RESEARCH PAPER

Attenuation of Metabolic Syndrome by Trifolium repens Flower Extracts via Antioxidant and Anti-inflammatory Mechanisms in Male Albino Rats

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ABSTRACT

Background: Metabolic syndrome (MetS) is a medical condition associated with several health issues, including insulin resistance, obesity, hypertension, and dyslipidemia. Individuals with MetS, particularly older adult suffer from many chronic diseases, including cardiovascular diseases (CVD), type 2 diabetes mellitus (T2DM), and hypertension. Trifolium repens (T. repens), known as white clover (flowers and seeds), is a traditional medicinal plant used in Egypt and other countries. Its flower nectar is a precursor to honey bees. The flower extracts, prepared using water and ethanol (WT and ET), contain numerous acidic phenols and flavonoid compounds with antioxidant and anti-inflammatory properties. Therefore, this study aimed to evaluate the effects of T. repens flower extracts on oxidative and inflammatory markers and compare them with those of metformin (MF) in rats with experimentally induced MetS.

Methods: This study involved 42 male albino rats weighing 120–150 g. Six rats were used as controls. MetS was induced in 36 male rats by providing 30% a high-fat diet and a 10% fructose solution in their drinking water for 12 weeks. From the beginning of week nine, the MetS-induced rats were categorized into six groups: MetS positive control, MetS + WT50 and 100, MetS + ET50 and 100, and MetS + MF (200mg/kg) as the reference group. At the end of week 12, blood samples were drawn for biochemical examination. Liver tissue was harvested for histological examination and used for semi-quantitative scoring of steatosis, fibrosis, and inflammation.

Results: Rats with MetS had higher body and organ weights (liver and internal fat), as well as elevated relative organ weights. Biochemical indicators, including liver function tests, were elevated, specifically alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities. Additionally, increased blood levels of the oxidative stress (OS) marker (MDA) were observed, alongside decreased antioxidant markers, such as glutathione (GSH) and glutathione peroxidase (GSHpx) activity, in rats with MetS. Inflammatory markers, including tumor necrosis factor-α (TNF-α) and C-reactive Protein (CRP), were substantially elevated and linked to a decrease in the anti-inflammatory marker interleukin-10 (IL-10) in the MetS group. Treatment with T. repense improved the last biochemical parameters. Histologically, MetS showed steatosis, ballooning degeneration, and inflammatory infiltration. The most improved histopathological analyses showed less liver damage in MetS + ET100, followed by MetS + WT100, and then MetS + MF rats than in untreated MetS rats.

Conclusion: Trifolium flower extracts reduced symptoms of metabolic syndrome by lowering oxidative stress, inflammatory markers, and pathological liver changes. These findings suggest that Trifolium extract could be used for the non-pharmaceutical management of metabolic syndromes.

Keywords: Trifolium flower extract, metabolic syndrome, oxidative stress, inflammation, pathological changes, rats

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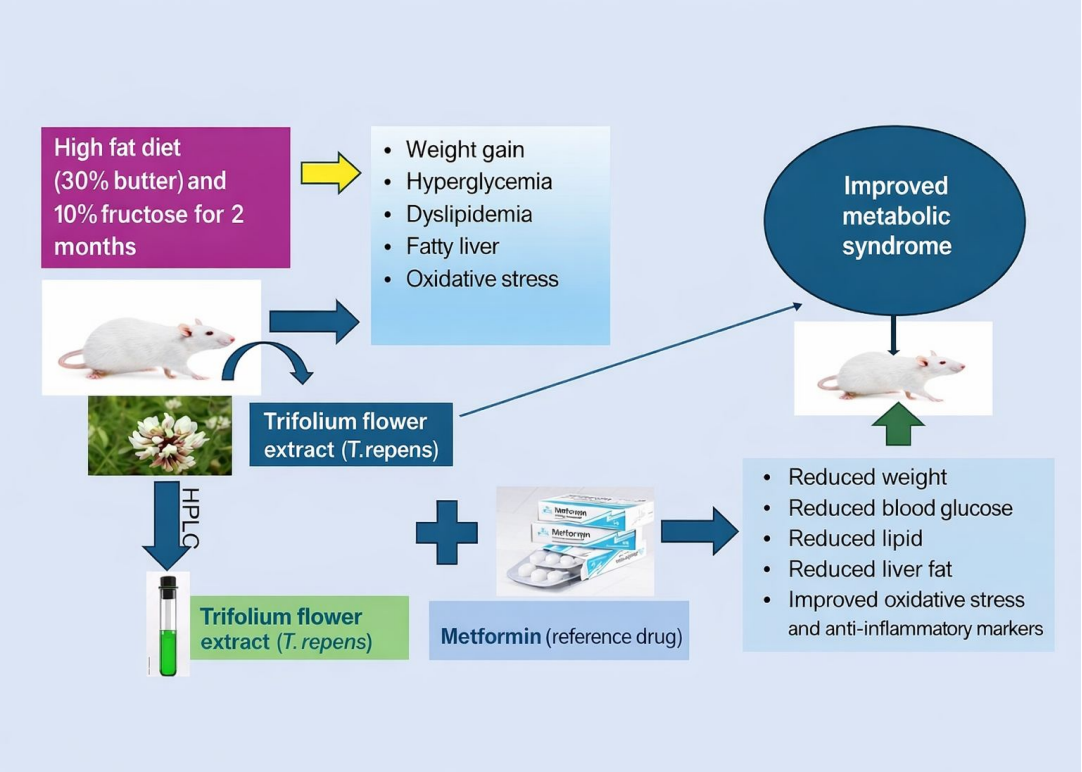
Graphical abstract



