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The protective effect of grape seed extract (*Vitis vinifera*) against mesenteric ischemia/reperfusion injury in rats: an in vivo, in vitro and molecular docking study

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ABSTRACT: In this study, we aimed to determine the protective effect of grape seed extract against mesenteric ischemia/reperfusion (I/R) injury in rats. In addition, an in silico molecular docking study was performed to compare the effects of natural compounds in grape seed extract on myeloperoxidase (MPO) enzyme at the molecular level. In addition to MPO activity, malondialdehyde (MDA) and glutathione (GSH) activities were also measured in the intestinal tissue samples. Serum cytokines, TNF-α and IL-1β, were measured to assess systemic tissue damage. Mesenteric GSH decreased significantly after ischemia/reperfusion, while MDA levels and MPO activity increased significantly. Binding interactions were detected by the binding mode of analysis, and the study compared the binding affinities of compounds at the active site of myeloperoxidase and glutathione peroxidase. As a result, it was found that procyanidins (B1-B4), which are predominantly present in GS extract, have the highest binding affinity and molecular interaction.

KEYWORDS: mesenteric ischemia/reperfusion; grape seed extract; myeloperoxidase; glutathione peroxidase; antioxidant; molecular docking.

1. INTRODUCTION

A group of disorders with high rates of morbidity and death, such as mesenteric ischemia, have grown more challenging to diagnose early and treat effectively with the development of medical science [1]. Although acute mesenteric ischemia (AMI) is not an isolated clinical disease, it is a combination of complex diseases such as acute mesenteric arterial thrombosis and embolism, mesenteric venous thrombus, and nonocclusive mesenteric ischemia [2]. Intestinal necrosis may first develop due to subclinical disease progression and difficulties in diagnosis [3]. Ischemia-reperfusion injury to the bowel can be caused by thoracoabdominal aortic aneurysm repair, cardiopulmonary bypass, and small bowel transplantation [4]. Severe metabolic disturbances can occur in intestinal ischemia due to overproduction of reactive oxygen species (ROS). In addition to reactive oxygen species, increased release of interleukin 1β (IL-1β) and tumor necrosis factor (TNF-α) may also contribute to the metabolic disturbances that occur during intestinal ischemia. In addition to reactive oxygen species, increased release of interleukin 1β (IL-1β) and tumor necrosis factor (TNF-α) may also contribute to the metabolic disturbances that occur during intestinal ischemia [4]. With its antioxidant action, grape seed extract has been demonstrated to prevent oxidative stress and tissue damage in kidney, heart, brain tissue, and liver IR damage in previous studies due to its high flavonoid content [5]. Flavonoids of grape seed are reducing agents which scavenge free radicals (superoxide and hydroxyl radical) by donating an electron [5]. Flavonoids scavenge superoxide anion (O-) and (OH-) originating from activated neutrophils and prevent lipid peroxidation [6, 7]. Another important way to elicit the antioxidant activity of flavonoids is to increase

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their antioxidant levels and inhibit enzyme activities that release oxidant molecules [7]. In previous in vitro experiments, grape seed flavonoids have been shown to non-competitively inhibit the enzyme myeloperoxidase, which specifically causes free radical formation [8]. According to the literature, the effects of compounds in GS extract on ischemia/reperfusion injury in other organs have been examined many times in previous studies [9-11]. However, no studies have been conducted to examine the effects of GS extract on mesenteric ischemia/post-reperfusion injury using an in silico approach. Therefore, myeloperoxidase and glutathione peroxidase enzymes were selected as targets in order to evaluate in silico the myeloperoxidase activity and GSH levels after administration of grape seed extract in mesentheric ischemia reperfusion model. The main aim of this study was to investigate the protective effect of GS extract against superior mesenteric artery injury in the small intestine and to demonstrate the inhibitory activity of the active components of GS using in vivo, in vitro and in silico molecular docking methods. Therefore, we believe this study has an important place in the literature.

2. RESULTS AND DISCUSSION

Serum levels of TNF-α and IL-1β were significantly higher in the mesenteric ischemia/reperfusion (I/R) group ($p<0.001$-0.0001, respectively) compared to the control group. Contrarily, the rise seen in the I/R group was dramatically decreased in the grape seed group and approached control values ($p<0.001$ for both TNF-α and IL-1β) (Table 1). Chemiluminescent (CL) levels of luminol and lucigenin in ileal tissue were greater in the I/R group ($p<0.01$-0.001, respectively) compared to the control group. It was seen that the group treated with GS considerably prevented this rise. Following application of GS extract, the chemiluminescence levels of luminol and lucigenin were both similar to control values ($p<0.01$; Table 2).

