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Estimation the Safety of Parenteral Resveratrol in Mice

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Abstract

Resveratrol is polyphenolic compound has many biochemical and biological effects on several organs. Therefore, resveratrol can be used to treat many diseases. The aim was to evaluate resveratrol safety when used in a parenteral single bolus dose. This study was conducted on 60 mice (30 males and 30 females) both sexes weighing 25-35g were divided into 6 groups (5animals per group) for each sex. All mice groups given 1% DMSO and five different doses of resveratrol (5, 2.5, 1.25, 0.625, 0.312) g/kg intra-peritoneally given to five groups respectively. The mice were continuously monitored during 14 days. The number of deaths, changes in general behavior, changes in physiological activity, and signs of toxicity were reported. On day 15 blood was collected using a jugular vein puncture to obtain blood samples for hematological and biochemical analysis. All mice were euthanized under anesthesia. The heart, lung, liver, kidney, and gonads were dissected and sent for histopathological study. The result showed that at dose 0.312gm/kg neither signs of toxicity nor death were detected. The LD50 dose was 1.18 g/kg for female and 1.07 g/kg for male mice. The body weight change, biochemical and hematological assay, revealed that at doses (1.25,0.625,0.312) g/kg for both sexes no significant changes had reported in comparison with the control group ($p>0.05$). Histopathological examination revealed that at doses 1.25 g/kg for both sexes no significant tissue changes had reported in comparison with the control group ($p>0.05$). In conclusion resveratrol at lower doses showed non-observed adverse effect while at high doses, showed dose dependent toxicity when used as single bolus dose intraperitoneally

Keywords: Acute toxicity, Intraperitoneally, Histopathology, Resveratrol, Biochemical assay

تقدير سلامة الريسفيراترول بالحقن في الفئران

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الخلاصة

الريسفيراترول هو مركب بوليفينوليك له العديد من التأثيرات البيوكيميائية والبيولوجية على العديد من الأعضاء. لذلك ، يمكن استخدام ريسفيراترول لعلاج العديد من الأمراض. كان الهدف هو تقييم سلامة ريسفيراترول عند استخدامه في جرعة بلعة مفردة بالحقن داخل الصفاق. أجريت هذه الدراسة على ٦٠ فأر (٣٠ ذكور و ٣٠ إناث). تم تقسيم كل فئران من الذكور والإناث إلى ٦ مجموعات (خمسة فئران لكل مجموعة). أعطيت جميع مجموعات الفئران ماده(الدي ام اس اوبتركيز اقل من ١ %)و خمس جرعات مختلفة من ريسفيراترول (٥ ، ٢,٥ ، ١,٢٥ ، ٠,٦٢٥ ، ٠,٣١٢) جم / كجم داخل الصفاق تعطى لخمس مجموعات على التوالي. تمت مراقبة الفئران بشكل مستمر خلال ١٤ يومًا. تم الإبلاغ عن عدد الوفيات والتغيرات في السلوك العام والتغيرات في النشاط الفسيولوجي وعلامات السمية. في اليوم الخامس عشر ، تم جمع الدم باستخدام ثقب الوريد الوداجي للحصول على عينات الدم لتحليل الدم والكيمياء الحيوية. تم قتل جميع الفئران تحت التخدير. تم تشريح القلب والرئة والكبد والكلى والغدد التناسلية وإرسالها لدراسة التشريح المرضي أظهرت النتائج أنه عند الجرعه ٠,٣١٢ جم/كجم لم يتم الكشف عن العلامات السميه والموت .كانت الجرعه التي تقتل ٥٠٪ من الحيوانات المختبريه للاناث هي ١,١٨ جم/كجم بينما للذكور تساوي ١,٠٧ جم/كجم. أظهر تغير وزن الجسم ، المقايسة البيوكيميائية والدمية ، أنه عند الجرعات ١,٢٥ ,٠,٣١٢,٠,٦٢٥ جم/كجم لم تسجل أي تغيرات معنوية مقارنه بمجموعه التحكم(قيمه بي اكبر من ٠,٠٥). عند الجرعه ١,٢٥جم/كجم لكلا الجنسين لم تسجل تغيرات معنوية في الأنسجة مقارنة بمجموعة التحكم(قيمه بي اكبر من ٠,٠٥). في الختام ، أظهر ريسفيراترول عند الجرعات المنخفضة تأثيرًا ضارًا غير ملحوظ بينما عند الجرعات العالية ، أظهر سمية تعتمد على الجرعة عند استخدامه كجرعة مفردة داخل الصفاق.

