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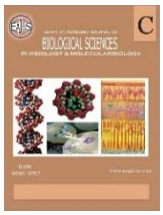

Protective Effect of Galangal Root Oil Against H_2O_2-Induced Oxidative Stress and Tnfα Activation in Rat Liver

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ABSTRACT

Galangal root oil (GRO) is an essential oil that is used in herbal medicine to overcome certain illness symptoms. The study aimed to evaluate the protective effect of GRO against oxidative stress induced by H2O2 in a rat model. 24 adult male albino rats (180-200g BW) were randomly divided into four groups (n= 6): control (0.1 normal saline.), oxidative stress (1% H2O2 in drinking water, (GRO0.1ml/rat orally), and finally GRO+H_2O_2 (GRO 0.1ml + 1%H2O2). The experiment lasted 30 days. liver tissues were collected for biochemical analysis (GSH-Px, MDA, and 8-OHdG), TNFα gene expression and histopathological and immunohistochemical (IHC) evaluation. Rats exposed to H_2O_2 showed significantly increased oxidative stress markers and decreased antioxidant activity, accompanied by marked cytological and pathological changes and strong TNFα expression. GRO supplementation alon leads to increase cell proliferation, reduced oxidative DNA damage and attenuated lipid peroxidation. Co-administration of GRO with H2O2 partially restored oxidative stress. toward control values and alleviated liver tissue damage. In conclusion, GRO exhibits protective effect against H2O2 induced oxidative stress and hepatotoxicity, potentially via modulation of TNFα activity.

INTRODUCTION

Stress triggered by intense stimuli is a state of disrupted homeostasis caused by a behavioural, environmental, or biological threat., The ability to adapt to such stress is an essential indicator of health or disease status(Li *et al.,* 2015). Reactive oxygen species (ROS) normally exist in cells to perform essential biological functions. Excessive production **of** reactive oxygen species (ROS) and reactive nitrogen species (RNS) can trigger cellular damage, a phenomenon known as oxidative stress. Under normal physiological conditions, ROS synthesis is tightly regulated in various cells and cellular compartments. (Singal *et al.,* 2011). Oxidative/nitrosative stress develops once the body fails to be able to produce and get rid of reactive oxygen and nitrogen species while doing so, and when cells produce less antioxidants (Skat-Rørdam *et al.,* 2025). Although often harmful, mild oxidative stress can play a beneficial role in certain physiological events. such as exercise, ischemia, and apoptosis during parturition (Zhang *et al.,* 2011). The liver's tissue is highly susceptible to oxidative damage. Parenchymal cells are the major cells in the liver that may be susceptible to oxidative stress-triggered impairment. The production of ROS by mitochondria, microsomes, and peroxisomes in parenchymal cells regulates PPARα, a gene associated with liver fatty acid oxidation.

Other liver cell types, including Kupffer cells, hepatic stellate cells, and endothelial cells might have been more at risk or prone to materials linked to oxidative damage (Abd-Eldayem *et al.,* 2025). Oxidative stress can induceTNF-α in Kupffer cells, leading to inflammation and apoptosis. In this instance of hepatic stellate cells, lipid peroxidation brought about by oxidative stress enhances a proliferation and production of collagen (Chen *et al.,* 2020). Cells have established a variety of safeguards to mitigate cellular harm to avoid disease (Shanmin e*t al.,* 2020). TNF alpha is a key cytokine involved in liver injury and repair, capable of inducing antioxidant and cytoprotective genes (Jing *et al.,* 2018). In its quiet state, TNF alpha is stored in the cytosol by gene expression in an intricate manner impacted upon the presence of ROS or positively charged stress, resulting in TNF alpha splitting and subsequently nuclear translocation that links the antioxidant response element (ARE) and promotes the transcription of antioxidant genes, such as heme oxygenase (HO)-1, superoxide dismutase (SOD), and catalase (Lopetuso *et al.,* 2018). Numerous research investigations have proven that naturally occurring compounds may serve as potential therapies for both avoiding and curing oxidative stress alongside its outcomes. Galangin (Gal), a natural flavonoid discovered in honey and the roots of Alpinia galngal, possesses several prospective beneficial properties, involving antioxidant, anti-inflammatory, and hepatoprotective signatures (Aladaileh *et al.,* 2021). Using a rat liver disorder model, GRO mitigated oxidative stress and saved the proliferation of hepatic stellate cells. The newest research revealed that GRO may suppress cyclophosphamide (CP)-induced oxidative stress, inflammation, and death in rat liver by stimulating unique pathways of signaling. Despite these promising effects, the protective potential of Galangal root oil (GRO) against oxidative liver injury and its underlying mechanisms remain unclear. Therefore, this study aimed to evaluate the hepatoprotective effects of GRO in H2O2-induced oxidative stress in rats and to explore the involvement of TNFα signaling.

