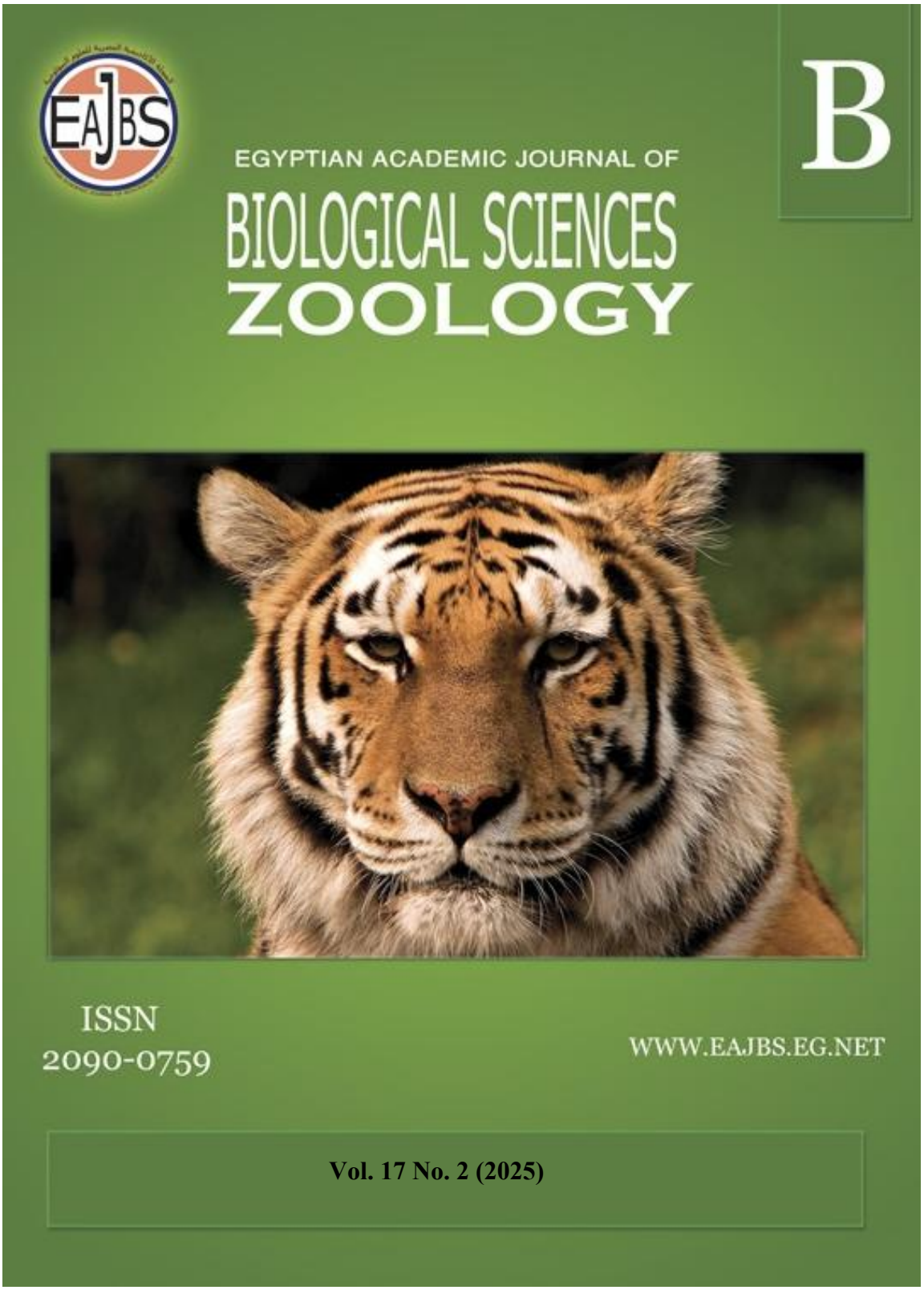

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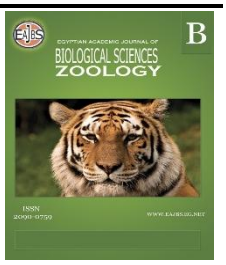

Influence of Galangal Root Oil on Some Heat Shock Proteins and Antioxidant Enzymes in H_2O_2-Induced Hepatic Damage in Rats

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ABSTRACT

The study examined how Galangal root oil affected heat shock proteins (HSPs) and antioxidant enzymes in rats with H_2O_2-induced liver injury. 24 adult male rats 200-230g BW were randomly assigned to four groups: the control group received 0.1 N.S. orally, and three treatment groups: Group 1 (G1) had liver damage from daily 2.5% H_2O_2 in drinking water, with water replaced twice daily after two weeks of acclimation. Group 2 (G2) got 0.2 ml of 100% Pure Therapeutic Grade Galangal Root Oil (GRO) orally each rat. Group 3 (G3) received 0.2 ml GRO/ H_2O_2. The trial lasted 30 days. Molecular analysis was performed on liver samples after the trial. H_2O_2 increased HSPs and decreased antioxidant enzyme expression throughout the research. This impact is enhanced by GRO alone/with H_2O_2. It was found that 2.5% H_2O_2 exposure has serious biological effects, while galangal root oil protects hepatocytes from severe oxidative stress via modifying heat shock protein and antioxidant enzyme regulatory genes.