Table 1. Serum cytokinelevels of control, I/R-GSE (Grape seed) ileum tissue in mesenteric ischemia/reperfusion (I/R) model in rats.

Serum cytokines	Control	I/R	IR-GS Extract
TNF-α	71.24 ± 3.36	174.7 ± 19.8 ***	73.7 ± 6.3 +++
IL-1β	108.7 ± 7.4	596.1 ± 72.4 ****	164.2 ± 12.7 +++

**p<0.01, ***p<0.001 compared to control group; + p<0.05, +++ p<0.001 compared to I/R group.

Table 2. Glutathione (GSH), malondialdehyde (MDA), myeloperoxidase (MPO) values of control, I/R-GSE (Grape seed) ileum tissue in mesenteric ischemia/reperfusion (I/R) model in rats.

Tissue Examinations	Control	I/R	I/R-GS Extract
Luminol CL(rlu/mg)	160.9 ± 11.66	299.5 ± 34.7 **	163.6 ± 15.9 ++
LucigeninCL (rlu/mg)	170.1 ± 19.2	316.3 ± 21.1 ***	212.5 ± 24.4 ++
GSH (mmol/g)	19.66 ± 2.37	13.05 ± 0.39 **	18.41 ± 1.20 +
MDA (nmol/g)	28.44 ± 4.66	53.31 ± 2.55 ***	28.78 ± 2.26 +++
MPO (U/g)	54.50 ± 2.93	145.40 ± 8.97 ***	68.99 ± 2.44 +++

**p<0.01, ***p<0.001 compared to control group; + p<0.05, +++ p<0.001 compared to I/R group.

The amount of glutathione decreased in the ileal tissues of rats in the I/R group compared to the control group ($p<0.01$). It was clear from the results that this decrease was significantly prevented in the grape seed extract treated I/R group ($p<0.05$; Table 2). In addition, when examining malondialdehyde levels, a significant increase was found in the group that underwent I/R in ileum tissue ($p<0.001$). According to the results, GSE considerably prevented this rise in MDA levels ($p<0.001$; Table 2). Furthermore, I/R significantly increased the infiltration of neutrophils into the ileum tissue, and consequently MPO values ($p<0.001$). In contrast, MPO activity was drastically dropped in the GS extract-treated group. The results were similar to the MPO values of the control group ($p<0.001$; Table 2).

The results obtained from molecular docking on myeloperoxidase binding sites were analyzed. In the study, the docking score of ascorbic acid, a potent antioxidant, was taken as a reference and compared with the results obtained from other ligands. The active GS compounds were tested and found to have a minimum binding energy between -8.6 and -10.8 kcal/mol. The results showed that gallate esters had better docking

scores than flavan-3-ols and resveratrol, suggesting that esterification of the backbone would increase their ability to scavenge free radicals.

Molecular interaction results showed that all tested compounds had a better binding affinity with the reference antioxidant molecule, ascorbic acid (-8.1 kcal/mol) (12). Therefore, these compounds could be good antioxidants. Binding affinities of all studied substances in Table 3. All substances interacted with the binding site of myeloperoxidase and glutathione peroxidase mainly via hydrogen bonding and electrostatic interactions with specific amino acid residues. The effects of grape seed extract and its major components on mesenteric ischemia/reperfusion injury in rats are summarized in Figure 1. The 2D and 3D binding interactions of flavan-3-ols (Figure 2), and procyanidins B1-B4 (Figure 3) against myeloperoxidase and glutathione peroxidase (Figure 4) were illustrated.

Table 3. The binding affinities ΔG (kcal/mol) of selected phenolic compounds (flavan-3-ols, and B-type procyanidins) of grape seed extract against myeloperoxidase and glutathione peroxidase active sites.

Name of Phenolic Compounds	Binding Affinity, ΔG (kcal/mol)	
	Myeloperoxidase	Glutathione peroxidase
(+)-Catechin	-9.1	-6.2
(+)-Gallocatechin	-9.8	-5.8
(+)-Gallocatechin-3-gallate	-10.2	-6.9
(-)-Epicatechin	-9.3	-6.4
(-)-Epicatechin-3-gallate	-10.4	-7.1
Procyanidins B1-B4	-10.8	-7.6
Resveratrol	-8.6	-5.8
Ascorbic acid (reference compound)	-8.1	-6.4

