. The goal of the pilot study was to illustrate the safe dose of GSE in mice model and to assess toxicity that may be initiated by the different concentrations of this plant. Forty-two mice were divided equally into 7 groups; groups 1 attended as control, were only received water, whereas animals of groups 2, 3, 4 were treated orally with 200, 400, and 800 mg/kg b.w. of black grape seed extract respectively, and the remaining groups including 5, 6, and 7 were treated orally with 200, 400, and 800 mg/kg b.w. of red grape seed extract respectively. The animals were observed daily for any sign revealing for activity alterations and toxicity along with their body weight measurement during the experiment for 21 days with histopathological examination. The results gained from this pilot study were recording that the 200 and 400 mg/kg b.w. doses of GSE were safe compared to the 800 mg/kg b.w.in both black and red grape seed extracts because the higher dose led to a reduction in body weight gain and produce changes in the mentioned organs.

Keywords: *Albino mice, Black grape seed, Histopathology, Red grape seed, Sharbazher village.*

Introduction

Plants can produce a large number of chemical compounds with significant biological effects and they have been used to manufacture numerous kinds of medicines since the creation of human being [1,2]

Grapes, *Vitis vinifera* berries, consider as conventional valued fruits in the world [3]. The primary composition of grape pomace is skin, steam, and seeds [4]. The fresh grape chemical composition is nearby 70-80% water and dissolved solids such as sugars, phenolic compounds, nitrogenous compounds, organic acids, minerals, aroma compounds, pectic substances [5]. A variety of bioactive compounds such as simple phenolics, flavonoids, anthocyanins, stilbenes, proanthocyanidins, and vitamin E are considered a distinctive mixture of phytochemicals in grapes [6]. Grape seeds consider as a waste product in industry and consist of protein, carbohydrates, lipids, and 5-8% of polyphenols reliant on the type of grapes [7]. Different cultivars have a different grape seed composition [8]. Additionally, revenues and seed quality affected by several environmental and biological factors, such as light, drought, high salinity, cold, metal ions, pollutants, xenobiotics, toxins, experimental manipulations, pathogenic infection, and aging of plants [9]. A multitude of flavonoids is contained in GSE [10]. The most abundant of these are the proanthocyanidins, which are oligomers of monomeric flavan-3-of units linked by carbon-carbon bonds [11].

The most plentiful biologically active phytonutrients among the polyphenols found in grapes are flavonoids, which are possessing cardioprotective, neuroprotective, antimicrobial, anti-aging, antioxidant, anti-inflammatory, and anti-cancer properties [12,13].

Oral toxicity studies dealing with grape seed safety in experimental animals are few in the Iraq/Kurdistan region. Therefore, the main objective of this pilot study was to determine the oral toxicity of acetone-extracted

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grape seed (black and red) extract at different doses in mice, through clinical observations and evaluation of histopathological changes in multiple organs.

Materials and methods

Grape seed sampling and extraction

The work was carried out on two different varieties of grape (*Vitis Vinifera*) the red and black, which were taken manually from Sulaimani (Sharbazher-Kurdistan region) in the middle of July. The grapes were isolated manually from the skin, dried in the open air away from direct sunlight, and grounded into powder by the electrical grinder. The powders were stored in dark glass containers at -20℃ until the use.

Each sample of (72.2 g) of red grape seed and (40.73 g) of black grape seed powders was suspended with 202 mL and 114 mL of (70%) aqueous acetone respectively in a 500 mL Erlenmeyer flask. The mixtures were left on a magnetic stirrer for 24 hours at room temperature. The resultant extracts were filtered on a Buchner funnel then firstly evaporated with a rotatory evaporator to remove acetone, finally were freeze-dried for 24 hours to remove the water [14].

GC–MS analysis

Gas Chromatography-Mass Spectrometry (GC-MS) analysis was used to ascertain the compounds present in the purified samples of the red and black types of grape seed (*Vitis Vinifera)* from Sulaimania (Sharbazhear-Kurdistan region). Our natural plant was evaluated by (GCMS-QP2010 Ultra) GC systems combined with a mass spectrometer [15].