INTRODUCTION

As the organ responsible for metabolism, detoxification, protein synthesis, and the production of several biochemicals needed for digesting, the liver is vital to life (Kalra *et al.*, 2023). The causes of liver injury include chemical metabolism, autoimmune inflammation, alcohol consumption, and drug intoxication. Slow-moving inflammation is the source of chronic liver disease, while fast inflammation is the cause of acute liver illness (Nishikawa & Osaki, 2015; Almazroo *et al.*, 2017). Because liver injuries impair liver function, normal body physiology is disrupted (Thompson & Karnsakul, 2023). Many hepatotoxic substances, including alcohol, acetaminophen, carbon tetrachloride (CCl_4) bromobenzene, and diethylenetriamine, can harm the liver. The majority of hepatotoxins do not directly harm the liver. Reactive intermediate metabolites and reactive oxygen species, which are created during the metabolism of hepatotoxins, were typically the cause of the noticeable toxicity impact (Ramachandran & Jaeschke, 2018; Francis & Navarro, 2024). When the concentration of these reactive intermediate metabolites and reactive oxygen species surpasses the hepatocytes' capacity to act as antioxidants, the liver is oxidatively damaged (Russmann *et al.*, 2009; Peng *et al.*, 2016). As per a 2016 study, $H_2O_2$was chosen since it is less detrimental to the

environment and one of the reactive oxygen species that can impair hepatocytes through oxidative damage (Liau *et al.*, 2016). Liver problems have been linked to a number of reactive oxygen species (ROS), involving hydrogen peroxide (H_2O_2), superoxide anion (O2-), hydroxyl radical (•OH), and hydroxyl ion (OH-). H_2O_2 was believed to be the primary precursor of reactive free radicals among them. A type of antioxidant enzyme called glutathione peroxidase (GPx) lets cells convert $H_2O_2$to H_2O (Shi *et al*., 2022).When the production of ROS surpasses the antioxidant defenses, it may lead to liver disorders. According to research (Arauz *et al.*, 2016), the primary cause of liver diseases such alcoholic liver disease, non-alcoholic steatohepatitis, viral hepatitis, and hemochromatosis is an increase in H_2O_2 levels.

Alpinia galangal roots are used to extract galangin, a flavonoid and derivative of curcumin. There is growing evidence that autophagy can be induced by galangin (Kong *et al.*, 2019). Because galangal root oil influences the redox state and promotes cellular health, it may be able to mitigate liver damage, especially through its active component, galangin (Emerald, 2024). By increasing liver cell autophagy and promoting lipid metabolism, it helps lessen fatty liver (hepatic steatosis)(Zhang *et al.*, 2020). By raising the activity of antioxidant enzymes and lowering lipid peroxidation, galangal extracts can also guard against oxidative stress, a significant cause of liver damage *(*Fatima *et al., 2023;* Zhou T.*et al*., 2014). Galangal offers a variety of functions, including antioxidant properties, which have been shown to neutralize toxic ROS and protect liver cells from oxidative damage (Al-Mosawy & Al-Salhie, 2021). Galangal can improve hepatocyte autophagy, which is a biological process that prevents damaged components and protects liver cells healthy (Zhang *et al.,* 2020). Investigations demonstrate that galangal may prevent fatty degeneration in the liver, which is a characteristic of NAFLD, as well as enhance liver function in cases of paracetamol-induced liver damage (Priyono *et al.*, 2024). Galangal's active constituents, such as galangin, isorhamnetin, and others, can inhibit the expression of pro-inflammatory cytokines that cause liver inflammation (Sivakumar & Anuradha, 2011). Furthermore, Galangal has demonstrated anti-cancer action against numerous cancer cell lines, including liver cancer, and may be effective in preventing or treating hepatocellular carcinoma (HCC) (Zhou Y.-Q.*et al.*, 2018). So, the aim of the study was to figure out the protective of effect of induced hepatic damaged by H_2O_2 on certain heat shock proteins and antioxidant enzymes.