MATERIALS AND METHODS

Animal and Sampling:

The project was authorized by ethical committee of Veterinary Medicine College of university of Mosul under reference number UM.VET.2024.148. Throughout the study, 24 adult male albino rats (180-200g BW) were assigned into four equivalent groups: the placebo group (0.1 N.S.), induced oxidative stress group (1% H2O2 daily with drinking water change twice a day), Galangal Root Oil -(Alpinia galangal)- essential oil(100% Pure Natural ;sheer essence company, USA) Galangal root oil (GRO) group (0.1ml/rat orally), and finally GRO 0.1ml + H2O2 group. The trial lasted 30 days. The animals were maintained in a laboratory animal house and research unit at the University of Mosul's College of Veterinary Medicine. They had been kept in plastic cages with a 12/12 light/dark cycle, humidity of 55%±5%, and free access to pellets and water. At the final stage of the trial, rats were euthanized by a cervical dislocation under anesthesia (a ketamine100 mg/kg+ xylazine mixture 20 mg/kg, respectively, i.m.) (Matsubara & Silva-Santos, 2024). The livers were taken apart, rinsed with cold PBS, and blotted dry to remove residual PBS prior being split into two separate samples (one for biochemical and gene expression analysis and the other for histopathology study).

Biochemical Analysis:

8-OHdG, and GSH-Px levels in liver homogenates have been measured using ELISA kits (Shanghai Ideal Medical Technology Co., Shanghai, China) using the directions given by the manufacturer. Simply rinse tissues in ice-cold PBS (pH 7.4) to flush out any excess blood and weighed prior processing. Tissues ought to have chopped and homogenized in PBS (500mg tissue: 1.5mL PBS) by a bead's homogenizer. Homogenates have been frozen and thawed to further break down the cells. The

homogenates are then centrifuged for 15 minutes at 12,000 RPM at 4°C to extract the supernatant. Transfer 100 µl of every single standard and sample inside the referring wells. Seal well and incubate for 2.5 hours at room temperature (4°C) with gentle shaking. The mixture was thrown away afterwards rinsed four times with 1X Wash Solution. Following a final rinse, eliminate any leftover solution by aspirating or decanting. Invert the plate and blot with clean paper towels. Add 100 µl of Biotinylated TNF alpha Detection Antibody to each well. Incubate for 1 hour at room temperature, gently stirring. 1X HRP-Streptavidin solution was added at 100 µl to each well. Incubation for 45 minutes at room temperature, softly agitating was done. Remove the solution. Resume the wash and add 100 µl of TMB One-Step Substrate Reagent to each well. Incubate at room temperature for 30 minutes in the dark, shaking gently. Finally, 50 µL of Stop Solution was put into each well. The wells were promptly read at 450 nm.

MDA has been estimated via a colorimetric technique (Mateos *et al.,* 2005) as described in the Rat MDA kit (Shanghai Ideal Medical Technology Co., Shanghai, China). In short, after generating standard concentrations, 50 ul of standards was added to the chosen wells. After that, add 40 ul of sample diluent to all wells having only samples, then add 10 ul of samples to each well, covering, and incubate at 37°C for 40 minutes. Following the incubation period rinse the wells at least five times with washing solution. HRP was added at 50 ul to all wells excepting the blank (standard tube 0) and was covered and incubated at 37°C for 40 minutes. The washing procedure was repeated. All wells received 50 ul of Chromogen A, followed by 50 ul of Chromogen B, and incubated for 20 minutes. Finally, 50 ul of Stop solution was applied to each well 10 and the plate was promptly scanned at 450 nm.

