Hepatoprotective effect of *Thymus vulgaris* extract on sodium nitrite-induced changes in oxidative stress, antioxidant and inflammatory marker expression

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The herb thyme (*Thymus vulgaris*) has multiple therapeutic uses. In this study, we explored how *T. vulgaris* leaf extract protects liver cells against sodium nitrite-(NaNO₂) induced oxidative stress. Mice were divided into four groups; each group received one of the following treatments orally: saline; *T. vulgaris* extract alone; NaNO₂ alone; or *T. vulgaris* extract + NaNO₂. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), reduced glutathione (GSH), superoxide dismutase (SOD), malondialdehyde (MDA), IL-1β, IL-6, TNF-α, and total proteins were measured in serum using standard methods. TNF-α, hemooxygenase-1 (HO-1), thioredoxin, SOD, and GSH synthase, all of which are linked to oxidative stress, were measured using quantitative real-time PCR (qRT-PCR). In mice treated with *T. vulgaris* extract, the effect of NaNO₂ on ALT and AST levels and total proteins was reduced, and its effect on antioxidant levels was reversed. Normally, NaNO₂ causes hepatocyte congestion and severe hepatic central vein congestion. Tissues in the mice treated with *T. vulgaris* were restored to normal conditions. Our results demonstrate that NaNO₂-induced hepatic injury is significantly reduced by pretreatment with *T. vulgaris* extract, which protects against hepatic oxidative stress and its associated genes at the biochemical, molecular, and cellular levels.

Nitrite is important to organ health. At very low concentrations, and within physiological levels, nitrite has antioxidant properties. When reduced to nitric oxide (NO), which is critical for vascular homeostasis, it plays a critical role in cell signaling, cellular immunity, and in the activation of certain regulatory proteins. These regulatory proteins are vital to the maintenance of normal human health.

Human beings use nitrates in a wide variety of ways, which makes them hard to avoid. In industry, nitrite and its salts are used in preservatives, colorants, and in manufacturing rubber products and dyes; in food, nitrite is employed for its anti-microbial properties; and in medicine, it is used to treat cyanide poisoning, ischemic heart disease, and as a vasodilator. Environmentally, improper waste disposal, over-reliance on nitrite-based fertilizers, and excessive use in food preservatives has led to chronic exposure and the accumulation of high nitrate-nitrite levels in humans.

Overexposure (due either to high concentrations or prolonged low-dose exposure) is toxic and can cause liver, kidney, or other organ failure, methemoglobinemia, and eventually death. Overexposure in humans occurs primarily through contaminated food or drinking water. After first affecting the GI tract, it passes into the circulation, where it reaches various other organs, causing further oxidative stress and toxicity. It has been observed that oxidative stress is a key mediator of nitrate-induced oxidative damage and that treatment with antioxidants can ameliorate or even reverse this. The possible mechanism by which NaNO₂ decreases antioxidant enzyme
activity is induction of reactive oxygen species (ROS), which mediate the oxidation of enzyme molecules or the binding of NO.

In the liver, NaNO2 induced hepatic toxicity through inhibition of antioxidant activity and increasing the levels of pro-inflammatory cytokines. Liver disease is often treated using synthetic chemical drugs, but these can cause serious undesirable side effects, including cirrhosis, cholestatic jaundice, and elevated serum transaminase levels. Consequently, there is a need to explore gentler and lower cost treatments for nitrite toxicity and liver disease. Many herbal compounds contain polyphenols and flavonoids, which have anti-inflammatory and anti-oxidative effects. This has led to increased pharmacological interest in certain herbal medications. Thyme (*Thymus vulgaris*), an herb commonly used in cooking throughout the world, has anti-septic, antimicrobial, carminative, and anti-oxidative properties and has been used to treat a range of human illnesses.

In traditional medicine, *T. vulgaris* has been used in a variety of ways because of its anti-septic, anti-spasmodic, anti-asthmatic, bronchodilator, expectorant, antitussive, carminative, anthelmintic, anti-microbial, and anti-oxidant qualities. It has been used to treat dyspepsia, chronic gastritis, diarrhea, and tonsillitis. *T. vulgaris* extracts are believed to contain important anti-inflammatory and immunomodulator factors with high free radical scavenging, anti-inflammatory, anti-platelet, anti-obesity, and anti-diabetic actions.

Monoterpenes and their hydrocarbons, as well as phenolics such as rosmarinic acid and flavonoid derivatives, are found in significant amounts in many species of thyme. The presence of these compounds renders thyme an important herb in research on antioxidants. The aim of our study was to examine the protective effects of *T. vulgaris*, at both the cellular and biochemical levels, on NaNO2-induced hepatic oxidative stress.

