Amendment effect of resveratrol on diclofenac idiosyncratic toxicity: Augmentation of the anti-inflammatory effect by assessment of Arachidonic acid and IL-1β levels

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ABSTRACT

Idiosyncratic drug induced liver injury (DILI) is one of the most serious hazards that could be induced due to the misuse of drugs. Diclofenac has long been well-known to prompt such complication. Resveratrol is one of the newest nutraceuticals that has been recently introduced in the medicinal field and proved to be effective in ameliorating many types of drug-induced hepatic injuries. The present study aimed to assess if combining resveratrol with diclofenac would result in hepato-protection and/or synergistic anti-inflammatory effect in an acute carrageenan induced paw edema model in rats. Results revealed that the combination groups showed augmented anti-inflammatory effect represented by decreased levels of interleukin-1β and arachidonic acid. Furthermore; combination groups diminished the inflammation-induced oxidative stress and nitrosative stress as shown in the reduction of GSSG/GSH ratio, MDA and NO_X levels. Moreover; combination groups also prevented the elevation in liver enzymes namely AST and ALT. In conclusion we can say that addition of resveratrol to diclofenac tablets could be beneficial for patients suffering from serious liver conditions.

INTRODUCTION

Liver injury due to both prescription and over-the-counter drugs is a growing public health problem. Drug induced liver injury (DILI) is usually categorized into "intrinsic" and "idiosyncratic". Intrinsic hepatotoxic drug is characterized by dose-dependent hepatotoxicity. In contrast, idiosyncratic DILI is not clearly related to drug dose, route, or duration of administration. Despite the extensive safety tests performed in the process of getting a drug to market, DILI remains enigmatic and cannotf be predicted in preclinical and clinical trials (Iruzubieta *et al*., 2015). Redox cycling and generation of reactive oxygen and nitrogen species have been postulated to explain cell damages including idiosyncratic toxicities of non-steroidal anti-inflammatory drugs (NSAIDs)(Droge, 2002, McMillian *et al*., 2004). Diclofenac is one of the most famous NSAIDs that is broadly prescribed for the treatment of a variety of rheumatoid disorders, including osteoarthritis, rheumatoid arthritis and acute muscle pain. Similar to other NSAIDs, diclofenac causes hepatic injury in patients. In fact, published cases of severe diclofenac hepatotoxicity amount to approximately 250 reports, with a case fatality rate of approximately 10%, but the number of total serious hepatic adverse reactions (mostly unpublished) reported to the health authorities is several folds higher. Worse even, the real incidence has been estimated to be 10- to 20-fold higher than all reported cases, due to notorious underreporting (Boelsterli, 2003, Ramm and Mally, 2013). The cause of diclofenac-induced liver dysfunction in patients likely involves a response to oxidative stress and mitochondrial dysfunction in conjunction with metabolite binding (Boelsterli and Lim, 2007, Vickers *et al*., 2009).

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Resveratrol (3,5,4-trihydroxystilbene) is a polyphenolic compound that has been detected in fruits and some flowering plants. Resveratrol was reported to work as an anti-inflammatory (Timmers *et al*., 2012). Moreover; *in vitro* antioxidant properties were identified for resveratrol as it could provide protective antioxidant action in primary rat hepatocyte cultures (Rubiolo and Vega, 2008). Additionally, the protective role of resveratrol against a number of hepatic injuries was reported by several authors (Ahmed *et al*., 2014, Farghali *et al*., 2013, Powell *et al*., 2014).

The aim of the present study was to investigate if resveratrol would augment the anti-inflammatory effect of diclofenac and at the same time protect against its adverse hepatic side effects.

MATERIAL AND METHODS

Animals

Adult Female Wister rats, weighing 130-150 g each, purchased from the animal house at the National Research Centre (NRC, Giza, Egypt). All animals received human care in compliance with the guidelines of the EU Directive 2010/63/EU for animal experiments. Upon arrival, the animals were kept in a quiet place, housed eight per cage and acclimatized to a colony room with controlled ambient temperature (22±1 °C), humidity (50±10%) and a 12 hour natural light/dark cycle. They were fed a standard diet, water was provided *ad libitum* and they were acclimated for 7 days before entry into the subsequent study.

They were allowed free access to water and food throughout the period of investigation. The experiments were performed with 8 rats per treatment group according to a randomized schedule.