Gene Expression:

Frozen specimens of liver have been sliced into tiny pieces to yield 100mg for total RNA, isolation using the (Addbio RNA Isolation kit ,Korea) according to the guidance. The RNA that was collected was subsequently tested for purity and concentration using a nanodrop instrument before being transformed to cDNA with an addbio RT reverse transcriptase kit and a thermal cycler in accordance with the attached protocol was run out. Running Rt-PCR for TNFα expression using specific primer (antisense TGGGCTCCCTCTCATC AGTTCC, sense CCTCCGCTTGGTGGTT TGCTAC, NM_012675.3) at 20ul total volume of PCR reaction containing 10ul syber green master mix, 0.5ul of both reverse and Forward primer, 2ul templet and 7ul RNA free PCR water. The relative expression of TNFα gene was normalized to the housekeeping gene actin beta (Actb) (antisense GGTCAGGTCATCACTATCGG CAATG, sense CAGCACTGTGTTGGCAT AGAGGTC, NM_031144.3) using the 2^−ΔΔCT method (Younis *et al.,* 2024).

Histopathological and in Situ Detection of TNFα:

Before beginning the process of preparing histology slides, liver samples had been fixed in 10% neutral buffer formalin for 48 hours. Specimens were fixed in paraffin, and slices of 5 µm were produced and stained with H&E. Hepatic tissue slides have been studied under a light microscope for histopathological investigation. Tissue samples were analyzed at magnifications of 20× and 40× and photographed using an Olympus microscope (model: BX43F Japan). The expression of TNF immunostaining is an approach used for identifying and illustrating TNF-alpha, a key proinflammatory cytokine, in tissue samples. It implies using antibodies that exclusively interact with TNF-α prior to exploring the attached antibody using a quantifiable marker, such as a colored stain or fluorescent label. Hepatic tissue sections (6 um thick) of each waxed block have been deparaffinized via xylene, hydrated in ethanol and phosphate-buffered saline (PBS, pH 7.4), followed by treatment using 0.3% hydrogen peroxide in anhydrous methanol for 60 min. To stop nonspecific antibody binding, trypsin-treated sections were incubated with rabbit serum for 20 minutes (Abdullah *et al.,*

2025). The sections were stained with primary antibodies using the avidin-biotin peroxidase complex (Swanson *et al.,* 1987). The anti-TNF-α antibody had been diluted 1:50 in PBS (pH 7.2) (Rabbit IgG poly clonal antibody kit, Elabscience, USA, catalog No. E-AB-33121). For optimal specificity, sections were treated with mouse serum instead of the primary according to manufacturer instructions. Following moderate contrasting with hematoxylin, samples were mounted and TNF-α immunohistochemistry was evaluated in relation to controls, hematoxylin and eosin sequential sections (Dong *et al.,* 2016 a).

Histological Scoring:

Histological scoring is the conversion of informal histological variations into numerical information for statistical evaluation. Table 1 highlights the key parts of the usual histological process that were investigated. A histologist evaluated the immune-stained sections without knowing the identities of the groups involved. To determine TNF alpha reactivity in hepatic sections, slides were evaluated at two powers (×40 and ×100) in the cytoplasm and nucleus. Five randomly chosen sites were assessed, and sections with heavy stains were given a random field. Slides have been evaluated analytically according to luminance and cell staining % at every point. The degree of intensity was assigned scores as "0" for no clarity, "1+" for fair staining, "2+" for acceptable staining, "3+" for highly intense staining, and "4+" for exceedingly powerful staining. The percentage of cells staining positive was separated into 10% groups. (Avallone *et al.,* 2021).