MATERIALS AND METHODS

Animal Treatment and Sampling:

This investigation employed 24 adult male rats 200-230g BW, conducted at the Laboratory Animal House of Veterinary Medicine College, University of Mosul. All rats were three months old and were housed in separate cages in a room kept at 20-24°C. There was unlimited access to food and drink. The Ethics Committee of the Veterinary Medicine College of the University of Mosul approved all animal-related procedures, which were carried out in compliance with its ethical rules UM.VET.2024.148) by institutional Animal Care and Use Committee of College of Veterinary Medicine, University of Mosul.

The rats were randomly assigned to four groups: the control group received 0.1 N.S. orally, and three treatment groups: G1 induced liver damage by H_2O_2 2.5% daily with drinking water changed twice a day after adaptive feeding for two weeks, G2 received Galangal Root Oil (GRO)-(Alpinia galangal)- Essential Oil 100% Pure Natural Undiluted Uncut Therapeutic Grade Oil (sheer essence company, USA) at dose 0.2ml/rat orally, and finally G3 received GRO 0.2mL H_2O_2 The trial lasted thirty. At the end of the trial, rats were euthanized via cervical dislocation shortly after being drugged with a ketamine/xylazine cocktail (100 and 20 mg/kg, respectively, i.m.) (Matsubara & Silva-Santos, 2024). After disassembling the livers, they were rinsed with cold PBS and dried to remove any remaining PBS before being processed for gene expression and kept in -08ºC until need.

Molecular Study:

Total RNA has been isolated from froze liver specimens that were first homogenized with a tissue lysis buffer and beads raptor homogenizer (100 mg tissues / 1 ml lysis buffer). The Adbio (Korea) total RNA isolation kit was utilized in line with the instructions provided with the product. Following the manufacturer's instructions for obtaining RNA, the thermal scientific Nano drop ONE system (Thermo Fisher, USA) was employed in order to evaluate RNA integrity as the initial stage in the PCR process. Reverse transcriptase (RT) was used for converting the isolated RNA to cDNA, and AddScript Rt Master (Addbio, Korea) was used to create the first and second strands of cDNA in a thermal cycler. This process included priming at 25°C for 10 minutes, reverse transcription at 50°C for 60 minutes, RT inactivation at 80°C for 5 minutes, and holding at 12°C.

The Step-One Applied Biosystems tool system, USA, served as the tool for the RT-PCR stages, which involved further analysing the cDNA samples for the mRNAs of certain heat shock proteins and antioxidant enzymes (Table 1) (Alchalabi, 2019). 25 µl were needed for each reaction, which included 2 µl of cDNA, 1 µl of sense and antisense specific primers each, 7.5 µl of PCR-grade water, and 12.5 µl of Add SYBER Master Addbio, Korea. Amplification was carried out in 40 cycles using the qPCR procedure, which included denaturation (15 seconds at 95°C), annealing (15 seconds at 60°C), and extension (30 seconds at 72°C) after the initial stage of three minutes at 95°C. Following each cycle, fluorescence was measured at 72 °C. A negative control run, in which no cDNA template was used, was part of each experiment. Using the standard methods for each target and monitoring gene, standard curves were created by plotting the Ct values (cycle threshold) against the log cDNA dilution. After PCR, it enabled relative quantification. We calculated the fold change using the 2^-(ΔΔCt) approach after receiving the data as Ct values.

Table 1 The primers sequences used throughout the study.

Gene name	**Forward sequence**	**Reverse sequence**
β-actin*	CAGCCTTCCTTCCTGGGTATG	AGCTCAGTAACAGTCCGCCT
HSP27	GAG GAG CTC ACA GTT AAG ACC AA	TTC ATC CTG CCT TTC TTC GT
HSP70	GGG CTC TGA GGA ACC AGA C	CAG CCA TTG GCG TCT CTC
HSP90α	TTT CGT GCG TGC TCA TTC T	AAG GCA AAG GTT TCG ACC TC
HSP90β	TGG TGG ATC CTT CAC TGT CC	TTT CTT CAC CAC CTC CTT GAC
GRP78	CATGCAGTTGTGACTGTACCAG	CTCTTATCCAGGCCATATGCAA
SOD	TAA GAA ACA TGGCGGTCC A	TGG ACA CAT TGG CCA CAC
CAT	CAG CGA CCA GAT GAA GCA	GGT CAG GAC ATC GGG TTT C
GST	TGT TAC AAC CCC GAC TTT GA	TCT TCT CAG GGA TGG TCT TCA
* β-actin was used as a house-keeping gene		

Statistical Analysis:

Using SPSS V.26 software, four-group comparisons were assessed using a one-way analysis of variance. The data in the text and graphics are presented as mean ± SE. At P value less than 0.05, differences were deemed statistically significant.