الكلمات المفتاحية : السمية الحادة ، داخل الصفاق ، التشريح المرضي ، ريسفيراترول، المقايسة البيوكيميائية.

Introduction

Resveratrol is a polyphenolic compound found in at least 70 plant species. Its phytoalexin has activity against viruses, bacteria, and fungi. Obtained by biotechnological synthesis from yeasts or by chemical methods. It is found in a discrete amount in several human foods such as grapes, pomegranate, mulberries, peanuts, apple, tomato, and dark chocolate[1,2,3]. Resveratrol has many biochemical and biological effects on several organs. For this reason, resveratrol can be used to

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treat many diseases. The curative effect of resveratrol is derived from its antimicrobial, anti-inflammatory, anti-viral anti-cancer, anti-oxidant, anti-hyper-lipidemic, anti-hypertensive, anti-diabetic. In addition to cardioprotective, neuroprotective, and androgen lowering effect on theca-interstitial cells of the ovary [1,4,5]. Also, it acts as phytoestrogen due to its similarity in structure to diethylstilbestrol. Other uses are calories restriction (weight loss), and anti-aging [2,6,7]. Moreover, it has a therapeutic effect on the liver in iron overload [8]. Many clinical studies demonstrated the above activity of resveratrol [3].

Acute toxicity is the capability of any material to cause severe biological injury or death soon following a single dose exposure; the goal of this study is aimed for acute toxicity testing and lethal dose required to kill 50% of tested animals (LD50) was estimated.

Materials and Methods

Materials

Resveratrol as a dry powder have been purchased from Hangzhou hyper chem. limited/China. Dimethyl sulfoxide (DMSO) is a solvent obtained from chem-lab NV, Belgium. 4% formaldehyde in phosphate buffer saline has been purchased from Edutek/India. Hematoxylin and Eosin stain purchased from BDH/England. All other kits used in biochemical and hematological have been obtained from Roche/Germany and CUSABIO/USA.

Samples preparation

Resveratrol CAS 501-36-0/99% freshly prepared as stock solution equivalents to 5.0, 2.5, 1.25, 0.625, 0.312 gm/kg by (by dissolving each concentration in separated volumetric flask in DMSO and then diluted gradually with Distilled water to give the required strength solution with concentration of DMSO 1% [9].

Experimental animals

Sixty Swiss albino mice weighing between 25-35 g had been purchased from the center for drug control and research in Baghdad/ Iraq. All handling and procedure process to the animal conducted with direction in the guide for the use and care of experimental animals of the animal ethics committee Al-Nahrain University/ College of pharmacy ". Animals were left over seven days in the animal care facility of Al-Nahrain University/ College of pharmacy in a light/ dark cycle with regular feeding with rodent chow and ad libitum. The environment of the place was well ventilated with fresh air and the temperature was set to standard levels (23 ± 2 °C).

Method

The study of acute toxicity was performed following the Organization of Economic Co-operation and Development (OECD) guideline for chemical testing[10]. Thirty male and thirty female Swiss albino mice weighing (25-35) g each were randomly distributed into control group and five treated groups, containing five animals per group. All animals were freely reach their water & food and were permitted to familiarize with the laboratory conditions for seven days before the test. All mice groups given 1% DMSO and five different doses of resveratrol (5,2.5,1.25,0.625,0.312) g/kg respectively. The acute toxicity testing was performed according to previous studies [11,12]. In which mice were continuously monitored for the first 4 h and then every hour for the next 24 h and at 6 hourly intervals for the next 48 h after administration of resveratrol. Then the number of death, changes in general behavior and other physiological activity, signs of toxicity such as changes in weight, skin, hair, eyes, mucous membranes, secretions and excretions, autonomic activity, and other CNS signs of toxicity such as (drowsiness, loss of gait, convulsion, tremor)were reported The observation period is 14 days. All mice were weighed and data collected at day zero (before any treatment had been received), at day 7 from the first dose, and on day 14[13]. Then on day 15 blood was collected using a jugular vein puncture(14)(approximately (1 ml) for hematologic analysis put in Ethylenediaminetetraacetic acid (EDTA) tubes and for clinical biochemistry assay, the blood for the hematological assay (hemoglobin (HGB) concentration and WBC count) was immediately analyzed using Diagon D-cell60. The blood for the clinical biochemistry assay (AST, ALT, ALP, bilirubin, creatinine, and urea) was centrifuged for 10 min at 3000 rpm to isolate plasma and deposited at -20 °C until reviewing for clinical biochemistry using COBAS/Roch apparatus. [15,11,16]. Then all animals were euthanized by cervical dislocation under light chloroform anesthesia. On the 15th day after administration of the treatment, the heart, lungs, livers, kidneys, and sex organs were dissected and fixed in a 10% neutral buffer formalin and processed effectively to study histopathological changes. Histopathologists using a Zeiss Imager M2 microscope fitted with an AxioCamHRc camera (Carl Zeiss Microscope) to observe histopathological changes