**Materials and methods**

**Chemicals and kits.** Kits for liver biomarkers, aspartate transaminase (AST), and alanine transaminase (ALT) were purchased from Clini Lab (Almanial; Cairo, Egypt). Kits for reduced glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), and total proteins were purchased from Bio-diagnostic (Giza, Egypt). Chemicals used, including NaNO2, and agarose, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Kits for RNA reverse transcription were purchased from Fermentas (Thermo Fisher Scientific, Waltham, MA, USA). The QIAzol reagents were purchased from QIAGEN (Valencia, CA, USA).

**Preparation of *T. vulgaris* extract.** *T. vulgaris* leaves were collected from Taif markets in April 2020. Plants were identified by Dr. Yassin Alsodani, a botanist at the College of Science, Taif University. The protocol and the methods used in this study was complied with relevant Taif University, Saudi Arabia and international guidelines and legislations. The extract was prepared according to the method used by Abdel-Aziem et al., the leaves were first air-dried, then powdered. The powder (100 g) was immersed in 500 mL ethanol (70%) and mixed. After 24 h, the mixture was filtered through Whatman paper No.1, then evaporated at 44 ± 1 °C in a vacuum. The solid extract (with a yield of 14.5% per 100 g of original material) was freeze-dried and stored at -20 °C.

**Measurement of total polyphenolic and flavonoid compounds.** Total polyphenols and flavonoids were estimated, using a method previously described by Singleton et al., who originally evaluated the total polyphenolic compounds in licorice extract using Foline-Ciocalteu reagent. This method has since been further developed and refined. We combined 200 μL of sample (in triplicate) with 1 mL Folin-Ciocalteu reagent (10% v/v) and 800 μL 7.5% (w/v) Na2CO3, which was then incubated for 30 min at 25 °C. The optical density was measured spectrophotometrically at 760 nm, calibrated using a gallic acid standard curve. Values are presented in terms of mg gallic acid equivalent per 1 g dry thyme extract. Total flavonoid content was determined using an aluminum chloride colorimetric method, based on a rutin calibration curve (mg rutin equivalent per 1 g dry thyme extract).

**Gas chromatography–mass spectrometry (GC–MS).** GC–MS analysis was performed using a Focus GC model (Thermo Electron Corporation, Waltham, MA, USA) under specific conditions: (1) DB-5 capillary column (30 m × 0.32 mm, 0.50 mm); (2) column temperature 60 °C (1 min) to 180 °C at 3 °C/min; (3) injector and detector temperature 220 °C; (4) split ratio 1:10; (5) carrier gas He; flow rate: 1.0 mL/min. A sample aliquot of 1 μL was diluted in chloroform at a ratio of 1:10. GC retention indices and ratios were compared on polar columns with specific standard mass spectra.

**Experimental design and animal handling.** Twenty-eight mice (7 weeks old; 35 ± 2 g) were kept at 12 hr /12 hr light dark cycle in the animal quarters at Turabah University College, Taif University, Saudi Arabia. Mice were given free access to food and water and handled daily for 7 days to accustom them to human contact. The Ethical Committee of Turabah University College, Taif University approved all procedures and animal use for this study (project TURSP2020-09). Moreover, the study was carried out in compliance with the ARRIVE guidelines. The animals were split into four groups as follows: Group 1, the negative control group (CNT), received only saline orally; Group 2, the *T. vulgaris* control group, received 0.5 g/kg bw *T. vulgaris* extract orally for 15 days; Group 3, the positive NaNO2 intoxication group, received 60 mg/kg bw NaNO2 orally on Day 14; and Group 4, the protective group, received *T. vulgaris* extract as for Group 2, and then NaNO2, on Day 14, as for Group 3. Figure 1 shows the details of the experimental protocol. We chose the dose of 60 mg/kg bw of NaNO2, because, while this dose is not lethal, it was nevertheless cause significant toxicity, damaging tissues and cells, which can be measured clearly using biochemical assays.

On Day 15, 24 h after the final treatment, the mice were anesthetized and then euthanized by decapitation. Liver samples were taken and preserved in Qiazol for RNA extraction and qRT-PCR; histological samples were...
prepared using Bouin’s solution; blood samples were collected and the extracted serum stored at -20 °C for later use.

**Biochemical assessments.** Serum ALT and AST levels were measured using a colorimetric spectrophotometer following the procedures in the user’s manual. MDA levels were assessed using the technique developed by Ohkawa et al.32. SOD was measured using the method of Beutler et al.33, reduced GSH using the method of Nishikimi et al.34, and total protein levels were determined using the method of Lowry et al.35. These colorimetric methods were performed using a Bio-Rad Smart Tech Spectrophotometer according to the procedures in the product inserts provided with each kit. The reduced glutathione (GSH) and oxidized glutathione (GSSG) kits were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). All chemicals used were of the highest purity and purchased from commercial suppliers in Saudi Arabia and Egypt.