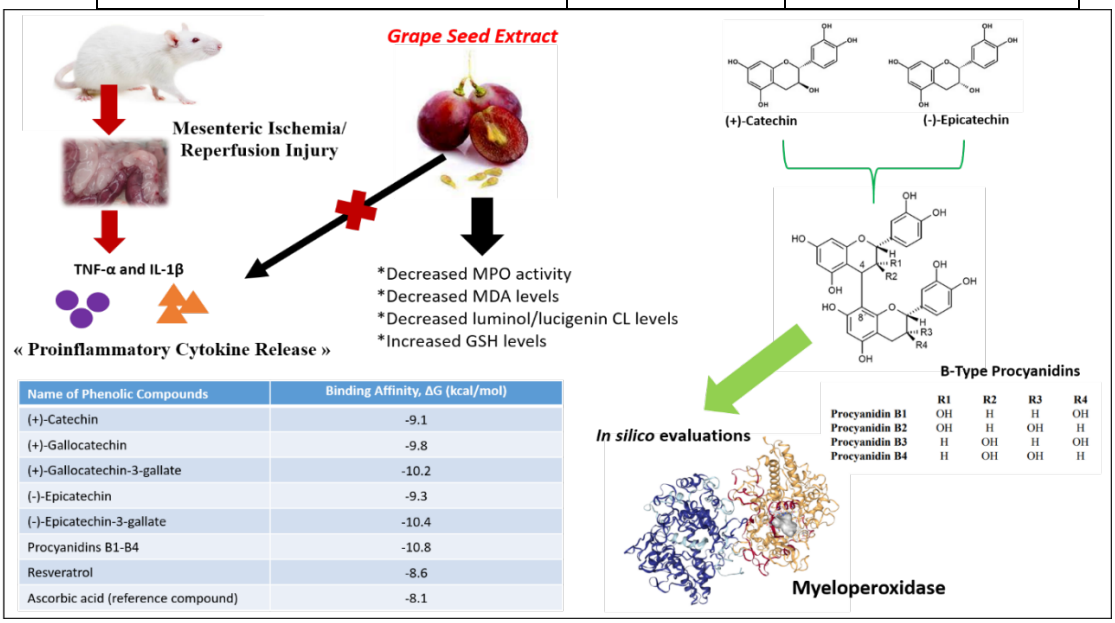

Figure 1. Effects of grape seed extract and its main constituents on mesenteric ischemia/reperfusion injury in rats.

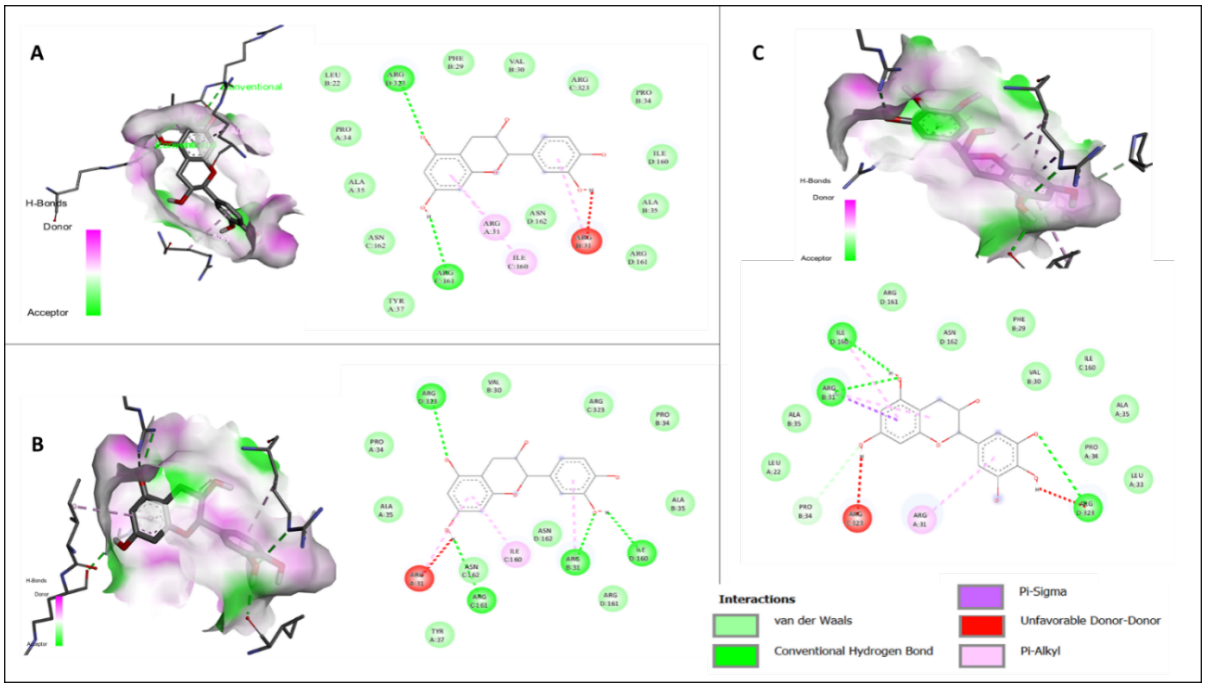

Figure 2. The 2D and 3D binding interactions of catechin (A), epicatechin (B) and gallocatechin (C) against myeloperoxidase (PDB ID: 1DNU). Green dotted lines denote hydrogen bonds between compounds and amino acids, whereas pink lines denote hydrophobic interactions.