Drugs and Drug Administration

Diclofenac Sodium (Voltaren®; 50 mg sugar coated tablets, Novartis) and Diclofenac Potassium (Cataflam®; 50 mg sugar coated tablets, Novartis); the tablets were freshly suspended in distilled water prior to oral administration. Trans-resveratrol was provided as a generous gift from (Jing Tea LLC), it was provided as 250 mg Harmoni-T micronized trans-resveratrol capsules for ingestion (trans-resveratrol in the capsules was extracted from the Asian plant *Polygonum Cupsidatum*). The powder in the capsules was freshly dissolved in distilled water just before oral administration.

Experimental Design

For the *in vivo* anti-inflammatory effect; rats were divided into groups (8 rats each) and were treated as follows; Group (1): Carrageenan group. Group (2): Resveratrol low (10 mg/kg) **R10**. Group (3): Resveratrol medium (20 mg/kg) **R20**. Group (4): Resveratrol high (40 mg/kg) **R40**. Group (5): Diclofenac sodium low (10 mg/kg) **V10**. Group (6): Diclofenac sodium medium (20 mg/kg) **V20**. Group (7): Diclofenac sodium high (40 mg/kg) **V40**. Group (8): Diclofenac potassium low (10

mg/kg) **C10**. Group (9): Diclofenac potassium medium (20 mg/kg) **C20**. Group (10): Diclofenac potassium high (40 mg/kg) **C40**. Group (11): Diclofenac sodium low + Resveratrol low (10 mg/kg + 10 mg/kg) **RV10**. Group (12): Diclofenac sodium medium + Resveratrol medium (20 mg/kg + 20 mg/kg) **RV20**. Group (13): Diclofenac sodium high + Resveratrol high (40 mg/kg+ 40 mg/kg) **RV40**. Group (14): Diclofenac potassium low + Resveratrol low (10 mg/kg + 10 mg/kg) **RC10**. Group (15): Diclofenac potassium medium + Resveratrol medium (20 mg/kg+ 20 mg/kg) **RC20**. Group (16): Diclofenac potassium high +Resveratrol high (40 mg/kg+ 40 mg/kg) **RC40**.

Experimental Procedure

Carrageenan- induced rat paw edema

The method developed by (Winter *et al*., 1962) was employed. Animals were deprived of food for 12 h prior to experiment and only water was given *ad libitum.* First group (carrageenan group) received distilled water (5ml/kg p.o). Other groups received the corresponding drug treatments in distilled water orally. Concomitantly; carrageenan suspension (0.1 ml of 1% w/v suspension in 0.9% saline solution) was injected into the sub planter region of right hind paw of animals. Immediately before carrageenan injection, the paw volume was measured (initial paw volume) using plethysmometer (Harvard Apparatus Co. Model No.LE7500, USA).

Thereafter, the paw volume was measured after 1, 2 and 3 h after carrageenan administration. The difference between initial (V_b) and subsequent readings (V_t) gave the change in edema volume for the corresponding time. % Edema of control (Ec) and of treated (E_t) were used to calculate percentage (%) inhibition and (%) edema volume by using following formula:

$$\textbf{\% Edema} = [(V_t–V_b)/V_b] \text{ x } 100,$$

$$\textbf{\% Inhibition} = [1- (E_t /E_C)] \text{ x } 100.$$

V_t= edema volume after different time intervals, V_b= basal edema volume, (Ec) = % Edema of control (carrageenan), (E_t)= % Edema of treated.

Biochemical Analysis

Immediately after the last measurement of paw edema rats were anesthetized using phenobarbital and the blood samples were collected and centrifuged. Serum was separated and used for further biochemical analyses. Blood samples were withdrawn from a group of eight normal animals to serve as normal control for the subsequent biochemical parameters

Determination of serum arachidonic acid (mg/ml)

Arachidonic acid was determined by using GC method of (Ren *et al*., 2013). Arachidonic acid was purchased from Sigma-Aldrich and stored at -35ºC till used. The extraction of serum samples was prepared with Folch's solution, chloroform: methanol

(2:1) then vortex for 2 min and centrifuged for 10 min. at 4000 rpm. Standard and samples were allowed to evaporate at room temperature prior to extraction. Esterification was done by mixing the supernatant with 2 ml of (95: 5 methanol: sulphuric acid) and heat in oven at 80 ºC for 2 h and extracted with 2 ml hexane.