**Cytokine measurements.** Several different kits for measuring parameters in mice, including mouse IL-1β (cat. No. E-EL-M0037), IL-6 (E-EL-M0044), and TNF-α (E-EL-M0049) were purchased from Elabscience (Houston, TX, USA). Serum cytokine levels were determined using Sandwich-ELISA kits in accordance with the product inserts and reference tables available at the Clinical Laboratory Sciences Department, Turabah University College, Taif University.

**Quantitative real-time PCR (qRT-PCR) and gene expression.** Total liver RNA was extracted and determined to be pure at 260/280 nm. Reverse transcription was performed using the QuantiTect reverse transcription kit with 1 µg total RNA, producing single-stranded complementary DNA (cDNA) using a two-step qRT-PCR reaction with a random primer hexamer. Amplification of cDNA was performed using SYBR Green master mix (Thermo Scientific, Waltham, MA, USA). Table 1 lists the primers used in thermal cycle qRT-PCR analysis, using the 2−ΔΔCt method. β-actin was used as a ‘house-keeping’ (internal standard) gene, against which cDNA was normalized. Reactions were run on and analyzed using a 7500 FAST Real-Time PCR Detection System (Applied Biosystems, Foster City, CA, USA). qRT-PCR conditions were: 95 °C for 10 min (1st denaturation stage) and 40 cycles of 95 °C for 15 s (2nd denaturation stage) followed by 60 °C for 1 min (annealing and extension stage).

Changes in intensity and expression of the specific genes listed in Table 1 under Analysis were determined using comparative cycle threshold (CT) values.

**Histological examinations.** After decapitation, typical tissue specimens from the livers of all animals were harvested according to a standardized necropsy protocol36 and immediately fixed in Bouin’s solution for 24 h. The fixed specimens were thoroughly washed under running tap water, dehydrated in an ascending series of ethanol and xylene, and embedded in paraffin before sectioning and staining. Staining was performed using hematoxylin and eosin (H&E) to assess histological changes in the liver tissue. The slides were then analyzed under a light microscope for the presence of pathological changes such as necrosis, inflammation, and fibrosis.
of ethanol concentrations (70%, 80%, 90%, and 100%; 2 h each), cleared in two changes of xylene (1 h each),
impregnated in two changes of soft paraffin (2 h each), embedded in paraffin blocks, cut into Sects. 4 μm thick,
and routinely stained with hematoxylin and eosin following standard processing and staining protocols. The
stained sections were examined microscopically and any histopathological alterations observed were recorded.
This was followed by multiparametric quantitative lesion scoring for the hepatic parenchyma in all groups
following the protocol described by Khalil et al. with a few modifications. For each animal, five randomly selected,
non-duplicated microscopic profiles (40 × objective) were captured using an AmScope digital camera attached
to an Olympus light microscope. These images were analyzed to allow calculation of the ratio of the area fractions
of the hepatic central veins, portal blood vessels, sinusoids, necrosis, hemorrhage, fatty change, and leukocyto-
cytic aggregations to the total areas using the image analysis software ImageJ (version 1.51v; Research Services
Branch, NIH, Bethesda, MD, USA). The proportion of hepatic cells that exhibited pyknosis, single-cell necrosis,
and vacuolar and hydropic degeneration with respect to the total number of hepatocytes per image was calcu-
lated subjectively. The frequency with which other lesions occurred (Kupffer cell hyperplasia, leukocytic inflamma-
tion, cholangiocyte hyperplasia and/or necrosis, and cholestasis) was determined by counting the number of
lesions per image. Eventually, the results were expressed as percentages (mean ± SEM).

Statistical analysis. Seven mice per group were selected and one-way ANOVA and Dunnett’s post hoc
descriptive tests were performed using SPSS software version 22 (SPSS, IBM, Chicago, IL, USA) for Windows.
The data are presented as mean ± standard error of mean; statistical significance is p < 0.05, indicated by letters
with different symbols**.

Ethical Statement. All experimental procedures were carried out under National Institutes of Health
Guidelines for the care and use of laboratory animals, and all procedures designed to minimize the suffering of
animals were followed.

Results
Analysis and detection of *T. vulgaris* content. Analysis (using gallic acid as a standard curve) revealed
that ethanolic *T. vulgaris* extract contained rosmarinic acid (38.23 mg/g dry weight thyme extract), luteolin-7-O-
glucoside (17.3 mg/g), and caffeic acid (1.96 mg/g). These results are shown in Table 2. Concentrations of phenol
and flavonoids were 221.65 and 115 mg/g, respectively, using rutin as a standard calibration curve (Table 1).

GC–MS analysis. Table 3 shows the major compounds contained in *T. vulgaris* extract. Ten major com-
ounds were detected in *T. vulgaris* extract following GC–MS analysis; the molecular weight (MW), retention
time (RT), and peak area vary among these compounds. The hydroxyl (OH), carbonyl (CO), acetyl (COCH₃),
and nitro (NO₂) functional groups are also shown in Table 3.