Introduction

Metabolic syndrome is a major public health issue that requires attention as it is significantly increasing worldwide [1,2]. It is categorized as a group of distinct metabolic risk factors rather than as a disease. These include abdominal obesity, elevated triglyceride levels (hypertriglyceridemia), high blood sugar levels (hyperglycemia), and low levels of high-density lipoprotein- cholesterol (HDL-C) [3]. The presence of these specific risk factors can significantly increase the risk of hepatic steatosis [1]. The rising incidence of MetS is associated with obesity, which is a major public health concern in the twenty-first century [2]. A complex interplay of lifestyle, environmental, and hereditary factors contributes to MetS. So, lifestyle changes are necessary for managing MetS. Regular exercise, healthy eating, and weight loss are essential components of effective treatment [4].

All over the world MetS is increased yearly among Arab populations, the MetS estimates are 39.8% according to the Adult Treatment Panel III (ATP III) criteria, 31.6% according to the International Diabetes Federation (IDF) criteria in Saudi Arabia, 17% in Oman, 40.5% in the United Arab Emirates (UAE) [5].30% in Tunisia, and 36.3% in Jordan [6]. In Egypt, the prevalence is 38.7% in men, 46.2% in women, and 42.5% in the general population [6][7]. As the proportion of elderly and senior populations continues to rise, the incidence of MetS is anticipated to increase. This trend is expected to lead to a substantial increase in healthcare expenditures for both outpatient and inpatient medical services [8]. MetS significantly leads to the development and progression of CVD, T2DT, hypertension and inflammation [9].

HFD is a typical dietary pattern in which lipids account for at least 35% of total calories [10]. There is growing evidence that HFD-induced inflammation hinders the metabolism of both energy and lipids, leads to insulin resistance, and promotes the development of atherosclerotic plaques and thrombi [11]. MetS models have been created using a variety of high-fat diets, including plant oils (e.g., soybean and maize oils) and animal fats (e.g., lard and butter). Dietary models with 30–70% diet-fat content increase blood levels of free fatty acids, body weight, and promote insulin resistance, dyslipidemia, and hyperglycemia [12,13].

The liver performs several functions, including primary detoxification of various metabolites and the synthesis of proteins, which play a significant role in regulating the body's metabolism [14] [15]. Liver function tests are ordered

for inpatients and outpatients with suspected liver disease. Elevated ALT and AST levels indicate hepatocellular disease [15]. Oxidative stress is implicated in acute and chronic liver injury and the pathogenesis of prevalent infectious or metabolic liver diseases, such as alcoholic fatty liver disease and non-alcoholic fatty liver disease (NAFLD). Moreover, OS plays a crucial role in the progression of liver disease to liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) [16]. The ability of HFD to modify inflammation may increase the risk of human diseases [17]. In metabolic syndrome, chronic inflammation is triggered by TNF-α, a detrimental adipokine produced by enlarged adipocytes. Elevated CRP levels are often associated with infections, tissue damage, and various inflammatory conditions [18]. Conversely, IL-10 is a key anti-inflammatory cytokine that plays an essential role in controlling immune responses and maintaining immune balance, which increases inflammation [19] .

However, according to World Health Organization (WHO) assessments, 80% of people prefer plant-based remedies for conditions such as diabetes, OS, cancer, and respiratory and dermatological disorders [20]. Biochemical substances found in abundance in plants, including phenols, fatty acids, saponins, essential oils, and alkaloids, have been shown to have therapeutic benefits but have received less research attention [21]. Although Trifolium species have not been highly valued, interest in them has increased recently [22]. Biologically active substances, such as phenolic acids and isoflavones, are abundant in Trifolium species which can be extracted by water or organic solvent (ethanol) [23]. Therefore, this study aimed to determine whether Trifolium extracts reduce OS and inflammation and protect liver histological structure in rats with MetS.

2-Materials and methods

2.1. Trifolium repens Flowers Collection and Extraction

Clover flowers were gathered from the Dakahlia Governorate. Following two rounds of washing with distilled water, the samples were dried in ambient air at room temperature. The weights were then recorded. Water and ethanolic (300 g each) extractions of *T. repens* flowers were performed at the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University. The Folin-Ciocalteu reagent (FCR) method was used to calculate the total phenolic content (TPC) [24], and the antioxidant activity of the extract was assessed in triplicate using the DPPH free radical-scavenging test. The mean values were considered [25]. To avoid deterioration, the concentrated extracts were labeled and stored in a refrigerator at 4 °C.

2.2. Metabolic Syndrome Induction and Experimental Design

Forty-two adult male albino rats weighing between 120 and 150 g were purchased from El-Nile Company for Pharmaceutical Products, Cairo, Egypt. The rats were housed in suitable cages measuring 40 × 32 × 40 cm, with a 12/12 light-dark cycle, regulated humidity, and a temperature of 22 ± 2 °C. The Al-Azhar University Animal Care Committee approved all surgeries. They were also given full access to water to help them acclimate before the two-week-long experiment began. Throughout the experiment, six rats were fed a regular diet and constituted the control group (Group 1).36 rats were fed HFD with the following macronutrient composition: 30 % fat (butter), 18 % protein, and 52% carbohydrates [26]. Additionally, the rats received 10% fructose in their drinking water [27] for three months to induce MetS. Before the final month of the experiment, the rats were divided into six groups as follows:

Group 2 (MetS, positive control): continued on HFD and 10% fructose in drinking water throughout the entire experimental period (last 4 weeks) without any treatment.