Finally samples were ready for injection in the GC set loop. SupelcoSP2330 column, 30mm X 0.32mm X 0.2µm film thickness, was used (Cat. No. 24073, Sigma-Aldrich, St. Louis, MO).

Gas chromatography was done with an Agilent Technologies 7890A GC. Hydrogen was the carrier gas with a column head pressure of 10 psi. Total flow rate at the split vent was 50 ml/min, the flow rate through the column was 2.5 ml/min and the septum purge was 2.5 ml/min. The injector was set at 220°C using the splitless injection mode, and 1 µl injections were made.

The temperature gradient started with a 70°C initial temperature, a linear increase to 170°C at 20°C/min, a slower linear increase to 170°C at 0.8°C/min to separate closely-eluting fatty acids, followed by an increase to reach 220°C at 20°C/min, and a final 2.5 min hold. The total run time was 20.1 minutes. The detector was flame ionization detector FID.

Determination of serum IL-1β(pg/ml)

IL-1β levels were determined by enzyme-linked immunosorbent assay (ELISA) kit(RayBio ® Rat IL-1 beta USA) according to (Sakamoto *et al*., 1994). The manufacturer's instructions (Invitrogen) were followed and finally the plate was read at 450 nm using ELISA reader.

Determination of serum GSH (mmol/l) and GSSG (mmol/l) levels by HPLC

Reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined by HPLC using the method of (Jayatilleke and Shaw, 1993)**.** The glutathione (GSH and GSSG) reference standards were purchased from Sigma Chemical Co. The samples were analyzed with the Agilent HP 1100 series HPLC apparatus (USA). The analytical column was aµ-Bondapak column (15 cm x 3.9 mm), UV detector and flow rate 1ml/min. The mobile phase consisted of 25 mmol sodium dihydrogen phosphate containing 5mmol tetrabutylammonium phosphate and methanol.

Determination of serum NO_x (µmol/l) by HPLC

Total nitrite and nitrate accumulation was performed for indication of NO_x production. The procedure for NO_x determination was according to the HPLC method of (Papadoyannis *et al*., 1999), Sodium nitrite and sodium nitrate were used for the reference standard preparation with stock concentration 1 mg/ml. The samples were analyzed with the

Agilent HP 1100 series HPLC apparatus (USA). The analytical column was an anion exchange PRP-X100 Hamilton, 150 X 4.1 mm by 10 mm. The mobile phase was a mixture of 0.1 M sodium chloride – methanol (45:55 v/v), with a flow rate 2 ml/min and wavelength of 230 nm, UV detector and flow rate 1 ml/min.

Determination of serum MDA (nmol/l) by HPLC

Lipid peroxidation was assessed by determining the malondialdehyde (MDA) content by using HPLC according to the method of (Karatas *et al*., 2002). The MDA standard was prepared by dissolving 25 ml 1,1,3,3 tetraethoxypropane (TEP) in 100 ml of water to give a 1 mM stock solution. The working standard was prepared by hydrolysis of 1 ml TEP stock solution in 50 ml 1% sulphuric acid and incubation for 2h at room temperature.

The resulting MDA standard of 20 nmol/ml was further diluted with 1% sulphuric acid to yield the final concentration of 1.25 nmol/ml, used as the standard for the estimation of total MDA.

The samples were analyzed with the Agilent HP 1200 series HPLC apparatus (USA). The analytical column was Supelcosil C18 (5 µm particle and 80 AO Pore size) (250X4.6 ID), UV detector and flow rate 1.2 ml/min.

Determination of serum ALT (u/ l) and AST (u/ l) levels

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were determined according to the method of (Reitman and Frankel, 1957) using colorimetric kits (SPECTRUM Egyptian company for biotechnology S.A.E). The manufacturer's instructions were followed and absorbances were measured at 505 nm using spectrophotometer to calculate the corresponding samples concentrations.

Statistical Analysis

Values were expressed as means ± S.E. Comparisons between means were carried out using one way ANOVA followed by Tukey's multiple comparisons test. P<0.05 was accepted as being significant in all types of statistical tests. Graph prism software (version 6) was used to carry out all statistical tests.