RESULTS

Effect of Galangal Root Oil on Certain HSPs mRNA Levels in Experimented Rats Exposed to H_2O_2

During oral H_2O_2 administration, the level of HSP27, HSP70 were considerably raised, and HSP27, HSP70 mRNA levels were over three-folds and 1-fold respectively, higher. In contrast, HSP27 and HSP70 were down-regulated in GRO and GRO+H2O2 liver samples, dropping to H2O2 levels nearly twice as low. nonetheless, up-regulated in contrast to the controls. Although mRNA level of HSP70 in rats subjected to GRO and H2O2+GRO did not

show statistical significance when compared to control rats, as well as mRNA level of HSP27in rats subjected to GRO and H_2O_2+GRO did not show statistical significance when compared together. After 30 days of treatment, HSP27 levels approached each other and HSP70 mRNA levels reached to control value (Figs. 1 &2).

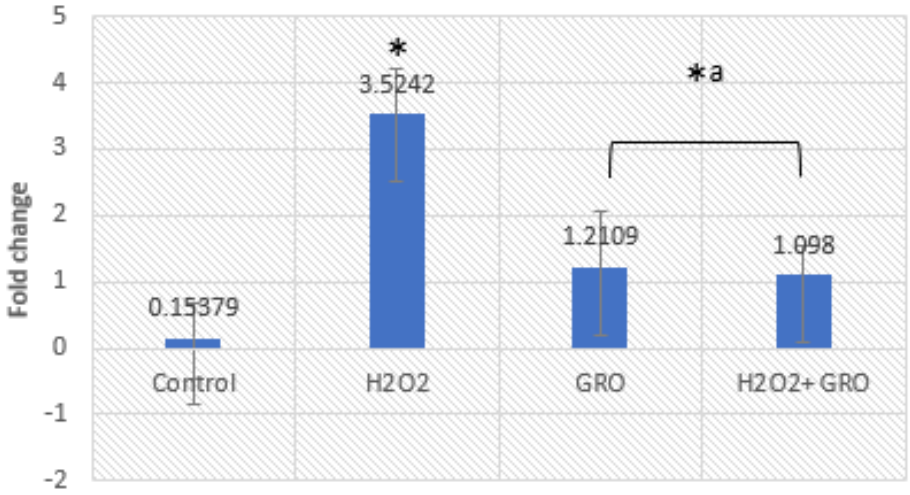

Fig.1: Fold change variation of HSP27 mRNA in liver samples of experimental rats.

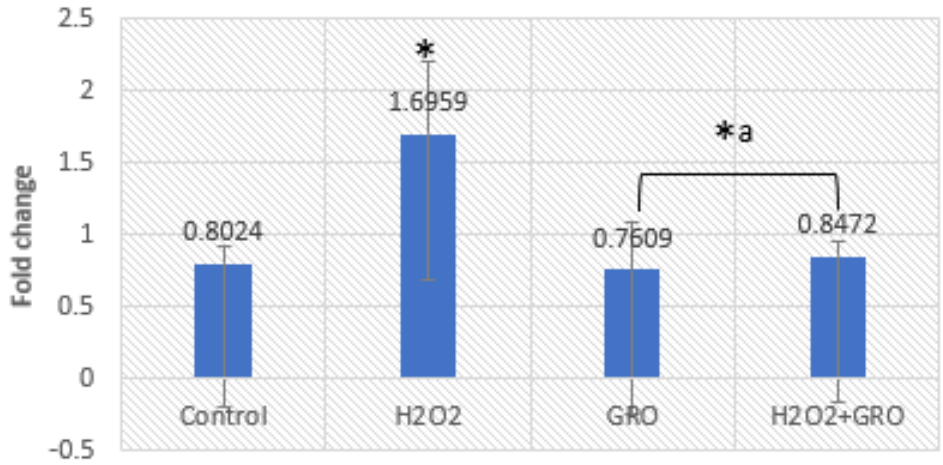

Fig. 2: Fold change variation of HSP70 mRNA in liver samples of experimental rats.

Real-time PCR was used to measure the levels of HSP90α. as depicted in Figure 3. In rats given GRO and H_2O_2+GRO compared to H_2O_2, HSP90α dramatically dropped after increasing by 2.7 times in H_2O_2 liver samples compared to the controls. After 30 days, HSP90α levels returned to the control level. In a comparable way, HSP90β rose up to three times in comparison to the controls following an H_2O_2 exposure group. When compared to H_2O_2, these elevations dramatically diminished in both GRO and H_2O_2+GRO liver samples before returning to control levels by day 30 (Fig. 4). After being exposed to H_2O_2 and receiving GRO treatment, all HSP90 types displayed a similar expression pattern.

