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Mast cells and pro-inflammatory cytokines roles in assessment of grape seeds extract anti-inflammatory activity in rat model of carrageenan-induced paw edema

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ABSTRACT

Objective(s): Reactive oxygen species (ROS)-produced oxidative disorders were involved at the pathophysiology of many inflammatory processes via the generation of pro-inflammatory cytokines and antioxidant defense system suppression. Although herbal antioxidants as mono-therapy relief many inflammatory diseases including, autoimmunity rheumatoid arthritis, but as combination therapy with other proven anti-inflammatory drugs in order to decreasing their toxic impacts has not yet been studied clearly, especially against chemical substances that's induced local inflammation with characteristic edema.

Materials and Methods: Grape seeds extract (GSE) at a concentration of 40 mg/kg B. wt alone or in combination with indomethacin (Indo.) at a dose of 5 mg/Kg B. wt orally given for 10 days prior (gps VI, VII, VIII) or as a single dose after edema induction (gps IX, X, XI) in rat's left hind paw by sub-planter single injection of 0.1 carrageenan: saline solution (1%) (gp. V) to assess the prophylactic and therapeutic anti-inflammatory activities of both through the estimation of selective inflammatory mediators and oxidative damage-related biomarkers as well as tissue mast cell scoring. Furthermore, both substances were given alone (gps II, III, IV) for their blood, liver and kidney safety evaluation comparing with negative control rats (gp. I) which kept without medication.

Results: A marked reduction on the inflammatory mediators, edema volume and oxidative byproducts in edema bearing rats' prophylactic and treated with grape seeds extract and indomethacin was observed. Indomethacin found to induce some toxicological impacts which minimized when administered together with GSE.

Conclusion: GSE is a safe antioxidant agent with anti-inflammatory property.

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Introduction

Inflammation is a localized protective response possesses several tissue injury and damage resulting in release of many pro-inflammatory cytokines, including interferon (INF-γ), prostaglandin E2, cyclooxygenase-2 (COX-2) IL-1,-6,-12 , tumor necrosis factor (TNF), and inducible nitric oxide synthase (iNOS) (1). These mediators play an important role in the starting and expansion of the inflammatory cascade (2).Viral, bacterial and parasitic infections as well as hypersensitivity reactions are considered the common causes of inflammation which also can be provoked directly either physically by trauma, ultraviolet or other ionizing radiation, excessive burns or cooling and/or chemically by corrosive such as acids, oxidizing irritants, alkalis and experimentally by carrageenan. Additionally, tissue infarction as a result of oxygen or nutrients deprivation caused by insufficient blood flow is considered as a potent inflammatory stimulus (3).

Over 50 years ago, inflammation is treated by non-steroidal anti-inflammatory drugs (NSAIDs) such as salicylates, naproxen, ibuprofen, and indomethacin which are differ in their structure but all have similar antipyretic, anti-inflammatory, analgesic properties and alleviate pain by reducing local inflammatory responses through inhibition of prostaglandin synthesis (4). Indomethacin emerged as one of the most extremely potent anti-inflammatory and analgesic medication through non-selective inhibition of cyclo-oxygenase (COX) enzyme which responsible for the conversion of arachidonic acid to prostaglandins (5). Although it was among the first NSAID drugs that used in treatment of several inflammatory disorders but as noted it poses a major health problems during its long standing clinical use and found to be agent with hematotoxic, hepatotoxic (6), nephrotoxic (7) and gastric ulcerogenic properties (8). Therefore, discovering of new natural medicinal products with potent anti-inflammatory activity and minimal adverse effects is required (9).

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Many herbal commercial preparations such as grape seeds extract (GSE) are being extremely used for edema inhibition due to the powerful antioxidant and anti-inflammatory properties of its ingredients mainly, proanthocyanidin which have the ability to inhibit the pro-inflammatory cytokines, COX-1 and 2, 5-lipoxygenase that increasing the plasma levels of prostacyclin and 6-keto-prostagladin (10-12). Moreover, it significantly decreases the concentrations of leukotrienes and leukotriene/prostacyclin ratio and also inhibit the cell DNA and oxidative stress damage by its free radical scavenging activity of ROS (13-14).

Mast cells (MCs) are myeloid progenitors cells widely localized in the mucosal and connective tissues in order to subsiding the inflammation as they releases pro-inflammatory molecules, like histamine, proteases, proteoglycans, chemokines, arachidonic acid, growth factors and, TNF-alpha. Therefore, mast cells scoring in inflamed tissues reflect a good or poor prognosis and efficacy of the anti-inflammatory remedies (15). Thus, this work planned to evaluate the prophylactic and therapeutic role of GSE alone or concurrently with indomethacin in controlling paw edema induced experimentally in rats by carrageenan depending upon MCs counting and measurement of other pro-inflammatory cytokines in addition to clarifying their safeness.

Materials and Methods

Drugs and chemicals

Indomethacin (Indo) in form of soft gelatin capsule with a concentration of 25 mg was obtained from PharcoPharmaceuticals, Alexandria, Egypt. A comercial Gervital capsules each contain 150 mg of grape seeds extract was produced by Arab Company for Pharmaceuticals& Medicinal Plants, Enshas El-Raml, Sharkia, Egypt. Carrageenan was purchased from Sigma Company. Other reagents, chemicals and stains of standard grade were purchased from El-Gomhouria Co for medical supplying and trading, Egypt.

Experimental protocol

At a controlled comparative experimental study, 110 adult male Sprague-Dawley rats (100–120 g) were purchased from the Laboratory Animals Unit of Veterinary Medicine Collage, Zagazig University. Rats were acclimated for two weeks before dosing at stainless-steel cages in comfortable environment and given balanced ration with basal standards and water *ad libitum*. The study was carried out following the guidelines of Animal Welfare and Ethics Committee for Animal Research of Zagazig University, Egypt. They were divided randomly into eleven equal groups.

Group (I): Kept as normal control.

Group (II): Received GSE (40 mg/kg B. wt) orally according to Wen-Guang *et al.* (16) for 10 days.