Table 1: Grading technique for immunohistochemistry expression in hepatic rat tissue sections

Criteria	-	+	++	+++	++++
TNFα	Negative -0 cells/field	Weak + 1–10 cells/field	Positive ++ 11–20 cells/field	Positive +++ 21–50 cells/field	Positive ++++ More than 50 cells/field

(Yang *et al.,* 2017)

Statical Analysis:

All parametric data were expressed as mean ±SE and analysed using Spss v.26, USA by ANOVA one-way test followed by LSD test at p value less than 0.05 that considered significant. While for nonparametric data Kruskal-Wallis Test was performed.

RESULTS

Effect of Galangal Root Oil (GRO) Supplementation on Liver Oxidative and Antioxidant Biomarkers:

Findings indicated that H2O2 usage had an adverse influence on GSH-Px in causing oxidative stress, which had a significant decline in value than those of the control and GRO groups (p =0.034, respectively). Moreover, GRO+H2O2 treatment leads to significantly low levels of GSH-Px than in control rats, although GRO alone remained constant at p = 0.06, as seen in Figure 1.

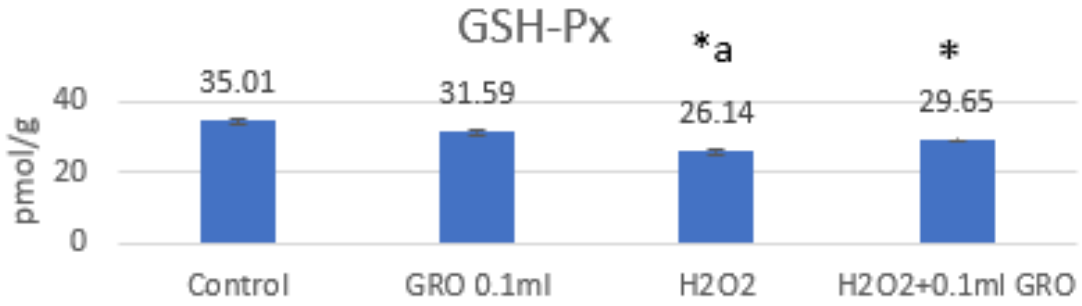

Fig. 1: Effect of GRO on GSH-Px levels in induced oxidative stress in hepatic tissues of rats

The oxidative DNA damage enzyme 8-OHdG assay in hepatocellular homogenates from experimental rats displayed significant increase in both the H2O2 and GRO+H2O2 groups as compared with the control and GRO groups (p = 0.001). Meanwhile, the GRO+H2O2 group had a significant drop in 8-OHdG level than the H2O2 group, as seen in Figure 2.

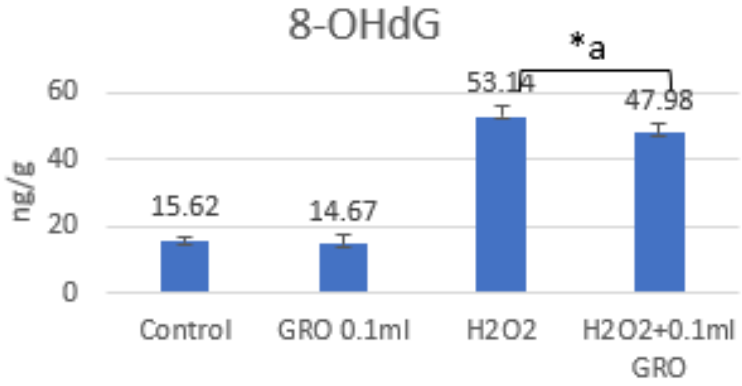

Fig. 2: Effect of GRO on 8-OHdG levels in induced oxidative stress in hepatic tissues of rats

MDA levels were significantly increased was clearly determined in the H2O2 and GRO+H2O2 groups in the hepatic homogenates when compared to those of the control and GRO rats' groups, each, (p=0.034), and supplying GRO with H2O2 exhibited a significant reduction in MDA value relative to the H2O2 group (p=0.042) (Fig. 3)