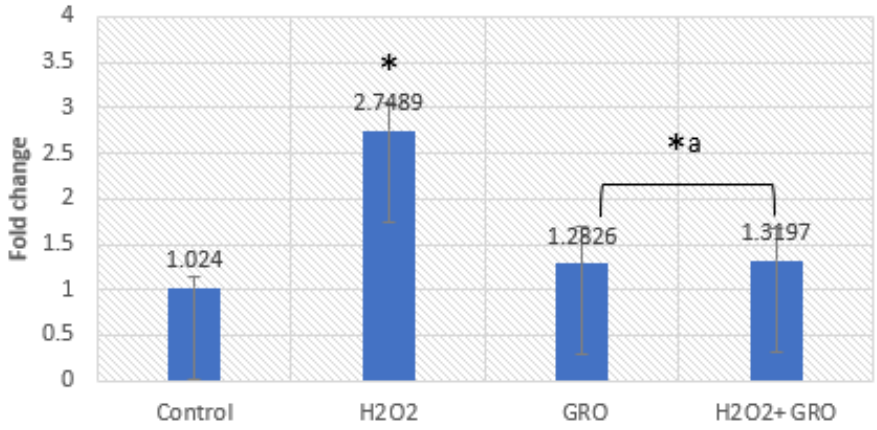

Fig. 3: Fold change variation of HSP90α mRNA in liver samples of experimental rats.

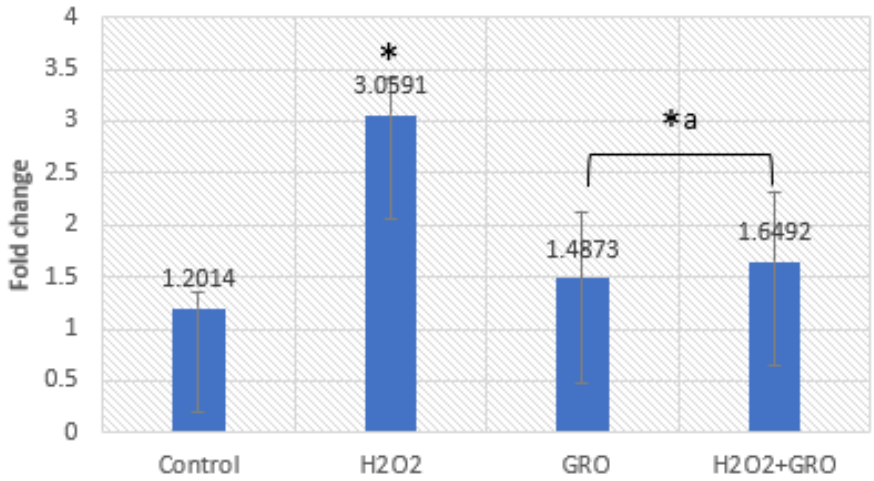

Fig. 4: Fold change variation of HSP 90β mRNA in liver samples of experimental rats.

Real-time PCR was performed to assess the impact of H_2O_2 and GRO alone and in combination on the expression of the glucose-regulated protein (GRP78) (**Fig.5**). Following H_2O_2 exposure, rats' levels of GRP78, sometimes referred to as HSPα5, were higher than those of the control, GRO, and H_2O_2+GRO groups. Rats exposed to GRO and GRO+ H_2O_2 did not exhibit differing amounts of GRP78. In contrast, GRP78 levels in the GRO and H_2O_2+GRO treated groups dropped sharply to values below those of H_2O_2. After 30 days, the reduced GRP78 levels returned to levels that were almost identical to the control.

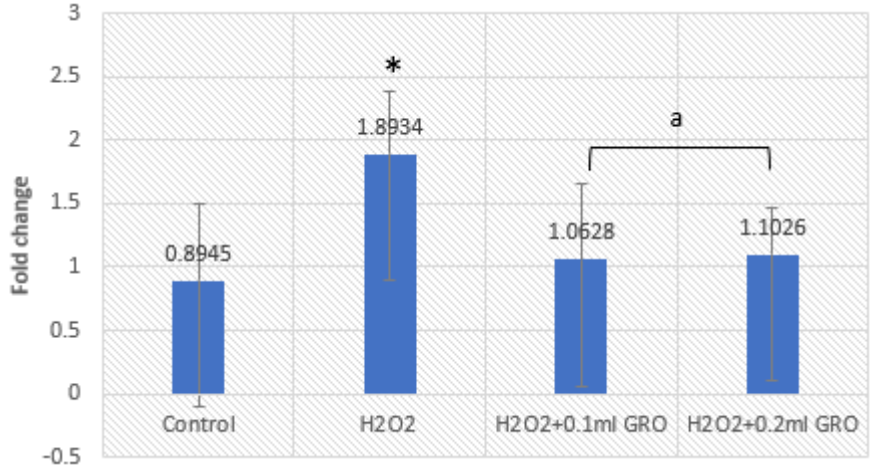

Fig.5: Fold change variation of GRP78 mRNA in liver samples of experimental rats.

