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Resveratrol in combination of Ibuprofen against acute carrageenan-induced inflammation and hepatic insult: rectificationof adenylate energy charge (AEC), anti-apoptotic, cell proliferation and DNA preservation potentials.

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Abstract : Inflammation is considered the quickest response to body organs damage and usually non-steroidal anti-inflammatory drugs (NSAIDs) are used in management of such condition, yet due to their undesirable side effects; other substitutes became of high demand. The present study aimed to investigate beneficial effects of combining resveratrol with ibuprofen in preventing carrageenan-induced inflammation and hepatic injury in rats. Results revealed that; combining resveratrol (40 mg/kg) with ibuprofen (40 mg/kg) lead to augmented anti-inflammatory and hepato-protective effects signified by partial prevention of carrageenan induced paw edema, reduced serum arachidonic acid, PGE2, ALT and AST levels after both single and repeated administrations, decreased hepatic TNF-α level and 8-OHDG content, enhancement in hepatic tissue cell energy performance, declined oxidative and nitrosative stresses. Finally both histochemical H & E studies as well as caspase-3 and PCNA immunuhistochemical examinations exposed the beneficial out comes from combining both treatments. As a conclusion; combining resveratrol with ibuprofen could be recommended over the use of ibuprofen alone in the treatment of inflammation.
Key wards : Carrageenan, Ibuprofen, Inflammation, Resveratrol.

Introduction

Inflammation is one of the first responses to cell and tissue damage and has been related with several physiological and pathological progressions by the initiation of immune responses within the damaged tissue. It results mainly by is the release of various inflammatory mediators for example; cytokines, prostaglandins, tumor necrosis factor alpha (TNF-α) bradykinin and histamine[1].

Carrageenans are highly sulfated polysaccharides that are obtained from red seaweeds (Rhodophycae). They have been widely used for decades as a thickener, stabilizer, or emulsifying agent in many processed foods and are also used in a variety of other products, such as cosmetics, toothpaste, room deodorizers, and pharmaceuticals. Since the biological properties of carrageenan were surveyed by Di Rosa in 1972; potential hazards of oral, intraperitoneal and subcutaneous carrageenan administration, including intravascular

coagulation, nephrotoxicity and liver histopathological changes have been investigated by several scientists. Single subcutaneous or intraperitoneal injection of carrageenan could induce serious liver injury and elevation in serum and tissue inflammatory markers levels that could persist for more than one week[2,3,4,5,6,7,8].

Nonsteroidal anti-inflammatory drugs (NSAIDs) has long been considered as the first line treatments to reduce inflammation[9]. Ibuprofen is an over the counter (OTC) nonsteroidal anti-inflammatory drug (NSAID) that is commonly used as an analgesic, anti-inflammatory and anti-pyretic agent. It acts principally by inhibiting the activity of cyclooxygenase, which is the key enzyme for the synthesis of prostaglandins. The resultant inhibition of prostaglandin production leads to a reduction in inflammation, temperature and pain, both centrally and peripherally[10,11,12]. Although usually tolerated by most patients, several case reports indicated the possibility of the occurrence of ibuprofen hepatotoxicity both at recommended dosages and at higher doses. Drug induced liver injury (DILI) in association with Ibuprofen was first reported in 1977. Subsequently it has been reported in various clinical situations where therapeutic doses of ibuprofen were associated with fatty liver, transaminitis and cholestatic hepatitis.There have been rare occasions where ibuprofen associated liver injury resulted in serious consequences requiring liver transplantation [13,14,15]. Consequently there is a prompt need to search for solutions to avoid those undesirable side effects where herbal supplements can be suitable candidates[1].

Resveratrol is a naturally occurring polyphenol that possesses several pharmacological activities including antioxidant, anti-inflammatory and hepatoprotective potentials. Resveratrol has been shown to prevent hepatic damage because of free radicals and inflammatory cytokines, induce anti-oxidant enzymes and elevate glutathione content. Resveratrol has also been shown to modulate varied signal transduction pathways implicated in liver diseases[16,17,18].

Women take more medications than men and therefore have a higher chance of experiencing side-effects and drug-drug interactions. Regrettably; females are under-represented in basic research as well as in animal tests, and more importantly, in human clinical trials. For many years, the Food and Drug Administration (FDA) guidelines specifically precluded participation of females in many drug studies. Laboratory animals are predominantly male, even in studies of diseases that disproportionately affect more women. Males are preferred because they are thought to be less variable due to their constant hormone levels. This variability should not be ignored as hormones can play a role in many inflammatory responses. Clinically, women have been reported to have a 1.5–1.7 fold greater risk than men of experiencing an adverse drug reaction (ADR). Specifically, acute liver failure is a rare but very serious ADR that occurs more frequently in women. Women largely predominate among patients with NSAID-induced hepatitis. Establishing more adequate drug doses on women may serve as a prevention method in the future[19,20,21,22,23].

The present study aimed to investigate the acute and sub-chronic anti-inflammatory activity of the combination of resveratrol and ibuprofen over the use of ibuprofen alone; as well as the beneficial outcomes from such combination in combating sub-acute carrageenan-induced hepatic insult in female rats.

2. Material and Methods

2.1. 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.

2.2. Drugs and drug administration

Ibuprofen (Profen; sugar coated tablets,Kahira Pharmaceuticals and Chemical Industries Company; Under License From Abbott Laboratories Limited- USA and its subsidiary in Pakistan); 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 Harmoni-T micronized trans-resveratrol capsules for ingestion. The powder in the capsules was freshly dissolved in distilled water just before oral administration.

2.3. 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 (20 mg/kg) **R20**. Group (3): Resveratrol high (40 mg/kg) **R40**. Group (4): Ibuprofen low (20 mg/kg) **I20**. Group (5): Ibuprofen high (40 mg/kg) **I40**. Group (6):Resveratrol low + Ibuprofen low (20 mg/kg + 20 mg/kg) **RI20**. Group (7): Resveratrol high + Ibuprofen high (40 mg/kg+ 40 mg/kg) **RI40**.

Treatments were orally administered on the first day concomitantly with carrageenan injection and then daily for the following three days at the same time as the first ingestions.

On the fifth day 24h. after the last ingestions; blood samples were withdrawn under anaesthesia; 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

All groups; including the normal control groupwere then killed by decapitation, livers were isolated and kept at -80 ^{0}C for further analyses.

2.4. Experimental procedure.

2.4.1. Carrageenan- induced rat paw edema:

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 beforecarrageenan 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:

% Edema = $[(V_t – V_b)/V_b]$x 100, **% Inhibition** = $[1- (E_t /E_C)]$ 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 [24].

2.4.2 Biochemical Analysis.

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

Alanine aminotransaminase (ALT) and Aspartate aminotransaminase (AST) activities were determined spectrophotometrically using commercial biochemical kits according to manufacturer's instructions. All of the samples and standards were assayed in duplicate, as suggested by the manufacturer (Biodiagnostic Co.,Egypt)[25].

2.4.2. 2. Determination of serum PGE2(pg/ml)

The PGE2 levels were measured using an enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions. All of the samples and standards were assayed in duplicate, as suggested by the manufacturer (Kono biotech Co.,LTD, China) [26].

2.4.2.3. Determination of serum arachidonic acid (pg/ml)

Arachidonic fatty acid was determined by using gas chromatography (GC)[27]**.** Arachidonic fatty acid were purchased in the triglyceride form and stored at −35°C until used. Fatty acid, solvents and other chemicals were obtained from Sigma-Aldrich. Standards for calibration were prepared in hexane:chloroform (1:1) and combined into a single fatty acid mixture. The extraction of serum samples were prepared with folch reagent,

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 derivatization. Esterification get by mixing the supernatant with 2ml of (95 methanol : 5sulphoric acid) then put in oven about 80 C° for 2 hrs then extracted with 2 ml hexane finally samples were ready for injection in the GC set loop.

2.4.2.4.Determination of tissue GSH (µmol /g tissue) and GSSG (µmol/g tissue) levels by HPLC:

The thiols compounds of oxidized and reduced glutathione were detected by HPLC system of Agilent HP1200series (USA) thatconsisted of quaternary pump, a column oven, Rheodine injector and 20µl loop, UV variable wavelength detector. The report and chromatogram taken from Chemstation program purchased from Agilent.30 cm × 3.9 mm C-18 µBondapak column was used. Theflow rate was 1ml/min and UV detection at wavelength 190 nm was applied. 0.0025 M sodium phosphate buffer, pH 3.5, containing 0.005 M tetrabutylammonium phosphate and 13% methanol was used as mobile phase. Samples were compared to glutathione (oxidized and reduced) reference standard purchased from Sigma Chemical Co. The results were expressed as µmol/g tissue [28, 29].

2.4.2.5. Determination of tissue MDA (nmol/g tissue) by HPLC:

For determination of MDA levels; the samples were analyzed on an Agilent HP 1200 series HPLC apparatus (USA) as described above. The analytical column was Supelcosil C18 (5 µm particle and 80 A^o pore size) (250 x 4.6 ID). The mobile phase was 82.5:17.5 (v/v) 30mM monobasic potassium phosphate (pH3.6)–methanol and the flow rate was 1.2 ml/min, wavelength 250 nm was applied for detection. MDA standard was prepared by dissolving 25 µl 1,1,3,3 tetraethoxypropane (TEP) in 100 ml of water to give a 1 mM stock solution. Working standard was prepared by hydrolysis of 1 ml TEP stock solution in 50 ml 1% sulfuric acid and incubation for 2 h at room temperature. The resulting MDA standard of 20 nmol/ml was further diluted with 1% sulfuric acid to yield the final concentration of 1.25 nmol/ml to get the standard for the estimation of total MDA [30,31,32].