Animals and grape seed extract treatments

Forty-two mice (Male and female *Mus Muscular* species, *BALB/c* strain) weighing 25-30 g, at 4 weeks age were purchased from the Animal House at the College of Veterinary Medicine, University of Sulaimani (Sulaimani, Iraq/Kurdistan region), the mice were provided tap water and standard food *ad libitum* and were permitted to acclimate for one week before the start of the experiment accommodated in temperature and light-controlled environment. All of the *in vivo* experimentation in this pilot study was performed humanely according to and the ethical approval that was obtained from the Ethics Committee at the College of Veterinary Medicine, the University of Sulaimani in number (01589).

Consequently, the mice were assigned into 7 groups (6 mice per group) as follows: Group 1 (control): the animal of which were received normal saline without treatment; Group 2, 3, and 4, the animal of which treated orally with 200, 400, and 800 mg/kg b.w. of black GSE, respectively; Group 5, 6, and 7, the animal of which were treated orally with 200, 400, and 800 mg/kg b.w. of red GSE respectively, All treatments were given as a single daily dose by oral gavages every two days with a third-day free treatment and this study continued for about 21 days.

Clinical observations and body weight measurements

The animals were examined daily during this pilot study (for 21 days) for signs of acute toxicity such as diarrhea, curved tail, falling of hair, mortality and any other sign indicative for activity alterations, and the body weights of mice were recorded 2 times throughout the pilot study (day 0 and at the third week).

Tissue sampling and histopathological examination

At the end of the experimental period, the mice were euthanized with (Xylazine-Ketamine: 0.1 mL/10 gm of body weight) as recommended dose intraperitoneally and cervical dislocation [16]. The liver, kidney, spleen, and lung tissues of the sacrificed mice were excised, cleaned by normal saline, cut into the 4mm, fixed in 10% neutral buffered formalin (PH 7.6) for 24 hours and undergone a series of histopathological processes, tissue slices of 4 µm thick were attained and stained using the standard H and E technique and envisioned by light microscope (Leica, Germany), connected with an image analyzer software (Am Scope, AmView, MU1000B) [17].

Statistical Analysis

Statistical analysis was performed using the ANOVA (One-way) analyses of variance. Results were presented as a mean±standard error (Mean±SE) and *P* values less than 0.05 were considered significant. All statistical explorations were accomplished using the SPSS software version 22 (SPSS Inc., USA).

Results

Gas chromatography-MASS Spectroscopy

The secondary metabolites present in red and black grape seeds were detected in GC-MS (Table 1and 2).

The Grape Seed (GSD) peak region percentage and peak area coverage are specified below.

Table 1: G-CMS components for red grape seed

Peak no.	RT (Min.)	Compound Name	Peak Area	Correct area	Peak Area (%)
1	3.439	Heptane, 3-methyl	4280	562353	19.620%
2	3.808	Octane	9767	1039837	36.279%
3	7.345	2-N-PROPYL-1-D1-AZIRIDINE	5538	168753	5.888%
4	11.304	Tridecane, 2-methyl	4642	87857	3.065%
5	14.960	Eicosane, 2-methyl-	3572	66809	2.331%
6	18.239	Cyclobutanone, oxime	3308	68064	2.375%
7	19.749	Di-isodecyl phthalate	5223	365445	12.750%
8	21.187	Octane, 2,4,6-trimethyl-	4653	93120	3.249%
9	22.193	1,2-Benzenedicarboxylic acid, bis 2-methylpropyl) ester	9067	212306	7.407%
10	22.554	4,4-Dimethylcyclooctene	3692	76959	2.685%
11	23.444	2-Acetyl-N-methylaniline	2537	58464	2.040%
12	23.856	Docosane	2972	66227	2.311%

Table 2: G-CMS components for black grape seed

Peak no.	RT (Min.)	Compound Name	Peak Area	Correct area	Peak Area (%)
1	3.445	Heptane, 3-methyl	4267	579464	3.736%
2	3.822	Octane	9823	998433	6.438%
3	7.349	2-N-PROPYL-1-D1-AZIRIDINE	5775	161076	1.039%
4	11.305	Heptadecane, 2-methyl-	5492	106189	0.685%
5	14.961	Hexadecane, 2-methyl-	4376	85425	0.551%
6	25.428	Bis(2-ethylhexyl) phthalate	115510	7027490	45.312%
7	26.876	Di-isodecyl phthalate	140559	6550999	42.240%

Clinical observations

The animals were healthy in general with no clinical signs of toxicity. There were no unusual changes in behavior or locomotors activity during the 21-day observation period. No deaths occurred during the stud.