Statistical analysis

All data were collected, tabulated and statistically analyzed using Social Sciences Software Statistical Package (SSPS) software version 20. The result was presented as Means ±SD one-way analysis of variance (ANOVA) followed by a t-test (2-tail) was used to compare between groups. The level of significance was set at the P values <0.05.

Results and Discussion

Table 1 and Table 2 show signs of acute toxicity of resveratrol in observation period and the number of dead for female and male mice respectively. At dose 5 g/kg and 2.5 g/kg of resveratrol all female and male mice died after the sign of toxicity (loss of gait, muscular fasciculation, convulsion, diarrhea, lacrimation, salivation finally muscle weakness, paralysis, dyspnea, and death). At 1.250 g/kg dose the symptoms of toxicity were less intense and the number of mortalities was decreased. This may be due to resveratrol has an OH group that binds to acetylcholine esterase enzyme (AChE) and suppresses its activity in a concentration-dependent manner, so excessive accumulation of acetylcholine at the neuromuscular junction and synapses causes symptoms of both muscarinic and nicotinic toxicity. Besides at dose 1.250 gm/kg, there is a symptom of dehydration (piloerection and sunken) [17], which may be due to loss of fluid through diarrhea. While (pale footpad and ear) may refer to shock or anemia that reversible in some mice which indicates the ability of detoxification[12], that resveratrol is extensively metabolized by phase II detoxification enzyme in the liver, its metabolism, and its metabolite are correlated with the presence of two genes (sulfotransferase and UDP-glucuronosyltransferase [18]. At the dose of 0.625 g/kg no signs of toxicity only in the first hours' diarrhea may be due to stress or side effect of resveratrol. This finding is in agreement with another study that reported the side effect of resveratrol is diarrhea regardless the route of administration [2], one mouse died from each group female and male were detected during 14 days of the acute toxicity trial span. At dose 0.312g/kg neither signs of toxicity nor death detected during 14 days of the acute toxicity. This result in agreement with previous studies that reported resveratrol has a dose-dependent inhibitory effect on both acetylcholine esterase and butyrylcholinesterase activity [20,21]. From these data concluded the dose of 0.312 g/kg consider the Non-Observed Adverse Effect Level (NOAEL) and this result was confirmed by a histopathological study that showed no morphological changes in the examination organs.

Table 1 .Signs of acute toxicity of resveratrol in observation period and number of dead female mice.

Dose g /Kg	T/D	Observance period	Sign of toxicity	No. of dead mice
5	5 /5	5 min-15min	loss of gait, muscular fasciculation, convulsion, dyspnea, lacrimation, and death (+++).	3
		15min-4h	hypoactivity, diarrhea atypical locomotion (back limbs falling abdominal contract, dyspnea, death (++).	1
		4 h-6h	atypical locomotion, piloerection, dyspnea, and death (+).	1
2.5	5 / 5	5 min-15min	loss of gait, muscular fasciculation, convulsion, dyspnea, lacrimation, and death (+++).	2
		15min-4h	Hypoactivity, diarrhea atypical locomotion (back limbs falling, dyspnea, and death (++).	1
		4 h-6h	atypical locomotion, piloerection, dyspnea, and death (+).	1
		6-24 h	atypical locomotion, piloerection, dyspnea, and death (+).	1
1.25	5 /3	10-15 min	loss of gait, muscular twitching and death (+).	0
		15 min-4h	atypical locomotion (back limbs falling) hypoactivity, hyperventilation, and death (++).	0
		6-24 h	hypoactivity, piloerection, atypical locomotion (back limbs falling) pale foot pads and ear finally death.	1
		24-48 h	Hypoactivity.	1
		48 h-14 d	no sign of. toxicity	0
0.625	5 / 1	1 -6 h	Hypoactivity.	0
		6-24 h	diarrhea (steaky stool in the anus), hypoactivity	0
		24h-48 h	Hypoactivity, sunken, piloerection, and death.	1
		48 h-14 d	no sign of. toxicity	0
0.312	5 / 0	1h-6 h	hypoactivity	0
		24 h-14 d	no sign of toxicity	0

T/D: number of mice treated/number of total deaths. the duration of observation =14 days. (+), (++), (+++) means slightly, moderately, and intensively increased respectively.