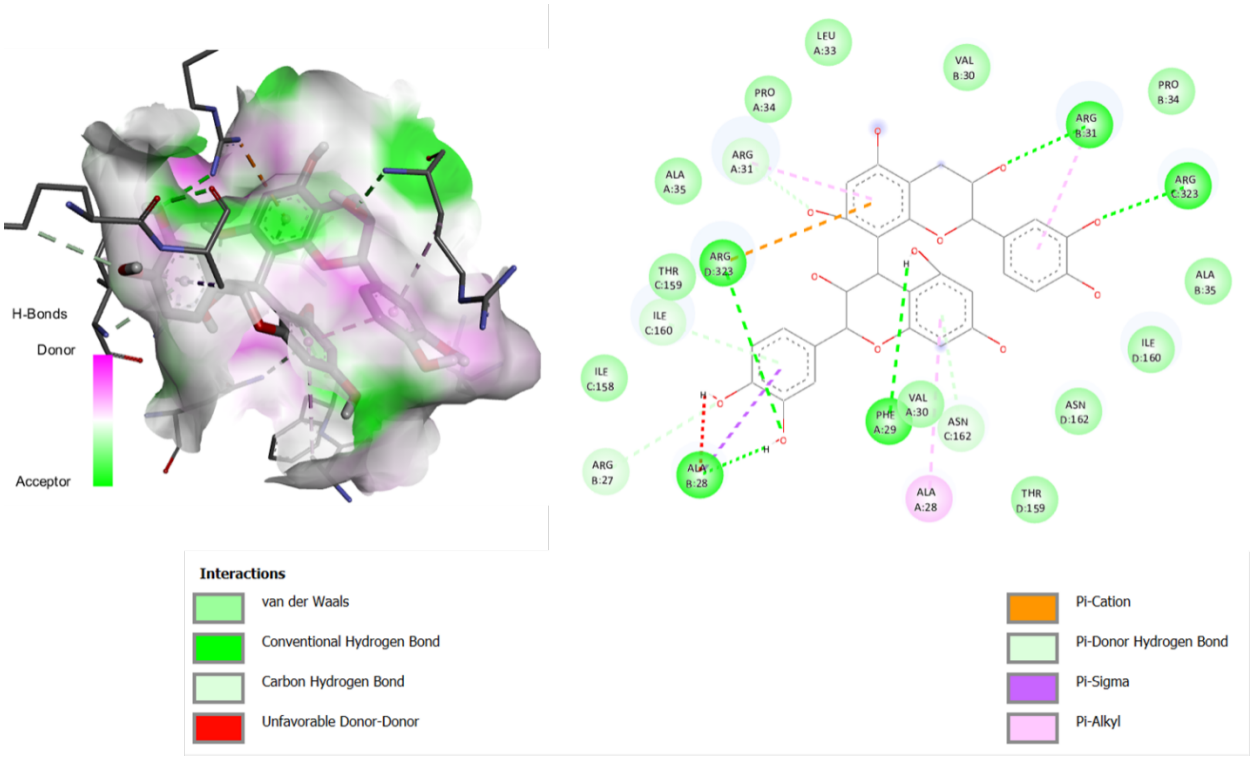

Figure 3. The 2D and 3D binding interactions of main B-type procyanidins (B1-4) in the active site of myeloperoxidase. Green dashed lines represent hydrogen bonds between compounds and amino acids, whereas pink/purple lines represent hydrophobic interactions. Electrostatic interactions are shown as orange lines.