2.4.2.6. Determination of tissue NOx (µmol/ g tissue) by HPLC:

LiverNO_x level was determined using Agilent HP 1200 series HPLC apparatus (USA) as described above. The analytical column was anion exchange PRP-X100 Hamilton, 150 x 4.1 mm, 10 µm. The mobile phase was a mixture of 0.1 M NaCl - methanol, at a volume ratio 45:55.The flow rate of 2 ml/min, wavelength adjusted to 230 nm. The resulting chromatogram identified the concentration from the sample as compared to that of the standard purchased from Sigma Aldrich [33].

2.4.2.7. Determination of tissue 8-OHDG (µg/ g tissue) by HPLC:

The separation of 8-OHDG was performed with an Agilent HP 1200 series HPLC apparatus (USA) as described above. The analytical column was Supelcosil C18 (5 µm particle and 80 A^o pore size) (250 x 4.6 ID). The eluting solution was H_2O/methanol at a ratio (85: 15) with 50 mM KH_2PO_4, pH 5.5 at a flow rate of 0.68 ml/min. the UV detector was set at 245 nm. The resulting chromatogram identified the concentration from the sample as compared to that of the standard purchased from Sigma Aldrich [34].

2.4.2.8. Determination of tissue ATP, ADP and AMP (µmol/ g tissue) by HPLC:

The separation of tissue ATP, ADP and AMPwas performed with an Agilent HP 1200 series HPLC apparatus (USA) as described above. The analytical column was Ultrasphere ODS EC 250 x 4.6 mm column. Mobile phase A consisted of 0.06 mol/l$K_2HPO_4$and 0.04 mol/lKH_2PO_4dissolved in deionized water andadjusted to pH 7.0 with 0.1 mol/lKOH, while mobile phase B consisted of 100 % acetonitrile.Flow rate of the mobile phase was 1.2 ml/min.ATP, ADP and AMP in the samples were identified by comparison withstandards purchased from Sigma Aldrich. The report and chromatograms were taken from chemstation program at wave length 254 nm [35, 36].

Total adenylate energy charge (AEC) was calculated according to the equation:

AEC = (ATP + 0.5ADP)/(ATP + ADP + AMP) [37].

2.4.2.9. Determination of tissue TNF- α (pg/ g tissue)

The TNF-α, levels were measured using an enzyme-linked immunosorbent assay (ELISA) according to manufacturer's instructions. All of the samples and standards were assayed in duplicate, as suggested by the manufacturer (Rat TNF-α ELISA KIT KOMA BIOTECH INC, Korea) [38]

2.4.3. Histopathological examination

Liver specimens were taken from all rats subjected to our study, which were sliced and fixed in 10% buffered formalin. Paraffin blocks were prepared from those samples after a serial of dehydration, clearing and embedding. The paraffin-embedded material was prepared in 5-µm-thick slices, which were stained with hematoxylin and eosin, mounted on microscope slides and examined by optical microscopy to evaluate the morphologic aspects. Furthermore, Section were taken on charged slides to be stained immunohistochemically by Caspase-3 and PCNA antibodies.

Immunohistochemistry for caspase-3 and PCNA.

Immunohistochemistry for caspase-3 and proliferating cell nuclear antigen (PCNA) was performed on formalin-fixed; paraffin-embeded tissue on positively charged slides. Sections mounted on charged slides were de-paraffinized in xylene, hydrated in graded alcohol, and pretreated for antigen retrieval in 10 mmol/l citrate buffer, pH 6.0, in a steamer at 98°C for 45 min. For caspase-3 staining was performed using commercial kit (PharMingen, San Diego, CA, USA). Caspase-3 stained figures were either immuno-positive apoptotic bodies or pre-apoptotic hepatocytes showing cytoplasmic and/or nuclear caspase-staining. Immunohistochemical detection of proliferating cell nuclear antigen (PCNA) was performed with a commercial kit (LSAB2 Kit; Dako, Kyoto, Japan). PCNA-positive hepatocytes were compared between the different groups. The use of PCNA protein determinations was used as qualitative measure of hepatic regenerative activity in rats.

Statistical analysis:

Statistical analysis was 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. Values were expressed as means ± S.E.

Results

Acute and sub-acute protection against carrageenan-induced inflammation.

Acute effect of combining resveratrol with ibuprofen on carrageenan induced paw edema.

Carrageenan injection resulted in severe paw edema that increased by time. Ibuprofen dose dependently exhibited an anti-inflammatory effect against carrageenan-induced inflammation. Resveratrol on the other hand dose dependently displayed milder anti-inflammatory activity that gradually increased by time. Furthermore; combining resveratrol with ibuprofen resulted in a synergistic anti-inflammatory response that increased by time (Table1).

Table 1. Acute effect of combining resveratrol with ibuprofen on carrageenan induced paw edema.

	% Edema			%Inhibition		
Time(h) / Groups	1st	2nd	3rd	1st	2nd	3rd
Carrageenan	83.37 ± 1.02	86.12 ± 0.74	89.79 ± 1.63			
I20	51.17 ± 1.47*	28.1 ± 1.04*	18.00 ± 0.90*	38.62	67.37	79.95
R20	53.13 ± 1.66*	34.78 ± 0.54*	26.95 ± 0.53*	36.27	59.61	69.99
RI20	49.26 ± 1.10*	27.49 ±1.28*	14.48 ± 0.34*	40.91	68.08	83.87
I40	34.76 ± 2.57*	24.52 ± 0.54*	13.19 ± 1.45*	58.31	71.53	85.31
R40	44.29 ± 2.40*	30.59 ± 1.43*	19.95 ± 0.67*	46.88	64.48	77.78
RI40	27.63 ± 1.08*	23.40 ± 0.34*	10.50 ± 0.40*	66.86	72.83	88.31

*significantly different from acute carrageenan group

Acute and sub-acute effects of combining resveratrol with ibuprofen on serum arachidonic acid and PGE2 levels

Serum samples collected on both day one and day five showed that; carrageenan significantly elevated the levels of both arachidonic acid and prostaglandin E2 as compared to normal control and this effect persisted from day one to day five. Ibuprofen dose dependently exhibited an anti-inflammatory effect against carrageenan-induced inflammation and this effect increased by sub-chronic administration for five days. Resveratrol on the other hand dose dependently displayed a mild anti-inflammatory activity that gradually increased by sub-chronic administration. Furthermore; combining resveratrol with ibuprofen resulted in a synergistic anti-inflammatory response that increased by sub-acute administration (Table 2).

Table 2. Acute and sub-acute effects of combining resveratrol with ibuprofen on serum arachidonic acid and PGE2 levels

Groups	Arachidonic acid (pg/ml)		PGE2 (pg/ml)	
	Acute	Sub-acute	Acute	Sub-acute
Normal	66.77 ± 2.02 $^{*@}$		53.65 ± 0.26 $^{*@}$	
Carrageenan	148.90 ± 2.51$^{\#@}$	243.60 ± 5.35 $^{\#*}$	181.2 ± 3.12 $^{\#}$	179.7 ± 5.38 $^{\#}$
I20	119.90 ±1.29 $^{\#*@}$	109.10 ± 2.26 $^{\#*@}$	133.4 ± 2.51 $^{\#*@}$	111.7 ± 1.60 $^{\#*@}$
R20	132.80 ± 2.21 $^{\#*@}$	125.20 ± 2.16 $^{\#*@}$	138.0 ± 1.83 $^{\#*@}$	128.6 ± 1.29 $^{\#*@}$
RI20	103.30 ± 0.67 $^{\#*@}$	77.69 ± 3.17 $^{*@}$	122.3 ± 1.66 $^{\#*@}$	104.1 ± 2.86 $^{\#*@}$
I40	113.20 ± 1.16 $^{\#*@}$	99.39 ±1.22 $^{\#*@}$	124.4 ± 0.25 $^{\#*@}$	101.1 ± 1.86 $^{\#*@}$
R40	117.00 ± 1.47 $^{\#*@}$	106.40 ± 1.38 $^{\#*@}$	127.7 ± 1.45 $^{\#*@}$	108.7 ± 1.45 $^{\#*@}$
RI40	80.44 ± 1.31 $^{\#*@}$	70.34 ± 1.13 $^{*@}$	83.42 ± 2.55 $^{\#*@}$	72.23 ± 1.55 $^{\#*@}$

significantly different from normal control, *significantly different from acute carrageenan group, @ significantly different from chronic carrageenan group

Sub-acute effect of combining resveratrol with ibuprofen on liver tissue level of TNF- α.

Carrageenan resulted in significant increase in liver tissue TNF-α level as compared to the normal control (654.2 ± 5.62 vs. 389.7 ± 4.71 pg/g tissue). Combining resveratrol with ibuprofen showed augmented anti-inflammatory response by dose dependently significantly reducing liver tissue TNF-α level as compared to carrageenan group (470.7 ± 2.2 and 441.5 ± 5.72 pg/g tissue) respectively (Figure 1).