Table 2. Signs of acute toxicity of resveratrol in observation period and number of dead male mice

Dose g /Kg	T/D	Observance period	Sign of toxicity	No. of dead mice
5	5 /5	5 min-15min	loss of gait, muscular fasciculation, convulsion, dyspnea, lacrimation, and death (+++).	3
		15min-4h	hypoactivity, diarrhea atypical locomotion (back limbs falling abdominal contract, dyspnea, death (++).	1
		4 h-6h	atypical locomotion, piloerection, dyspnea, and death (+).	1
2.5	5 / 5	5 min-15min	loss of gait, muscular fasciculation, convulsion, dyspnea, lacrimation, and death (+++).	2
		15min-4h	Hypoactivity, diarrhea atypical locomotion (back limbs falling, dyspnea, and death (++).	1
		4 h-6h	atypical locomotion, piloerection, dyspnea, and death (+).	1
		6-24 h	atypical locomotion, piloerection, dyspnea, and death (+).	1
1.25	5 /3	10-15 min	loss of gait, muscular twitching and death (+).	1
		15 min-4h	atypical locomotion (back limbs falling) hypoactivity, hyperventilation, and death (++).	1
		6-24 h	hypoactivity, piloerection, atypical locomotion (back limbs falling) pale foot pads and ear finally death.	1
		24-48 h	Hypoactivity.	0
		48 h-14 d	no sign of. toxicity	0
0.625	5 / 1	1 -6 h	Hypoactivity.	0
		6-24 h	diarrhea (steaky stool in the anus), hypoactivity	0
		24h-48 h	Hypoactivity, sunken, piloerection, and death.	1
		48 h-14 d	no sign of. toxicity	0
0.312	5 / 0	1h-6 h	hypoactivity	0
		24 h-14 d	no sign of toxicity	0

T/D: number of mice treated/number of total deaths. the duration of observation =14 days. (+), (++), (+++) means slightly, moderately, and intensively increased respectively.

Figure 1 shows the dose-response curve of resveratrol for female mice groups.and calculate the LD50 dose through the equation Y=40.375 ln(x)+43.003 and it was 1.18 g/kg. Figure 2 shows the dose-response curve of resveratrol for male mice groups shows the lethal dose that kills fifty percent of male mice (LD50) of resveratrol calculated through the equation Y=40.377 ln(x)+47.003 and it was 1.07 g/kg. The data concluded from both figures shows that resveratrol has dose dependent toxicity. The more toxic substance has lower LD50. Figure 1& 2 also showed the present of mortality was 100 percent in the first two doses while the percent of mortality was decreased in slight variation between male and female which may be referred to gender effect.

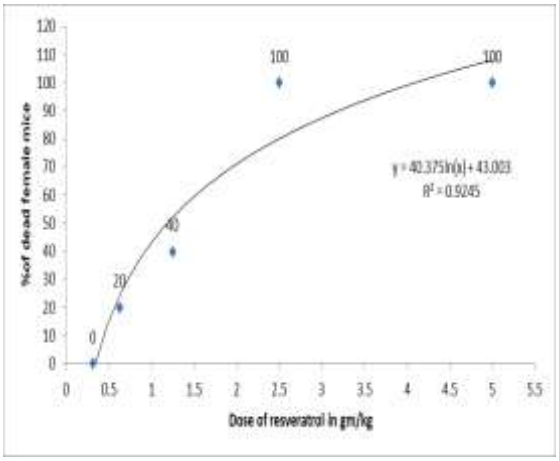

Figure 1 .Dose-response curve of resveratrol for female mice groups.

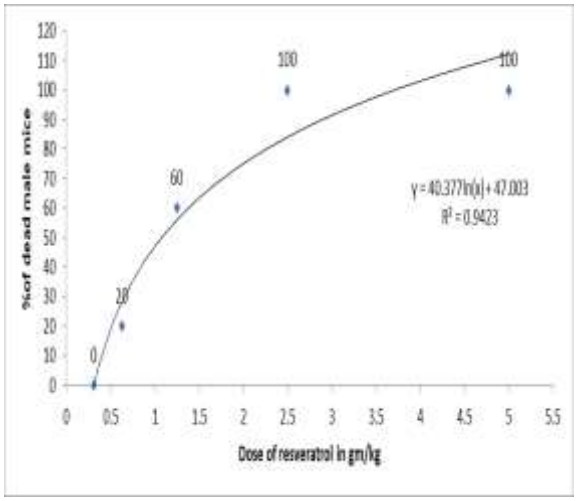

Figure 2. Dose-response curve of resveratrol for male mice groups.