Group (III): Received Indo. (5 mg/Kg B. wt) orally according to Sandra *et al*. (17) for 10 days.

Group (IV): Received both Indo. (5 mg/Kg B. wt) and GSE (40 mg/kg B. wt) orally for 10 days.

Group (V): Received a single injection of 0.1 ml of carrageenan-saline solution (1%, w/v) into the plantar surface of the hind paw of left leg, at the 10th day of the experiment according to Sandra *et al* (17).

Group (VI): Received GSE (40 mg/kg B. wt) orally for 10 days. After the last dose of GSE by 2 hr, paw edema was induced.

Group (VII): Received Indo. (5 mg/Kg B. wt) orally for 10 days using a stomach tube. After the last dose by 2 hr, paw edema was induced.

Group (VIII): Received Indo. (5 mg/Kg B. wt) and GSE (40 mg/kg B. wt) orally for 10 days. After the last dose by 2 hr, paw edema was induced.

Group (IX): Received a single dose of GSE (40 mg/kg B. wt) orally after carrageenan injection with 1 hr at the 10th day from the starting of the experiment.

Group (X): Received a single dose of Indo. (5 mg/Kg B. wt) orally after carrageenan injection with 1 hr at the 10th day from the starting of the experiment.

Group (XI): Received a single dose of Indo. (5 mg/Kg B. wt) and GSE (40 mg/kg B. wt) orally after carrageenan injection with 1 hr at the 10th day from the starting of the experiment.

The paw volume of groups (V-XI) was measured after 1, 2, 3, 4 and 5 hr post- carrageenan exposure (18).

Sampling

At the end of the experiment, blood was collected from rat's retro-orbital venous plexus of eye medial canthus and then divided into two portions; the first portion was collected into EDTA tube for hemato - logical studies at groups (I-IV). The second portion was taken on a clean centrifuge tube to separate serum for clinico-biochemical analysis of inflammatory cytokines, serum turbidity for groups (I&V-XI), liver and kidney function tests for groups (I-IV). Furthermore, 0.5 g of the left hind paws tissue of rats in groups (I&V-XI)was taken then freezed at -20 °C for oxidative stress biomarkers assay and another part was sent at 10% neutral formalin for histo - pathological and histochemical examination besides stomach, intestine, liver and kidneys of rats in groups (I-IV).

Hematological study

Hemogram including, red blood cells (RBCs) count, hemoglobin (Hb) concentration, packed cell volume (PCV) and blood indices such as mean cell volume (MCV), mean cell hemoglobin concentration (MCHC) as well as total and differential leukocytic count were carried out using an automatic cell counter (Sysmex KX-21N, Japan).

Inflammatory cytokines assay

Selective pro-inflammatory cytokines were measured quantitatively using rat specific Enzyme-Linked Immunosorbent Assay (ELISA) kits of Thermo Scientific™ (USA) with CAT. No. KRC3012 for TNF-α (19), NRC0011 for IL1β (20), ER3IL6 for IL6 (21), ERCRP for CRP (22), meanwhile, rat IgE ELISA kit (MBS564118) of MyBioSource for estimation of IgE titer (23).

Serum turbidity test

Briefly, serum turbidity was performed as previously reported by Kosersky *et al.* (24) by using 0.1 ml of clear serum thoroughly mixed with 2.9 ml of 0.067 mol/l Sorensen's phosphate buffered solution (2 ml 0.067 mol/l mono-potassium phosphate and 98 ml 0.067 mol/l di-sodium phosphate, pH 5.2). The mixture was allowed to stabilize at 25 °C for 15 min before being incubated in water bath at 69 °C for 30 min then cooled in an ice bath and the absorbance was measured at wavelength of 645 nm.

Oxidant and antioxidant status

Paws tissues (0.5 g) were grounded at a mortar with liquid nitrogen then 4.5 ml of phosphate buffer saline was added. The mixtures were homogenized for 15 min on ice cold Universal Laboratory Aid homogenizer (MPW-309, Mechanika Precyzyjna, Warsaw, Poland). Homogenates were filtered, centrifuged at 4 °C and the supernatants were collected into clean eppendorf tubes then freezed at -20 °C (25) until used in the colorimetric assessment of oxidative stress byproducts for each mg protein (26) on UV-Vis Spectrophotometer (OPTIMA, PHOTOMECH. 301-D+, Japan) using reagent kits of Biodiagnostic Co, Cairo, Egypt with CAT No GR 25 11 for reduced glutathione (27), CAT No SOD 25 21 for superoxide dismutase (28) and CAT No MAD 25 29 for lipid peroxidation; malondialdehyde (29).

Liver and kidney function

Liver and kidney damaging byproducts were estimated using colorimetric kits of Diamond Diagnostics, Cairo, Egypt for alanine and aspartate aminotransferases enzymes (30), serum and plasma total proteins (31), albumin (32), creatinine (31),urea (33) and Spectrum Bioscience kits (REF:216001) for alkaline phosphatase (34).

Histopathological examination

Edematous paws were fixed in 10% buffered formalin for 1 hr then dehydrated in a graded series of ethanol and embedded in paraffin wax. Sections of 5 µ thickness were stained with hematoxylin and eosins (HE) stain. Other sections were dewaxed in xylene rehydrated then rinsed with 0.5N HCl (pH 0.5) for 5 min, stained for 30 min with 0.5 w/v Toluidine blue in 0.5N HCl to observe mast cells (35).

Stomach, intestine, liver, and kidney specimens immersed directly in 10% neutral buffered formalin, preserved in 70% ethyl alcohol, dehydrated into a graded series of ethanol, cleared in 3 changes of xylene, and then embedded in paraffin wax. Paraffin blocks were sectioned into 4-5 µm thick sections and then subjected to HE stain according to Bancroft and Gamble (36).

Statistical analysis

The collected data were analyzed by means of one way (ANOVA) F-test using the software statistical program (SPSS, ver 16.00, USA). Data are expressed as the mean ± SE, and results are statistically significant at $P \leq 0.05$ (37).