RESULTS

Effect on carrageenan induced paw edema

Carrageenan resulted in pronounced inflammation represented by exaggerated paw edema that increased from hour to hour. All treatments significantly prevented paw edema as compared to carrageenan group where combination groups showed comparable reduction in paw edema levels as compared to the corresponding standalone dose levels (Table1).

Table 1: Effect of adding resveratrol to diclofenac on carrageenan induced paw edema in rats.

Groups	Time(h) 1st	% EDEMA 2nd	3rd	% INHIBITION 1st	2nd	3rd
Carrageenan	73 ± 0.91	77.99 ±1.35	87.85±2.54			
R10	54.67 ± 1.32*	42.6 ±1.04*	33.23±1.19*	25.11	45.38	62.17
R20	40.42 ± 0.89*	32.96 ± 1.63*	27.47±0.88*	44.63	57.74	68.73
R40	31.52 ± 1.15*	28.27 ±1.07*	26.46±1.32*	56.82	63.75	69.88
V10	54.15 ± 1.17*	43.17 ±1.26*	33.22±0.74*	25.82	44.65	62.19
V20	39.53 ± 1.5*	32.38 ±1.41*	25.95±1.35*	45.85	58.48	70.46
V40	31.32 ± 2.27*	27.82 ±0.56*	25.37±1.6*	57.10	64.33	71.12
RV10	50.02 ± 0.57*	42.89 ±0.99*	32.01±1.1*	31.48	45.01	63.56
RV20	39.97 ± 1.83*	32.16 ±1.48*	24.25±0.91*	45.25	58.76	72.40
RV40	27.82 ± 1.42*	27.11 ±1.43*	23.89±1.44*	61.89	65.24	72.81
C10	52.91 ± 0.51*	42.95 ±0.77*	28.92±0.34*	27.52	44.93	67.08
C20	38.24 ± 2.5*	30.1 ±1.32*	22.81±0.88*	47.62	61.41	74.04
C40	30.99 ± 1.02*	27.49 ±0.63*	22.32±0.67*	57.55	64.75	74.59
RC10	48.48 ± 0.88*	38.1 ±0.85*	26.23±1.31*	33.59	51.15	70.14
RC20	35.2 ± 1.06*	28.57 ±0.97*	20.88±0.7*	51.78	63.37	76.23
RC40	25.92 ± 2.24*	26.52 ±1.24*	20.32±1.32*	64.49	66.00	76.87

* Significantly different from Carrageenan group.
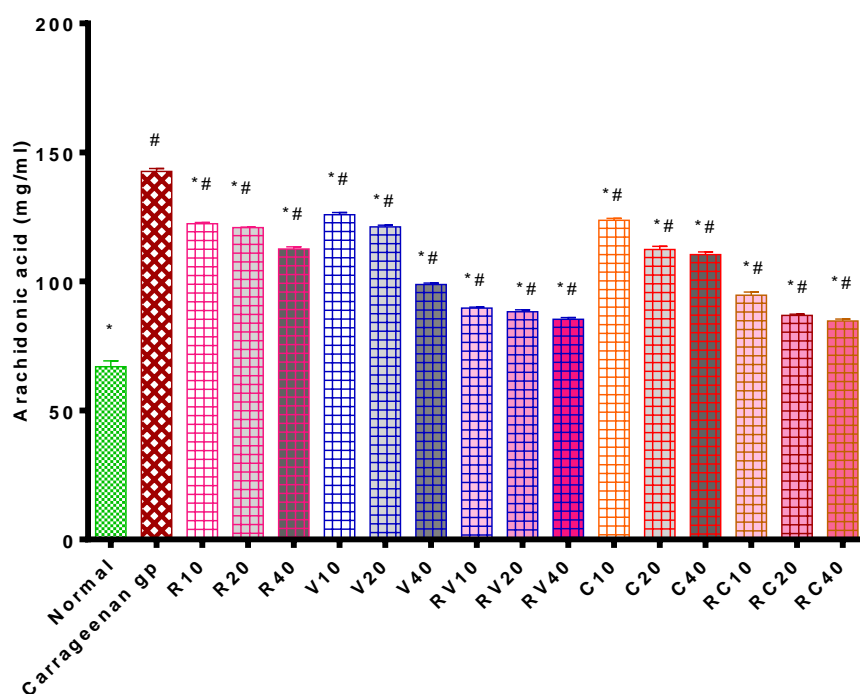
Fig. 1: Effect of adding resveratrol to diclofenac on arachidonic acid level.
* Significantly different from Carrageenan group, # Significantly different from normal control.