Body weight measurements

All groups gained weight during the pilot study period compared to day 0 of the study. The mean body weight of the mice of all groups showed a significant increase (P<*0.05*) in their body weight measurements in comparison to the initial day, no-significant variations (P<*0.05*) were seen in the mean of the body weight gain of mice in the treatment groups of 200 mg/kg b.w. and 400 mg/kg b.w. in comparison with that of the control groups. While significant reduction (P<*0.05*) in the mean body weights gain was recorded for 800 mg/kg b.w. groups in the last week of an experiment in comparison with that of the mice in the control and other treated groups (Table 3 and 4).

Table 3: Body weight gain (gram) of mice in black GSE treated groups.

			A black grape seed extract		
Experiment duration		**Control group**	**200 mg/kg b.w.**	**400 mg/kg b.w.**	**800 mg/kg b.w.**
	Day 0	26.00±0.04	26.93±0.00	27.00±0.04	28.00±0.15
	Week 3	35.00±0.57	34.95±0.44	35.05±0.62	34.08±0.02
	Weight gain	9.00±0.53A	8.02±0.41A	8.05±0.58A	6.08±0.13B

The body weight gains are expressed by mean ± standard error, bodyweight values, values marked by different letters are significantly different (p<*0.05*).

Table 4: Bodyweight gain (gram) of mice in red GSE treated groups.

			A red grape seed extract		
Experiment duration		**Control group**	**200 mg/kg b.w.**	**400 mg/kg b.w.**	**800 mg/kg b.w.**
	Day 0	27.00±0.02	27.10±0.00	28.13±0.05	28.70±0.15
	Week 3	35.00±0.57	35.15±0.00	35.90±0.82	34.98±0.64
	Weight gain	8.00±0.55A	8.05±0.00A	7.77±0.77A	6.28±0.49B

The body weight gains are expressed by mean ± standard error, bodyweight values, values marked by different letters are significantly different (p<*0.05*).

Histopathological Results

Regarding histopathological findings, the liver section of control, and 200 mg/kg b.w. treated groups in the black and red GSE exhibited normal histomorphological features including, central vein, sinusoidal capillaries with normal appearance of hepatocytes (Figure- 1a-d), in comparison to 400mg/kg b.w. treated groups in the black and red GSE, the liver cells showed karyolitic features (Figure- 1e and f), while the hepatocytes in 800mg/kg b.w. treated groups in both types of GSE undergo coagulative necrosis, characterized by eosinophilic cytoplasm with features of karoryolysis and karyorrhexis of the nucleus and kupffer cell proliferation (Figure- 1g and h). The microscopical section of the kidney in the control group showed; normal and intact appearance of glomeruli, proximal and distal convoluted tubules with normal renal vasculature in control, and 200mg/kg b.w. in black and red GSE treated groups (Figure- 2a-d). While in 400 mg/kg b.w. in black and red GSE treated groups the epithelial lining of collecting tubules showed slightly swollen. In the 800mg/kg b.w. in both types of GSE treated groups, the kidney showed dilation of Bowman's capsule, glomerular atrophy with the segmentation of glomerular capillaries and increasing the mesangial cells, also moderate swollen of the epithelial lining of convoluted tubules with interstitial hemorrhage (Figure- 2e-h). Microscopical section of the spleen revealed normal histological appearance in control and 200mg/kg b.w. in black and red GSE treated groups, whereas in 400mg/kg b.w. in both types of GSE treated groups showed mild-moderate lymphocytic hyperplasia in the white pulp region and congestion in the red pulp area if compared to the 800mg/kg b.w. in black and red GSE treated groups that showed moderate lymphocytic hyperplasia in white pulp region and congestion in the red pulp area (Figure- 3a-h and 6a-h).The histopathological finding of lung parenchyma in black and red GSE treated groups revealed normal histological structures of bronchi, bronchioles, alveolar ducts, alveolar sac, and alveoli with normal vasculatures in control and 200mg/kg b.w. treated groups, but the minimum-mild proteinous fluid was found in the alveolar lumen of 400 and 800mg/kg b.w. black and red GSE treated groups in addition to vascular congestion particularly in the 800mg/kg b.w. black and red GSE treated groups.