Results

Anti-inflammatory activity study

Clinical signs

As shown in Figure 1, signs of inflammation (redness, hotness and swelling) were noticed in the left hind paw of rats at gp. (V) which injected with 1% carrageenan and also showed a significant increase in the paw edema volume (36.35±0.73, 54.36±0.87, 67.58±0.81, 68.85± 0.33, 58.71±3.92), respectively after 1, 2, 3, 4, 5 hr post-injection. The percentage of inflammation associated with the fourth hr, given that paw edema to be more pronounced after 4 hr of carrageenan injection. On the other hand, indomethacin and/or GSE treated groups either before or after carrageenan injection (groups VI-XI) showed an improvement in the edema volume comparing with groups (V). This improvement was more pronounced in the protective groups (VI- VIII) with percentage difference (36.46, 30.38, 19.45%), respectively than the curative groups (IX-XI) which showed percentage difference 65.91, 48.59 and 48.75%, respectively at the 5th hr. Moreover, indomethacin in combination with GSE showed powerful synergistic anti-inflammatory action than the monotherapy of both.

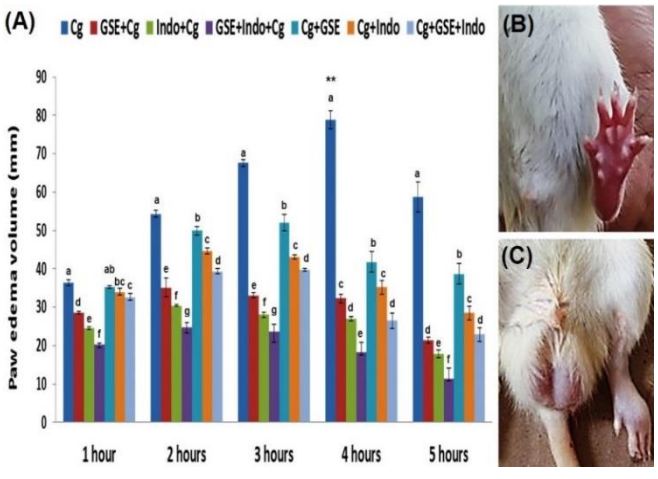

Figure 1. Representative photo showing edema inhibition activity of grape seeds extract (A) in groups (V-XI) after 1, 2, 3, 4, and 5 hours from carrageenan injection which caused swelling and redness of the injected paw (B, C)

Table 1. Selective serum inflammatory markers of rats in groups (V-XI) comparing with the control group (I)

Parameters / Treatments	TNF –α (pg/ml)	IL-1 (pg/ml)	IL-6 (pg/ml)	IgE (ng/ml)	CRP (ng/ml)
Prophylactic					
Cn	5.27^{e}±0.30	3.30^{e}±0.16	10.21^{e}±0.25	10.23^{e}±0.43	1.71^{e}±0.14
Cg	12.58^{a}±0.19	11.79^{a}±0.51	17.00^{a}±0.40	16.00^{a}±0.54	9.53^{a}±0.84
GSE + Cg	8.13^{c}±0.34	6.90^{c}±0.62	13.05^{c}±0.38	12.65^{c}±0.26	5.13^{c}±0.22
Indo+ Cg	10.20^{b}±0.26	8.75^{b}±0.13	15.80^{ab}±1.00	14.00^{b}±0.62	6.69^{b}±0.61
GSE+ Indo+Cg	6.69^{d}±0.16	5.88^{d}±0.17	12.20^{d}±0.51	11.95^{cd}±0.39	3.20^{d}±0.40
Therapeutic					
Cn	5.27^{e}±0.30	3.30^{e}±0.16	10.21^{d}±0.25	10.23^{e}±0.43	1.71^{e}±0.14
Cg	12.58^{a}±0.19	11.79^{a}±0.51	17.00^{a}±0.40	16.00^{a}±0.54	9.53^{a}±0.84
Cg+GSE	11.20^{b}±0.70	10.33^{b}±0.45	16.10^{b}±0.35	15.00^{b}±0.36	8.11^{b}±0.39
Cg+Indo	10.00^{c}±0.40	9.25^{c}±0.37	14.00^{c}±0.62	14.11^{c}±0.17	5.00^{c}±0.78
Cg+ GSE+ Indo	8.03^{d}±0.83	7.00^{d}±0.48	13.16^{c}±0.88	13.27^{d}±0.15	3.66^{d}±0.44

Each value represents the mean of 5 rats±SE
All data having different letters are differ significantly at the same column at $P \leq 0.05$
Cn: Control; Cg: Carrageenan; GSE: Grape seeds extract; Indo: Indomethacin ; TNF –α:Tumor necrosis factor-alpha; IL-1: Interleukin-1; IL-6: Interleukin-6; IgE: Immunoglobulin-E; CRP : C– reactive protein.

Inflammatory cytokines changes

Data of table (1) revealed a significant elevation in serum TNF-α, IL-1, IL-6, IgE and CRP (138.70, 257.27, 66.50, 56.40, 455.55%), respectively in group (V) compared to the control group (I). On the other side, prophylactic groups (VI-VIII) showed a significant reduction in the aforementioned parameters comparing with group (V) but not return to the normal control values. The improvement was marked in GSE/Indo/Cg group (VIII) (111.76, 179.09, 47.01, 39.58, 370.17%), moderate in GSE/Cg group (VI) (84.44, 148.18, 38.68, 32.74, 257.30%) and mild in Indo/Cg group (VII) (45.16, 92.12, 11.75, 19.55, 166.08%). Furthermore, therapeutic groups (IX-XI) showed moderate reduction in the above mentioned parameters compared to the prophylactic groups (Cg/GSE/Indo group (XI); 86.33, 145.15, 37.61, 26.68, 343.27& Cg/Indo group (X); 48.95, 76.96, 29.38, 18.47, 264.91& Cg/GSE group (IX); 26.18, 44.24, 8.81, 9.77, 83.04%), respectively.