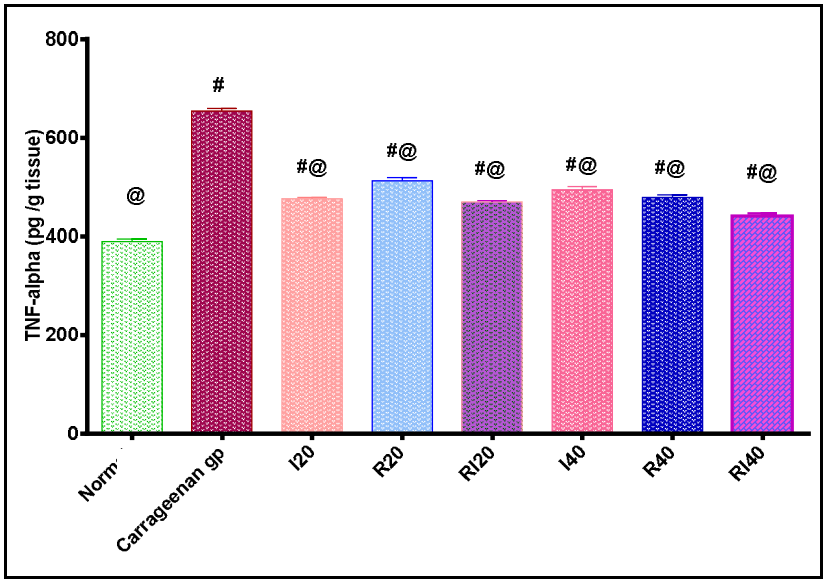

Figure 1. Sub-acute effect of combining resveratrol with ibuprofen on liver tissue level of TNF- α (pg/ g tissue).

significantly different from normal control, @ significantly different from chronic carrageenan group

Acute and sub-acute protection against carrageenan induced liver insult

Acute and sub-acute effects of combining resveratrol with ibuprofen on serum ALT and AST levels.

Carrageenan resulted in significant elevation in ALT and AST levels that persisted and increased from day one to day five as compared to normal control. Ibuprofen treatment alone was found to be nearly non-protective against carrageenan-induced elevation in liver enzymes and moreover even insulting by itself as the dose and duration of administration increased. On the other hand; resveratrol dose-dependently showed significant protection against the increase in the liver enzymes levels. Finally; combining resveratrol with ibuprofen dose dependently slightly protected against carrageenan- induced elevation in ALT and AST levels as compared to the use of ibuprofen alone. (Table 3).

Table 3. Acute and sub-acute effects of combining resveratrol with ibuprofen on serum ALT and AST levels.

Groups	ALT (u/l)		AST (u/l)	
	Acute	Sub-acute	Acute	Sub-acute
Normal	48.36 ± 0.50 *@		90.20 ± 1.97*@	
Carrageenan	63.68 ± 1.53 #@	78.43 ± 1.58 #*	118.1± 1.05 #	119.2 ± 0.73 #
I20	56.02 ± 0.62 #*@	76.02 ± 2.37 #*	99.51± 2.10 #*@	112.2 ± 0.88 #
R20	53.52 ± 0.79*@	68.95 ± 1.09 #@	97.95 ± 2.24 #*@	95.59 ± 1.05 *@
RI20	54.31 ± 0.50*@	75.45 ± 1.52#*	98.92 ± 1.84 #*@	108.9 ± 1.21 #*@
I40	63.94 ± 2.26 #@	83.18 ± 0.71 #*	105.6 ± 2.05 #*@	118.3 ± 0.707 #
R40	52.69 ± 1.29 *@	66.03 ± 2.33 #@	92.60 ± 1.28 *@	91.46 ± 0.82 *@
RI40	62.78 ± 1.50 #@	69.10 ± 2.02 #@	93.22 ± 1.15 *@	99.22 ± 1.56 # *@

significantly different from normal control, *significantly different from acute carrageenan group, @ significantly different from chronic carrageenan group.

Sub-acute effect of combining resveratrol with ibuprofen on liver tissue cell energy performance.

Carrageenan resulted in significant disruption in hepatic cells energy represented by increased AMP/ATP ratio as well as significant decrease in adenylate energy charge (AEC) as compared to the normal control. Combining resveratrol with ibuprofen dose dependently reversed that disruption in cell energy; decreasing the AMP/ATP ratio as well as normalizing the AEC (Table 4).

Table 4. Sub-acute effects of combining resveratrol with ibuprofen on liver tissue cell energy performance.

Groups	ATP (umol/g tissue)	ADP (umol/g tissue)	AMP (umol/g tissue)	AMP/ATP	AEC
Normal	28.74 ± 0.18 @	15.39 ± 0.18 @	9.47 ± 0.25 @	0.33 ± 0.01@	0.68 ± 0.003 @
Carrageenan	11.05 ± 0.18 #	7.57 ± 0.20 #	4.69 ± 0.24 #	0.43 ± 0.02 #	0.64 ± 0.01#
I20	15.62 ± 0.22 #@	9.73 ± 0.37 #@	6.13 ± 0.12 #@	0.39 ± 0.01 #	0.65 ± 0.004 #
R20	14.99 ± 0.20 #@	9.26 ± 0.23 #@	5.33 ± 0.14 #	0.36 ± 0.01 @	0.66 ± 0.002 @
RI20	19.98 ± 0.26 #@	12.82 ± 0.22 #@	6.92 ± 0.06 #@	0.35 ± 0.004 @	0.66 ± 0.002 @
I40	16.67 ± 0.46 #@	10.5 ± 0.28 #@	6.05 ± 0.20 #@	0.36 ± 0.01 @	0.65 ±0.01 #
R40	17.85 ± 0.27 #@	11.18 ± 0.24 #@	6.18 ± 0.21 #@	0.33 ± 0.01 @	0.67 ± 0.01 @
RI40	24.08 ± 0.45 #@	13.7 ± 0.25 #@	8.27 ± 0.22 #@	0.34 ± 0.01 @	0.68 ± 0.01 @

@ significantly different from carrageenan control, # significantly different from normal control.

Sub-acute effect of combining resveratrol with ibuprofen on liver tissue 8-OHDG level.

Carrageenan resulted in significant increase in the 8-OHDG level indicating the incidence of hepatic tissue DNA fragmentation (526.4 ± 5.95 vs. 220.1 ± 6.15 ug/g tissue) as compared to the normal control. Ibuprofen alone at the lower dose showed pronounced protection against hepatic tissue increase in the 8-OHDG level. This protection decreased as the dose increased indicating that sub-acute administration of ibuprofen

could itself be insulting to the liver tissue. On the other hand resveratrol combination with ibuprofen resulted in augmented hepatoprotective effect (Figure 2).

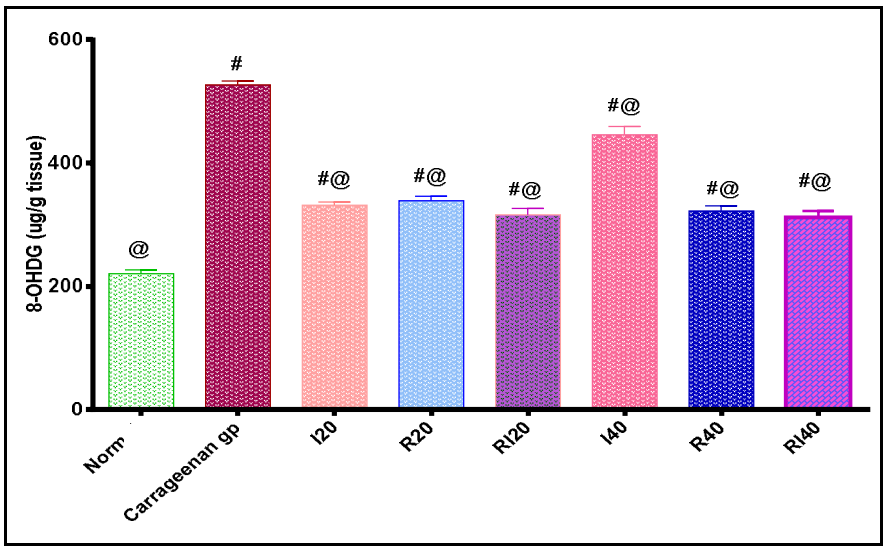

Figure 2. Sub-acute effects of combining resveratrol with ibuprofen on liver tissue 8-OHDG level.

@ significantly different from carrageenan control, # significantly different from normal control.

Sub-acute effects of combining resveratrol with ibuprofen on liver tissue oxidative and nitrosative stresses parameters.

Sub-acute effect of combining resveratrol with ibuprofen on liver tissue GSH, GSSG, MDA and NOx levels

Carrageenan resulted in significant increase in the level of the oxidized from of glutathione (GSSG) (0.69 ± 0.02 vs. 1.83 ± 0.05 umol/g tissue) and decrease in the level of the reduced form of glutathione (GSH) (15.83 ± 0.38 vs. 35.19 ± 0.85 umol/g tissue) and consequently increasing the GSSG/GSH ratio and furthermore; elevated MDA and NOx levels (41.76 ± 1.67 vs. 13.35 ± 0.29 nmol/g tissue) and (1.78 ± 0.06 vs. 0.52 ± 0.03 umol/g tissue) respectively as compared to normal control, indicating pronounced oxidative stress and nitrosative stress. Combining resveratrol with ibuprofen dose dependently significantly decreased GSSG/GSH ratio, MDA and NOx levels as compared to carrageenan control indicating augmented protection against oxidative and nitrosative stresses (Figures 3, 4, 5).