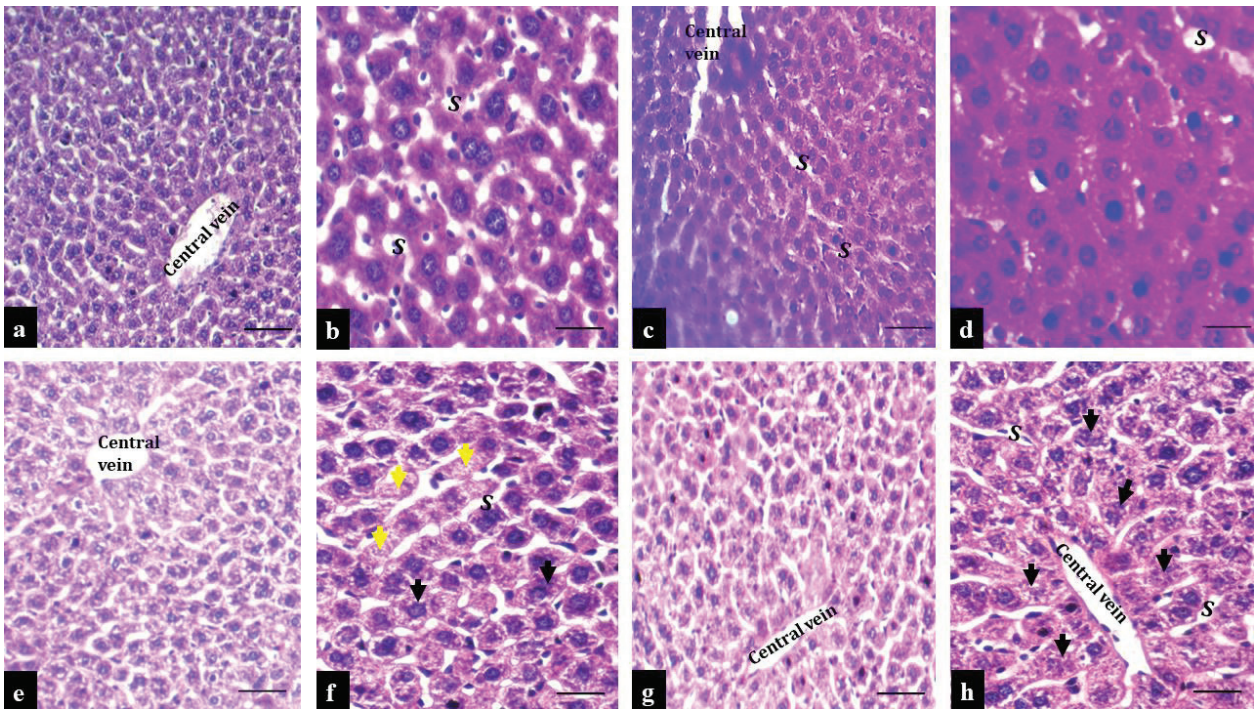

Figure 1- Histomicrograph of liver sections in control, black and red GSE treated groups. a and b: The normal liver histology in the control group, c and d: The normal histological features of liver parenchyma in 200mg/kg b.w. treated groups, e, and f: The hepatocytes showed slightly swollen (black arrows) with karyolysis in few hepatocytes (yellow arrows) in 400mg/kg b.w. treated groups, g and h: The hepatic cells undergo coagulative necrosis as indicated by black arrows in 800mg/kg b.w. treated groups, (S) sinusoidal capillaries, (H&E stain, scale bar 50 µm, scale bar 20 µm).