Effect on arachidonic acid

Inflammation significantly increased arachidonic acid compared to normal control (142.6 ± 1.2 *vs*. 66.97 ±2.22). All treatments significantly lowered arachidonic acid as compared to carrageenan group where combination groups showed significant reduction in arachidonic acid levels as compared to the corresponding standalone dose levels where; levels for resveratrol combinations with diclofenac sodium *vs*. diclofenac sodium alone were (89.68 ± 0.39, 88.31± 0.7 and 85.33 ± 0.66 *vs*. 125.9 ± 0.83, 121.2 ± 0.66 and 98.81 ± 0.54) respectively and levels for resveratrol combinations with diclofenac potassium *vs*. diclofenac

potassium alone were (94.67 ± 1.26, 86.81± 0.5 and 84.68 ± 0.76 *vs*. 123.7 ± 0.76, 112.2 ± 1.23 and 110.4 ± 1.04) respectively (Figure1).

**Effect on IL-1β**

Inflammation significantly increased IL-1β level compared to normal control (142.6 ± 1.2 *vs*. 66.97 ± 2.22). All treatments significantly lowered IL-1β level as compared to carrageenan group where combination groups showed comparable reduction in IL-1β levels as compared to the corresponding standalone dose levels (Figure 2).
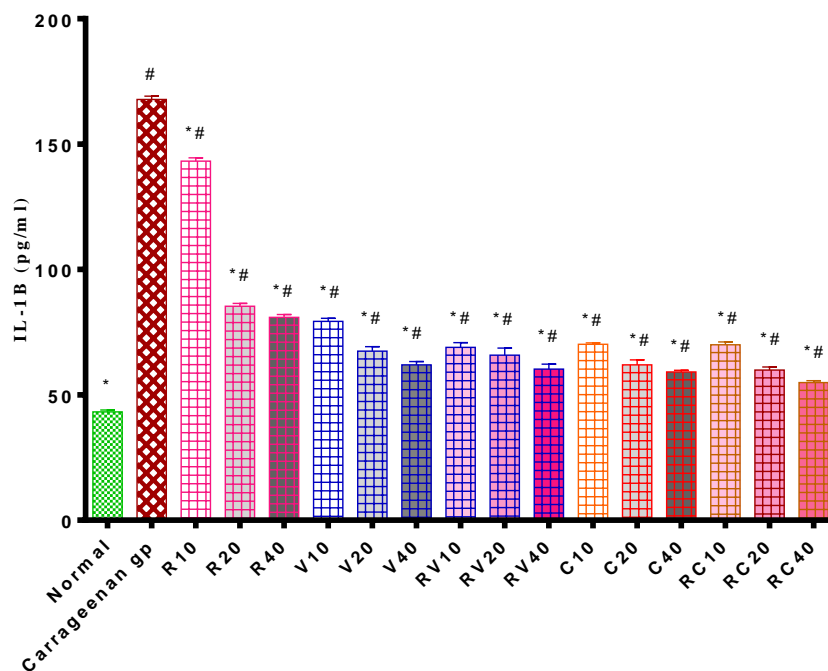
Fig. 2: Effect of adding resveratrol to diclofenac on IL-1β level.
* Significantly different from Carrageenan group, # Significantly different from normal control.
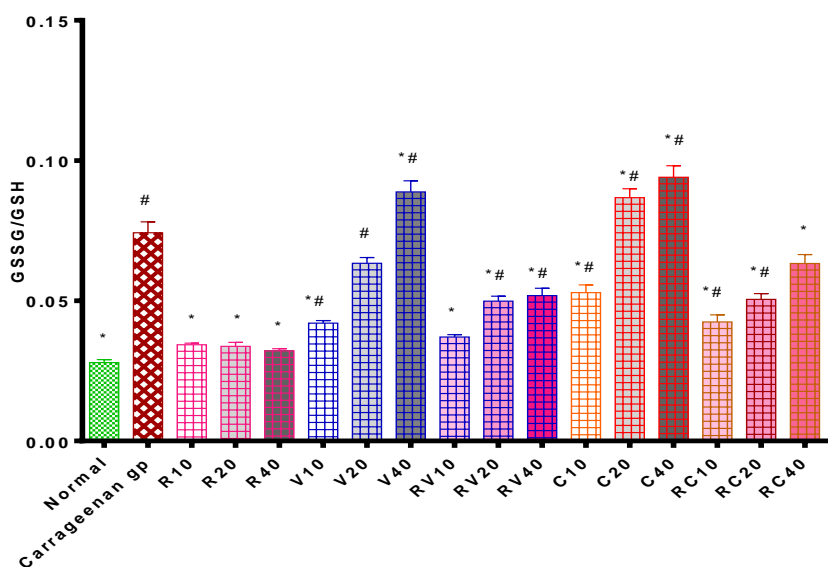