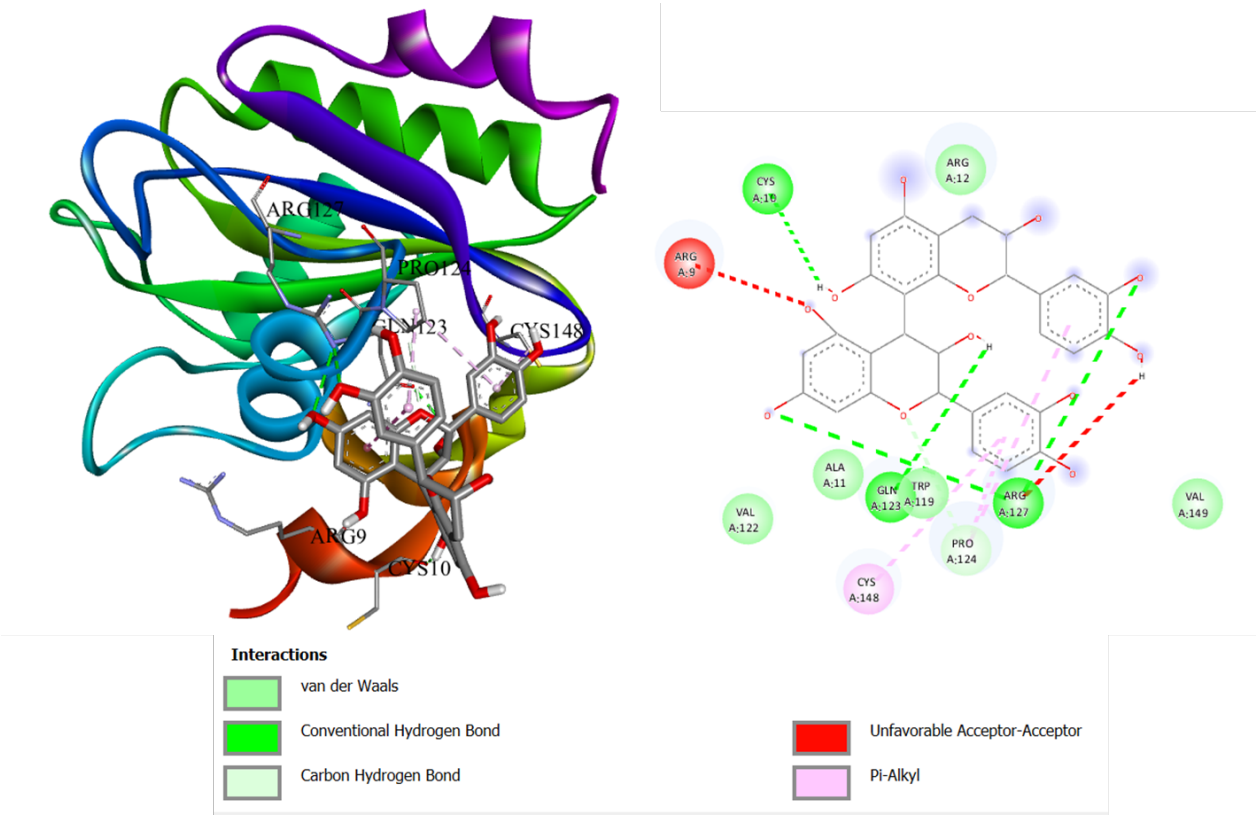

Figure 4. The 2D and 3D binding interactions of main B-type procyanidins (B1-4) in the active site of glutathione peroxidase. Green dashed lines represent hydrogen bonds between compounds and amino acids, whereas pink lines represent hydrophobic interactions.

Acute mesenteric ischemia (AMI) is a chronic or emergency vascular pathology of the small or large intestine causing an acute abdomen. Due to challenges and delays in diagnosis, it has a significant mortality rate despite medical and surgical treatment [13]. Intestinal ischemia injury arises as a result of unmet tissue metabolic demand due to vascular insufficiency and the inability to clear waste metabolites from the tissue. Ischemia leads to acute cell enlargement, interstitial edema, cell dysfunction and ultimately cell death [14]. Restoration of blood flow, or reperfusion, is essential for the repair of intestinal tissue damaged by ischemia. Although beneficial, reperfusion of an ischemic organ or tissue can lead to the development of cellular damage [15]. Prevention of I/R injury during surgery is crucial as it is a complex procedure including multiple intracellular signaling pathways, cell mediators, pathological and physiological issues [16]. Reactive oxygen species are the main cause of tissue damage caused by I/R [17, 18]. Therefore, treatment approaches aim to prevent free radical formation or to remove existing free radicals to reduce free radical damage. Membrane damage, DNA degradation, protease activation, lipid and protein peroxidation, and cell death leading to apoptosis and necrosis are effects of reactive oxygen species [19].

The cytokines TNF-α and IL-1β, produced by macrophages, are critical in controlling inflammation and the inflammatory response [20]. In previous studies, it was found that rats receiving I/R showed a greater inflammatory response [21]. It has been reported that proinflammatory cytokines (TNF-α and IL-1β) weakening intestinal barrier function, disrupting intestinal continuity and altering mucus production may be among the causes of this response [21]. In our investigation, rats given I/R had considerably greater amounts of TNF-α and IL-1β than the IR/GS and control groups. The fact that the addition of GS prevented these high levels relative to the IR group suggests that GS prevents neutrophil infiltration and activation, which are factors in tissue damage and the inflammatory response.