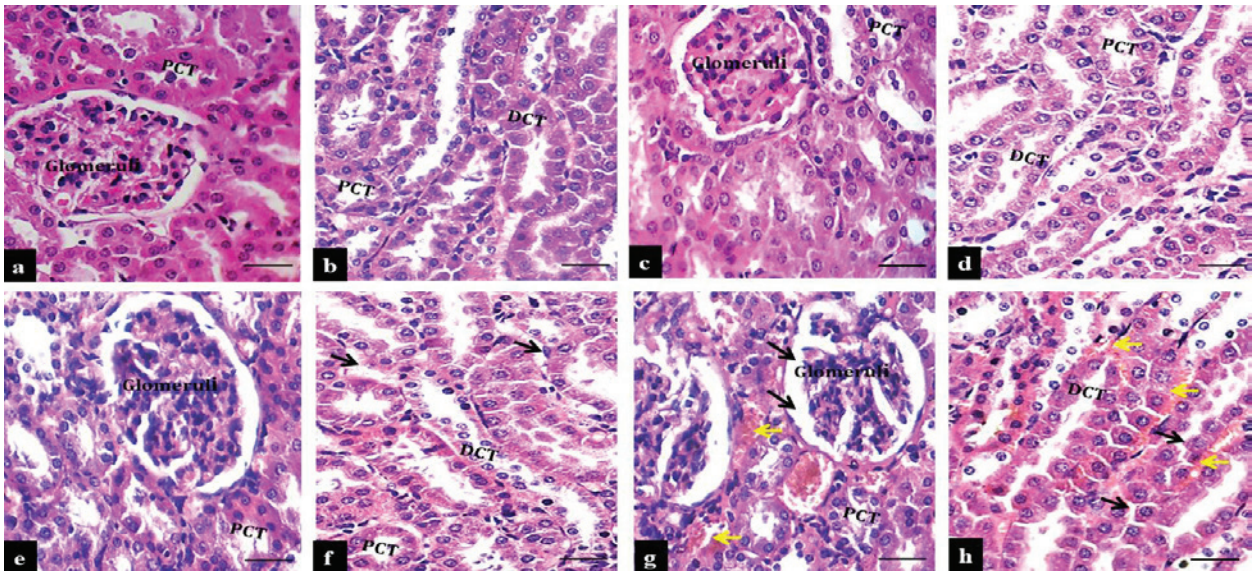

Figure 2- Histomicrograph of kidney sections in control, black and red GSE extract treated groups. a and b: The normal kidney histology in the control group, c and d: The normal histological structures of kidney parenchyma in 200mg/kg b.w. treated groups, e, and f: The epithelial lining of PCT and DCT showed slightly swollen (black arrows) in 400mg/kg b.w. treated groups, g and h: The swollen of the Bowmans' space (blackhead arrows), segmentation of glomerular capillary tuft with mesangial hypercellularity and glomerular atrophy, the epithelial lining of PCT and DCT showed moderately swollen (black arrows) with interstitial hemorrhage indicated by yellow arrows in 800mg/kg b.w. treated groups, (DCT) convoluted tubules, and (DCT) distal convoluted tubules, (H&E stain, scale bar 50 µm, scale bar 20 µm).