Fig. 3: Effect of adding resveratrol to diclofenac on GSSG/GSH ratio.
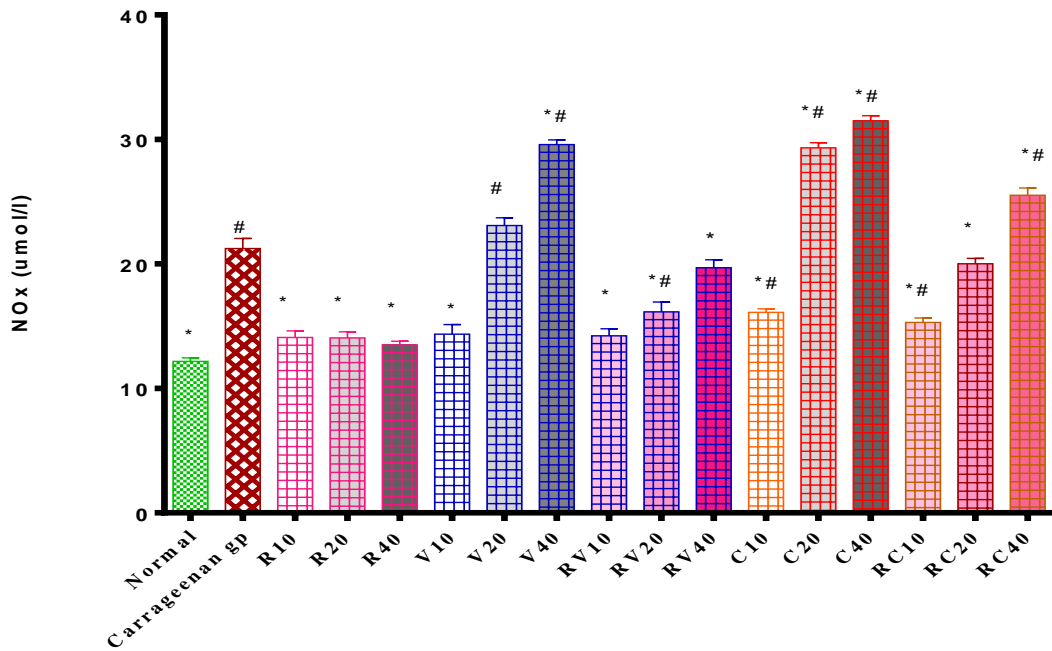* Significantly different from Carrageenan group, # Significantly different from normal control.

Effect on GSSG/GSH

Inflammation resulted in a significant increase in the conversion of reduced glutathione to the oxidized form indicating pronounced oxidative stress compared to normal control (0.074 ± 0.0039 vs. 0.028 ± 0.001).

Resveratrol; at the three dose levels showed normalization in the GSSG/GSH ratio. Individual NSAIDs treatments showed noticeable dose dependent oxidative stress as seen by the elevation in the GSSG/GSH ratio. All combination treatments lowered GSSG/GSH ratio as compared to their corresponding individual NSAID treatment (Figure 3).

NO$_X$ and MDA

Inflammation resulted in a significant increase in NOx and MDA levels indicating marked nitrosative stress and oxidative stress (21.24 ± 0.81 vs. 12.17 ± 0.28) and (344.4 ± 9.76 vs. 202.8 ± 4.94) respectively as compared to normal control. Resveratrol; at the three dose levels showed normalization in the NOx and MDA levels. Individual NSAIDs treatments showed prominent dose dependent nitrosative stress and oxidative stress as seen by the elevation in the NOx and MDA levels. All combination treatments lowered NOx and MDA levels as compared to their corresponding individual NSAID treatment (Figures 4, 5).