Serum hepatic biomarkers. We observed elevated ALT and AST levels and lower total proteins in the
NaNO₂-intoxicated groups (Groups 3 and 4), which indicated liver damage and dysfunction. In the group pre-
treated with *T. vulgaris* extract (Group 4), which received *T. vulgaris* extract for 14 days and then NaNO₂, for 24 h,
we observed less pronounced changes in ALT and AST levels and total proteins (Table 4). Pretreatment with *T. vulgaris* extract normalized and returned ALT and AST levels and total proteins to their relative control levels.

Impact of *T. vulgaris* extract on oxidative stress biomarkers. Increased levels of MDA (a biomarker
of lipid peroxidation), along with lower SOD serum levels, indicated tissue damage in the NaNO₂-intoxicated
mice (Group 3). Group 2 mice, who received *T. vulgaris* only, exhibited moderate increases in SOD and GSH.
In the NaNO₂-intoxicated mice (Group 4) pretreated with *T. vulgaris* extract, we observed alleviated GSH and
SOD levels and a decrease in MDA levels, providing evidence for the ameliorative effect of the *T. vulgaris* extract

### Table 1. Primers used for quantitative real time PCR in mice.

| Gene          | Accession number | Product size (bp) | Direction | Primer Sequence (5′–3′)                |
|---------------|------------------|------------------|-----------|---------------------------------------|
| Thioredoxin   | NM_011660.3      | 137              | Sense     | 5′-TGGAGAGGGGCTCATGCTG-3′             |
|               |                  |                  | Antisense | 5′-GTCGATCCATGTTGGCCATAG-3′           |
| HO-1          | NM_010442.2      | 126              | Sense     | 5′-GGCCGAGATTTCGCACT-3′              |
|               |                  |                  | Antisense | 5′-GAGCTCCATACCCGACTG-3′             |
| SOD           | NM_011434.2      | 157              | Sense     | 5′-AGGACGATGTCGCAATG-3′              |
|               |                  |                  | Antisense | 5′-GGCTCCACAGTCTCAGT-3′              |
| GSH synthase  | NM_008180.2      | 139              | Sense     | 5′-CAACGAGAAACCACGCTCC-3′            |
|               |                  |                  | Antisense | 5′-CGATTCAGGCCCAGAACA                |
| TNF-α         | NM_013693.3      | 131              | Sense     | 5′-CACCATGAGCCAGAAGAAGCA-3′          |
|               |                  |                  | Antisense | 5′-CTGCCACAGGCGAAATGAG-3′            |
| β-actin       | NM_007393.5      | 140              | Sense     | 5′-CCAGGCCTCTCTCTTGGGTA-3′           |
|               |                  |                  | Antisense | 5′-CAATGCCGGGTACATGGTG-3′            |
against NaNO₂-induced hepatic toxicity (Table 5). The effect of T. vulgaris extract on the glutathione system was evaluated by determining the ratio of GSH to oxidized glutathione (GSSG) in serum from NaNO₂-intoxicated mice (Table 5). The T. vulgaris extract had a positive effect on the glutathione status in NaNO₂-injected mice by causing a concomitant increase in GSH levels and decrease in GSSG levels, which restored the GSH/GSSG ratio to control levels. The profound decrease in the GSH/GSSG ratio induced by NaNO₂ was normalized by T. vulgaris treatment for 15 days.

Table 2. Total Phenolic and flavonoids compounds identified in ethanolic extract of Thymus vulgaris for triplicate samples.

| No | Name                  | Concentration  |
|----|-----------------------|----------------|
| 1  | Caffeic acid          | 1.96 ± 0.53    |
| 2  | Rosmarinic acid       | 38.23 ± 2.33   |
| 3  | Luteolin              | –              |
| 4  | Luteolin-7-O-glucoside| 17.3 ± 1.034   |
| 5  | Luteolin-7-O-rutinoside| –              |
| 6  | Apigenin              | –              |
| 7  | Apigenin-7-O-glucoside| –              |
| 8  | Apigenin-7-O-rutinoside| –              |
| 9  | Hesperidene           | –              |
| 10 | Hesperidine-7-O-glucoside| –        |
| 11 | Hesperidine-7-O-rutinoside| –       |
| 12 | Total Phenols         | 221.56 ± 6.8   |
| 13 | Total flavonoids      | 115 ± 2        |

Table 3. Compounds identified by Gas Chromatography-Mass Spectrometry (GC-MS) in ethanolic extract of Thymus vulgaris.