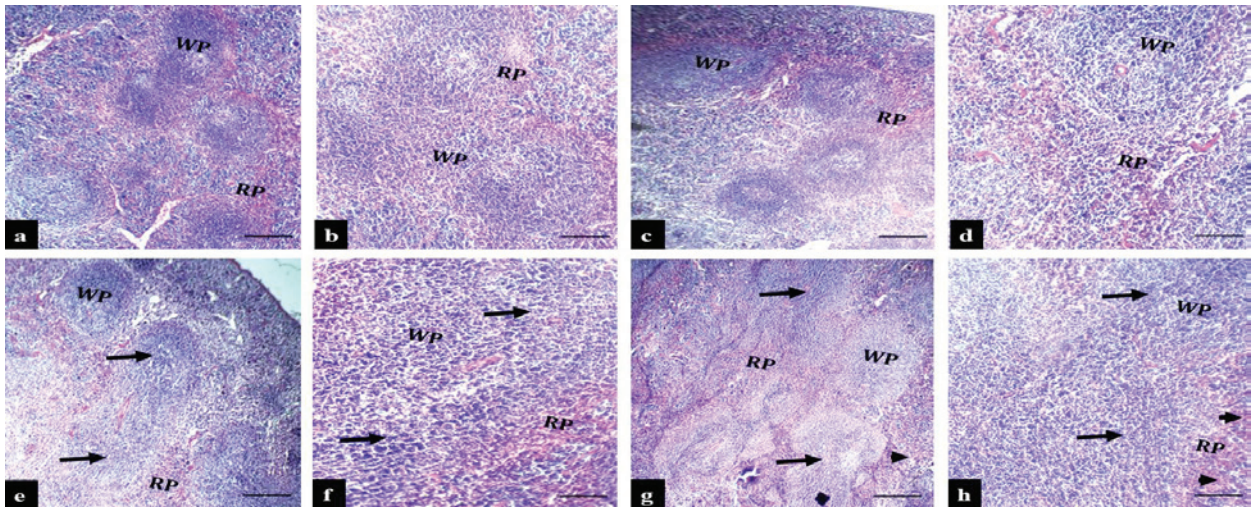

Figure 3- Histomicrograph of spleen parenchyma in control, black and red GSE treated groups. a and b: The normal spleen structures in the control group, c and d: Normal microscopical features of the spleen in 200mg/kg b.w. the treated groups, e, and f: Slightly lymphocytic hyperplasia (black arrows) in the white pulp regions (black arrows) in 400mg/kg b.w. treated groups, g and h: Mild-moderate lymphocytic hyperplasia (black arrows) in the white pulp regions and congestion of the red pulp sinusoids as indicated by blackhead arrows in 800mg/kg b.w. treated groups, (WP) white pulp, and (RP) red pulp, (H&E stain, scale bar 50 µm, scale bar 20 µm).