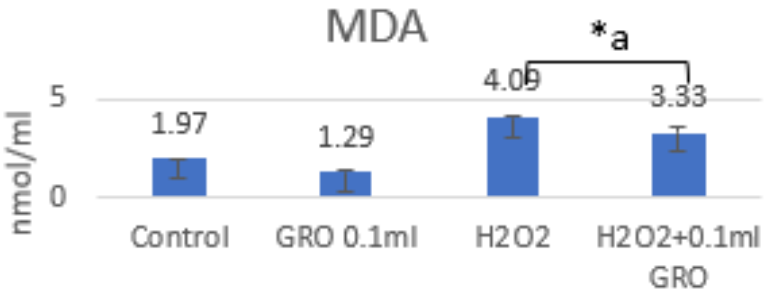

Fig. 3: Effect of GRO on MDA tissue level in induced oxidative stress in hepatic tissues of rats

Effect of GRO Supplementation on TNFα mRNA Expression in Rats' Liver Tissue Exposed to Oxidative Stress:

A production of inflammatory factors is a pathological hallmark of hepatic damage. H2O2 administration upregulated the mRNA and protein expression of proinflammatory cytokines TNF-α, but GRO intake significantly decreased their expression. TNFα gene expression study demonstrates an upregulation of proinflammatory proteins in liver cells in both H2O2 and GRO+H2O2 groups as opposed to the control group. At the same time, the GRO group displayed an overexpression of this marker compared to the control animals approximately 2-folds (P=0.037). Giving GRO with H2O2 for 30 days increases TNFα levels compared to the GRO group (p=0.041), as shown in Figure 4

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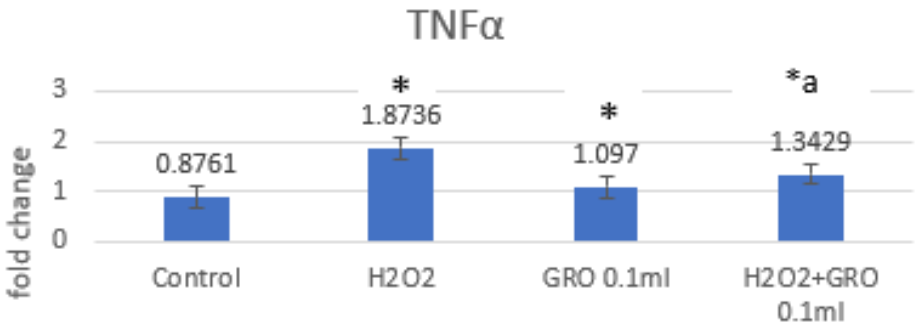

Fig. 4: Effect of GRO supplementation on TNFα mRNA expression in hepatic cells of experimental rats.

Effect of GRO Supplement on Liver Pathological Changes in Stressed Rats:

The histopathological changes that were shown in liver samples of experimental rats varied between mild to severe changes. Microscopy showed that H2O2 exposure induces severe morphological alterations in the liver, including poorly defined hepatocyte borders, cytoplasmic vacuolization, and cellular degeneration. However, GRO supplementation can help to reduce these effects and protect against H2O2 liver injury. Specifically, by monitoring hepatocyte morphological alterations, we discovered that the degree of degeneration and disorder of the cell arrangement was more severe in the H2O2 group, and the intercellular space was larger than in the GRO/ GrO+H2O2 group. Hepatocytes in the GRO+H2O2 group were relatively more compact than those in the H2O2 group, as was also visually apparent. Hemorrhage and moderate fatty change have been noted along with aggregation of mononuclear cells around blood vessels and infiltration of inflammatory cells, primarily macrophages, in the liver parenchyma. Moreover, dilatation of sinusoids were observed in the livers of rats treated with 1% H2O2 for 30 days. Additionally, in-situ expression of TNFα was distinctly observed in the same samples, characterized by intense staining using TNFα antibody. The observed changes were notably diminished in rats administered GRO 0.1 ml orally, exhibiting reduced vacuolation and dilation, despite the presence of inflammatory cell infiltration around the sinusoids and between hepatocytes. TNFα local expression exhibited modest staining in GRO and GRO+H2O2 groups compared to H2O2 liver sections. The GRO supplement stimulates hepatocyte proliferation and increases TNFα expression, as indicated by positive staining Figure 5.

