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Impact of *Trifolium repens* flower extracts on morphological and biochemical changes in male rats with metabolic syndrome

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Abstract

Metabolic syndrome (MetS) is a serious health condition that causes several diseases. It is characterized by insulin resistance, obesity, hypertension and dyslipidemia. Trifolium repens (T. repens, family Fabaceae), commonly known as white clover, is used in traditional medicine in Egypt and other countries. This study aimed to determine the effects of T. repens (T) flower extracts (water and ethanolic; W and E) on morphological, biochemical, and redox status changes associated with MetS induction in male rats. ***Methods:*** *This study was conducted on 42 male albino rats weighing 120–150 g. Six rats were used as a control group and fed a normal balanced diet. MetS was induced in male rats (36) by feeding them a high-fat diet (HFD; a regular chow diet for laboratory rats adjusted to 30% fat content) and 10% fructose solution in their drinking water. The MetS-induced rats were divided into a MetS positive control group, MetS + W50 and 100T, MetS+ E50 and 100 T, and MetS + metformin (MF) drug as the reference group.*

Morphological parameters, including body and organ weights, were measured. Biochemical parameters (fasting blood glucose and insulin levels, glycated hemoglobin A1c (HbA1c), serum lipid profile, and kidney function tests) were assessed. In addition, a lipid peroxidation marker, malondialdehyde (MDA), antioxidant markers (glutathione, GSH), and glutathione peroxidase (GSHpx) activity were measured.

Results: *MetS-induced rats had higher body weights and organ weights (liver, pancreas, heart, and internal fat), as well as elevated relative organ weights. Induced MetS Rats had elevated fasting blood glucose, serum insulin, and HbA1c levels. The serum lipid profile showed enhanced total cholesterol (TC) and triglycerides (TG), while high-density lipoprotein cholesterol (HDL-C) decreased compared to those in the control group. Only serum creatinine had elevated concentrations, but not urea concentration in kidney tests. The serum oxidant marker MDA increased and the anti-oxidant marker GSHpx activity also increased in the MetS-induced group compared to those in the control group. In contrast, T. repens flower extracts showed enhanced morphological and biochemical changes in MetS-induced rats, nearly similar to those of the control and reference groups.*

Conclusion: *Both HFD and fructose in drinking water successfully induced MetS in a male albino rat model with desired metabolic changes. T. repens flower extracts played a significant role in improving the side effects of MetS in male albino rats.*

Keywords*: Trifolium repens, white clover, metabolic syndrome, morphological changes, biochemical changes, redox status, male rats*

Introduction

Metabolic syndrome (MetS) is a disease of the twenty-first century. This is a serious nutritional metabolic syndrome [(1)]. Currently, more than 30% of the adult population has this syndrome, and this percentage has

increased in recent years [2,3]. MetS in humans may be due to a sedentary lifestyle and dietary, neuroendocrine, or genetic changes [4]**.** Long-term HFD consumption is an important risk factor for MetS, which may increase oxidative stress (OS) [5]**.** It is an imbalance between the production of free radical species and antioxidant defense systems, which can cause damage to cellular biomolecules including lipids, proteins, and DNA [6]. Previous studies have demonstrated that chronic consumption of an HFD increased body and organ weights in humans [7], rats [8], and rabbits [9]. Fat is one of the three main macronutrients and the most calorically dense macronutrient [10]. Different types of high-fat diets have been used to develop MetS models, either from animal-derived fats, such as butter or lard, or plant oils, such as corn oil and soybean oil. Diet models containing 30-70% fat increase body weight; cause hyperglycemia, insulin resistance, and dyslipidemia; and increase free fatty acids in the blood [11, 12]

Phytochemicals have numerous health benefits. It can normalize body weight and fat in mouse and rat models of diet-induced MetS [13] and humans [14]. *Trifolium* species are rich in biologically active compounds such as isoflavones [15]. All Trifolium species are known to act as traditional medicines [16]. There is a lack of literature on the biological compounds in *T. repens* and their medicinal properties, although the flowers of *T. repens* contain several important phenolic compounds [17]. Therefore, this study aimed to use *T. repens* flower extracts to reduce or prevent the symptoms of MetS in rats after HFD and fructose intake in drinking water for three months.

Materials and methods

Trifolium repens flowers collection and extraction

T. repens flowers were collected from the Al Addakahlia governate in April and May 2023. The samples were washed twice with distilled water and air-dried at room temperature till complete drying. Three successive, constant weights were recorded.

T. repens flower water and ethanolic (300 g for each) extractions were performed at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University. The total phenolic content (TPC) was determined using the Folin-Ciocalteu reagent (FCR) method [18], while the antioxidant activity of the extract was determined by the DPPH free radical scavenging assay in triplicate, and average values were considered [19]. The concentrated extracts were labeled and stored in a refrigerator to prevent degradation.

Metabolic syndrome induction and Experimental design

Forty-two adult male albino rats weighing 120 – 150 g were obtained from the animal farm of El-Nile Company for Pharmaceutical Products (Cairo, Egypt). The animals were housed in suitable cages (40 × 32 × 40 cm for every 5 rats) at controlled humidity, temperature 22±2 °C, and a 12/12 light-dark cycle. All procedures were approved by the Animal Care Committee of the Al-Azhar University. Rats were kept on a normal diet with the following macronutrient composition: 4.6 % fat, 23 % protein and 72.4 Carbohydrates and had free access to water for acclimatization before starting the experiment for two weeks. Six of them were fed a normal diet until the end of the experiment (Group 1). 36 rats fed HFD with the following macronutrient composition: 30 % fat (butter), 18 % protein, and 52% carbohydrates and were supplied with 10% fructose solution in their drinking water for three months. In the last month, the MetS-induced animals were classified according to the different treatments as follows: the positive control MetS-induced group was fed a HFD and 10 % fructose solution in drinking water during the experimental period (three months).