Bodyweight changes were tabulated at day zero (before any dose given), day seven, and day 14, statistically analyzed in mean ±SD and summarize in Table 3 and three figures. Figure 3 represents bodyweight changes between male and female mice at dose 1.250gm/kg and shows no significant changes in body weight compared to the control group ($P > 0.05$). Figure 4 represents body weight changes between male and female mice at dose 0.625gm/kg and shows no significant changes in body weight compared to the control group ($P > 0.05$). Figure 5 represents body weight changes between male and female mice at dose 0.312gm/kg and shows no significant changes in body weight compared to the control group ($P > 0.05$). The change in animal body weight has been used as a reliable predictor of the drug or chemical's side effects on the animal. [22],and the loss in body weight from the control would reflect the toxicity of the material[11,23] also the change in animal body weight may indicate drug change the metabolic events and growth rate of the tested treated mice groups[24].From this results concluded resveratrol has no toxic effect on the metabolic events and growth rate at these single doses because there are no significant changes in body weight of treated mice at (1.250 g/kg,0.625 gm/kg, and 0.312gm/kg).

Table 3. Body weight changes weakly.

Group	sex	Mean± SD		
		Weight at Day 0 (g)	Weight at Day 7(g)	Weight at Day 14(g)
Control	M	28.6 ± 2.70	31.66 ± 3.3	32.98 ± 3.3
	F	29. 2± 1.9	31.96 ± 1.8	34.48 ± 2.1
Group I	M	30.38 ± 5.3	37 ± 0.1	39.55 ± 1.7
	F	30.9±3.8	32.7±0.8	33.98±0.95
Group II	M	27.32±4.1	27.72±2.3	30.5±1.50
	F	28.6±5.5	30.5±3.3	31. 87±2.9
Group III	M	33.14±4.8	32.1±6.0	34.12±5.4
	F	28.6±4.8	29.3±4.9	31±4.7

M refer to male, F refer to female.
Group 1: 1.25 g/kg Resveratrol, Group II: 0.625g/kg Resveratrol, Group III: 0.312g/kg Resveratrol

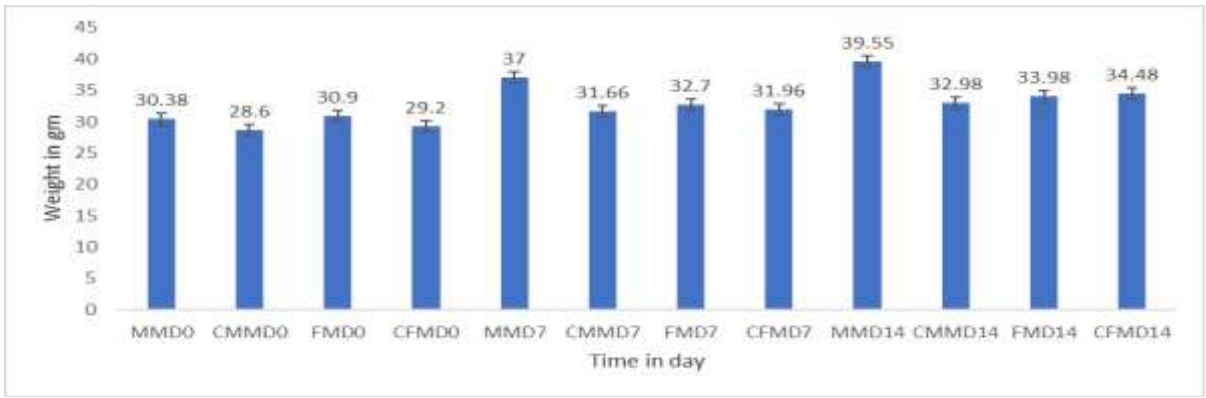

Figure 3 Weight changes between male and female mice have received 1.25gm/kg resveratrol and their Controls at day zero, seven, and on day fourteen. where MMD0, CMMD0, FMD0, CFMD0, MMD7, CMMD7, FMD7, CFMD7, MMD14, CMMD14, FMD14, and CFMD14 represent, male mice day zero, its control, female mice day zero, its control, male mice day seven, its control, female mice day seven, its control, male mice day fourteen, its control, female mice day fourteen and Its control, respectively.