Specific chemicals such as luminol or lucigenin react with ROS in the chemiluminescence (CL) process to generate light [22]. For highly sensitive in vitro monitoring of ROS generation, CL represents a well-established principle. CL can theoretically be used to measure ROS generation since light emission can be seen continuously [22, 23]. Luminol and lucigenin are the two most commonly used CL enhancers in studies [24]. It is generally accepted that lucigenin is more sensitive to superoxide, while luminol is mainly reactive with hydrogen peroxide [25]. A wide range of ROS-related studies use the bivalent hydrophilic fluorescent cation lucigenin (N,N'-dimethyldiacridinium) as a standard CL probe [24-26]. Lucigenin is monovalently reduced to

the corresponding radicals. As a result, dioxetane is formed. The dioxetane then splits into two acridone molecules. One of the acridone molecules released is electronically excited and releases a photon with a blue-green spectral range [27]. It is important to remember that lucigenin itself does not generate chemiluminescence with reactive oxygen species [25]. Therefore, the reduction of lucigenin to the CL-producing substance most likely occurs only intracellularly in the in vivo system [26]. As a result, lucigenin is extremely susceptible to ROS being produced in cells.

The same chemiluminescence mechanism was used in this study. Consequently, it was observed that the increase in luminol and lucigenin chemiluminescence levels in group I/R-GSE ileal tissue was greatly reduced. The findings of this study demonstrated that GSE improved GSH levels in rat small intestinal tissue and protected the gut from oxidative damage. Activation and invasion of neutrophils are strongly influenced by pro-inflammatory cytokines. The contribution of neutrophils to tissue damage during reperfusion is described by the activity of the neutrophil-specific enzyme MPO [27]. Therefore, the presence of MPO in the small intestine is a sign of acute inflammation and neutrophil infiltration. The enzyme MPO released by neutrophils accelerates tissue damage and overproduces reactive species. In our study, GSE administration reduced both neutrophil activation, which initiates the oxidative pathway leading to gut injury, and increased levels of MPO.

Reactive species have both positive effects on the organism and adverse consequences when they are present in excess in cell components such as lipids [28]. Lipid hydroperoxides are formed when these dangerous species interact with the double bonds of polyunsaturated fatty acids [28]. MDA is a secondary oxidation product of polyunsaturated fatty acids and is high in many diseases thought to be related to reactive oxygen species injury [28]. Therefore, it is often used as a marker for injuries. Our research showed that GS extract inhibits the rise in MDA levels, a factor in intestinal damage in I/R and a key marker of lipid peroxidation.

Grape seed extract has been shown to be beneficial in a number of cardiovascular, cerebrovascular and neurological disorders due to the compounds with antioxidant properties [29]. Resveratrol, an important phenolic compound found in GS extract, is known to significantly improve subacute intestinal I/R injury with reduced nitric oxide production as well as iNOS expression when administered intravenously at low doses [30]. In addition, resveratrol has been shown to have a protective effect against oxidative damage caused by the model of testicular torsion/detorsion in rats (31).

Resveratrol has been found to protect against oxidative stress in the liver, spleen, and heart by preventing lipid peroxidation and DNA damage caused by excessive ROS formation [31]. On the other hand, the procyanidins in grape seed extract belong to the flavonoids, a special class of polyphenolic compounds [29]. High molecular weight polymers known as procyanidins consist of the monomeric unit flavan-3-ol (+) catechin and (-) epicatechin) [29]. The most typical dimers discovered in *Vitis vinifera* are the procyanidins B1–B4 (32).

Procyanidins have a wide range of biological, pharmacological and therapeutic properties [32]. In addition to anti-cancer, anti-viral, anti-allergic, anti-inflammatory, cardioprotective, immune-stimulating, estrogenic, antibacterial and free-radical scavenging properties, proanthocyanidins also block the enzymes lipoxygenase and phospholipase A2 [33]. The molecular properties of procyanidins, in particular the presence of phenolic hydrogen atoms, which act as quenchers of singlet oxygen and scavengers of hydrogen donor radicals, are the reason for their antioxidant effects [32].

In addition, the procyanidins B1-B4 in grape seed extract are identified by the C4-C8 bond. Previous studies have shown that dimeric procyanidins with the C4-C8 bond have higher free radical scavenging activity than those with the C4-C8 bond [32]. B-type procyanidins showed the highest binding affinity (-10.8 kcal/mol for myeloperoxidase and -7.6 kcal/mol for glutathione peroxidase) of all compounds. As a result, there is a promising correlation between our in silico results and previous experimental studies in the literature.