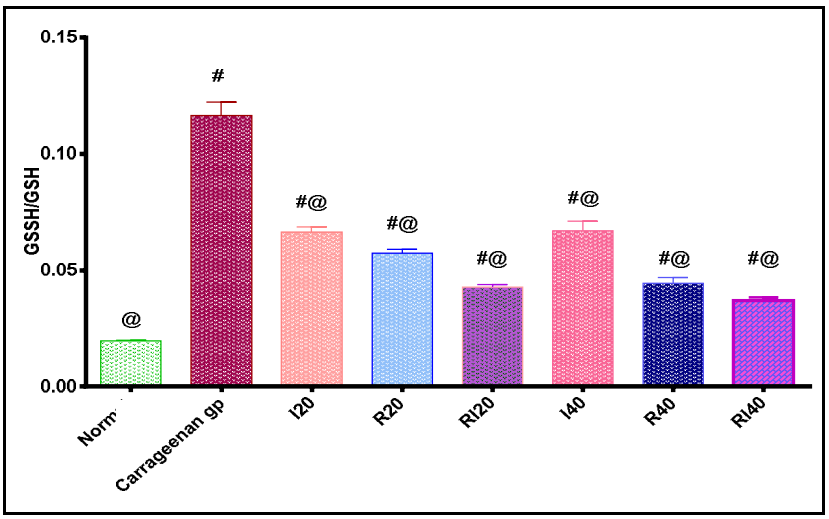

Figure 3. Sub-acute effect of combining resveratrol with ibuprofen on liver tissue GSSG/ GSH ratio.

@ significantly different from carrageenan control, # significantly different from normal control.

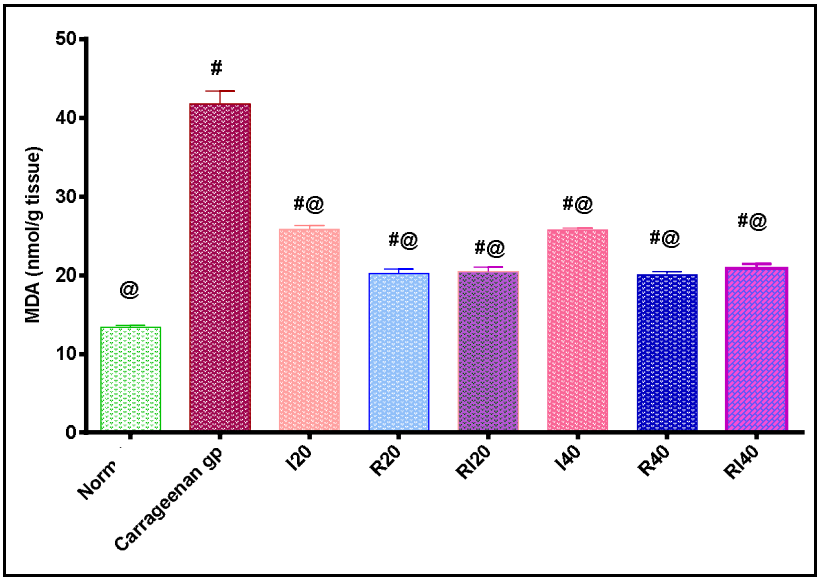

Figure 4. Sub-acute effect of combining resveratrol with ibuprofen on liver tissue MDA (nmol/g tissue) level.

@ significantly different from carrageenan control, # significantly different from normal control.

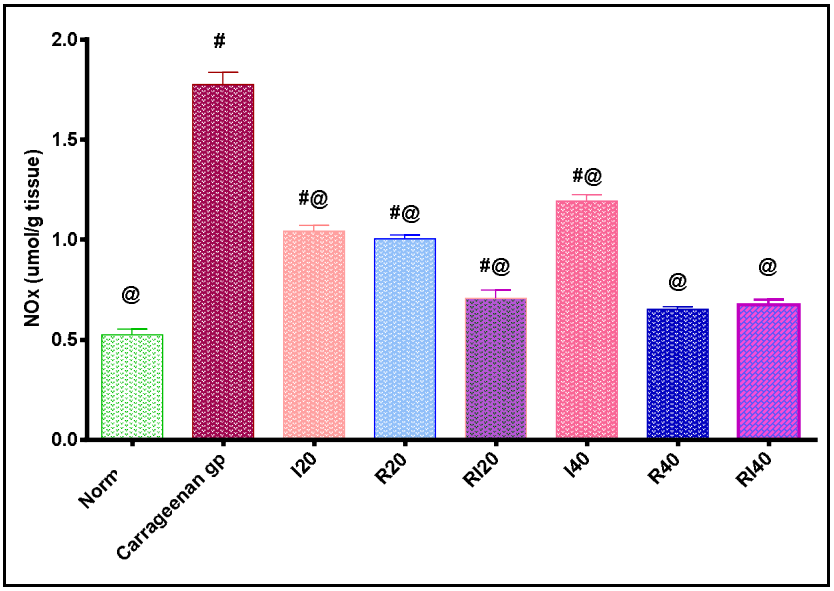

Figure 5. Sub-acute effect of combining resveratrol with ibuprofen on liver tissue NOx (µmol /g tissue) level.

[@]significantly different from carrageenan control, [#] significantly different from normal control.

Histochemical and immunohistochemical studies

Carrageenan sub-planter injection resulted in serious hepatic tissue insult represented by clearly seen vacuolar cytoplasmic degeneration along the three hepatic zones, large bi-nucleated acidophilic cell, areas of necrosis and nuclear DNA fragmentation as seen on H&E stain. Furthermore; immunohistochemical evaluation of Caspase-3 demonstrated positively stained hepatocytes along the three hepatic zones with the nuclei showing chromatin condensation in some of them. Moreover; assessment of PCNA showed scattered faintly positive nuclei along the hepatic lobule.

Ibuprofen at the lower dose (20 mg/kg p.o) showed areas of necrosis in the hepatic tissue along with congestion in central vein and more vacuolar degeneration in zone 2 than zone 3 in H &E stained sections. As for the immunuhistochemical evaluations; minimal stained nuclei were seen with caspase-3 as well as PCNA negative stain in the group ingesting the low dose of ibuprofen as no regeneration took place without a

hepatoprotective drug administration. As for the group ingesting resveratrol at the low dose level alone; thickening of central vein wall without congestion and less areas of necrosis than the group ingesting ibuprofen (20 mg/kg) were revealed in H &E stain. Caspase-3 stain showed mixed pattern of staining with positive and negative stained nuclei. Moreover; PCNA stain showed few positive stained cells with granular nuclei. So we can say that the cells are affected but not fully saved by the low dose of resveratrol. Combination of ibuprofen with resveratrol at the low dose levels in H &E stain showed areas of necrosis and vacuolar degeneration and the results were better than the group ingesting the lower dose of ibuprofen alone and close to that ingesting resveratrol at the low dose level alone. The results were confirmed at the level of Caspase-3 where; minimal scattered faintly stained cells less than 1-2 per high power field were observed. PCNA stain revealed faint positively stained nuclei indicating active cellular division and DNA repair. So the combination group in low dose has a favorable picture than low dose ibuprofen and we can say that itsmorphology lies in between the low dose of ibuprofen and the low dose resveratrol.

On the other side ibuprofen at the high dose (40 mg/kg) showed vesicular nuclei alongside with cytoplasmic vacuolar degeneration and extensive areas of necrosis in H &E stain. The overall picture was near to carrageenan control where the hepatocytes were much affected by the toxic effects of carrageenan as well as the effect of added ibuprofen. The picture was additionally proved on immunohistochemistry level where; caspase-3 stain showed many positive cells with fragmented chromatin and moreover; PCNA showed few stained cells with mild faint homogenous stain with minimal granular distribution.

The group ingesting resveratrol at the higher dose (40 mg/kg) showed vacuolar degeneration of cytoplasm and fragmented nuclei but no areas of necrosis. Results of this group is better than ibuprofen at the low and high dose as well as resveratrol at the low dose level and the RI20 combination. On immunohistochemistry level; caspase-3 stain revealed relatively strongly stained cells expressing spontaneous apoptosis as a protective physiological process. PCNA stain showed moderate positivity with weak intensity revealing cellular regeneration in a higher rate than in case of ingesting resveratrol at the lower dose.

The group ingesting ibuprofen in combination with resveratrol in the high dose showed overcrowded cells as well as minimal areas of necrosis, results were better than using either treatments alone at the high dose level. On immunohistochemical analysis levels; minimal scattered faintly stained cells less than 1-2 per five high power fields in case of caspase-3 stain were observed. PCNA stain revealed high positivity indicating active cellular division and DNA repair. Finally combinations of resveratrol and ibuprofen at the high dose levels revealed much better results than all groups (Figures 6, 7, 8).

Figures pathology

Figure 6 (a). Normal hepatic tissue X100:

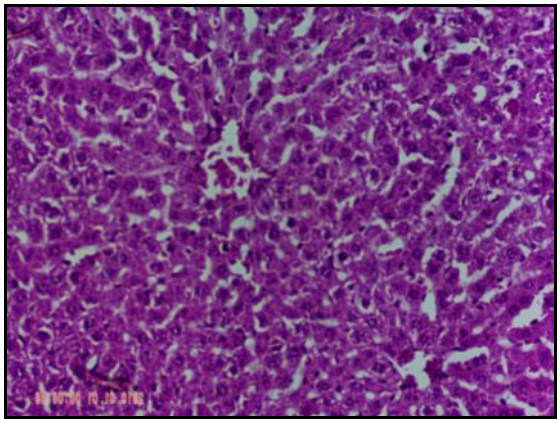

Figure 6. (a): Normal hepatic tissue showing normal hepatic architecture

Figure 6 (b). Carrageenan control X100:

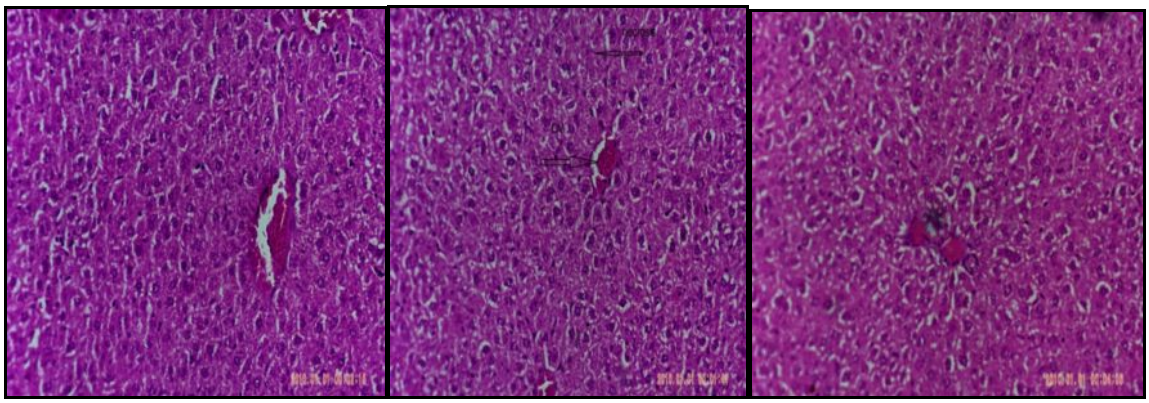

Figure 6 (c). Carrageenan control X400:

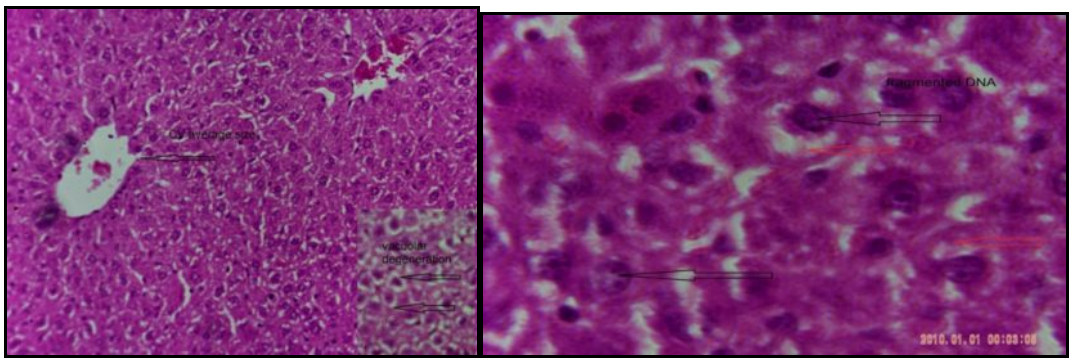

Figure 6. (b, c): Carrageenan control showing vacuolar cytoplasmic degeneration along the three hepatic zones, large bi-nucleated acidophilic cell, areas of necrosis and nuclear DNA fragmentation.

Figure 6. (d). Ibuprofen (20 mg/kg) X100:

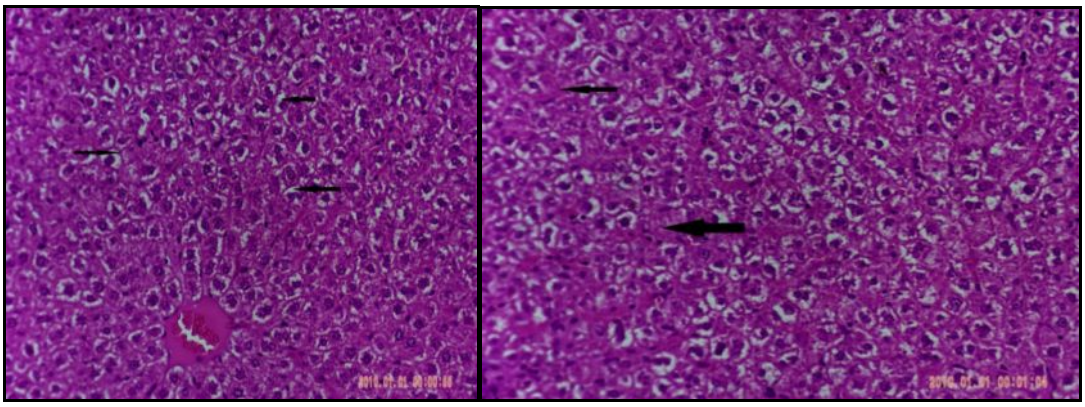

Figure 6. (d): Ibuprofen (20 mg/kg) group showing congested central vein, vacuolar degeneration more in zone two than zone three and areas of necrosis.

Figure 6. (e). Resveratrol (20 mg/kg)X100: Figure 6.(f).Resveratrol (20 mg/kg)X400:

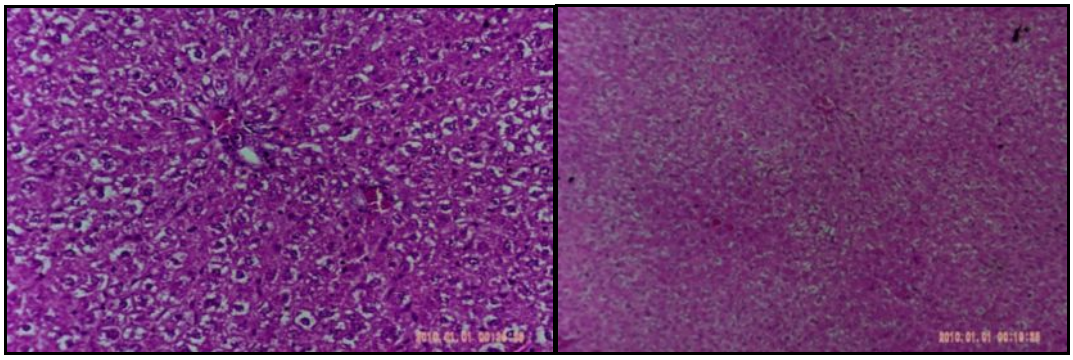

Figure 6. (e, f): Resveratrol (20 mg/kg) group showed thickening of central vein wall without congestion. At higher power examination revealed areas of necrosis less than carrageenan and I20 groups but more than RI20 group.

Figure 6. (g). RI (20 mg/kg)X100: **Figure 6. (h). RI (20 mg/kg)X400**:

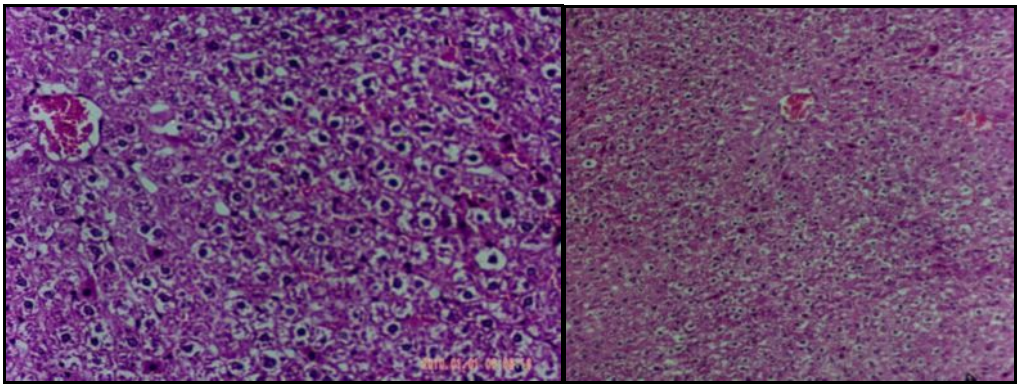

Figure 6. (g, h): RI (20 mg/kg) showing mild necrosis and vacuolar degeneration.

Figure 6. (i). Ibuprofen (40 mg/kg) X100: Figure 6. (j). Ibuprofen (40 mg/kg) X400:

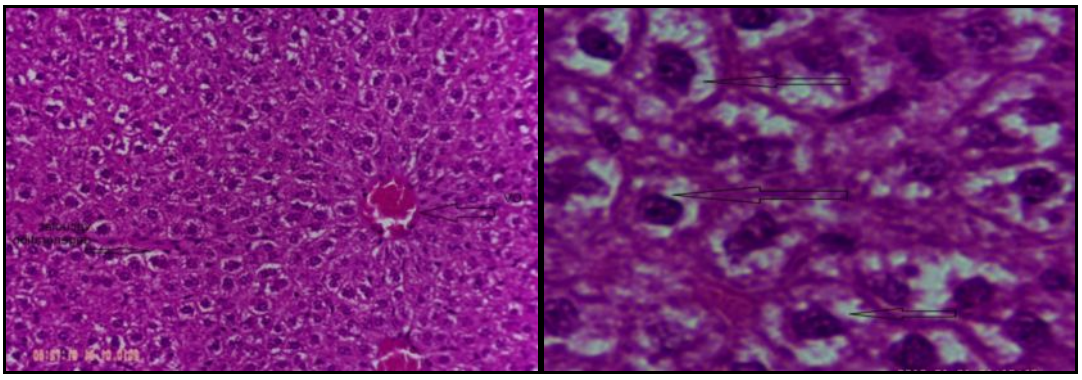

Figure 6. (i, j): Ibuprofen (40 mg/kg)group showing vesicular nuclei, cytoplasmic vacuolar degeneration and extensive areas of necrosis.