Turbidity results

As shown in Figure (2A, B), a significant decrease in serum turbidity was recorded in groups (V-XI) compared with the control group (I). Meanwhile groups (VI-VIII and XI) showed a significant improvement comparing with group (V). This improvement was more pronounced in the prophylactive groups (gps.VI-VIII) than the treated group (gp. XI).

Oxidative stress biomarkers changes

Comparing with the control group (I), Table 2 showed a significant decrease in paw tissue SOD activity and GSH concentration at percentage 66.05 and 82.23%, respectively with a significant increase in the MDA level by a percentage 353.28% in gp.(V).

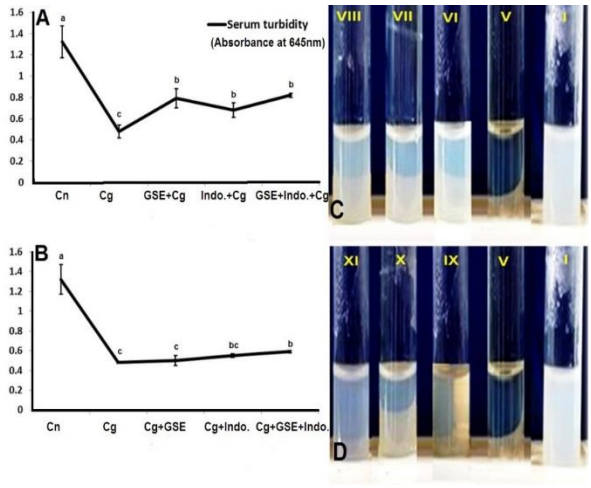

Figure 2. Representative photo showing the changes in serum turbidity (A,C); in the prophylactic groups (V-VIII) and (B,D); therapeutic groups (V,IX-XI) compared to the control group(I)

On the contrary, a significant elevation in the SOD activity and GSH concentration, beside a significant decrease in the MDA level were noticed in the prophylactic groups (VI-VIII).The improvement was marked in GSE/ Cg group (VI) (48.81, 51.73, 246.30%), moderate in GSE/Indo/Cg group (VIII) (37.30, 39.76, 168.77%) and mild in Indo/Cg group (VII) (24.67, 22.39, 77.48%). Moreover, therapeutic groups (IX-XI) showed moderate improvement in the oxidative damage markers compared to the prophylactic groups (Cg/GSE/Indo group (XI); 27.56, 63.70, 126.04% & Cg/GSE group (IX); 25.20, 34.74, 75.92% & Cg/Indo group (X); 21.77, 34.36, 33.31%),respectively.

Table 2. Paw tissue oxidative damage biomarkers of rats in groups (V-XI) comparing with the control group (I)

Parameters / Treatments	MDA (mmol/g)	SOD (U/g)	GSH (ng/g)
Prophylactic			
Cn	24.34^{e}±1.67	32.14^{a}±0.53	2.59^{a}±0.11
Cg	110.33^{a}±1.11	10.91^{e}±0.81	0.46^{d}±0.12
GSE + Cg	50.38^{d}±3.85	26.60^{b}±1.21	1.80^{b}±0.07
Indo+ Cg	91.47^{b}±2.68	18.84^{d}±2.04	1.04^{bc}±0.10
GSE+ Indo+Cg	69.25^{c}±2.97	22.90^{c}±1.08	1.49^{b}±0.19
Therapeutic			
Cn	24.34^{d}±1.67	32.14^{a}±0.53	2.59^{a}±0.11
Cg	110.33^{a}±2.11	10.91^{e}±0.81	0.46^{d}±0.12
Cg+GSE	91.88^{b}±4.32	19.01^{c}±0.39	1.36^{c}±0.29
Cg+Indo	102.22^{ab}±6.39	17.91^{d}±0.46	1.35^{c}±0.15
Cg+ GSE+ Indo	79.65^{c}±2.79	19.77^{b}±0.24	2.11^{b}±0.21

Each value represents the mean of 5 rats±SE
All data having different letters are differ significantly at the same column at $P \leq 0.05$
Cn: Control; Cg: Carrageenan; GSE: Grape seeds extract; Indo: Indomethacin; MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH: Reducedglutathione

Histological and histochemical alterations

Microscopical examination of HE-stained paw tissue of rats in control group (I) revealed no cytological alterations (Figure 3A). However, carrageenan injected paw in rats of group (V) showed marked inflammation and edema infiltration with marked cartilage damage (Figure 3B). GSE/ Cg group (VI) and Indo/Cg group (VII) showed mild inflammation and mild cartilage damage (Figure 3C, D). GSE/Indo/Cg group (VIII) showed no inflammation and slight decrease in the thickness of the cartilaginous plate (Figure 3E). Cg/GSE group (IX) showed mild inflammation, infiltration and cartilage damage (Figure 3F). Cg/Indo group (X) showed minimal inflammation and cartilage damage (Figure 3g). Cg/GSE/Indo group (XI) showed minimal inflammation, infiltration and cartilage damage (Figure 3H).

Toluidine blue–stained paw tissue of control group (I) showed negative mast cell distribution (-ve score) with no inflammation and no infiltration and no cartilage damage (Figure 4A). Meanwhile, group (V) showed marked inflammation, marked infiltration, marked loss of toluidine blue staining and dilated blood vessels in the inflamed synovial tissue (Figure 4B) with massive mast cell presence (+++ score ≤ 20 HPF) (Figure 4C). GSE/ Cg group (VI) showed minimal inflammation, minimal infiltration, minimal to mild loss of toluidine blue staining with mild mast cell presence (+ score ≤ 5 HPF) (Figure 4 D). Indo/Cg group (VII) showed mild inflammation, infiltration, and mild loss of toluidine blue staining with moderate mast cell presence (++score≤10 HPF) (Figure 4E). GSE/Indo/Cg group (VIII) showed minimal inflammation, minimal infiltration, with slightly increased cellularity and mild mast cell presence (+ score≤ 5 HPF) (Figure 4F). Cg/GSE group (IX) showed moderate inflammation, infiltration and cartilage damage and moderate mast cell presence(++ score ≤ 10 HPF) (Figure 4 G). Cg/Indo group (X) and Cg/GSE/Indo group (XI) showed minimal inflammation, infiltration and cartilage damage and mild mast cell presence (+ score ≤ 5 HPF) (Figure 4 H, I).