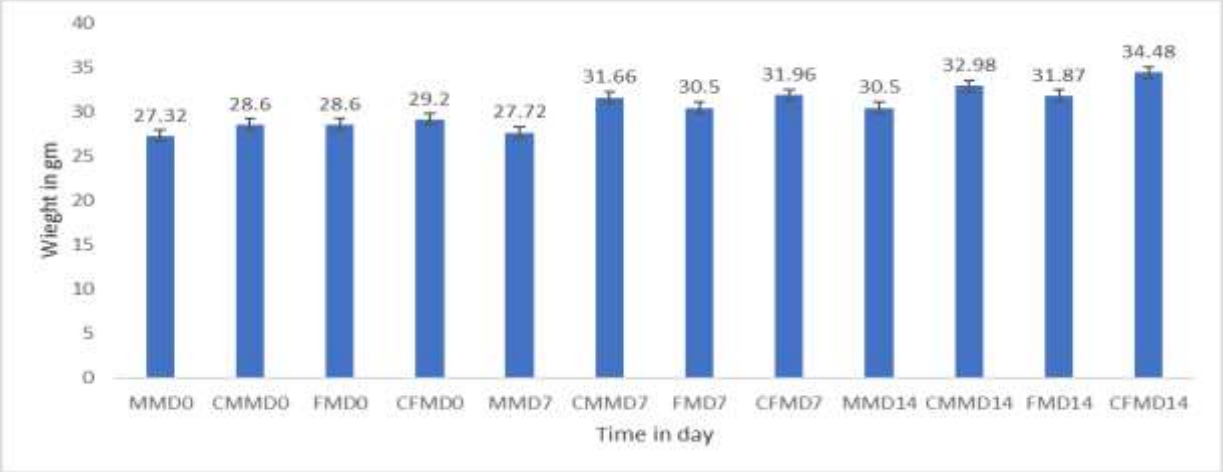

Figure 4 Weight changes between male and female mice have received 0.625 gm/kg resveratrol and their Controls at day zero, seven and on day fourteen. where MMD0, CMMD0, FMD0, CFMD0, MMD7, CMMD7, FMD7, CFMD7, MMD14, CMMD14, FMD14, and CFMD14 represent, male mice day zero, its control, female mice day zero, its control, male mice day seven, its control, female mice day seven, its control, male mice day fourteen, its control, female mice day fourteen and Its control, respectively.

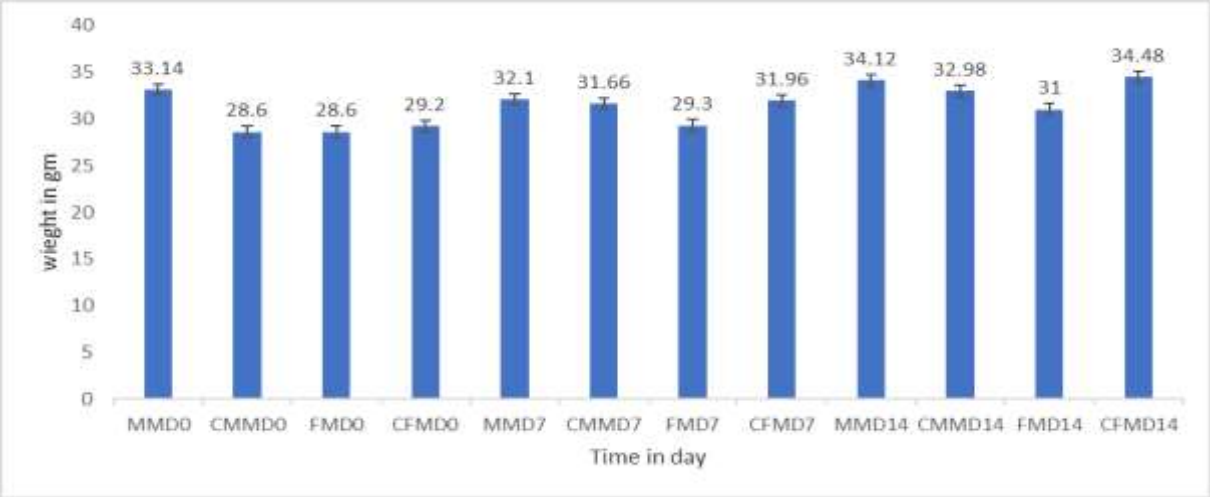

Figure 5 Weight changes between male and female mice have received 0.312 gm/kg resveratrol and their Controls at day zero, seven, and on day fourteen. where MMD0, CMMD0, FMD0, CFMD0, MMD7, CMMD7, FMD7, CFMD7, MMD14, CMMD14, FMD14, and CFMD14 represent, male mice day zero, its control, female mice day zero, its control, male mice day seven, its control, female mice day seven, its control, male mice day fourteen, its control, female mice day fourteen and Its control, respectively.