Effect of Galangal Root Oil on Antioxidant Enzyme Gene Expression Levels in Experimented Rats Exposed to H_2O_2

Using real-time PCR, the impact of H2O2 and GRO alone or with H2O2 on the expression of antioxidant enzyme mRNA was ascertained. After 30 days of treatment with GRO alone or with H_2O_2, the levels of SOD expression that were dramatically reduced by H_2O_2 exposure returned to values that were almost identical to the control. Conversely, there was no discernible difference in GRO alone or in combination with H_2O_2 between these two groups and the controls; nonetheless, the SOD levels were not much lower than those of the controls. Figure 6.

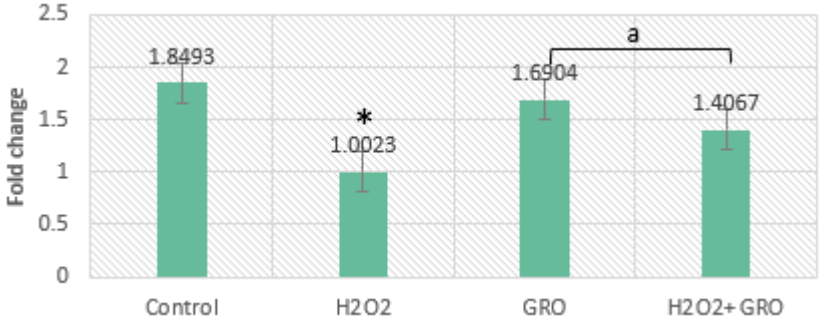

Fig. 6: Fold change variation of SOD mRNA in liver samples of experimental rats.

Through real-time PCR, the influence of H2O2 and GRO alone or with H_2O_2 on catalase (CAT) was assessed (Fig. 7). After 30 days of investigation, the livers of rats treated with GRO alone or with H_2O_2 revealed catalase expression levels that were close to the control levels. Rats exposed to H_2O_2 showed significantly lower levels of catalase in their liver samples when compared to the control. Comparing GRO with H_2O_2 markedly raised catalase levels in comparison to those exposed to H_2O_2, and the catalase levels that had dropped after H_2O_2 exposure returned to control levels after 30 days of GRO therapy.

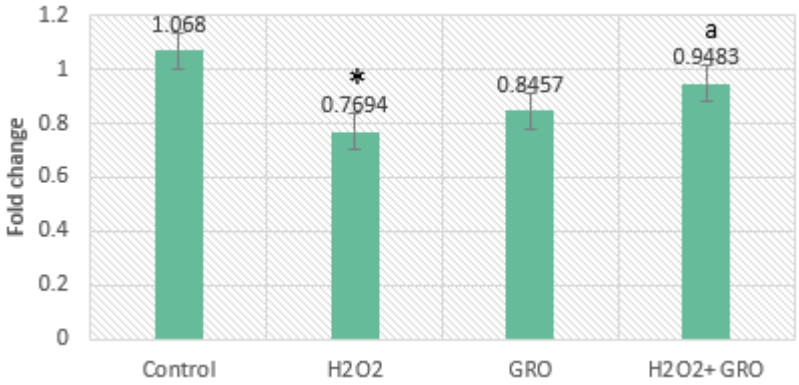

Fig. 7: Fold change variation of CAT mRNA in liver samples of experimental rats.

Finally, real-time PCR was used to assess the amounts of Glutathion-S-transferase (GST)mRNA in the livers of control rats and H_2O_2, GRO, and H_2O_2+GRO rats. GST levels were found to have grown by more than twofold after 30 days of H_2O_2 exposure (Fig.8). Compared to the control levels and GRO alone or with H_2O_2 after 30 days of treatment, which declined sharply and gradually returned to the control levels, the elevated GST levels after H2O2 exposure were noticeably higher. On the other hand, GST levels were greater than the controls in liver samples of H_2O_2+GRO exposure, but considerably lower in liver samples of GRO alone and H_2O_2+GRO exposure.