Group 3 (WT50) was fed as group 2 and treated with water extract at a dose of 50 mg/kg body weight (Bwt).

Group 4 (ET 50) was fed as group 2 and treated with ethanolic extract at a dose of 50 mg/kg body weight (Bwt).

Group 5 (WT100):was fed as group 2 and treated with water extract at a dose of 100 mg/kg body weight (Bwt).

Group 6 (ET100): was fed as group 2 and treated with ethanolic extract at a dose of 100 mg/kg body weight (Bwt).

Group 7(MF): was fed as group 2 and treated with a reference drug, metformin (MF) at a dose of 200 mg/kg Bwt[28].

All treatments were performed daily using a gastric tube, starting from the beginning of the third month.

2.3. Body weight assessment

Initial and final Bwts evaluations (g)were conducted, and the animals were weighed weekly.

The following formula was used to calculate the percentage (%) change in Bwt [29]:

$$\%\text{change of the Bwt} = \frac{final\ Bwt - initial\ Bwt}{initial\ Bwt} \times 100$$

At the end of the study period, the body and organ weights (liver and internal fat) were measured. Relative organ weights (liver and internal fat) were calculated as a percentage using the following formula: Relative organ weight (%) = organ weight (g)/body weight (g) × 100 [30].

2.4. Blood Collection and Biochemical Measurements

The rats had unrestricted access to drinking water and underwent a 12-hour fasting period at the end of the 12-week study in the Medical Physiology Laboratory, Faculty of Medicine, Al Azhar University (boys' branch), Cairo, Egypt. Heparinized capillary tubes were used to draw blood from the retroorbital venous plexus of each rat's eye. From each rat, blood was collected in clean, dry glass tubes. Serum was separated from each blood sample by centrifugation for ten minutes at 3000 RPM. Supernatants were collected in Eppendorf tubes and stored at -20°C till analysis.

All biochemical measurements were done using kits from Biodiagnostic Company, Cairo, Egypt.

2.4.1 Liver Function Enzymes activity

The activities of AST as well as ALT were measured by Tietz [31],

2.4.2. Redox State Parameters.

Malondialdehyde (MDA), one of the peroxidation end products formed by the reaction of fatty acids with free radicals, was measured spectrophotometrically according to Placer *et al*. [32]. GSH was assessed spectrophotometrically, following the method outlined by Beutler *et al*. [33]. GSH-Px activity was determined using the method described by Paglia and Valentine [34].

2.4.3. Inflammatory Process parameters:

An enzyme-linked immunosorbent test (ELISA) was used to determine both TNF-α [35] and IL-10 [36] levels. CRP was measured according to Kasperska *et al.* [37] by the turbidimetric latex agglutination method.

All kits were purchased from Biodiagnostic Company. (Cairo, Egypt) .

2.5. Histological Examination

Autopsy samples from the livers of different groups of rats were submerged for 48 h in a 10% formalin solution. After washing with tap water, the samples were serially dehydrated with ethyl alcohol. After clearing in xylene, the specimens were embedded in paraffin wax for tissue block preparation. These blocks were sectioned at 4 µm using a rotary LEITZ microtome. Following paraffin removal, the tissue sections were placed on glass slides and stained with hematoxylin and eosin(H&E) [38]. Histological examinations were performed at RCMB (Al-Azhar University, Cairo, Egypt) using a LABOMED Fluorescence light microscope LX400 (cat no: 9126000; USA) [39].

2.5.1. Histopathological Scoring: A semi-quantitative scoring system was used in liver sections to rate the extent of steatosis, fibrosis, and inflammatory infiltration on a scale of 0 to 3, with 0 absent, 1 mild, 2 moderate, and 3 severe [40].

2.6.Statistical analysis

Each set of six rats is represented as the mean ± standard error (SE) of the biochemical data. The Statistics Package for the Social Sciences(SPSS) statistical software, version 25, was used to analyze differences between the control group and the control-positive group, as well as between the control-positive group and each of the treatment groups. One-way analysis of variance (ANOVA) followed by an independent t-test was employed to assess the data. A p-value of < 0.05 was considered statistically significant. It was highly significant when the p-value < 0.01.

3-RESULTS

3.1. Extraction of *T. repens*

The extraction yield of *T. repens* flowers revealed that the ethanolic extract yield (29.62 g/kg) was higher than that of the water extract (24.43 g/kg) (**Table 1).**

Table 1: Extraction yield (g/kg) and total phenolic content (mg gallic acid/g) in *T. repens* flowers.

Sample	Extraction yield (g/Kg) (±) SE	Total phenolic content (mg/g) (±) SE
Water Extract	24.43±0.95	65.04±1.69
Alcoholic Extract	29.62±1.06	74.13±2.05

High-performance liquid Chromatography (HPLC) analyzed *T. repens* of water flower extract identified Caffeic acid> Gallic acid>Naringenin>Vanillin>quercetin> chlorogenic acid>Rutin>Hesperetin>Kaempferol>Rosmarinic

acid>Methyl gallate>Pyrocatechol>Catechin>Ferulic acid>Coumaric acid>Daidzein >Cinnamic acid. While HPLC in T.repens ethanolic flower extract revealed ordered as follows: Caffeic acid >Naringenin > chlorogenic acid>Rutin >Kaempferol > Gallic acid>Catechin >quercetin >Vanillin Methyl gallate >Ferulic acid >Cinnamic acid >Rosmarinic acid >Daidzein Pyrocatechol >Hesperetin >Coumaric acid. **(Table 2).**

Table 2: HPLC fractions of *the water and ethanolic extracts of T. repens*.