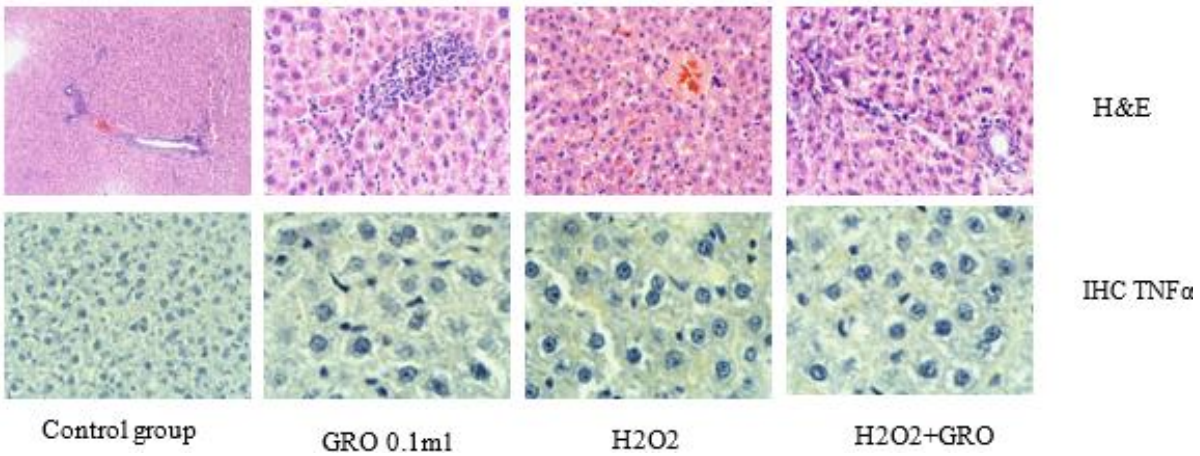

Fig. 5: liver sections showed aggregation of inflammatory cells and cytoplasmic vacuolations in hepatocyte exposed to oxidative stress with high intense TNFα staining. GRO with /without H2O2 lead to decrease these changes.

DISCUSSION

H2O2 1% via oral administration induces severe liver damage due to oxidative stress, lipid peroxidation, and inflammation triggered by proinflammatory factor upregulation and antioxidant repression (Cichoz-Lach & Michalak, 2014). GRO Galangal root oil (Alpinia galanga) has hepatoprotective properties, such as antioxidant activity and regulation of lipid metabolism (Al-Amarat *et al.,* 2021). As an outcome, we predicted that GRO could offer protection against a damaged liver. H2O2 1% impacts the oxidant-antioxidant equilibrium by diminishing GSH-Px activity while enhancing the activity of 8-OHdG, an oxidative DNA damage enzyme linked in tissue injury and free radical generation. likewise, oxidative stress suppresses the activity of antioxidant enzymes (SOD and GSH-Px) and the expression of antioxidant genes (Nrf2, Ho-1) in the liver, contributing to lipid peroxidation and inflammation (Sadasivam *et al.,* 2022). GRO 0.1ml has antioxidant and hepatoprotective properties by upregulating Nrf2 expression and antioxidant enzyme activity (Aladaileh *et al.,* 2019). This finding is consistent with previous reports showing that GRO reduced hepatic Keap1 and enhanced Nrf2 and HO-1 mRNA expression, leading to increased antioxidant enzyme activity via inhibition NF-κB and Keap1, s. (Kim *et al.,* 2024, Sadasivam *et al.,* 2022). Oxidative stress triggers inflammatory genes and pathways in the liver, leading to the production of proinflammatory cytokines, including TNF-α, which play crucial roles in several cellular processes (Dong *et al.,* 2016 b). Oxidative stress causes Kupffer cells in the liver to secrete proinflammatory cytokines due to increase intestinal permeability to harmful proteins; the permeability of the intestinal barrier is identified as "leaky gut" or "intestinal wall leakage illness (Abdel Moneim *et al.,* 2014;Yu *et al.,* 2022; Huang & Kong, 2021)." The disorder, which primarily arises by oxidative stress, alcohol or ongoing allergen exposure, and dysbiosis, results in the occurrence of several pathologic disorders, including obesity, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and cirrhosis (An *et al.,* 2022; Huang & Kong, 2021). In contrast, Alpinia galanga, which contains flavonoids, increases the expression of intestinal tight junction proteins, which reduces intestinal permeability and inflammation (Murugan *et al.,* 2022).