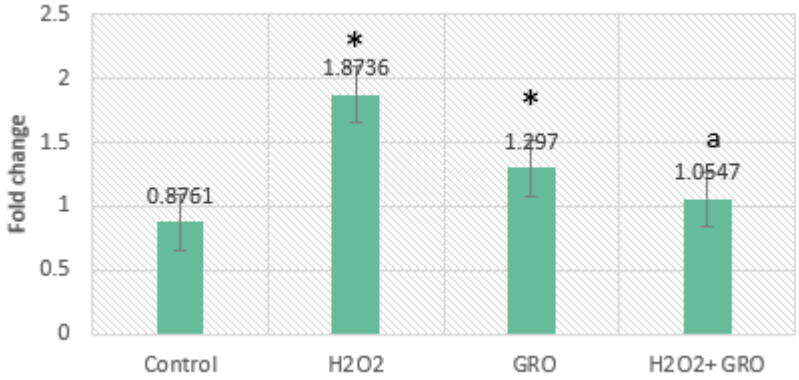

Fig. 8: Fold change variation of GST mRNA in liver samples of experimental rats

DISCUSSION

The present research assessed the impact of GRO, alone and in together with H_2O_2, on the expression of HSPs and antioxidant enzymes in liver damage caused by H_2O_2. The livers of rats exposed to H_2O_2 and GRO, both individually and in combination with H_2O_2, were assessed for mRNA expression of heat shock proteins and oxidative indicators (antioxidant enzymes). Our findings demonstrated that H_2O_2 exposure elevated the levels of certain heat shock proteins (HSPs) and antioxidant enzymes in the exposed group, significantly reduced the levels to below those observed in the GRO and GRO+ H_2O_2 groups, and then returned to control levels after 30 days of treatment.

A significant up-regulation of HSPs caused by stimuli like elevated temperatures or other stresses is known as the heat shock response. It is triggered as a defense against the non-specific toxicity brought on by the production of aberrant proteins and changed cellular processes (Garbuz, 2017; Singh *et al.*, 2024). Consequently, the stress elicited by H_2O_2 exposure produced an elevation of HSP expression, enabling the animal model to endure the toxicity caused by H_2O_2. The heat shock response is an evolved conserved process that guards cells or organisms toward the negative impacts of various stressors, like heat, chemicals, toxic substances, UV rays, and oxidizers. Heat shock response activates heat shock genes and proteins, such as HSP100, HSP90, HSP70, HSP60, and small HSPs (Morimoto *et al.*, 1997). HSPs play important roles in physiological activities such as protein folding, misfolded protein elimination, apoptosis, and cell signaling regulation. The current study is in agreement with (Younis *et al.*, 2024) who proved that different concentrations of H_2O_2 were linked to hepatic oxidative injury, indicated by a blood elevation above one fold in Tp53 and 8-OHdG levels as well as to its effect on HSPs and antioxidant enzyme expression. This parameter was linked to endogenous DNA damage, leading to structural alterations in DNA and a loss of its functionality due to upregulation to HSPs and down regulation of antioxidant enzymes (Younis *et al.*, 2024,). A study by (Huang *et al.*, 1999) indicated that hydrogen peroxide causes liver injury and hepatic oxidative damage (Huang *et al.*, 1999), these two studies in similar with our findings that hepatic damage leas to upregulation to HSPs as well as the inflammatory process. Other study performed in 2019 by (Alchalabi, 2019) who proved that microwave radiation exposure which is another source of stress on the biological system, leads to oxidative stress via influencing on HSPs and antioxidant enzyme expression in a rats model at the cellular level as a consequence of cellular toxicity resulting from oxidative stress induction. This could be due to the production of free radicals and the resulting onset of inflammation have been implicated in the damaging mechanism. This opinion is supported by that measured oxidative stress parameters and apoptotic indicators have shown that these actions have been observed in both in vivo in a mouse model and in vitro in the L02 Liver cell line by study in (Huang *et al.*, 1999). This aligns with our findings regarding H_2O_2 administration, which induces hepatic

damage by increasing cellular oxidative stress through the elevation of free radicals, including reactive oxygen species (ROS). The relationship between heat shock proteins (HSPs) and antioxidant enzymes was established by (Alchalabi, 2019), who demonstrated a strong linear correlation between the expression of antioxidant enzymes and heat shock proteins. Moreover, a significant linear relationship was observed between the expression of catalase and GST genes and the GSH-Px1 gene expression. Free radicals were primarily generated by liver cells, Kupffer cells, and neutrophils. The application of H_2O_2 has resulted in liver damage through oxidative reactions. This opinion also support our findings which in similar with (Abdellatif *et al.*, 2003). Other supportive studies by (Kiang & Tsokos, 1998; Calabrese *et al.*, 2006) who stated that The oxidation of the protein moiety, which impacts HSP, is caused by the production of free radicals linked to the oxidation of protein biomolecules. The generation of free radicals correlates with an increase in HSP synthesis. When exposed to H_2O_2, HSP90α and HSP27 are altered; HSP90α is decreased and HSP27 is increased in a concentration-dependent manner. HSP is a protein that regulates cellular homeostasis in both normal and pathological situations. This gives an explanation to our outcomes that GRO administration alone andor with H_2O_2 leads to decline in HSPs levels compared to rats receiving H_2O_2 orally up to 30-days