| Item | RT (min) | Peak Area % | Class name                                                                 | Molecular weight | Molecular Formula |
|------|----------|-------------|-----------------------------------------------------------------------------|------------------|------------------|
| 1    | 9.7      | 2.51        | Pyrrole derivative chelated with Nickel                                      | 715              | C₄₄H₂₇N₅NiO₂    |
| 2    | 11.58    | 2.54        | 5-ethyl-6-methylheptan-2-yl-10,13-dihydro-1H-cyclopenta[a]phenanthrene-3-ol   | 414              | C₉₀H₁₀O    |
| 3    | 11.69    | 2.44        | 3-(tert-butylidimethylsilyl)oxy)-14-methyl-7,8,9,11,12,14,15,16,17-decaydro-6H-cyclopenta[a]phenanthrene-16,17-diy bis(2,2,3,3,3-pentafluoropropionate) | 694              | C₉₅H₁₄F₄O₈Si |
| 4    | 27.99    | 2.9         | 10-hydroxy-3a,6,6a,9a,9ai-pentamethyl-1- (6-methylheptan-2-yl)hexadehydrocyclopenta[7,8]phenanthro[8a,9-b](oxiren-7-yl)acetate | 502              | C₁₁₂H₁₂O₈    |
| 5    | 28.29    | 2.56        | 1-(2-hydroxy-7,9a,11b-trimethylhexadehydrocyclopenta[1,2]phenanthro[8a,9-b](oxiren-9-yl)ethanone | 346              | C₂₀H₁₄O₇    |
| 6    | 32.39    | 2.97        | 8-methoxy-10a,12a-dimethyl-5-methylene-1-(6-methylheptan-2-yl)hexadehydrocyclobuta[m]cyclopenta[a]phenanthren-6(4H)-one | 454              | C₁₅H₁₀O₄    |
| 7    | 41.69    | 2.44        | N-(2-(2-acetyl-7-methoxy-1H-indol-3-yl)ethyl)benzenesulfonamide              | 372              | C₁₇H₁₇N₂O₃S   |
| 8    | 44.62    | 2.69        | dimethyl 3-[(5-hydroxy-1-methoxycarbonyl)-1-methyldecahydrophenalen-2-yl]butyl-1-methoxy-7-methylphenanthrene-2,6-dicarboxylate | 568              | C₂₁H₁₆O₂    |
| 9    | 46.61    | 2.96        | Ethyl 7-[(4-hydroxy-3-methoxy-5-nitrophenyl)-5-methyl-4-dihydro-1,2,4-triazolo[1,5-a]pyrimidine-6-carboxylate | 375              | C₁₃H₁₀N₂O₆   |
| 10   | 50.18    | 3.16        | 5,7,8,8’-tetrahydro-6,6’-dimethyl-[2,2’-binaphthalene]-1,1’ ,4,4’-tetraone | 406              | C₁₄H₁₀O₄    |

Table 4. Protective effects of Thymus vulgaris extract against NaNO₂ induced alteration in liver biomarkers in mice. Values are means ± standard error (SEM) for 7 different mice per each treatment. Values are statistically significant at *p < 0.05 versus control; #p < 0.05 versus control and thyme extract groups. $p < 0.05 versus NaNO₂. ALT: alanine transaminase; AST: aspartate transaminase.

|                  | ALT (U/l) | AST (U/l) | Total proteins (mg/dl) |
|------------------|-----------|-----------|------------------------|
| Control          | 17.3 ± 0.6| 24.9 ± 0.9| 81.6 ± 6.9             |
| Thyme Extract    | 23.5 ± 5.1| 20.4 ± 1.8*| 78.3 ± 2.5             |
| NaNO₂            | 93.5 ± 4.3*| 88.1 ± 3.3*| 42.5 ± 4.5*             |
| Thyme Extract + NaNO₂ | 44.5 ± 1.5*| 41.6 ± 4.7*| 66.5 ± 4.8*             |

against NaNO₂-induced hepatic toxicity (Table 5). The effect of T. vulgaris extract on the glutathione system was evaluated by determining the ratio of GSH to oxidized glutathione (GSSG) in serum from NaNO₂-intoxicated mice (Table 5). The T. vulgaris extract had a positive effect on the glutathione status in NaNO₂-injected mice by causing a concomitant increase in GSH levels and decrease in GSSG levels, which restored the GSH/GSSG ratio to control levels. The profound decrease in the GSH/GSSG ratio induced by NaNO₂ was normalized by T. vulgaris treatment for 15 days.
Mitigated impact of *T. vulgaris* extract on inflammatory cytokines. To examine the destructive impact of NaNO₂ on the immune state of mice, we measured the levels of pro-inflammatory cytokines. NaNO₂ injection increased serum levels of IL-1β, IL-6, and TNF-α in the NaNO₂-intoxicated mice (Groups 3 and 4). Pretreatment of NaNO₂-intoxicated mice with *T. vulgaris* (Group 4) mitigated these effects (Table 6), which eventually returned to normal levels. *T. vulgaris* extract normalized all altered pro-inflammatory cytokines.