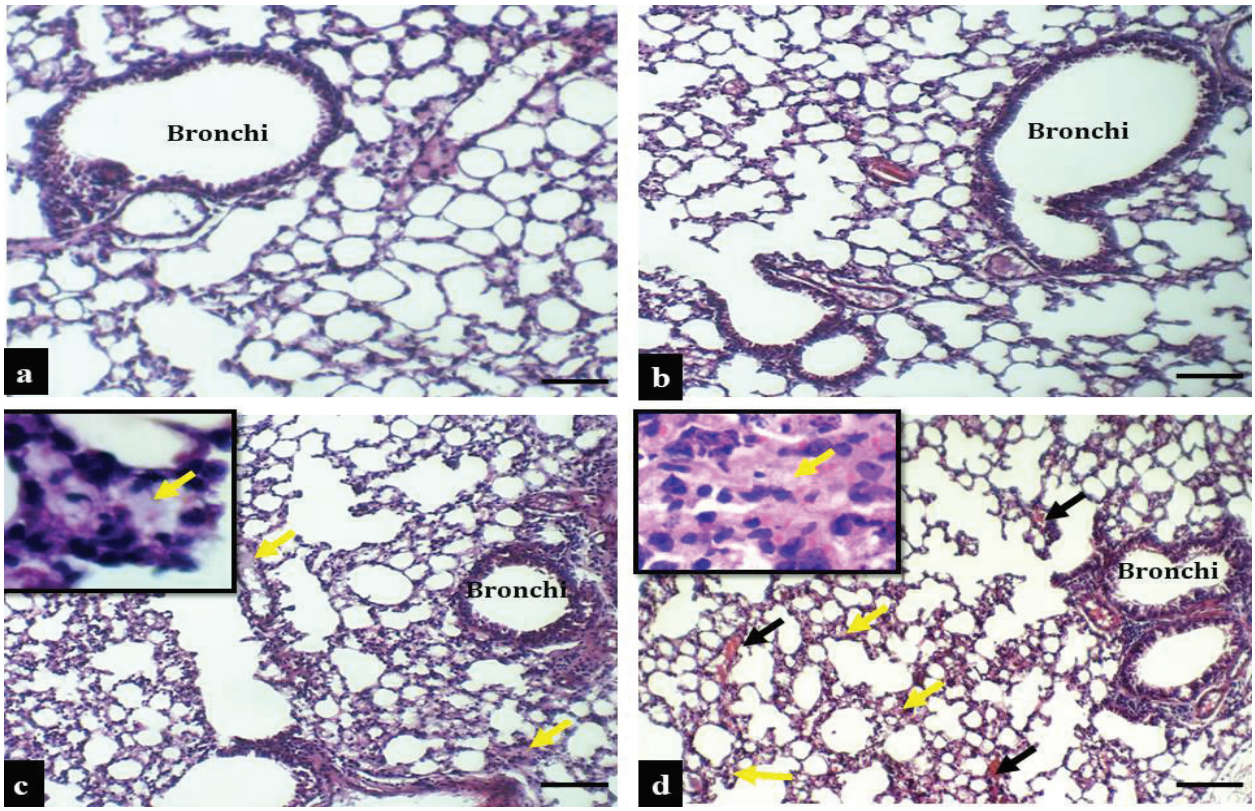

Figure 4- Histomicrograph of the lung parenchyma of control, black and red GSE treated groups. a and b: In the control, and 200mg/kg b.w. treated groups, the lung showed normal histological constructions of bronchi, bronchioles, and alveoli with normal vascular appearance, c: There is slight transudate fluid in the alveolar lumen (Inset and yellow arrows) in 400mg/kg b.w. treated groups, d: The lung parenchyma exhibited mild accumulation of eosinophilic fluid in the alveolar lumen (Inset and yellow arrows) with vascular congestion as indicated by black arrows in 800mg/kg b.w. treated groups, (H&E stain, scale bar 50 µm, scale bar 20 µm).

Discussion

For the continuous development of phytochemicals under-regulated plant cell cultures, prospective replacements are generally considered [18,19]. In recent years, plant cell biotechnology of grapes and particularly of grape cell suspensions have enjoyed great scientific and industrial consideration [20]. New research aims to decode the benefits of grapes as a rich source of essential phytonutrients with remarkable beneficial impacts on human health [21].

No mortality was reported with no significant changes in body weight gain or physical appearance, and no abnormal histopathological changes in the liver, kidney, spleen, and lung were observed during this pilot study in that groups received the 200, and 400mg/kg b.w. doses of both black and red GSE and the outcomes attained from this study are, in agreement with the results of other studies that revealed using of the GSE in experimental animals did not result in any disturbances in clinical activities or body weight loss [22,23].

However, the significant decrease in body weight gain was found in mice of group 800mg/kg b.w. of GSE, Yamakoshi, et al, (2002), who reported a similar result in which the high dose of GSE may lead to slightly significant weight loss in an animal model [24], while the observed result may disagree with the Mittal et al, (2003), study who documented that the high dose of GSE did not interfere with physical activity and significant difference in body weights or other signs of clinical toxicity [25]. Also, the other study on Sprague-Dawley rats documented no significant decrease in body weight was observed even after 90 days of oral administration of GSE [26]. The decrease in body weight gain may correlate with the high antioxidant activity in induced to enhance the lipase effect and enhancing lipolysis [27].

Additionally, it is obvious from the present study the histopathological changes in 800mg/kg b.w. dose groups of GSE represented by the mild-moderate swollen in the hepatocytes with a moderate degree of necrosis, segmentation of glomerular capillaries and moderate swollen of the epithelial lining of convoluted tubules with interstitial hemorrhage in kidney organ, the histological changes in spleen revealed mild-moderate lymphocytic hyperplasia in the white pulp region and congestion in the red pulp area, and the lung parenchyma showed mild accumulation of eosinophilic fluid the alveolar lumen. Our findings disagree with the other studies that mentioned no significant histopathological lesions in multiple organs after administration of GSE high dose [28,29].

Conclusion

Consequently, it is concluded that the results of this study support the health of GSE dietary components for human use. No observed level of adverse effects (NOAEL) was deemed approximately in a dose of 400 mg/kg b. w. /day for administration in both types of GSE. While a mild-moderate changes microscopically was seen in the mice's organs that treated by 800mg/kg b.w. black and red GSE treated groups.

Conflict of Interest: Nil

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