The redox balance is thrown off, excessive oxidative stress is brought on, and cellular constituents like DNA, proteins, and lipids are harmed by an imbalance of free radicals and antioxidants. Our results show that GST levels were elevated after exposure to H_2O_2, whereas SOD and CAT levels were lowered; however, these values were reversed after treatment with GRO alone or with H_2O_2. This result could be linked to the excessive utilization of enzymatic antioxidants during H_2O_2 -induced oxidative stress, suggesting that the hepatic antioxidant capacity was depleted. Treatment of H_2O_2 group rats with 0.2 ml of GRO led to enhanced antioxidant and HSP levels in hepatic tissues by end of the trial. The antioxidant properties of Alpinia galangal root oil may account for this effect. The findings align with those of (Aziz *et al.*, 2024) and (Tian *et al.*, 2022), who demonstrated that Alpinia galangal extract exhibits antioxidant properties and significant radical scavenging capability across various tissues and cell cultures. Galangal extract significantly enhances cognitive function in diabetic rats, reduces degenerative changes in the hippocampus, and demonstrates both preventive and therapeutic effects on diabetic encephalopathy. Transcriptomic analyses of hepatic tissues from rats administered H_2O_2 and H_2O_2 combined with GRO indicated that H_2O_2 adversely affects enzymatic antioxidant genes by downregulating their expression at the cellular level in hepatic tissues. Moreover, administering GRO to rats subjected to H_2O_2 diminished the biological effects of H_2O_2 on genes regulating antioxidants in hepatic tissues, suggesting that Alpinia galangal root oil possesses active compounds that influence critical genetic pathways, particularly those related to antioxidant defense. This *aligns* with (Yu *et al.*, 2016), who demonstrated that A. galangal extract mitigates pathological changes in the hippocampus, enhances cognitive function in diabetic rats, and provides preventive or therapeutic effects on diabetic encephalopathy by augmenting antioxidant cellular capacity in brain tissues.

Furthermore, other supportive studies to our findings revealed that Galangal include chemicals such as flavonoids and phenols, which possess antioxidant qualities (Eram *et al.*, 2020). These antioxidants can neutralize detrimental free radicals that may injure liver cells (Laksmitawati *et al.*, 2022). Additionally, Galangal's anti-inflammatory properties may contribute to liver protection, as inflammation can lead to liver damage (Üremiş *et al.*, 2025). Other studies indicate that galangal extract can normalize elevated liver enzyme levels in the blood, commonly associated with liver damage (Eram *et al.*, 2020). Additionally, galangal extract has been demonstrated to restore the liver's intrinsic antioxidant defense system, essential for safeguarding against oxidative stress. Galangal extract may assist in mitigating hepatocyte apoptosis induced by hepatic injury (Parthasarathy & Evan Prince, 2021).

Finally, the Galangal root oil and its extracts exhibit hepatoprotective properties, potentially mitigating liver damage caused by diverse sources. Galangin, a chemical present in

galangal, has been demonstrated to mitigate fatty liver (hepatic steatosis) via promoting autophagy in hepatocytes, as seen by research conducted on rats. Moreover, galangal extracts have demonstrated potential in mitigating liver damage induced by anti-tubercular medications and in safeguarding against liver cancer, likely through the reduction of inflammation and oxidative stress.

Conclusion

The study indicates that exposure to 2.5% H_2O_2 is highly detrimental, resulting in significant biological impacts, while galangal root oil safeguards hepatocytes from excessive oxidative stress by modulating the regulatory genes of heat shock proteins and antioxidant enzymes. By upregulating specific enzymatic antioxidant genes and downregulating HSP genes in liver tissues, Galangal root oil enhances antioxidant capacity, mitigating the bioeffects of H_2O_2 by disrupting oxidant and antioxidant mechanisms, thereby protecting the liver from substantial damage due to H_2O_2 toxicity in a rat model.

Declarations:

Ethical Approval: The Ethics Committee of the Veterinary Medicine College of the University of Mosul approved all animal-related procedures, which were carried out in compliance with its ethical rules UM.VET.2024.148) by the Institutional Animal Care and Use Committee of College of Veterinary Medicine, University of Mosul.

Competing interests: The authors declare no conflict of interest.

Author's Contributions: Raya Ghassan for laboratory works, Ali Alchalabi for data curation and Methodology, formal analysis and validation and writing original draft and Waad Sabri Reviewing and editing**.**

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Availability of Data and Materials: The datasets generated during and/or analysed during the current study are present in the manuscript file in the form of figures and tables.

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