The molecular structures of resveratrol, flavan-3-ols and their gallate esters contain a significant amount of hydroxyl groups. Therefore, as can be seen from the interaction schemes of the myeloperoxidase active site, they normally interact via hydrogen bonds. found that all compounds play a crucial role for the amino acid residue ARG323 and that the molecules interact through this residue via hydrogen bonding. In addition, we found that flavan-3-ols bind to the active site of the enzyme through amino acid residues ARG31, ILE160, and ILE161 through hydrogen bonding, pi-alkyl, and pi-sigma interactions.

Gallate esters interact with the amino acid residue CYS153 in the enzyme's active site through hydrogen bonding and electrostatic pi-sulfur bonding, in addition to interactions with flavan-3-ols. Furthermore, we show that the procyanidins with the highest docking values frequently interact with amino acid residue ALA28 through hydrogen bonding, electrostatic interactions, and hydrophobic forces. On the other hand, B-

type procyanidins showed the highest binding affinity in the glutathione peroxidase active pocket as in myeloperoxidase and interacted mainly with CYS10, GLN123, ARG127 amino acid residues through hydrogen bonds due to hydroxyl groups in their molecular structure. In addition, B-type procyanidins interacted with the amino acids PRO124 and CYS148 in the active site through pi-alkyl bonds.

In our study, I/R-induced mesenteric damage dramatically increased plasma levels of TNF-α and IL-1β, which was supported by biochemical studies. As a result of GS extract treatment, it was observed that reperfusion-induced oxidative damage and inflammatory parameters in mesenteric tissue were reversed and protection against oxidative damage was provided.

3. MATERIALS AND METHODS

3.1 Experimental animals

A total of 24 Wistar albino rats were used in the study. The rats were divided into 3 groups, each with 8 animals between 200 and 250 g. After shaving the anterior abdominal wall, Betadine was used to disinfect the skin before a midline incision was made to expose the anterior abdominal wall and access the abdomen. Grape seed extract (Solgar, 100 mg) used in this study was obtained from *Vitis vinifera* and contains 95% oligomeric proanthocyanidins and monomeric polyphenols (catechin, epicatechin, epigallocatechin). 100 mg grape seed extract capsule was suspended in 5 ml water and 1 ml was given per rat. Grape seed extract was suspended in water and administered to the I/R -GS group 15 days before I/R and repeated orally. None of the animals died during these procedures. There were three main groups in this study, each containing 8 rats:

1) Group 1 (Control group): Without performing an additional operation, 2 ml heart blood and small intestine tissue samples were taken after 60 minutes.

2) Group 2 (I/R group): Mice underwent ischemia and exploration of the superior mesenteric artery for 45 minutes before undergoing reperfusion. 60 minutes after reperfusion, 2 ml of blood was taken from the heart and tissue samples from the small intestine.

3) Group 3 (I/R-GS): Rats received 100 mg/kg GS extract orally 15 days before mesenteric ischemia and 30 minutes before ischemia. After 60 minutes of reperfusion, tissue samples were taken from the small intestine and 2 ml of blood from the heart.

3.2 Serum examinations

Plasma levels of interleukin (IL)-1β and tumor necrosis factor alpha (TNF-α) were measured using rat cytokine-specific enzyme-linked immunosorbent assay (ELISA) kits (Biosource International, Nivelles, Belgium).

3.3 Tissue examinations

3.3.1 Chemiluminescence assay

Luminol and lucigenin are important biomarkers for evaluating the effect of reactive oxygen species. Chemiluminescence levels of these markers were also evaluated in mesenteric I/R. Measurements were performed first in the I/R group and then in the I/R-GS group. At room temperature, measurements were taken with a Junior LB 9509 luminometer (EG&G Berthold, Germany). Samples were placed in vials with 0.5 M PBS containing 20 mM HEPES as a buffer solution, with a pH of 7.2. Following the addition of enhancers, lucigenin or luminol, at a final concentration of 0.2 mM, ROS were quantitated. While lucigenin is selective for oxygen free radicals, luminol detects a group of reactive species, including OH, H_2O_2, and HOCl radicals. Counts were made once per minute within the 5 min counting period. Wet tissue weights were then adjusted and relative light units (rlu/mg tissue) were used to express the counts.