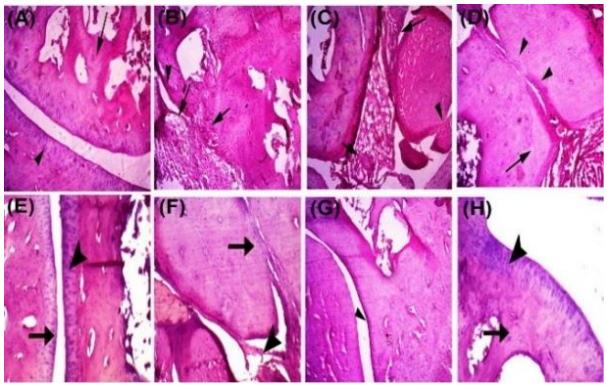

Figure 3. HE-stained paw tissue of rats in negative control group (I) showing no inflammation (arrow), no cartilage damage with thickened intact cartilaginous plate (arrowhead) (Figure 3A). However, carrageenan injected paw in rats of group (V) showing marked inflammation and edema infiltration (arrow) with marked cartilage damage (arrowhead) (Figure 3B). GSE/ Cg group (VI) showing mild inflammation (arrow) and mild cartilage damage (arrowhead) (Figure 3C)& Indo/Cg group (VII) showing mild inflammation (arrow) and no cartilage damage (arrowhead) (Figure 3D). GSE/Indo/Cg group (VIII) showing no inflammation (black arrow) and slightly decreased thickness of cartilaginous plate (black arrowhead) (Figure 3E). Cg/GSE group (IX) showing mild inflammation, mild infiltration (arrow) and cartilage damage (arrowhead) (Figure 3F). Cg/Indo group (X) showing minimal inflammation, cartilage damage (arrowhead) (Figure 3G). Cg/GSE/Indo group (XI) showing minimal inflammation, minimal infiltration (arrow), cartilage damage (arrowhead) (X100)
Cn: Control; Cg: Carrageenan; GSE: Grape seeds extract; Indo: Indomethacin

Safety evaluation study

Hematological changes

Regarding to erythrogram and leukogram (Table 3), In comparison with the control group (I), rats of group (III) showed marked leukocytosis, neutrophilia, monocytosis with microcytic hypochromic anemia represented by significant decreases in the values of RBCs, Hb, PCV, MCV and MCHC. However, GSE/Indo-exposed group (IV) showed marked elevation in the erythrogram variables and indices with significant reduction in total leukocytes, neutrophils and monocytes counts compared to group (III). While group (II) showed non-significant changes in hemogram compared to the control group.

Table 3. Toxic effects of grape seed extract and indomethacin on blood picture of rats in groups (I-IV)

Erythrogram	RBCs(x10⁶/µl)	Hb (g/dl)	PCV(%)	MCV(fl)	MCHC(%)	
Cn	10.00^{a}±0.29	16.00^{a}±0.89	52.33^{a}±1.45	52.34^{a}±2.97	30.65^{a}±0.39	
GSE	10.17^{a}±0.17	16.10^{a}±0.77	53.00^{a}±2.08	53.20^{a}±2.71	30.49^{a}±0.53	
Indo	9.03^{b}±0.11	12.00^{b}±0.82	42.50^{b}±1.17	47.06^{b}±1.13	28.23^{b}±0.18	
GSE+Indo	10.28^{a}±0.26	15.17^{a}±0.90	49.67^{a}±1.45	50.80^{a}±0.88	30.07^{a}±0.37	
Leukogram (x10³/µl)	WBCs	Neutrophils	Lymphocytes	Eosinophils	Monocytes	Basophils
Cn	10.21^{c}±0.29	2.24^{c}±0.24	6.65^{a}±0.33	0.95^{a}±0.19	0.24^{c}±0.09	0.12^{a}±0.01
GSE	10.13^{c}±0.40	2.20^{c}±0.19	6.60^{a}±0.06	1.00^{a}±0.22	0.21^{c}±0.03	0.11^{a}±0.10
Indo	12.20^{a}±0.34	3.74^{a}±0.05	6.52^{a}±0.49	0.85^{a}±0.23	0.95^{a}±0.05	0.14^{a}±0.01
GSE+Indo	11.52^{b}±0.27	3.04^{ab}±0.12	6.74^{a}±0.48	0.90^{a}±0.10	0.73^{b}±0.03	0.12^{a}±0.12

Cn: Control; Cg: Carrageenan; GSE: Grape seeds extract; Indo: Indomethacin; RBCs: Red blood cells; Hb: Hemoglobin; PCV: Packed cell volume; MCV: Mean corpuscular volume; MCHC: Mean corpuscular hemoglobin concentration; WBCs: White blood cells