Ameliorative effect of *T. vulgaris* extract on quantitative expression of liver genes. Nitrite toxicity induced liver damage and hepatic dysfunction as shown by the increases in hemeoxygenase-1 (HO-1), an oxidative stress biomarker, and TNF-α, an inflammatory cytokine (Fig. 2A,B). Gene expression for TNF-α
mRNA was downregulated in the group pretreated with *T. vulgaris* extract (Fig. 2A). Conversely, the HO-1 gene was upregulated in the *T. vulgaris*-treated group (Fig. 2B). The ameliorative effects of *T. vulgaris* extract on both TNF-α and HO-1 expression and its potential to restore and recover gene alterations caused by NaNO2 intoxication are shown in Fig. 2A,B.

**Figure 3.** Ameliorative effect of *T. vulgaris* extract on mRNA expression of thioredoxin (A), SOD (B), and GSH synthase (C) in NaNO2-treated mice. Graphic presentation of liver mRNA expression by qRT-PCR analysis of thioredoxin, SOD, and GSH synthase in different groups of mice relative to β-actin. *p* < 0.05 versus the control group; *p* < 0.05 versus the control and *T. vulgaris* extract groups; and *p* < 0.05 versus the NaNO2-treated group.

Impact of *T. vulgaris* extract on quantitative expression of antioxidant genes in liver. qRT-PCR results showed that NaNO2 downregulated thioredoxin, SOD, and GSH synthase mRNA expression (Fig. 3A–C), leading to oxidative stress in the liver. The *T.vulgaris*-treated group (Group 2) showed positive correlation in the antioxidant genes examined, as *T. vulgaris* upregulated thioredoxin, SOD, and GSH synthase mRNA expression, demonstrating that the extract has important antioxidative properties (Fig. 3). In the group pretreated with *T. vulgaris* extract for 14 days followed by NaNO2 intoxication for 24 h (Group 4), the altered mRNA expression of the examined genes returned to within the normal range as a result of pretreatment.

Histopathological examination. Upon microscopic examination of the specimens taken from the control and *T. vulgaris*-treated animals, we observed normal hepatic histological architectures: roughly hexagon-shaped hepatic lobules with sinusoids converging from the periphery to the central vein and portal canals present at approximately three of the six angles of the lobule. The hepatic parenchyma between the portal canals consisted of hepatocytes arranged in cell plates (Fig. 4A,B). Exposure to NaNO2 induced a wide variety of hepatopathic histological alterations, including circulatory changes (congestion of the central and portal veins and...
sinusoids), hepatocyte degeneration and necrosis (pyknosis, cellular swelling associated with vacuolations due to vacuolar and hydropic degenerations or fatty changes, and single cell necrosis), and inflammatory changes (intralobular and/or leukocytic infiltration or aggregations) (Fig. 4C,D). Interestingly, the biliary system did not show any noticeable histological alterations. T. vulgaris showed marked hepatoprotective effects against the NaNO₂-induced hepatopathy. Although the hepatic tissue sections from the NaNO₂ + T. vulgaris-treated animals were not completely histologically normal, we observed a significant reduction in the severity and frequency of the NaNO₂-induced histological changes. The most frequent lesions in this group were vacuolar vascular congestion, sinusoidal dilatation, hepatocyte vacuolation, and tiny mononuclear cell aggregations (Fig. 4E,F).

Figure 4. Representative photomicrograph of hematoxylin and eosin stained hepatic tissue sections showing the effects on the hepatic histology of exposure to NaNO₂ and/or T. vulgaris. (A) and (B), normal histological photomicrographs in the control and T. vulgaris-treated mice, respectively. (C) and (D), livers of the NaNO₂-treated mice showing portal congestion (arrows), inflammatory cell infiltrate (ellipses), cellular swelling associated with hydropic degeneration (red arrowheads), and single-cell necrosis (black arrowheads). (E) and (F), livers of NaNO₂ + T. vulgaris-treated mice showing focal mononuclear cell aggregates (ellipses), fatty change (black arrowheads), congested blood vessels (black arrows), and sinusoids (blue arrows).
as IL-1β, thereby ameliorating the NaNO2-induced inflammatory response. The inflammatory response causes increase in protein expression49. As such, HO-1 plays an important role in mechanisms of oxidative stress and lipid peroxidation in agreement with previous reports11,30. Antioxidant defenses are affected by the expression of genes such as thioredoxin, SOD, and GSH46, and oxidative stress is regulated by several other downstream genes52. The antioxidant mechanism is regulated by downstream genes that control oxidative stress, such as Nrf2 inflammation50. By controlling IL-1β activation51, HO-1 regulated the expression of inflammasome-associated genes53, which play a leading role in sepsis and fever. NaNO2 intoxication has the same effect54, yet our study found that cytokine levels in mice pretreated with thymus extract were significantly lower than in the NaNO2-treated group and downregulation in the NaNO2-treated groups, confirmed the potential of thymus extract, we examined the role of HO-1 signaling. Most reports have indicated that HO-1 plays a role in the activation and expression of some antioxidant and anti-inflammatory cytokine genes54,55. All other conditions were determined by the same method. The overall hepatic lesion scoring changes induced by NaNO2 and possible amelioration by thymus extract are shown in Table 7.