Name	Water sample (Conc.µg/g)	Ethanolic sample (Conc.µg/g)
Caffeic acid	4286.67	17592.53
Catechin	28.97	806.59
Chlorogenic acid	446.89	2612.33
Cinnamic acid	4.60	338.95
Coumaric acid	21.04	88.68
Daidzein	16.86	211.59
Ferulic acid	25.58	448.43
Gallic acid	1341.79	1271.10
Hesperetin	220.80	89.23
Kaempferol	176.99	1424.33
Methyl gallate	72.34	452.19
Naringenin	1260.79	5135.69
Pyro catechol	61.81	99.02
Querectin	646.19	770.37
Rosmarinic acid	157.50	281.68
Rutin	280.74	1940.97
Vanillin	659.61	576.06

3.2. Morphological Results

3.2.1. Body Weight Assessment:

The initial body weights of the groups did not differ significantly. BW increased substantially ($P < 0.05$) after two months of MetS induction compared to the control group. After one month of treatment, MetS-induced rats still recorded higher body weight compared to the control group, while MetS-induced rats treated with different *T. repens* flower extracts and MF showed a highly significant decrease ($P < 0.01$) in the final body weight compared with those in the MetS-induced group **(Table 3).**

3.2.2. Liver absolute and relative weights

After 12 weeks of the experiment, the absolute and relative liver weights were significantly higher ($P < 0.01$) in the MetS group than in the control group. In contrast, relative liver weights were significantly decreased ($P < 0.01$) in all MetS-induced treated (*T repens* extracts or MF) groups compared to the MetS-induced group only **(Table 3).**

3.2.3. Fat Absolute and relative weights

There were highly significant increases ($P < 0.01$) in absolute and relative fat weights in the MetS group compared to those in the control group, but absolute and relative fat weights were significantly decreased ($P < 0.01$) in all treated groups (*T repens* extracts or MF) compared to those in the MetS group **(Table 3).**

Table 3: Initial, final body weights (g), percentage body weight change, liver and fat absolute (g), and relative weights (%) in the control, MetS, and different treatment groups.

Parameter	Control	MetS	MetS + WT 50	MetS +ET 50	MetS +WT 100	MetS +ET 100	MetS +MF
Initial body weight	142.00±4.24	138.00±3.01	140.00±2.40	138.00±2.88	140.00±2.84	141.67±4.26	140.00±3.70
Final body weight	272.00±10.79	295.00±3.83*	262.50±8.87^{++}	262.33±8.90^{++}	238.17±5.29^{++}	252.67±4.50^{++}	219.00±4.33^{++}
Body weight change %		8.46 %	-11.02 %	-11.07 %	-19.26 %	-14.35 %	-25.76 %
Absolute liver	5.80 ±0.15	7.77 ±0.54 **	7.30± 0.55	6.93± 0.57	6.05± 0.15	7.02± 0.17	6.73± 0.29

The relative weight of the liver	2.20 ±0.07	3.03 ±0.02 **	2.27± 0.10++	2.40± 0.06++	2.45± 0.02++	2.40± 0.03++	2.40± 0.06 ++
Absolute fat	3.62 ±0.19	7.87 ±0.23 **	5.87± 0.35++	4.38± 0.17++	3.08± .05++	3.72± 0.16++	2.17± 0.22 ++
The Relative weight of fat	1.02 ±0.0.09	3.03 ±0.03 **	2.27± 0.10 ++	1.48± 0.0.07 ++	1.27± 0.03++	1.62± 0.07++	0.90± 0.01 ++

The values represent the mean ±SE of six rats in each group. Using ANOVA and t-test, *p<0.05, **P<0.01 when compared to the control group, and +p<0.05, ++p<0.01 when compared to the MetS group.

Table 4: Liver function enzyme activities in the control, MetS, and different treatment groups.

Parameter	**Control**	**MetS**	**MetS+WT 50**	**MetS+ET 50**	**MetS+WT 100**	**MetS+ET 100**	**MetS+MF**
AST(U/L)	93.2 ±1.42	120.00 ±4.43**	91.00 ±6.31++	86.23 ±2.61++	84.20 ±3.26 ++	83.20 ±5.57 ++	90.02 ±1.47++
ALT (U/L)	31.4 ±1.70	44.83± 1.07**	31.60 ±1.61++	28.03 ±1.89++	28.20 ±1.86 ++	27.10± 1.93++	26.00 ±2.59++

The values represent the mean ±SE of six rats in each group. Using ANOVA and t-test, *p<0.05, **P<0.01 when compared to the control group, and +p<0.05, ++p<0.01 when compared to the MetS group.

3.2.4. Redox State Parameters

MDA levels were substantially higher (P<0.01) in the MetS group than in the normal group, but there was a highly significant increase (P<0.01) in all treated groups compared to the MetS group. Although GSH level was not significantly different, it decreased in the MetS group compared to the normal group, but increased in all treated groups in comparison with the MetS induction group. GSHpx was also measured. All treated groups exhibited a substantial increase (P < 0.01), in contrast to the MetS induction group, while the MetS induction group demonstrated a highly significant reduction (P < 0.01) relative to the normal group (Table 8).

Table 5: serum malondialdehyde, glutathione, and glutathione peroxidase activity in control, MetS, and different treatment groups.