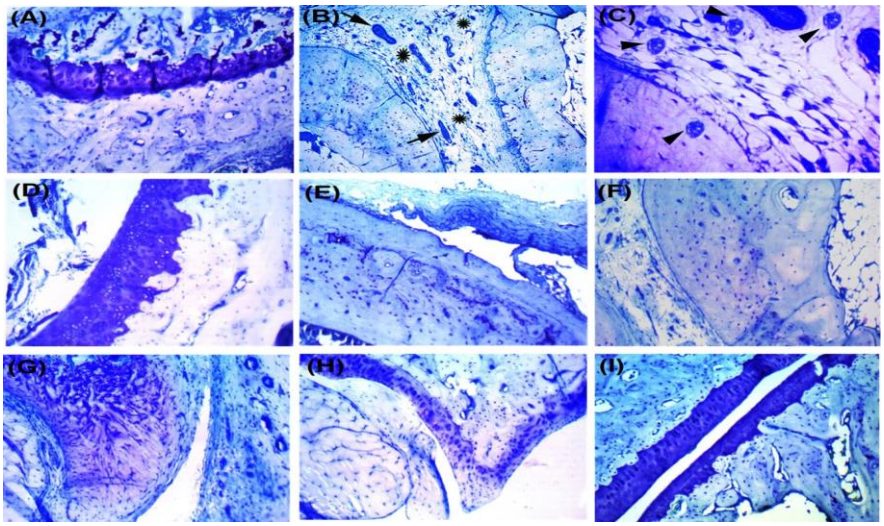

Figure 4. Toluidine blue–stained paw tissue of normal control group (I) showed negative mast cell distribution with no inflammation, no infiltration and no cartilage damage (Figure 4A) (X100). Meanwhile, carrageenan injected rats (V) showing marked inflammation , marked infiltration (asterisks), marked loss of toluidine blue staining and dilated blood vessels in the inflamed synovial tissue (arrow) (Figure 4B) (X100) with massive abundant mast cells (arrowheads) presence (Figure 4C) (X400). GSE/ Cg group (VI) showing minimal inflammation, minimal infiltration, minimal to mild loss of toluidine blue staining with mild mast cell presence (Figure 4D) (X100). Indo/Cg group (VII) showing mild inflammation, infiltration, and mild loss of toluidine blue staining with moderate mast cell presence (Figure 4E) (X100). GSE/Indo/Cg group (VIII) showing minimal inflammation, minimal infiltration, with slightly increased cellularity and mild mast cell presence (Figure 4F) (X100). Cg/GSE group (IX) showing moderate inflammation, infiltration and cartilage damage and moderate mast cell presence (Figure 4G) (X100). Cg/Indo group (X) and Cg/GSE/Indo group (XI) showing minimal inflammation, infiltration and cartilage damage and mild mast cell presence (Figure 4H, I) (X100). Cn: Control; Cg: Carrageenan; GSE: Grape seeds extract; Indo: Indomethacin

Table 4. Hepato-nephro-toxic impacts of grape seed extract and indomethacin in rats at groups (I-IV)

Parameters Treatments	ALT (U/l)	AST (U/l)	ALP (IU/l)	T. proteins (g/dl)	Albumin (g/dl)	Urea (mg/dl)	Creatinine (mg/dl)
Cn	27.65^{c}±1.33	16.00^{c}±1.73	23.00^{c}±1.73	6.30^{a}±0.26	3.63^{a}±0.09	30.00^{c}±2.51	0.93^{c}±0.52
GSE	27.00^{c}±3.51	15.00^{c}±1.15	23.67^{c}±1.33	6.23^{a}±0.14	3.53^{a}±0.24	28.80^{c}±2.23	0.87^{c}±0.37
Indo	73.44^{a}±5.45	48.00^{a}±2.31	60.00^{a}±3.05	5.23^{b}±0.35	2.40^{bc}±0.17	58.77^{a}±2.08	4.70^{a}±0.91
GSE+Indo	31.00^{b}±0.96	20.00^{b}±1.00	28.00^{b}±1.16	5.77^{ab}±0.24	2.80^{b}±0.23	39.47^{b}±5.09	2.00^{b}±0.32

Each value represents the mean of 5 rats±E
All data having different letters are differ significantly at the same column at $P \leq 0.05$
Cn: Control; Cg: Carrageenan; GSE: Grape seeds extract; Indo: Indomethacin.; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase;T. proteins: Total proteins

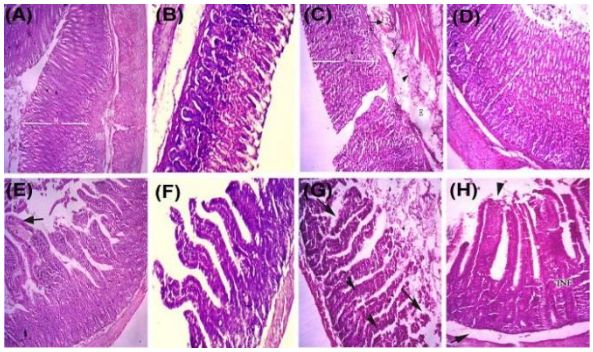

Figure 5. HE-stained liver and kidney sections of normal control and GSE-treated rats in groups (I, II) showing no structural alterations (Figure 5A, B, E, F). Meanwhile, indomethacin-treated group (III) showing distorted hepatic lobular architecture, remarkable dilated portal areas (arrow head) with mild hydropic degeneration of hepatocytes (arrow) and congested vessels (asterisk) (Figure 5C), markedly degenerated glomeruli and shrunken capillary tuft with increased cellularity (arrowhead) in addition to mild hydropic degeneration of tubules (arrow) and areas of focal congestion (asterisk) (Figure 5G).The co-exposed group (IV) showing preserved hepatic lobular architecture, unremakable portal areas with no inflammatory infiltrate and mild hydropic degeneration of hepatocytes (arrow head) and dilated sinusoids(Figure 5D), mild glomeruli degeneration and shrunken capillary tuft with increased cellularity (arrowhead) in addition to mild hydropic degeneration of tubules (arrow) (Figure 5H) (X400)

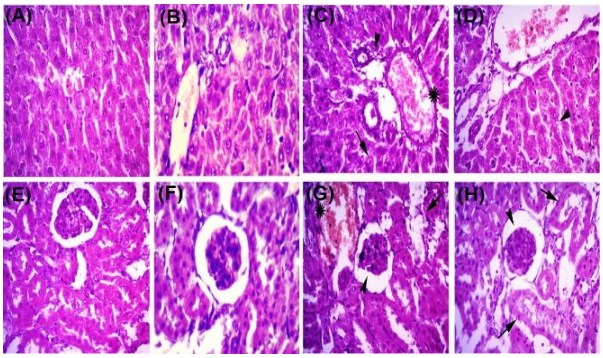

Figure 6. HE-stained stomach and intestinal sections of normal control and GSE-treated rats in groups (I, II) showing showing intact epithelium and unremarkable mucosa (M), muscularis mucosa (MM) and submucosa (Sm) (Figure 6 A, B), preserved villous architecture with no pathological changes (arrow) (figure 6E, F). However, indomethacin-treated group (III) showing ulceration of epithelium (white arrow) and mild inflammatory infiltrate in mucosa with slightly decreased thickness (arrow head), mild sub-mucosal edema (E) and congestion of blood vessel (black arrow) (Figure 6C), erosion (arrow head) and moderately fragmented intestinal villi with inflammatory infiltrate (arrow) (Figure 6G). The co-exposed group (IV) showing showing intact superficial epithelium and mild inflammatory infiltrate in mucosa with preserved mucosal thickness (white arrowhead) (Figure 6D), preserved intestinal villous architecture with no erosion (arrow head), intact villi with inflammatory infiltrate and minimal vacuolar degeneration (arrow) (Figure 6H) (X100)