Figure 6.(k).Resveratrol (40 mg/kg)X100: Figure 6. (l). Resveratrol (40 mg/kg)X400:

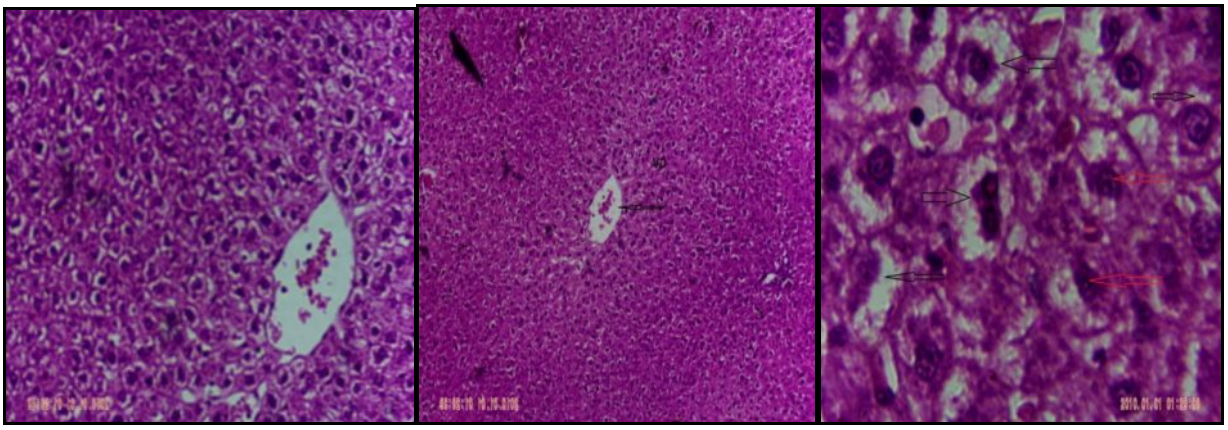

Figure 6. (k, l): Resveratrol (40 mg/kg) group showing vacuolar degeneration of cytoplasm and fragmented nuclei but no areas of necrosis.

Figure 6. (m). RI (40 mg/kg)X100: **Figure 6. (n). RI (40 mg/kg)X400:**

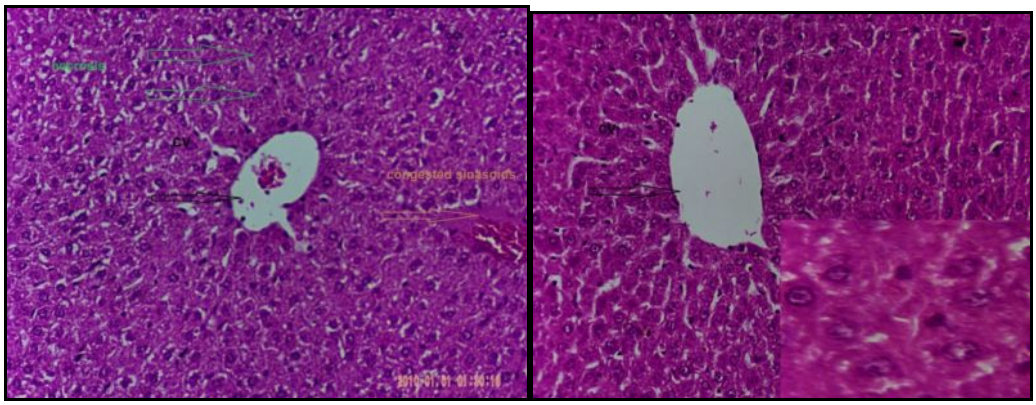

Figure 6. (m, n): RI (40 mg/kg) group at lower power, photomicrography revealed non congested central vein, overcrowded cells as well as minimal areas of necrosis. At higher power, cytoplasm is preserved in most of cells, nuclei are vesicular and active.

Immunohistochemical figures

Casepase-3 stain

Figure 7. (a). Normal hepatic tissue

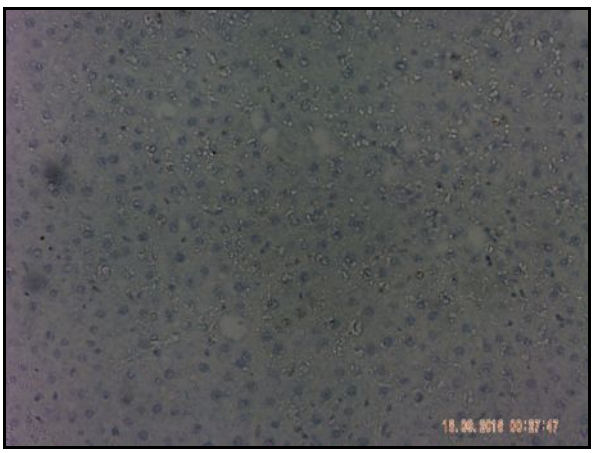

Figure 7. (a): Normal hepatic tissue photomicrography showing negative staining for caspase-3

Figure 7. (b). Carrageenan control X100:

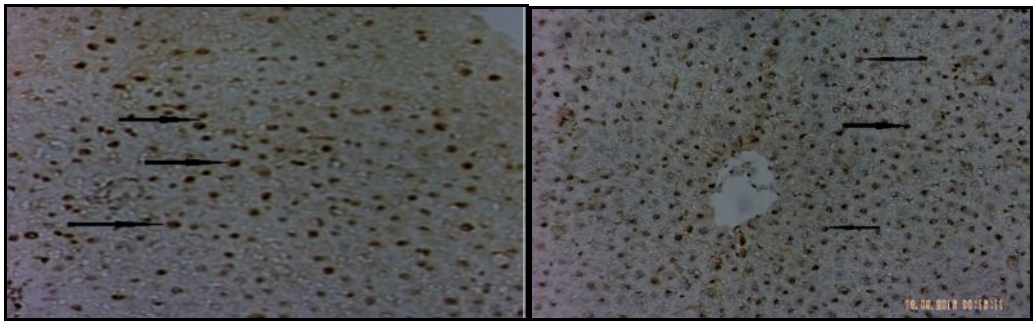

Figure 7. (b). Carrageenan control groupshowed positively stained hepatocytes along the three hepatic zones where the nuclei showing chromatin condensation in some of them.

Figure 7. (c). Ibuprofen (20 mg/kg) X100:	**Figure 7. (d). Ibuprofen (20 mg/kg) X 400:**	**Figure 7. (e). Resveratrol (20 mg/kg) X100:**

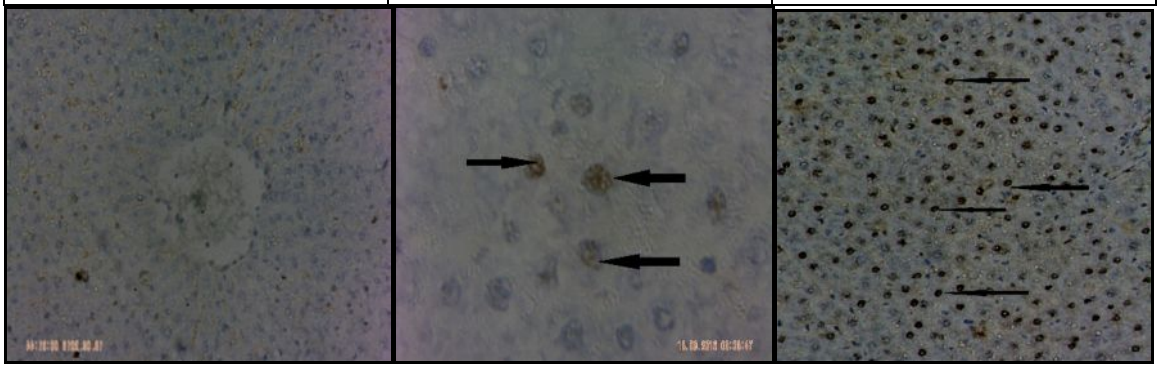

Figure 7. (c, d). Ibuprofen (20 mg/kg) group showed minimal stained nuclei with caspase-3 indicating that low dose of ibuprofen could be tolerated by liver cells.	**Figure 7. (e). Resveratrol (20 mg/kg)group showed mixed pattern of staining with positive and negative stained nuclei indicating that the cells are affected but not fully saved by the low dose of resveratrol.**

Figure 7. (f). RI (20 mg/kg) X100Figure 7. (g). RI (20 mg/kg) X400

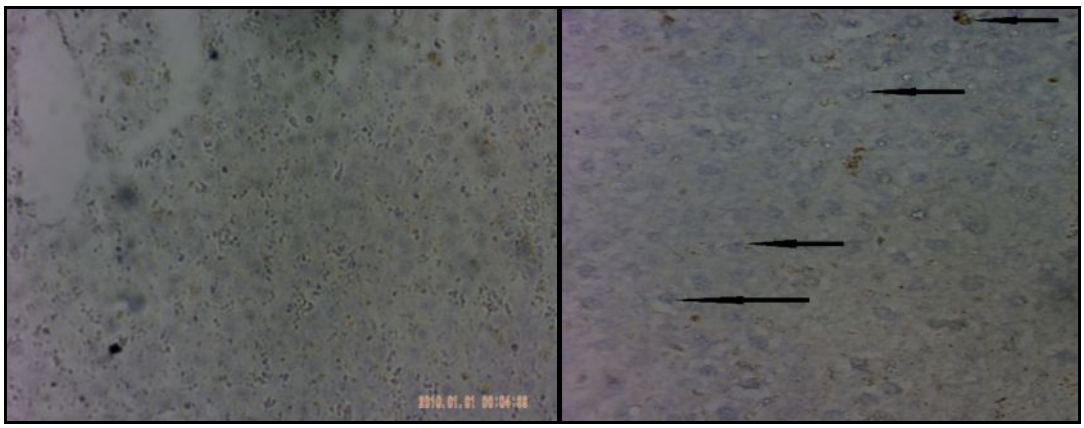

Figure 7. (f, g). RI (20 mg/kg) group showedminimal scattered faintly stained cells less than 1-2 per high power field.