Parameter	**Control**	**MetS**	**MetS+WT 50**	**MetS+ET 50**	**MetS+WT100**	**MetS+ET 100**	**MetS+MF**
MDA (nmol/mg)	2.33 ±0.15	3.05± 0.16**	2.87± 0.18++	2.03± 0.19++	2.1 ±0.18++	2.00± 0.17++	2.05± 0.18++
GSH Red (nmol/mg)	24.00± 2.02	20.06 ±1.72	21.78 ±1.9	22.38 ±1.9	23.6±1.7	25.2± 1.8	26 ±1.5
GSHpx(U/L)	26.80± 0.75	17.27 ±0.05 **	28.38 ±0.11 ++	28.00 ±0.13 ++	29.20± 0.22++	29.20 ±0.22 ++	26.92 ±0.11 ++

The values represent the mean ±SE of six rats in each group. Using ANOVA and t-test, *p<0.05, **P<0.01 when compared to the control group, and +p<0.05, ++p<0.01 when compared to the MetS group.

3.2.5. Inflammatory Process parameters

TNF-α and CRP levels in the MetS induction group were significantly higher (P < 0.01) than those in the normal group. In contrast to the MetS induction group, there was a significant reduction (P < 0.05) in TNF-α levels in the MetS+ET100 and MetS+MF groups and a significant reduction (P <

0.05) in CRP levels in all treated groups. In contrast to the MetS induction group, there was a substantial increase ($P < 0.05$) in MetS+WT100, MetS+ET100, and MetS+MF in IL-10, but a significant decrease ($P < 0.05$) in MetS (Table 8).

Table 6: serum TNF-α, C-reactive protein, and IL-10 in the control, MetS, and different treatment groups.

Parameter	Control	MetS	MetS+WT50	MetS+ET50	MetS+WT100	MetS+ET100	MetS+MF
TNF-α (pg/ml)	4.50±0.18	5.92±0.11**	5.28±0.23+	4.66±0.12+	4.30±0.17+	4.2±0.18+	4.32±0.17+
CRP (mg/l)	3.58±0.26	5.68±0.2**	4.62±0.18+	4.68±0.13+	4.10±0.10+	4.18±0.19+	4.44±0.15+
InterleukinIL-10(pg/ml)	2.96±0.14	2.34±0.12*	2.86±0.10	2.94±0.16	3.11±0.13+	3.12±0.15+	3.10±0.13+

The values represent the mean ±SE of six rats in each group. Using ANOVA and t-test, *p<0.05, **P<0.01 when compared to the control group, and +p<0.05, ++p<0.01 when compared to the MetS group.

3.2.6.Histological Scoring

As shown in Table 7, a highly significant increase ($P < 0.01$) in liver steatosis was observed in the MetS group compared with the normal group. In contrast, all treated groups showed a highly significant decrease ($P < 0.01$) compared with the MetS-induced group.

Fibrosis: A highly significant increase in fibrosis ($P < 0.01$) was observed in the liver sections of the MetS-induced group compared to the normal group. In contrast, a highly significant reduction ($P < 0.01$) was observed in all treated groups compared with the MetS-induced group.

Inflammatory infiltration: A highly significant increase ($P < 0.01$) was observed in the MetS group compared to the normal group, whereas a highly significant reduction ($P < 0.01$) was observed in all treated groups compared to the MetS group.

Table 7: Semi-quantitative morphometric analysis of liver sections in the control, MetS, and different treated groups.

Parameter	Control	MetS	MetS+WT100	MetS+ET100	MetS+MF
Degree of steatosis	0.33±0.21	3.00±0.00**	1.33±0.21++	0.67±0.21++	0.67±0.21++
Fibrosis	0.00±0.00	2.67±0.21**	2.00±0.00++	1.00±0.00++	0.65±0.22++
Inflammatory infiltration	0.33±0.21	3.00±0.00**	1.33±0.21++	0.67±0.21++	0.67±0.21++

The values represent the mean ±SE of six rats in each group. Using ANOVA and t-test, *p<0.05, **P<0.01 when compared to the control group, and +p<0.05, ++p<0.01 when compared to the MetS group.

3.4. Light microscopic results

3.4. Light microscopic results

The histopathological results of the liver

Figure 1 showed Normal architecture of liver tissue components in basic hepatic lobules. Hepatocytes are arranged in radial cords around a central vein and separated by blood sinusoids. a central vein (CV) surrounded by an inflammatory infiltrate. Hepatocyte with an enlarged nucleus and steatosis (lipid accumulation). Dilated hepatic portal area with inflammatory cell infiltration, along with ballooning degeneration of hepatocytes, is shown in Figure 2A, B. Mild lymphoplasmacytic infiltrates were observed in the perivascular and periportal regions. A central vein surrounded by mildly normal hepatocytes and reduced-swelling hepatocytes compared to the positive control group. The hepatic duct and hepatic portal vein appear dilated. Hepatocytes with less inflammatory

infiltration compared to the positive control group, as observed in Figure 3C, D. Treatment group (ET 100) hepatocytes appear near normal hepatocytes, where a central vein is surrounded by hepatocytes arranged in radiant cords and sinusoids near normal. Moreover, the hepatocytes in the less-infiltrated group were compared to the positive control group, as shown in Figure 4. Decreased infiltration, especially in the perivascular region. Infiltration is decreased compared to the positive control group

Figure 1: Liver section of the control group showing Normal appearance, hepatic lobules (H). central vein (CV), and blood sinusoids (BS).H&E, x400. The image scale bar represents 100µum.