Clinico-biochemical results

Compared to the control group (I) as shown in Table 4, rats of group (III) exposed to indomethacin for 10 days showed marked hypoproteinemia, hypoalbuminemia with elevated serum levels of ALP, AST, ALT, urea and creatinine. On the other hand, GSE/Indo-exposed group (IV) showed marked elevation in serum total proteins and albumin with significant reduction in urea and creatinine concentrations as well as serum ALP, AST, ALT activities compared to group (III). While group (II) orally received GSE for 10 days showed non-significant changes in liver and kidney functions estimating parameters comparing with normal control group.

Histopathological findings

Microscopical examination of HE-stained liver, kidney, stomach and intestine of groups (I, II) showed no structural alterations (Figure 5&6 A, B, E, F). Meanwhile, group III showed distorted hepatic lobular architecture, remarkable dilated portal areas with mild hydropic degeneration of hepatocytes and congested vessels (Figure 5C), in addition to markedly degenerated glomeruli and shrunken capillary tuft with increased cellularity in addition to mild hydropic degeneration of tubules and areas of focal congestion (Figure 5G), besides ulceration of gastric epithelium and mild inflammatory infiltrate in gastric mucosa with slightly decreased thickness, mild sub-mucosal edema and congestion of blood vessel (Figure 6C),also intestinal erosion and moderately fragmented villi with inflammatory infiltrate (Figure 6G).

The co-exposed GSE/Indo-group (IV) showed preserved hepatic lobular architecture, unremakable portal areas, mild hydropic degeneration of hepatocytes and dilated sinusoids (Figure 5D), mild degeneration of glomeruli and shrunken capillary tuft with increased cellularity in addition to mild hydropic degeneration of tubules (Figure 5H), intact superficial gastric epithelium and mild inflammatory infiltrate in mucosa with preserved mucosal thickness (Figure 6D) and also showed preserved villous architecture of intestine with no erosion, intact villi with inflammatory infiltrate and minimal vacuolar degeneration(Figure 6H).

Discussion

Inflammation is an immunological defense process resulted from burns, traumatic injuries, chemical agents, microbial organisms, and other stimulus exposure (1). Although, using of non-steroidal (NSAID) or steroidal (SAID) anti-inflammatory drugs may be helpful in the treatment of many inflammatory disorders (38) but their adverse impacts pose major health problems therefore; therapies with natural origin agents and low adverse effects are needed (39). These natural antioxidants have the ability to detoxify and neutralize the free radicals by suing the ROS generation so they protect against oxidative stress induced cellular damage (40). GSE is a medicinal candidate with multi-therapeutic uses as anti-inflammatory (41) and anti-ulcer potentials (42) through its top ingredient; proanthocyanidins which

exhibit oxygen free radical scavenging and pro-inflammatory cytokines inhibition activities (43).

In the current study, carrageenan in rats resulted in redness; swelling, hotness and painful edematous paw tissue with a significant increase in edema volume till reach the maximum after four hours post-injection as previously obtained by Young-Hua *et al* (44). This might be due to the acute inflammatory response induced by carrageenan which characterized by the exudation of tissue fluids and plasma resulting in edema formation and concurrent accumulation of leukocytes mainly neutrophils (45). Pre-treatment of inflamed rats with indomethacin (5 mg/kg B. wt) for 10 days before edema induction caused a significant decrease in edema volume as found by Nishanthini *et al.* (46) through prostaglandin over-production. Also, the decrease in edema volume in animals received GSE (groups VI, VIII, IX&XI) may be due to its anti-inflammatory action by inhibition of pro-inflammatory cytokines such as 5-lipoxygenase, COX1, 2 and increasing the plasma levels of 6-keto-prostagladin and prostacyclin (10-12). Moreover, GSE significantly lower the cellular concentrations of leukotrienes, leukotriene prostacyclin ratio and also prevent the cellular oxidative and DNA damage via its water-and fat-soluble free radical scavenging activity (13) which increase the cells membrane integrity against ROS by activating the antioxidant enzymes activity (14, 47).

Regarding to the results of inflammatory markers, rats of group (V) showed a significant increase in serum levels of TNF –α, IL-1, IL-6, IgE and CRP. On the same trend, others reported significant increases in serum levels of TNF –α, IL-1, IL-6, IgE and CRP in rats after 4 hr from carrageenan injection (48-50). The administration of GSE alone before (group VI) or after (group IX) injection of carrageenan or in combination with indomethacin (group XI), revealed a significant improvement in the aforementioned parameters compared with carrageenan induced paw edema in rats of group (V) which may be due to its flavonoids which exert their anti-inflammatory effects by modulating the inflammatory cells through the inhibition of T lymphocyte proliferation, pro-inflammatory cytokines (TNF- α and IL-1) production (51) and DNA oxidative damage prevention (14). Furthermore, a significant improvement in serum levels of TNF –α, IL-1, IL-6, IgE and CRP was recorded during the using of indomethacin as prophylactic (group VII) or treatment (groups X&XI). On the same ground, Saba *et al.* (52) and Mohammad *et al.* (48) said that treatment with indomethacin in carrageenan-induced paw edema rats decreased the tissue level of IL-6 and TNF-α.