Figure 7. (h). Ibuprofen (40 mg/kg)X100:Figure 7.(i). Resveratrol (40 mg/kg)X100:

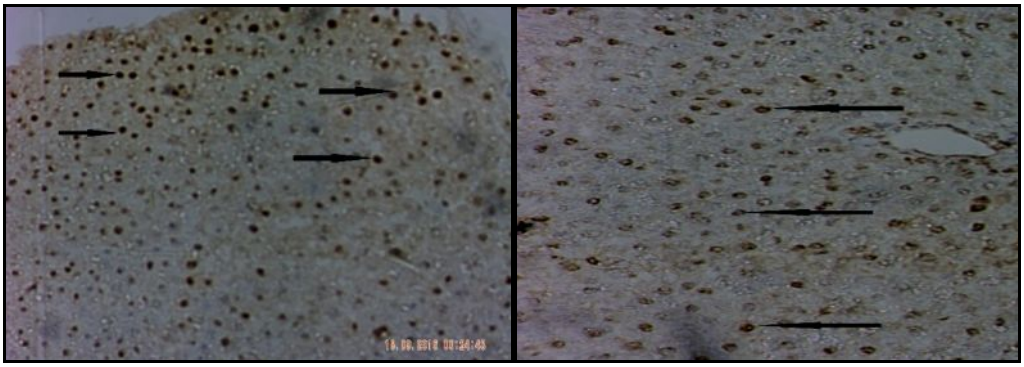

Figure 7. (h). Ibuprofen (40 mg/kg) group showed many positive cells with fragmented chromatin.	**Figure 7. (i). Resveratrol (40 mg/kg)group showedrelatively strongly expressed caspase-3 stain; as part of the cells underwent spontaneous apoptosis as a protective physiological process**

Figure 7. (j). RI (40 mg/kg) X100: **Figure 7. (k). RI (40 mg/kg) X400:**

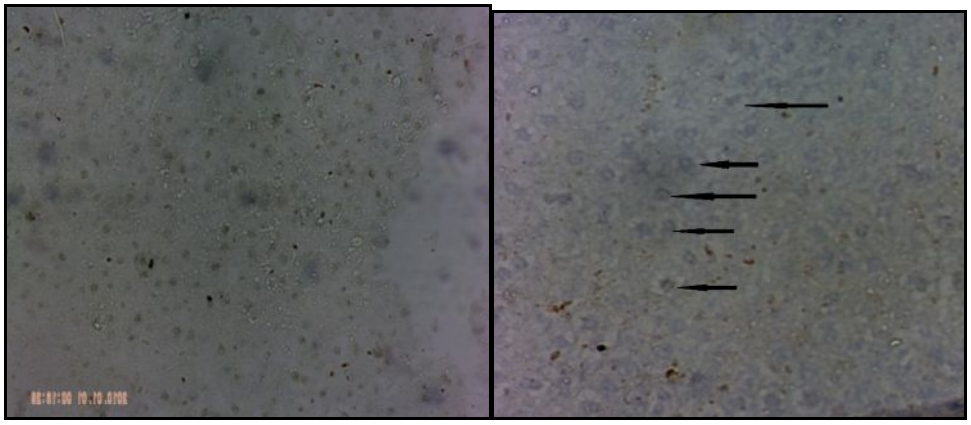

Figure 7. (j, k). RI (40 mg/kg)group showed minimal scattered faintly stained cells less than 1-2 per five high power fields.

PCNA

Figure 8 (a). Normal hepatic tissue X100:

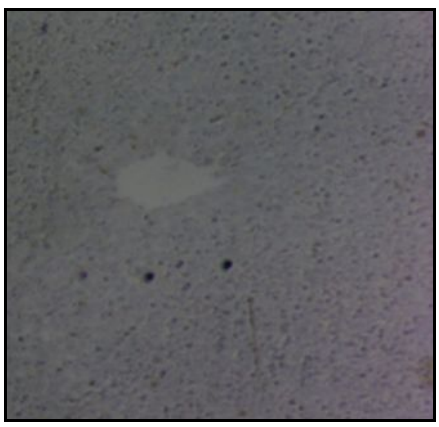

Figure 8 (a). Normal hepatic tissue showing scattered positive cells indicating active regeneration of liver cells.

Figure 8 (b). Carrageenan control X100:

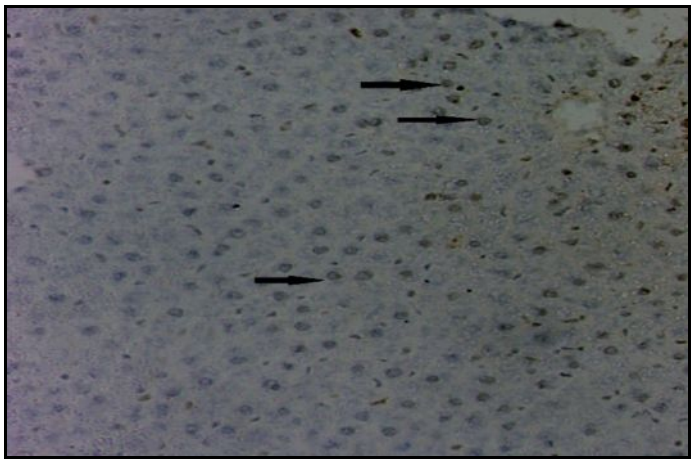

Figure 8 (b). Carrageenan controlshowed scattered faintly positive nuclei along the hepatic lobule.

Figure 8 (c). Ibuprofen (20 mg/kg) X100:Figure 8 (d). Resveratrol (20 mg/kg)X100:

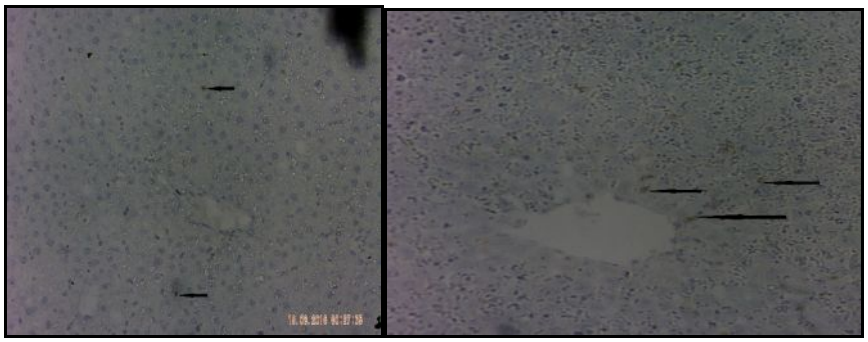

Figure 8 (c). Ibuprofen (20 mg/kg)group showed negative stain as no regeneration took place without a hepatoprotective drug administration.	**Figure 8 (d). Resveratrol (20 mg/kg) group showed few positive stained cells with granular nuclei.**

Figure 8 (e). RI (20 mg/kg) X100:

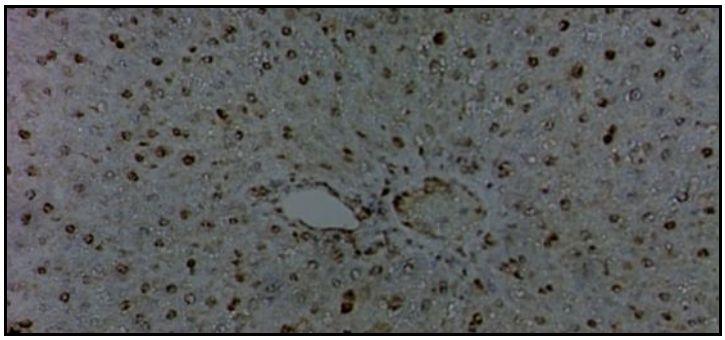

Figure 8 (e). RI (20 mg/kg)groupshowed faint positivity stained nuclei indicating active cellular division and DNA repair.

Figure 8 (f). Ibuprofen (40 mg/kg) X100:Figure 8 (g). Resveratrol (40 mg/kg)X100:

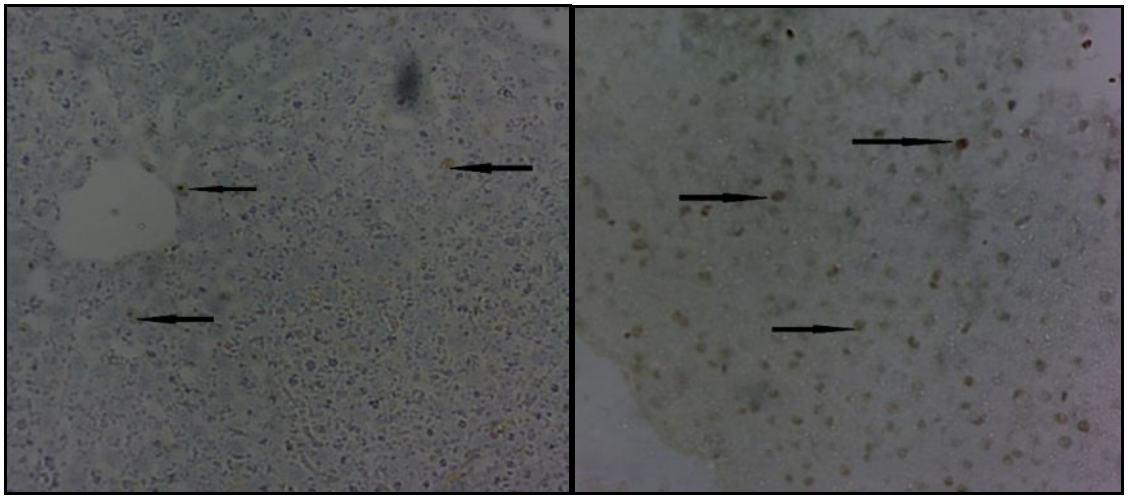

Figure 8 (f). Ibuprofen (40 mg/kg) group showed PCNA few stained cells with mild faint homogenous stain with minimal granular distribution.	**Figure 8 (g). Resveratrol (40 mg/kg) group showed PCNA moderate positivity with weak intensity revealing cellular regeneration in a higher rate than R20.**