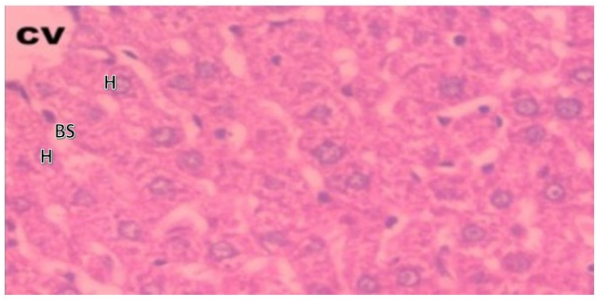
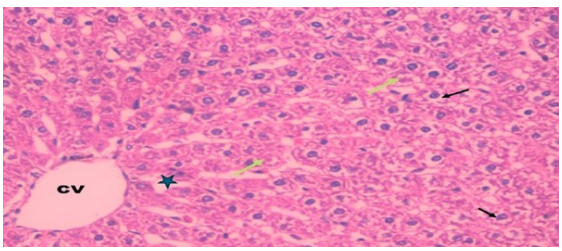


Figure (2A, B): Liver section of MetS (Control Positive group) showing a central vein (CV) and inflammatory infiltrate (★). Enlarged nucleus (green arrow) and steatosis (black arrow), as seen in Figure 2A. Dilated hepatic portal area (HPT) with inflammatory cell infiltration (HD), and ballooning degeneration (green arrow), is shown in Figure 2B. H&E, x400. The scale bar represents 100 µm.

A



B

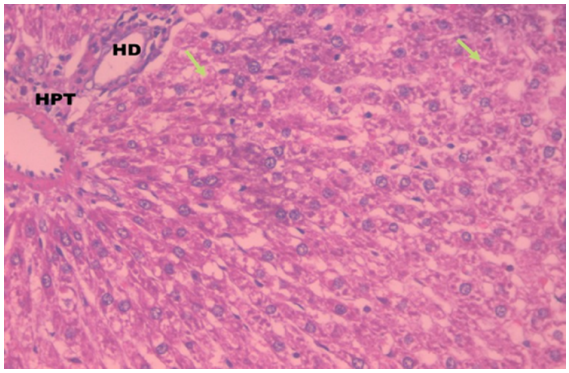
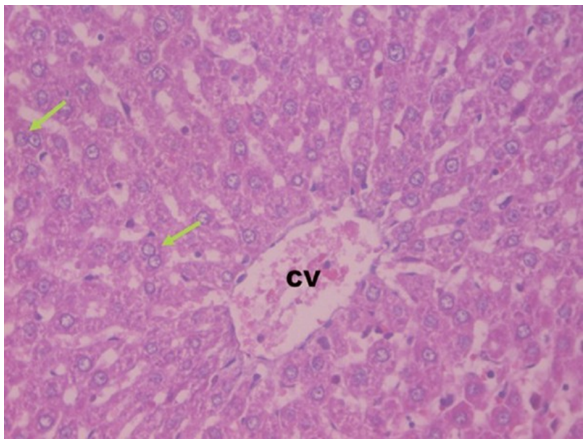


Figure (3C, D): Liver sections of the MetS +WT100 group showed A central vein (CV), reduced-swelling hepatocytes (green arrow) (Figure 3C). The hepatic duct (hd) and portal vein (hpv) with less inflammatory infiltration (red arrow) (Figure 3C).**H&E, x400. The image scale bar represents 100µum.**

C



D

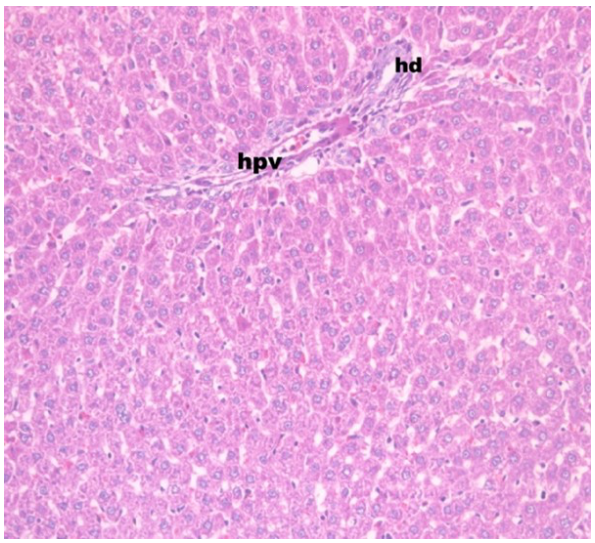


Figure (4): Liver sections from the MetS +ET100 group show anear normal architecture, central vein (CV), radiant cords, and sinusoids near normal (green arrow) with less infiltration (yellow arrow), compared to the positive control group. H&E, x400. The image scale bar represents 100µum.

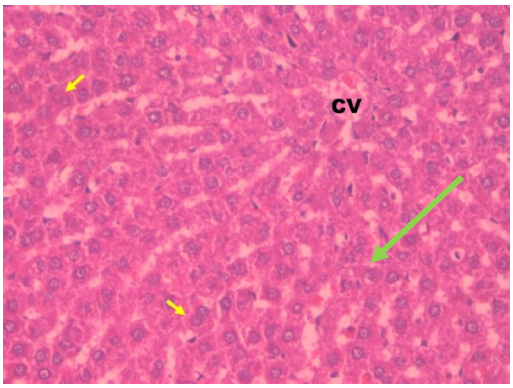
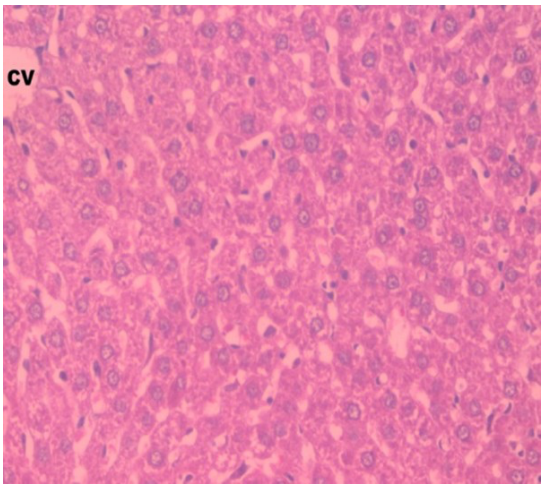


Figure (5): Liver section of the MetS+MF group shows an improved number of normal hepatocytes (green arrow), and infiltration (red arrow) is decreased compared to the positive control group. H&E, x400. The image scale bar represents 100µum.