According to the Table 4 that shows there are no significant changes between male and female mice concerning Hematological and biochemical changes compared to their control ($P > 0.05$)) Hematological and biochemical changes are of essential importance for the detection of pathophysiological changes in animals. Moreover, deviations in hematological parameters are capable of signifying toxicity-induced hemolysis [(11)]. Also, Hb level can signify renal failure (impairment erythropoietin synthesis) and may indicate toxicity that induces hemorrhage or hemolysis. Enzymatic and non-Enzymatic biochemical parameters (e.g., Alanine transaminase (ALT), Alkaline phosphatase (ALP), Aspartate transaminase (AST), and bilirubin) which are often used to indicate liver damage. The enzyme Alanine transaminase (ALT) and Aspartate transaminase (AST) are the mitochondrial enzyme mostly found in the liver, skeletal muscles, and kidneys. So, elevate AST level indicates either liver damage or cardiac infarction and also, may indicate muscle injury [(25)] bilirubin and albumin are a good marker for liver function while urea and creatinine parameters are used to indicate kidney damage. [(26)] So, the results of this study indicate the resveratrol is safe to the liver and kidney which are the main organs for xenobiotic detoxification.

Table 4 The serum profile and hematological assay after 14 days for Group I: 1.25 g/kg resveratrol, Group II: 0.625g/kg resveratrol, Group III: 0.312g/kg resveratrol for both sex of mice after administration as a single intra-peritoneal route.

Serum and blood profile	Control male	Control female	Group I Male: 1.25 g/kg Resveratrol Male	Group Male II: 0.625g/kg Resveratrol	Group III Male: 0.312g/kg Resveratrol	Group I Female: 1.25 g/kg Resveratrol Male	Group Female II: 0.625g/kg Resveratrol	Group III Female: 0.312g/kg Resveratrol
AST U/L	264±8.20	199.4±2.1	267±2.82	266±9.89	264.6± 1.27	223.9± 1.55	202.05± 1.34	200± 2.54
ALT U/L	44.65±6.1	46.5±9.89	47.2±9.47	45.8±9.75	45± 1.41	49.75± 4.59	48.25± 3.88	47.25± 7.00
ALP U/L	64 ±2.54	47±4.24	67.7 ±0.28	66.1±0.98	65.15± 0.21	50.75± 2.47	48.5± 9.19	47.75± 3.18
Bilirubin T mg/dl	0.15±0.07	0.1±0.14	0.2±0.14	0.15±0.07	0.05± 0.07	0.2± 0.14	0.15± 0.07	0.1± 0.14
Albumin g/Dl	2.9±0.42	2.85±0.35	3±0.28	2.85±0.49	2.75± 0.35	3.05± 0.07	2.95± 0.07	2.7± 0.42
Creatinine mg/l	0.3±0.14	0.35±0.07	0.45±0.35	0.4±0.28	0.35± 0.21	0.45± 0.35	0.4± 0.14	0.35± 0.21
Urea mg/dl	31.4±2.26	32±2.12	32.4±0.56	31.55±3.0	31.5± 2.12	33.5± 0.28	32.85± 0.35	32.3± 0.98
WBC $\times 10^3$/L	5.9±0.14	4.95±0.77	6.95±0.49	6.1±0.98	6± 0.14	5.95± 0.49	5.35± 0.77	5.2± 0.14
HGB g/dl	14.95±0.6	14.85±0.2	14±1.27	14.3±0.56	14.7± 0.56	14.15± 1.34	14.55± 0.77	14.65± 0.63