### Table 7. Analysis and lesion scoring in the hepatic tissues of mice in response to NaNO2 and thymus vulgaris extract. Values are expressed as means ± SEM for 7 different mice per each treatment. Values with different letters means significant at p < 0.05 betweengroups.

| Lesion                        | Control    | Thymus extract | NaNO2 | GA3 + NaNO2 |
|-------------------------------|------------|----------------|-------|-------------|
| Central vein                  | 2.2 ± 0.4  | 2.17 ± 0.03‡  | 2.7 ± 0.1*| 2.4 ± 0.06* |
| Portal blood vessels          | 1.4 ± 0.06*| 1.33 ± 0.07‡  | 3.9 ± 0.13| 2.2 ± 0.09* |
| Sinusoidal spaces             | 6.3 ± 0.12*| 6.7 ± 0.1*†   | 9.01 ± 0.2| 8.0 ± 0.1*  |
| Fatty change                  | 0†         | 0              | 0.6 ± 0.1*| 0.18 ± 0.09*|
| Focal leukocytic aggregation  | 0†         | 0              | 0.77 ± 0.029| 0.29 ± 0.1* |
| Focal necrosis                | 0†         | 0              | 0.1 ± 0.007| 0.04 ± 0.01*|
| Vascular and hydropic degeneration| 0*      | 0              | 26.2 ± 4.9| 17.3 ± 4.6* |
| Hepatocyte pyknosis           | 0*         | 0              | 2.1 ± 0.4 | 0.6 ± 0.2*  |
| single-cell Nécrosis          | 0*         | 0              | 1.6 ± 0.4 | 0.4 ± 0.1*  |
| Cholestasis                   | 0*         | 0              | 0.0*     | 0.17 ± 0.002*|
| cholangioocyte hyperplasia    | 0*         | 0              | 0        | 0*          |
| Von Kupffer cell hyperplasia  | 0*         | 0              | 4.6 ± 0.23| 3.2 ± 0.3*  |
| Inflammatory infiltrate       | 0*         | 0              | 23 ± 0.52| 9.1 ± 2.9*  |

Discussion

Our study confirmed that NaNO2 intoxication increases the levels of ALT and AST in serum, decreases total proteins, affects cytokine levels and gene expression (leading to an imbalance in antioxidant mechanisms), and causes histopathological changes, resulting in severe oxidative stress in liver tissues. However, pretreatment with T. vulgaris extract prevented or reversed these effects, confirming the antioxidant effect of T. vulgaris against NaNO2-induced hepatic dysfunction.

The elevated levels of ALT and AST, decreased total serum proteins, and the histopathological alterations that we observed, are all indicators of toxicity and also well-known quantitative measures of liver activity39. While functional liver damage can be indicated by high serum levels of AST, a more liver-specific indicator is the enzyme ALT. ALT catalyzes the conversion of alanine into pyruvate and glutamate, making serum levels of ALT a more reliable indicator of liver damage40. Our findings demonstrated that the membrane structure and integrity of the liver cells, which would otherwise have been destroyed by NaNO2, remained undamaged and uncongested, providing strong evidence for the protective effect of T. vulgaris extract also described previously17,41.

Sharma et al.42 noted that hepatoprotective plants are characterized by significant levels of polyphenols and flavonoids, major compounds also found in T. vulgaris. Flavonoids promote the expression of enzymes involved in the production of glutamylcysteine synthetase and thioredoxin, leading to increased intracellular GSH levels43,44. The antioxidant assays used in this study, which targeted five different types of ROS molecules (MDA, SOD, GSH, GSSG, and GSH/GSSG), confirmed the strong antioxidant effect of the T. vulgaris extract. Our findings are in agreement with those of previous studies that reported the high phenolic content and strong antioxidative effect of T. vulgaris extract17. Inhibition and decreased function of the antioxidant system causes the accumulation of H2O2, and hepatic cell decomposition40. Treatment with T. vulgaris extract significantly reversed these decreases in antioxidant enzyme activity.