DISCUSSION

Numerous metabolic abnormalities were developed in rats fed HFD and high-fructose drinking water. The last published data from this study revealed that the rats had hyperglycemia, accompanied by hyperinsulinemia and insulin resistance. Additionally, they had hypertriglyceridemia and decreased HDL-C levels, confirming the induction of MetS [3]. This study aimed to examine the risks associated with MetS and to provide effective strategies for reducing them. Medicinal plants contain a wealth of physiologically active compounds with a range of pharmacological effects on animals. Approximately 60–80% of the global population relies on traditional medical practices to treat common ailments. Additionally, a main goal of drug development research is to identify plant-based antioxidants that effectively neutralize reactive oxygen species (ROS), as plants are rich in compounds such as phenols, saponins, and fatty acids [41].

Our findings are consistent with those of others, who have shown that HPLC can be used to quantify polyphenols in plant extracts. This study validated the substantial impact of using alcohol as an extraction medium compared to water. Ethanol enhances the antioxidant capacity of clover extracts. Jakubczyk *et al*. [42] found that ethanolic extracts exhibited superior antioxidant activities compared to aqueous extracts.

Phytochemicals have many health benefits. It can normalize BW and fat in mouse and rat models of diet-induced MetS [43] and in humans [44]. Trifolium (*Fabaceae*) is an important plant species. All Trifolium species are recognized for their traditional medicinal use [45,46]. In the current study, HPLC fractionation of T. repens flower extracts (table 2) showed many active biochemical compounds, such as phenols (e.g., pyrocatechol and vanillin), Flavones (like naringenin), flavonols (like kaempferol and rutin), phenolic acids (including gallic acid, chlorogenic acid, caffeine, coumaric acid, ferulic acid, rosmarinic acid, ellagic acid, methyl gallate), isoflavones (e.g., daidzein), as well as catechin (a flavonoid but not specifically under flavones or flavonols), and cinnamic acid (a derivative of phenolic acid). These phytochemicals could serve as therapeutic agents for addressing the side effects of MetS. Previous studies have used these phytochemicals to protect against oxidative damage and to reduce metabolic and inflammatory markers associated with MetS [47].

Our study revealed the presence of a large group of polyphenolic compounds, including flavonoids, flavanones(e.g., naringenin and hesperetin), and flavonols (e.g., quercetin). The presence of unique isoflavonoids (e.g., daidzein) in clover plants affects the development of various chronic diseases. The bioavailability of daidzein has been the subject of numerous investigations, including studies on abdominal obesity and hypertension in rats (48, 49). Therefore, the consumption of isoflavones may impact the development of diseases and specific circumstances that contribute to the development of metabolic syndrome [50]. The primary phenolic molecule

identified in our plant, quercetin, exhibits several bioactivities, including antibacterial and antioxidant properties [51]. Its role in the prevention of various pathogeneses is attributed to its ability to inhibit OS and inflammation, as well as to enhance endogenous antioxidant defense mechanisms [52].

Our results demonstrated that rats with MetS gained more BW than rats in the control group. This finding is supported by Marques *et al.* [53] and Moreno-Fernández *et al.* [54], who found significant increases in body weight in Wistar male rats after feeding HFD (high-fat/high-sucrose and high-fat/high-fructose). These findings may stem from an imbalance between energy expenditure and uptake, leading to the accumulation of fat in white adipose tissue. In the present study, the final body weights of MetS-induced rats treated (for one month) with *T. repens* flower extracts (W and E) or the MF drug showed a significant reduction in the following groups: MetS+W50T, MetS+E50T, MetS+W100T, MetS+E100 T, and MetS+MF, compared to the MetS group. Rufino *et al.* [55] found that body weight decreased significantly in T. repens, suggesting that flavonoids may have anti-obesity properties. These effects include appetite control, reduced meal intake, and decreased intestinal fat absorption.

After 12 weeks of the experiment, both absolute and relative liver weights were higher in rats with MetS than in those without MetS. Increased levels are associated with lipid accumulation in hepatocytes resulting from insulin resistance. The influx of free fatty acids into the liver stimulates triglyceride synthesis and intracellular fat accumulation, leading to larger hepatocytes [56]. Relative liver weight decreased in all MetS-induced groups treated with *T. repens* (W and E). These effects may result from the phenolic compound content in the flower extracts, which can serve as a source of beneficial bioactive compounds (quercetin) with hepatoprotective properties [57].

Absolute and relative fat weights increased significantly in MetS-induced rats compared with the control group, in agreement with Buettner *et al*. [58]. They reported that HFD feeding can induce obesity, adipose tissue, and metabolic disorders in rodents that resemble human MetS. In contrast, absolute and relative fat weights significantly decreased in all treated groups with T. repens flower extract and the MF drug compared with the MetS induction group. These results are consistent with those of Oliveira *et al.* [59]. The anti-obesity effects of flavonoids in *T. repens* occur by modulating proteins, genes, and transcription factors that contribute to reduced lipogenesis, increased lipolysis, and increased energy expenditure. Previous results in this paper indicated that water and ethanolic extractions of T. repens positively affected body and organ weights, as well as MF, according to Jakubczyk et al. [42].