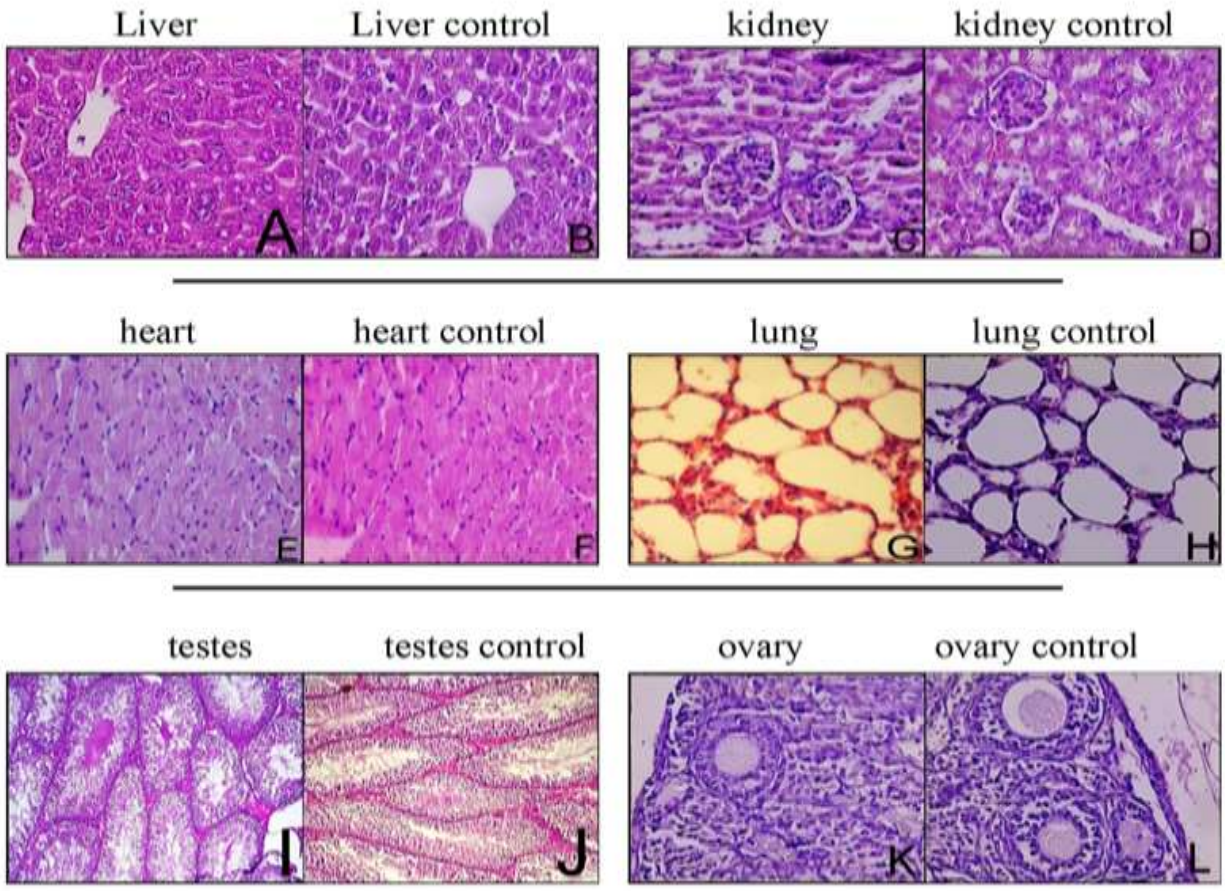

Figure 6 .shows selected images with total magnification 400 H&E stain

Figure 6 shows selected images with total magnification 400 H&E stain were (A) Represent liver tissue for mice received resveratrol shows no hepatic tissue changes such as hepatocyte

degeneration, no fatty changes or necrosis.no interstitial inflammatory cell infiltration or fibrosis. When compare to control Liver tissue (B). (C) Represent renal tissue exposed to resveratrol shows neither interstitial inflammatory cell infiltrate nor fibrosis, and normal glomeruli when compared with control renal tissue (D). (E) Represent heart tissue for mice exposed to resveratrol shows there was no inflammatory cell infiltrate in the interstitial or perivascular spaces, and no myocyte damage or necrosis compare to control heart tissue. (F). (G) Represent the lung tissue of mice exposed to the resveratrol displaying there was no capillary obstruction, no alveolar epithelial cell necrosis, no interstitial or intra-alveolar edema or hemorrhage, no inflammatory cell penetration in the interstitial space, and no hyaline membranes lining the alveolar ducts compare to control lung tissue. (H) . (I) Represent Testis tissue from mice given resveratrol and found normal seminiferous tubule thickness in the slice, normal spermatogenesis, no tubular wasting, no Leydig cell hyperplasia, and no thickening of the seminiferous tubules' basement membrane compare to appearance and structure of the control testis tissue (J). (K) Represent Resveratrol-treated ovary tissue exhibits typical stages of vesicular follicle maturation and no modifications in the ovarian stroma when compared appearance & structure to control ovary tissue (L). These data concluded resveratrol has no toxic effect on tissue when has been given in a single dose of 1.250 g/kg intraperitoneally.

Conclusion:

It was concluded that Resveratrol at lower doses showed non-observed adverse effect while at high doses, showed dose dependent toxicity when used as single bolus dose intraperitoneally

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