Hydrogen peroxide absorption was linked to liver oxidative damage, as evidenced by a 4–5-fold increase in 8-OHdG levels, indicating oxidative DNA damage. This feature was caused by endogenous DNA damage, which causes cellular breakdown and functional loss. This result is consistent with (Younis *et al.,* 2024). The pathogenic pathway includes the generation of free radicals, which contribute to the onset of inflammation. These changes were detected in in vitro cell culture of the L02 liver cell line and in vivo in an animal model, as validated by analyses of oxidative stress parameters and apoptotic markers. The free radicals were primarily produced by liver cells, Kupffer cells, and neutrophils. As a result, the use of H2O2 has caused liver damage due to oxidative processes. This opinion is similar to (Valavanidis *et al.,* 2009). The mediated rise of reactive oxygen species (ROS) can lead to significant cellular damage via lipid peroxidation (LPO), protein oxidation, depletion of antioxidants, and DNA damage. LPO can disrupt membrane fluidity and permeability and inactivate membrane-bound proteins, eventually leading to destruction of the membrane (Li Y. *et al.,* 2018), LPO can alter membrane fluidity and permeability, inactivate membrane-bound proteins, and ultimately result in membrane destruction (Sivakumar & Anuradha, 2011). GRO supplementation successfully mitigated oxidative stress-related increases in ROS, LPO, NO, and DNA damage, while enhancing GSH levels and antioxidant enzyme activity. Thus, attenuation of oxidative/nitrative stress and restoration of antioxidant defenses represent a central part

of the hepatoprotective mechanism of GRO (Aladaileh *et al.,* 2019). Thus, GRO's hepatoprotective mechanism involves reducing oxidative/nitrative stress and restoring antioxidant defenses via its composition of flavonoids (Amić *et al.,* 2003).

TNF-alpha has played a key role in inducing and worsening oxidative stress in the liver. It is a pro-inflammatory cytokine that induces oxidative stress by activating NADPH oxidase, which produces more reactive oxygen species (ROS). Oxidative stress, in turn, can intensify the effects of TNF-alpha, resulting in a feedback loop that exacerbates liver injury (Tan *et al.,* 2020). TNF-α has been shown to have both beneficial and detrimental effects, such as in host defense and toxic shock. TNF-α has a dual function in liver injury, causing both aggravation and alleviation, making it difficult to create preventative therapy(Tan *et al.,* 2020;Tiegs & Horst, 2022; Lu *et al.,* 2022). GRO inhibited NF-κB, BAX, and caspase-3, in the liver of rats with H_2O_2-induced oxidative stress. In a similar, GRO inhibited apoptosis in caused liver damage (Li *et al.,* 2018).

Conclusion

This study provides in vivo evidence that oil (GRO) exerts protective effects against -H2O2-induced oxidative damage. GRO inhibited H_2O_2-induced ROS generation, LPO, DNA damage, inflammation, and apoptosis in rats liver. GRO increased antioxidant defenses, activated TNF signaling, and reducing its overexpression under oxidative stress while supporting hepatocyte survival."

Declarations:

Ethical Approval and Consent to Participate: The animal study protocol was approved by the Institutional Animal Ethics Committee (UM.VET.2024.148) of the Veterinary Medicine College of the University of Mosul. All procedures were performed in accordance with the institutional guidelines for animal care and use, and efforts were made to minimize animal suffering and reduce the number of animals used.

Competing interests: The authors have no competing interests to declare that are relevant to the content of this article.

Availability of Data and Materials: All data generated or analyzed during this study are included in this published article.

Authors' Contributions: Authors equally shared in the data collection, wrote, revised the manuscript, and approved its publication.

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