Fig. 4: Effect of adding resveratrol to diclofenac on NO$_x$ level.
[*] Significantly different from Carrageenan group, [#] Significantly different from normal control.
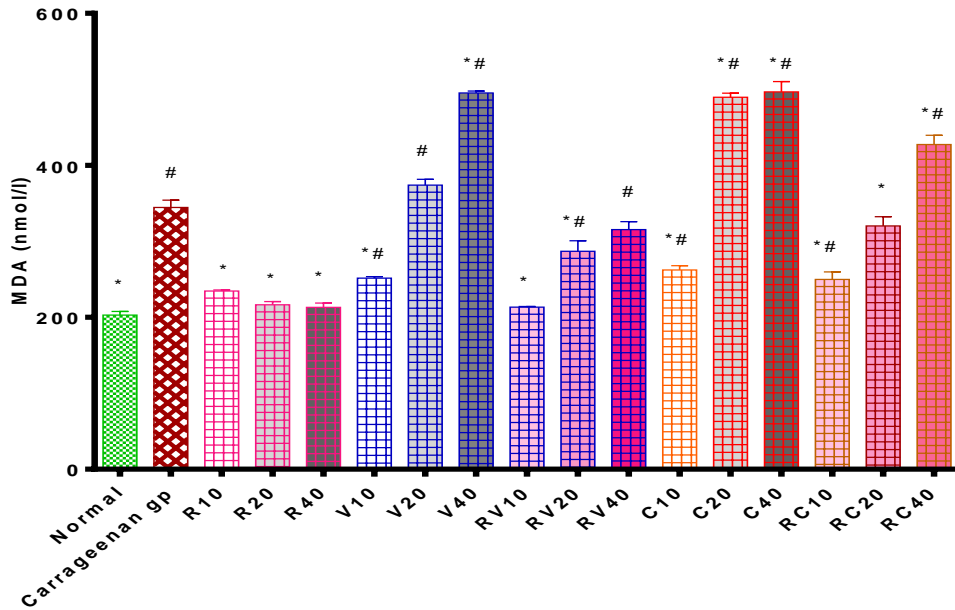
Fig. 5: Effect of adding resveratrol to diclofenac on MDA level.
* Significantly different from Carrageenan group, # Significantly different from normal control.

Effect on ALT and AST

Inflammation resulted in significant increase in liver functions levels as compared to normal control. Standalone NSAIDs treatments showed dose dependent elevation in the ALT and AST levels as compared to normal control and even the carrageenan group at the high dose levels. Combination groups showed lower levels in both ALT and AST levels as compared to their corresponding standalone treatments indicating hepato-protective effect (Table 2).

Table 2: Effect of adding resveratrol to diclofenac on ALT and AST.

Groups	ALT (unit/l)	AST (unit/l)
Normal	47.66 ± 0.63[*]	106.5 ±1.21[*]
Carrageenan	56.43 ± 0.38[#]	123.7 ±1.74[#]
R10	50.89 ± 0.38[*]	118.3 ±2.63[#]
R20	50.85 ± 0.66[*]	112.1 ±1.39[*]
R40	49.95 ± 0.43[*]	107.3 ±1.62[*]
V10	52.36 ± 0.42[*#]	121.7 ±0.72[#]
V20	53.98 ± 0.58[#]	127.8 ±1.10[#]
V40	59.11 ± 0.43[#]	138.1 ±1.12[*#]
RV10	51.51 ± 0.62[*#]	120.6 ±0.76[#]
RV20	51.9 ± 0.65[*#]	124.3 ±0.77[#]
RV40	52.34 ± 0.32[*#]	125.2 ±1.24[#]
C10	50.9 ± 0.90[*]*	121.6 ±0.60[#]
C20	52.16 ± 1.39[*#]	134.0 ±2.24[*#]
C40	59.45 ± 0.88[#]	146.4 ±1.13[*#]
RC10	50.23 ± 0.41[*]	119.4 ±1.29[#]
RC20	51.59 ± 0.72[*#]	125.1 ±1.89[#]
RC40	54.94 ± 0.88[#]	125.9 ±1.13[#]

*Significantly different from Carrageenan group, # Significantly different from normal control.