In the present study, serum turbidity of paw edema rats was found to be significantly reduced compared with normal rats as a result of decreasing serum albumin concentration as negative acute phase protein and increasing plasma fibrinogen level as positive acute phase protein following carrageenan injection. Treatment with indomethacin and GSE increased the turbidity significantly compared with carrageenan induced paw edema. Also, reduction in serum turbidity of positive control rats is known to be caused by denatured products (53), immune response (54) and increasing the serum lysozyme levels in paw inflammation (53).

In fact, inflammation stimulate the iNOS enzyme which is responsible for over-production of nitric oxide (NO) at the area of inflammation (55). NO combined with superoxide anion forming peroxynitrite; an oxidizing agent able to promote the lipid peroxidation as MDA which causes oxidative deterioration of polyunsaturated lipids was occurred producing radical products that induce cellular damage (56, 57). The infiltrating inflammatory cells also generate ROS and free radicals including, hydroxyl radical superoxide anion, hydrogen peroxide and singlet oxygen. Superoxide dismutase (SOD) enzyme capable for catalyzing the dismutasable conversion of superoxide to oxygen and hydrogen peroxide. Then hydrogen peroxide is catalyzed to water by catalase and glutathione system. SOD and other enzymatic antioxidative agents activities decline in severe inflammatory and oxidative stress conditions (58). Additionally, glutathione, a non-enzymatic reducing molecule that traps free radicals and prevents oxidative damage, is also diminished in inflammatory conditions (59). The entire above can discuss the results of the present study which showed a significant increase in MDA level with a significant decrease in levels of SOD and GSH at rats injected with carrageenan, similarly as reported by others (49, 60, 50). On the other hand, inflamed rats received GSE showed marked suppression in MDA and activation of SOD and GSH through its ability to scavenging the free radicals and its antioxidant property (16,13).These findings agreed with others results (14, 16, 61-64). Also, using of indomethacin against the carrageenan induced paw edema improved the oxidative stress markers. This was parallel to the results recorded by Mahaveer *et al* (65).

The obtained results were confirmed by the histopathological findings which showed marked inflammation and edema infiltration with marked cartilage damage, and massive presence of mast cell (are known to regulate angiogenesis, vascular homeostasis, vasodilation, adaptive and innate immune responses, and toxin neutralization as it has wide variety of secretory granules (50-200) that store inflammatory mediators, including histamine, cytokines, heparin, neutral proteases and chondroitin sulfate) in the inflamed tissue (66). Administration of GSE alone or in combination with indomethacin prior and after edema induction capable to restore the damaged tissue towards the normal control (67, 68).

By follow up the toxic impacts of both tested substances on liver and kidney; GSE alone induce no adverse effects on liver and kidney but indomethacin elevated the serum AST, ALT, ALP, creatinine, urea levels and caused hypoproteinemia and hypoalbuminemia when administered for 10 days. This may attributed to the ability of NSAIDs to inhibit both COX-1 and COX-2 (Isoenzymes mediated prostaglandin synthesis in inflammation and carcinogenesis) which are located within the hepatic and renal tissues so, their blockade affect almost of liver and kidney functions (69, 70). These results are partially agreed with those reported by Abatan *et al.* (6) and Silva *et al* (71). Such biochemical changes in the present work are the outcome of nephropathy which is manifested by markedly degenerated glomeruli and shrunken capillary tuft with increased cellularity and mild hydropic degeneration of tubules (7) in addition to, dilated bile ducts in portal areas, minimal inflammatory infiltrate, mild hydropic degeneration of hepatocytes with moderately dilated sinusoids and congested vessels and also, distorted hepatic lobular architecture (6).

On the other hand, the using of GSE in combination with indomethacin (groups IV&VIII) significantly attenu ated the adverse effect of indomethacin on kidneys. This supported the previously obtained results by Yanarates *et al* (72). The improvement was confirmed by presence of mild degeneration of glomeruli and shrunken capillary tuft with increased cellularity in addition to mild hydropic degeneration of tubules. The protective effect of GSE against liver damage was observed as Ahmed and Fatani (73) and Ahmad and Khan (74) documented that treatment with GSE significantly attenuated the liver damage. Moreover, they added that GSE prevent the leakage of intracellular enzymes. Also, Somaia *et al.* (64) recorded that GSE (100 or 200 mg/kg B. wt) significantly decreased the elevated serum activities of AST, ALT, and ALP. Our results were confirmed by the presence of preserved hepatic lobular architecture, unremarkable portal areas, and mild hydropic degeneration of hepatocytes and dilated sinusoids.

By monitoring the erythrogram, results revealed that oral administration of indomethacin (5 mg/kg B. wt) for 10 days in rats caused a significant decrease in RBCs count, Hb concentration, PCV, MCV and MCHC as reported by others (71, 75, 76). From our opinion the recorded microcytic hypochromic anemia may be due to nutritional deficiency (inadequate digestion and absorption by damaged intestine) or bleeding ulcers present in stomach. These findings were confirmed by the presence of ulceration in the epithelium of the stomach, intestinal epithelial erosion with fragmented villi and marked sub-epithelial edema were seen in intestine (7). On the contrary, GSE (40 mg/kg B. wt/10 days) administration for 10 days cause no significant changes on erythrogram as seen by other researchers (77-79). Additionally, leukogram showed that rats received GSE showed non-significant changes in total and differential leukocytic count (16, 77, 80). Meanwhile, indomethacin induce leukocytosis with neutrophilia and monocytosis. The recorded leukocytosis is a result of increasing the phagocytic cells (neutrophil and monocyte) in order to tissue destruction (81).

Conclusion

GSE and indomethacin in combination evoked powerful anti-inflammatory activity against carrageenan-induced local inflammatory edema in rats. Furthermore, indomethacin is a potent, fast acting anti-inflammatory drug and gives better results than GSE when used as treatment but GSE as prophylactic is better than indomethacin as it caused gastrointestinal, hepatic, renal and hematological alterations which were subsided by GSE.

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Conflict of interest

The authors declare that no conflict of interest exists.

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