Figure 8 (h). RI (40 mg/kg) X100:

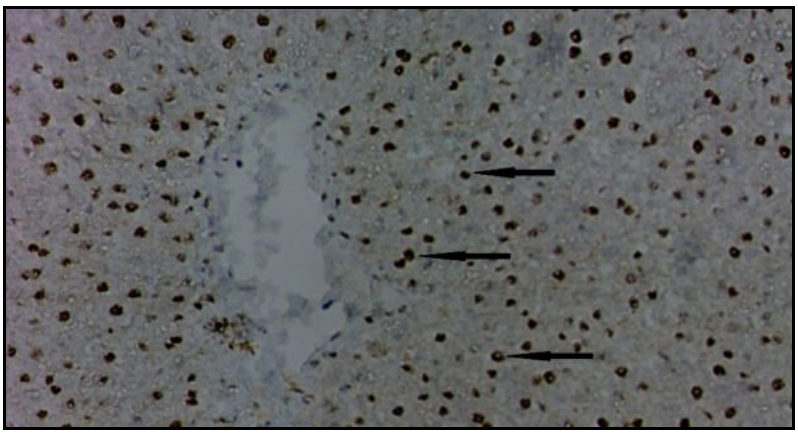

Figure 8 (h).RI (40 mg/kg) group showedPCNA high positivity indicating highly active cellular division and DNA repair.

Discussion

Inflammation is an important sign of many pathological diseases including rheumatoid arthritis, periodontitis and cancer. It progresses through the prostaglandin (PG) and leukotriene (LT) pathways starting with the release of arachidonic acid (AA) from the membrane lipid bilayer[39]. Arachidonic acid (AA) pathway leads to the generation of the proinflammatory biochemical mediators and is a critical component of inflammation. The first and key step in the activation of this pathway is the generation of a large pool of AA from membrane phospholipids by the action of phospholipase A2 (PLA2). Subsequently, AA is metabolized by lipoxygenase (LOX), cyclooxygenase (COX) and their respective downstream enzymes resulting in the production of eicosanoids such as leukotrienes (LTs), prostaglandins (PGs) and thromboxanes (TXs) [40]. Furthermore; tumor necrosis factor-α (TNF-α) is a cell signaling protein (cytokine) possessing a major role in systemic inflammation and is responsible for the acute phase reaction [41]. Biochemical energy is the fundamental element to maintain the turnover of the bio-molecular structures and the functional metabolic viability of all unicellular organisms. The concentration levels of ATP, ADP and AMP reflect roughly the energetic status of cells, and a determined ratio between them was proposed by Atkinson as the adenylate energy charge (AEC) [42]. Attenuation of energy deficits indicated by increased ATP and adenylate energy charge levels, and decreased

AMP/ATP ratio could be used as reliable markers indicating protection against tissue injury[43].Moreover; oxidative and nitrosative stresses represent well-established and reliable indicators of existence of tissue insult [1, 44].Another parameter that assess the occurrence of tissue insult is the percent of tissue DNA damage. An important cause of DNA damage concerns the attack by reactive oxygen species, leading to DNA hydroxylation. Oxidizing DNA to form 8-hydroxy-2-deoxyguanosine (8-OHDG) adducts; a major species of oxidative DNA damage [45]. 8-OHDG content is considered a sensitive biomarker of the oxidative DNA damage and repair[46]. Proliferating cell nuclear antigen (PCNA) is a cell cycle marker protein that functions as sliding clamp during DNA replication [47].PCNA is involved in the DNA damage tolerance pathway known as post-replication repair. [48].PCNA is used in the study of the dynamics of replication and repair in living cells by detecting its introducing translational fusions. This is done by examining cells undergoing repair and showing PCNA positive stain [49]Caspase-3 has long been recognized as a key protease mediating cell demolition during apoptotic cell death. Numerous nuclear substrates for caspase-3 have been recognized although procaspase-3 is mainly localized in the cytoplasm. Recent studies revealed that caspase-3 could to an extent increase the production of PGE2 via a caspase-3-activated signaling pathway involving cytosolic calcium-independent phospholipase A2 and arachidonic acid [50,51,52]

Carrageenans are classically used as agents for the induction of experimental inflammation and inflammatory pain. Studies in mammals have demonstrated that carrageenan exposure predictably causes inflammation, including development of ulcerations, polyps, colitis, and colorectal tumors, and since then; carrageenan has been used in thousands of cell-based and animal experiments to cause inflammation, primarily to study mediators of inflammation and anti-inflammatory therapeutics. Moreover; carrageenan was found to induce acute and chronic hepatic injury in many animal models. The mechanism underlying carrageenan-induced hepatic injury could be explained by the fact that carrageenan could be detected within Kupffer cells, where it persists for several months; leading to the impairment of Kupffer cells' activity. It is possible that damage to hepatic parenchymal cells is caused by proteolytic enzymes released from carrageenan-damaged Kupffer cells. Furthermore; it was demonstrated that single intraperitoneal carrageenan injection into mice (1 mg/0.2 ml/mouse) resulted in prominent activation of natural killer (NK) cells in the liver, lungs, and spleen. A time-kinetic study showed sequential activation of NK and natural killer T (NKT) cells in the liver on days 3–7 after the injection. In parallel with the activation of NK and NKT cells in number, NK and NKT cytotoxicities were augmented. At this time, liver injury was induced, accompanied by massive hepatic necrosis and the elevation of transaminases. The In-vivo elimination of NK cells reduced the liver injury induced by carrageenan. These findings suggest that not only phagocytic cells but also primitive lymphocyte (mainly NK cells) subsets might be important targets for the acute toxicity of carrageenan [2, 7, 53, 54].

In the present study single sub-planter injection of carrageenan resulted in severe paw edema, accompanied with a major increase in serum and liver inflammatory biomarkers namely; AA, PGE2 and TNF-α that lasted for several days after carrageenan injection. Additionally; carrageenan injection resulted in pronounced oxidative and nitrosative stress, noticeable decrease in adenylate energy charge (AEC) and marked increase in 8-OHDG level. Besides; histopathological and immuneohistochemical examination revealed definite hepatic tissue injury.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are notably used worldwide for the treatment of inflammatory diseases [55]. They exert their efficacy through inhibition of cyclooxygenase (COX), a key enzyme for the biosynthesis of proinflammatory molecules, such as PGE2, prostaglandin I2, and thromboxane [56].Unfortunately NSAIDs share a common spectrum of clinical toxicities; of the most hazardous and particularly important in patients are the renal, hepatic, and cardio-vascular toxicities. Small elevations of one or more liver tests may occur in up to 15% of patients taking NSAIDs, and notable elevations of ALT or AST (approximately ≥3 times the upper limit of normal) have been reported in approximately 1% of patients in clinical trials of NSAIDs [57].Ibuprofen, as an example of NSAID showed moderate hepatotoxicity in rats[58]. It is one of the NSAIDs that are commonly known to produce hepatotoxicity through inducing cholestatic hepatitis [59]. Four weeks chronic treatment with ibuprofen (0.4 g/kg/day i.p) demonstrated a sequence of pathologies, not formerly stated in ibuprofen-treated rats, mainly; hepatic encephalopathy, hepatomegaly, increased AST and ALT serum values [60].

Resveratrol has long been acknowledged to possess anti□oxidative, anti□inflammatory and hepatoprotective properties [17, 61].Several studies highlighted the beneficial outcomes from using resveratrol in the protection against and treatment of liver injury either alone or in combination with other medications or

nutraceuticals. It was demonstrated that; resveratrol pretreatment protected against [D-galactosamine (800 mg/kg) + lipopolysaccharide (LPS; 0.5 ug/kg)] induced liver failure in rats. Among the mechanisms responsible for the hepatoprotective effect of resveratrol in this model were the reduction in nitrosative stress and modification of oxidative stress parameters which led to overall improvement in hepatotoxic markers and morphology after the hepatic insult[62].Previous researchers stated that resveratrol was able to reduce inflammation via inhibition of prostaglandin production, cyclooxygenase-2 activity, and nuclear factor-κB (NF-κB) activity and due to the probability thatinflammatory mediators may stimulate hepatic stellate cell (HSC) activation, it is possible that the anti-inflammatory effect of resveratrol may contribute to its anti-fibrotic activity in the injured liver via inhibition of HSC activation[63, 64]. Several in vitro studies have documented the inhibition of stellate cell activation by resveratrol using rat HSCsand cultured human liver myofibroblasts[65, 66]. The hepatoprotective and anti-fibrogenic effects of resveratrol have been reported recently in several models of rat liver injuries [67, 68].

In the present study; ibuprofen ingestion resulted in reversal of carrageenan induced inflammatory signs but failed to show discernible protection against the carrageenan-induced liver insult especially as the ingested dose increased. On the other hand combining resveratrol with ibuprofen resulted in augmented anti-inflammatory and hepato-protective effects represented by acute protection against carrageenan induced paw edema, pronounced decline in serum arachidonic acid, PGE2, ALT and AST levels after both single and repeated administration, reduction in hepatic TNF-α level and 8-OHDG content, improvement in hepatic tissue cell energy performance, amelioration of oxidative and nitrosative stresses. Finally both histochemical and immunuhistochemical evaluations showed the beneficial out comes from combining both treatments.

Conclusion

The present study revealed that combining resveratrol with ibuprofen to combat inflammation could be beneficial by augmenting the anti-inflammatory effect as well as protection against the hepatic insult that could occur when using ibuprofen alone.

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