Our study showed that NaNO2 intoxication reduced antioxidant activity and increased both oxidative stress and lipid peroxidation in agreement with previous reports14,39. Antioxidant defenses are affected by the expression of genes such as thioredoxin, SOD, and GSH46, and oxidative stress is regulated by several other downstream genes, including HO-147. Hyun48 showed that many plant extracts play a critical role in increasing antioxidant activity and reducing ROS. Overexpression of a particular hemoxygenase-1 reported here in thymus control group and downregulation in the NaNO2-treated groups, confirmed the potential of T. vulgaris extract. HO-1 is normally involved in the regulation of mitochondrial biogenesis, neurogenesis, and angiogenesis48,49, causing an increase in protein expression49. As such, HO-1 plays an important role in mechanisms of oxidative stress and inflammation50. By controlling IL-1β activation48, HO-1 regulated the expression of inflammatory-associated genes51. The antioxidant mechanism is regulated by downstream genes that control oxidative stress, such as Nfr2 and HO-147,53. Our findings confirmed that HO-1 expression plays a key role in the regulation of hepatic oxidative stress regulated by T. vulgaris extract. To explore the potential mechanism underlying the hepatoprotective effect of T. vulgaris extract, we examined the role of HO-1 signaling. Most reports have indicated that HO-1 plays a role in the activation and expression of some antioxidant and anti-inflammatory cytokine genes54,55. All were coincided with our reported findings.

Our study confirmed that T. vulgaris extract regulated the expression of pro-inflammatory cytokines, such as IL-1β, thereby ameliorating the NaNO2-induced inflammatory response. The inflammatory response causes increased production of IL-1β, IL-6, and TNF-α cytokines56, which play a leading role in sepsis and fever. NaNO2 intoxication has the same effect57, yet our study found that cytokine levels in mice pretreated with T. vulgaris extract also described previously17,41.
extract returned to normal. Therefore, *T. vulgaris* extract prevented NaNO₂-induced hepatic toxicity by ameliorating disrupted serum levels of IL-1β, IL-6, and TNF-α.

Reactive oxygen species (ROS) play a vital role in maintaining various human physiological processes, but at high levels, they can be cytotoxic and cause liver damage. Therefore, mechanisms exist to maintain appropriate ROS levels in the liver and other organs, thereby preventing oxidative stress and promoting redox homeostasis. Our study found that while NaNO₂ intoxication induced lipid peroxidation, pretreatment with *T. vulgaris* reversed these effects. In part, this was due to the recovery of depleted GSH, which plays a significant defensive role against oxidative stress.

Hydroperoxides are converted into alcohols and water in a process catalyzed by thioredoxin, a cytosolic protein. Thioredoxin has several antioxidant functions, including scavenging for free radicals, removing hydrogen peroxide, protecting against oxidative stress, as well as broader functions in genetic transcription, DNA and protein repair, immunostimulant roles, apoptosis, and cell proliferation. As a thiol-specific antioxidant, thioredoxin shares many of the same functions as glutathione systems and the two are understood to act in tandem in the management of oxidative stress. In our study, NaNO₂ downregulated thioredoxin and GSH synthase expression, but these returned to normal in mice pretreated with *T. vulgaris* extract.

In addition to biochemical alterations, the hepatotoxic effect of NaNO₂ has been further implicated in a wide variety of degenerative, circulatory, and inflammatory alterations. The etiopathogenesis of these hepatopathic morphological alterations are likely to be multifactorial and mediated by NaNO₂-induced oxidative damage and inflammation due to the formation of ROS and lipid peroxidation. The latter is associated with lysosomal and mitochondrial membrane rupture and subsequent release of digestive proteases and activation of the apoptosis-related Bax and caspase-3 genes.

Treatment with NaNO₂ caused hepatic dysfunction, oxidative stress, and alteration in the levels of inflammatory cytokines. Pretreatment with *T. vulgaris* extract alleviated these responses to toxicity and restored the levels and mRNA expression of the proteins and genes. The collective summary for the protective impact of *T. vulgaris* on NaNO₂-induced liver dysfunction are clearly shown in Fig. 5.

**Figure 5.** Schematic representation of the ameliorative effect of *T. vulgaris* extract on liver dysfunction. NaNO₂ intoxication induced hepatic dysfunction, decreased antioxidant activity, and an increase in the levels and expression of inflammatory cytokines. These results of treatment with NaNO₂ were normalized by pretreatment with *T. vulgaris* extract.
Conclusion

*T. vulgaris* extract exerted a protective effect against oxidative stress in liver tissues caused by NaNO2 intoxication. The effect was observed in different antioxidant systems and through the regulation of pro-inflammatory cytokines. In short, the common herb thyme (*T. vulgaris*) can be used in the prevention and treatment of hepatic oxidative stress.

Data availability

Data are available upon request.

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Author contributions
M.M.S.: designed, supervise experiments, analyzed data and wrote the paper. A.A.: Analyzed data, Real time PCR, Chemistry analysis. M.M.M.M.: Histopathology and Revised paper.

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Competing interests
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