In the present study, a significant increase in liver enzymes was linked to obesity and its associated comorbidities. Metabolically dysfunctional steatohepatitis (MASH) is a severe form of metabolic dysfunction-associated steatotic liver disease (MASLD). These disorders are associated with inflammatory changes and liver damage [60]. Liver enzyme levels are decreased and improved in *T. repens* because this extract contains antioxidants, such as phenolic acids (e.g., gallic acid), that protect liver cells from damage, as well as anti-inflammatory compounds. Its mechanisms involve direct free-radical quenching, metal chelation, and the upregulation of endogenous antioxidant pathways [61]. Additionally, naringenin is a flavonoid with broad preventive effects against liver disease. It causes lipid metabolism, inflammation, fibrogenesis, and liver cancer. In models of non-alcoholic fatty liver disease, it reduces steatosis by regulating lipid and cholesterol synthesis and oxidation [62].

The results of the current study revealed that feeding rats a diet containing 30% HFD and 10% fructose in drinking water was associated with many symptoms of MetS. Histopathological results showed that, after 12 weeks of the experiment, hepatocytes in rats with MetS exhibited greater ballooning than those without MetS. These findings concur with those of Perumpail *et al*. [63], who demonstrated a high correlation between the characteristics of MetS and nonalcoholic fatty liver disease (NAFLD). Increased liver fat is associated with steatotic liver disease. The term "hepatic steatosis" refers to the accumulation of lipids in hepatocytes. This usually occurs when there is an imbalance between lipid synthesis and intake, and between the export and oxidation processes that dispose of them. The liver receives more free fatty acids when lipolysis is inhibited, particularly in the presence of insulin resistance [64]. In the present study, liver function improved in all MetS-induced groups treated with *T. repens* (W and E). Several isoflavonoids, such as daidzein in the flower extracts, possess antioxidant and anti-inflammatory

properties and can serve as sources of bioactive compounds with hepatoprotective effects, potentially reducing liver necrosis [65].

OX results from a high-fat diet, as it increases the generation of reactive oxygen species (ROS). MDA is a byproduct of lipid peroxidation caused by ROS, and elevated MDA levels indicate increased oxidative damage [66]. This process damages cell membranes and other structures, contributing to various metabolic disorders [67]. GSH-Px is an enzyme that helps neutralize ROS by converting lipid peroxides and hydrogen peroxide into lipid alcohols and water, respectively. A high-fat diet increases OS, prompting the body to upregulate GSH-Px activity as a protective mechanism. The impact of HFD on GSH-Px activity can vary among individuals [68]. While some tissues may show a decrease in activity due to overwhelming OS, others may exhibit an increase as part of the body's adaptive response to counteract elevated ROS levels [69]. The current study revealed a decrease in MDA levels but an increase in GSH-Px levels in *T. repens,* most likely as a result of caffeic acid (one of the components of *T. repens).* It is a polyphenolic compound known to improve redox status. According to Pavlíková [70], it contains antioxidants that can inhibit obesity-associated inflammation [71]. Caffeic acid can positively influence the gut microbiota, which, in turn, can help reduce OS and inflammation. Caffeic acid can improve overall metabolic health and reduce the negative effects of a HFD [72]. The simultaneous decrease in MDA but increase in GSH-px reflects a biological response in which the body attempts to balance oxidative damage (indicated by MDA) with an enhanced antioxidant defense (indicated by GSH-px) in induced MetS [73].

MetS is characterized by a chronic, low-grade inflammatory state, reflected in elevated levels of pro-inflammatory cytokines such as TNF-α and CRP, and decreased levels of anti-inflammatory cytokines, primarily IL-10. The liver is the primary source of CRP production in response to inflammatory cytokines, and abdominal obesity and increased visceral fat are associated with elevated CRP levels in the blood. This is attributed to the increased secretion of a group of adipokines with inflammatory effects by fat cells, in addition to the association of this condition with insulin resistance [74]. Fat cells are a significant source of TNF-α production, and their levels are elevated in the adipose and muscle tissues of individuals with insulin resistance. TNF-α contributes to disrupting the insulin signaling pathway within fat cells and inhibits lipoprotein lipase activity, thus limiting fat storage and promoting the production of triglyceride-rich lipoproteins. This results in impaired lipid metabolism and elevated triglyceride levels in the blood. In addition, inflammatory processes affect insulin sensitivity and the regulation of cholesterol uptake and excretion within phagocytic cells [75]. The anti-inflammatory cytokine IL-10, produced in several organs, including the spleen, plays a crucial protective role against pathological inflammation in the liver. Low levels of IL-10 have been linked to an increased risk of metabolic syndrome and worsening of liver cirrhosis [74]. This study has shown that caffeic acid (CA), extracted from Trifolium flowers, possesses anti-inflammatory properties by reducing OS and enhancing antioxidant defenses. It also elevates IL-10 levels, thereby enhancing its anti-inflammatory effect [76,77].

Conclusion:

Trifolium flower extracts possess antioxidant properties; therefore, they can help reduce OS, which plays a key role in the development of various metabolic disorders, including harmful effects on the structure and function of different organs. Lowering oxidative stress benefits inflammation by decreasing levels of TNF-α and CRP. These biochemical improvements were supported by histopathological examinations, where Trifolium flower extracts were used as a hepatoprotective therapeutic in MetS.

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