3.3.2 Malondialdehyde and glutathione assays

GSH and MDA levels in the tissue samples were measured by homogenization with ice-cold 150 mM KCl. In accordance with a previously reported approach [34] utilizing the formation of thiobarbituric acid-

reactive compounds, the existence of lipid peroxidation was checked at the MDA levels. Lipid peroxidation was calculated as nmol MDA/g tissue and presented as MDA equivalents using an absorbance value of 1.56 x 10^5 M^{-1} cm^{-1}.

A modified version of Ellman's technique was used to measure GSH [35]. Briefly, 2 mL of 0.3 mol/L $Na_2HPO_4.2H_2O$ solution was added to 0.5 mL of supernatant after centrifugation at 2000 g for 10 min. Immediately after the addition of 0.2 mL Elman's reagent, measurements were made of the absorbance at 412 nm. A 1.36 x 10^5 M^{-1} cm^{-1} extinction coefficient was used to calculate the GSH level. The units of measurement were mol GSH/g tissue.

3.3.3 MPO activity

Using techniques that Hillegass et al. had previously described, MPO activity was assessed in intestinal tissues [36]. The pellets were suspended in 50 mM potassium phosphate buffer that contained 0.5% HETAB (hexadecyltrimethylammonium bromide) after the tissue samples had been homogenized in the potassium phosphate buffer. Samples were centrifuged at 41,400 x g for 10 minutes after three freeze-thaw cycles with sonication in between. Then 2.3 mL of the reaction mixture comprising 50 mM PB, 20 mM H2O2 solution and o-dianisidine were obtained in 0.3 mL aliquots. The amount of MPO required to produce an absorbance change measured at 460 nm for three minutes was considered as a unit of enzyme activity. MPO activity was expressed in U/g tissue.

3.4 Molecular docking study for peroxidases

Before the molecular docking process, the 2D structures of grape seed flavan-3-ols and their gallate esters; (+)-Catechin, (+)-gallocatechin, (-)-epicatechin, (+)-gallocatechin-3-gallate and (-)-epicatechin-3-gallate as well as B-type dimeric procyanidins (procyanidins B1-B4) were downloaded from PubChem database and converted to mol2 file format using Open Babel 3.1 program.

The X-ray crystallographic protein structure of myeloperoxidase enzyme (PDB: 1DNU) and phospholipid hydroperoxide glutathione peroxidase 4 (GPx4) (PDB: 5L71) were retrieved from the Protein Data Bank (PDB). Protein preparation was performed according to established worldwide methodology and practice [37]. Using AutoDock 4.6 software, the protein was constructed by introducing polar hydrogen atoms after previously bound ligands were eliminated. Prior to docking, certain cofactors and water molecules were chosen and eliminated. After refinement of the structure, they have been converted to the PDBQT file format for docking. A central grid box of size 60 × 40 × 60˚A pointing to x, y and z coordinates respectively was created and used as such for all molecular docking simulations.

In order for ligands to be energetically favorable and to determine the correct arrangement of molecules in space, the energy must be minimized. For the minimization process, the AutoDock software uses Lamarckian genetic algorithm [38]. A conformal cluster analysis was performed, which allowed a root mean square deviation (RMSD) of 2.0 Å in the docked results. During docking process, eight different conformations were created for each ligand molecule. Discovery Studio 3.0 software was used to select conformations with the lowest and most favorable free energy of binding to assess target protein-ligand interactions. The binding energies (ΔG) of the studied compounds were calculated. The binding sites, hydrogen bonds, electrostatic and hydrophobic interactions of the ligands are illustrated using different colors, bars, stripes and lines.

3.5 Statistical Analysis

Results were generated using GraphPad Prism 9.0 (GraphPad Software, San Diego, CA, USA). For the comparison of data, analysis of variance (ANOVA) and Tukey's multiple comparison tests were employed. The mean ± standard deviation (SD) were used to express all data. Statistics were deemed to be significant at a p-value of <0.05.

4. CONCLUSION

The I/R-induced impairment was greatly reduced by the administration of GS extract, which also dramatically reduced the I/R-induced increases in mesenteric lipid peroxidation, MPO activity and serum

cytokines, while at the same time replenishing the reduced levels of GSH. Grape seed extract has activities such as inhibiting neutrophil infiltration, stabilizing the oxidant-antioxidant status and regulating the formation of inflammatory mediators. Therefore, it shows protective effect against reperfusion-induced oxidative damage. This implies a potential role in the future management of ischemia-reperfusion-induced organ failure. Molecular in silico analyzes used in the study demonstrated the effects of resveratrol, flavan-3-ols and procyanidins in grape seed extract and the data collected supports the experimental results.

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Conflict of interest statement: The authors declared no conflict of interest.

Ethical Approval: The study was approved by the Marmara University, School of Medicine, Animal Care and Use Committee (65.2018.mar).

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