DISCUSSION

Models of inflammation to decipher whether drug-induced hepatotoxicity is augmented during episodes of inflammation may serve as useful models to predict idiosyncratic adverse drug reactions(Vickers *et al*., 2009).

In the present study, carrageenan was injected into the sub planter region of the right hind paw of animals to induce acute inflammation. Carrageenan injection resulted in severe paw edema, prominent increase in both arachidonic acid and interleukin-1β levels as well as pronounced oxidative stress and elevation in AST and ALT levels.

The inflammation is the result of interleukins secretion. Interleukins mobilize leukocytes by making them migrate from blood circulation towards the place of inflammation. These interleukins bind to their receptors at the membrane of monocytes to activate them to macrophages in order to eliminate pathogens or damaged cells and tissues. The synthesis of pro-inflammatory lipid mediators from arachidonic acid can occur via several pathways, including the prostaglandin H synthase (PHS) pathway, the cyclooxygenase (COX) pathway, and the lipoxygenase (LOX) pathway. Most anti-inflammatory drugs target the production of inflammatory mediators from arachidonic metabolism through cyclooxygenases 1 and 2 (COX-1 and -2), which are involved in the synthesis of pro-inflammatory leukotrienes and prostaglandin E2 (PGE2) (Lancon *et al*., 2016).One possibility to reduce inflammation is to inhibit the production of interleukins. NSAIDs inhibit cyclooxygenase (COX) isoforms, COX-1 and COX-2, which play a key role in the conversion of arachidonic acid to eicosanoids (Peng *et al*., 2016).

Results of our research revealed that; both forms of diclofenac salts and in the three dose levels (10, 20 and 40 mg/kg)dose dependently reversed carrageenan induced paw edema and significantly reduced the levels of the inflammatory biomarkers but on the other hand; elevated the liver enzymes, nitrosative and oxidative stress biomarkers levels in a grave way. Diclofenac possesses a weak acid group (carboxyl) which non-selectively inhibits COX enzymes at the arachidonate-binding site; which accounts for its anti-inflammatory effect. In the meantime; diclofenac use has been associated with hepatotoxicity, which is linked with the formation of reactive metabolites and the lipid nature of the carboxyl group. Mild liver dysfunction is reported in∼15% of patients, marked by increases of the transaminases alanine aminotransferase (ALT) and aspartate aminotransferase (AST). In ∼4% of patients the blood transaminase levels are ≥ 3 × the upper limit of normal (ULN), and in 1% of patients the levels are ≥ 8× ULN. In rare instances, patients have been presented with severe hepatocellular necrosis and inflammation and hematological toxicity(Vickers *et al*., 2009).

Meanwhile; resveratrol groups (10, 20 and 40 mg/kg)resulted in anti-inflammatory effect that was comparable to that of the standalone diclofenac groups and moreover; showed hepato-protective effect as seen by the reduction in both ALT and AST levels.

These findings are in agreement with previous investigations as the anti-oxidant, anti-inflammatory as well as the hepato-protective properties of resveratrol either alone or in combination with other natural products has previously been reported(Csaki *et al*., 2009, Lee *et al*., 2015, Liu *et al*., 2015, Pan *et al*., 2016). Moreover, it has been recently shown that resveratrol inhibits the expression of pro-inflammatory genes in human monocytes by stimulating the synthesis of anti-inflammatory micro RNAs (Tili *et al*., 2010a, Tili *et al*., 2010b).Besides; Resveratrol was proved to be a potent inhibitor of NO_x and cytokine release(Steiner *et al*., 2016). Finally; resveratrol-diclofenac combination groups and at all dose levels showed superior effects as compared to the standalone groups, which proves our hypothesis that combining resveratrol with diclofenac would be beneficial not only through augmenting diclofenac's anti-inflammatory effect but also by hindering the possibility of idiosyncratic DILI.In conclusion we would recommend the use of the middle dose combination (Diclofenac 20 mg/kg + Resveratrol 20 mg/kg) for patients suffering from liver injuries and in need of taking an anti-inflammatory as the overall results obtained from this combination were the most favorable.

Conflict of Interests: There are no conflicts of interest.

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