The third, fourth, fifth, sixth, and seventh groups were also fed as the second group; however, at the beginning of the third month, the rats were treated as follows:

The third group was treated with W50 mgT /kg body weight (Bwt)

The fourth group was treated with E50mg T/kg Bwt

The fifth group was treated with W100mg T/kg Bwt

The sixth group was treated with E100 mgT/kg Bwt and

The seventh group was treated with metformin (MF) drug (200mg/kg /kg Bwt)[20]

All treatments were performed daily by using a gastric tube.

Body and relative organ weights.

The body weight of the rats in each group was recorded weekly. At the end of the study period, body and organ weights (liver, kidney, heart, and internal fat) were measured.

Relative organ weight was calculated as a percentage, as follows:

Relative organ weight (%) = organ weight (g)/body weight (g) × 100 [21]

Blood sampling

At the end of 12 weeks, the rats were fasted overnight for 12 hours with free drinking water. Blood was collected from the retroorbital venous plexus of the eyes of each rat using a heparinized capillary tube. Blood was collected in two clean, dry glass tubes. The first tube contained EDTA to measure glycosylated hemoglobin (HbA1c), and the second tube had normal blood and was allowed to clot to obtain serum. Blood samples were centrifuged at 3000 RPM for 10 min to obtain the serum. The supernatant sera were collected into Eppendorf tubes and stored at -20°C for biochemical analysis.

Serum biochemical parameters

Blood sugar levels were measured using the glucose oxidase method [22], whereas serum insulin levels were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Abcam, catalog number: ab273188) [23]. The level of insulin resistance was estimated using the homeostatic model assessment of insulin resistance (HOMA-IR), which was calculated using the following equation [24]:

HOMA – IR = FSI (μU/ml) × FSG (mmol/L) / 22.5. The insulin sensitivity index (ISI) was calculated as Ln (FBG × FSI) $^{-1}$ [25].

Where FSI is the fasting serum insulin level and FSG is the fasting serum glucose concentration.

Serum lipid profile

Total cholesterol (TC), triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C). Friedwald *et al*. [26] equations were used to calculate serum low-density lipoprotein cholesterol (LDL-C) and very-low-density lipoprotein cholesterol (VLDL-C). After calculating serum LDL and VLDL levels, the TC/HDL (risk factor 1) and LDL-C/HDL-C (risk factor 2) ratios were calculated.

The Friedwald equation for VLDL-C and LDL-C is as follows:

VLD L-C=TG/5 LDL-C= TC – HDL-C - (TG/5)

Kidney Function tests: specific markers related to renal function including levels of urea and creatinine in the sera were assessed spectrophotometrically according to Kielstein *et al.* [27] and Bagshaw and Gibney [28] respectively.

Serum redox state

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.* [29], GSH was assessed spectrophotometrically according to the method of Beutler *et al*. [30] and GSHpx activity was determined according to the method of Paglia and Valentine [31]. All the kits were purchased from Biodiagnostic Co. Cairo, Egypt.

Statistical analysis

The statistical variation between the control (normal and positive) and test groups were evaluated by t-test using the statistical software SPSS (Statistical Package for Social Science) Statistical program version (25.0).

RESULTS

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

Table (1) Extraction yield (g/kg) and extracted 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**

Table 2. High-performance liquid Chromatography (HPLC) analyzed *T. repens* of water flower extract identified coffei acid>Gallicacid>Naringenin>Vanillin>quercetin>chlorogrnicacid>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: coffeic acid >Naringenin >chlorogrnicacid >Rutin >Kaempferol >Gallicacid >Catechin >quercetin >Vanillin Methyl gallate >Ferulic acid >Cinnamic acid >Rosmarinic acid >Daidzein Pyrocatechol >Hesperetin >Coumaric acid.

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

name	**Water sample (Conc.µg/g)**	**Ethanolic sample (Conc.µg/g)**
Gallic acid	1341.79	1271.10
Chlorogenic acid	446.89	2612.33
Catechin	28.97	806.59
Methyl gallate	72.34	452.19
Coffeic acid	4286.67	17592.53
Syringic acid	0.00	0.00
Pyro catechol	61.81	99.02
Rutin	280.74	1940.97
Ellagic acid	0.00	0.00
Coumaric acid	21.04	88.68
Vanillin	659.61	576.06
Ferulic acid	25.58	448.43
Naringenin	1260.79	5135.69
Rosmarinic acid	157.50	281.68

Daidzein	16.86	211.59
Querectin	646.19	770.37
Cinnamic acid	4.60	338.95
Kaempferol	176.99	1424.33
Hesperetin	220.80	89.23

Morphological changes

The body and organs' weights

There were no significant differences in the initial body weights between the groups. After 2 months of MetS induction, body weights significantly increased ($P < 0.05$) compared to those in the control group. After one month of treatment, the MetS-induced rats with different *T. repens* flower extracts and MF significantly decreased ($P < 0.01$) the final body weights compared to those in the MetS-induced group (Table 3).

Liver (absolute and relative) weights: After 12 weeks of the experiment, absolute and relative liver weights were significantly higher ($P < 0.01$) in the MetS-induced 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 (Table 4).

Pancreas (absolute and relative) weights: There were highly significant decreases ($P < 0.01$) in the absolute weight of the pancreas in the MetS+E50T, MetS+W100T, and MetS+E100T groups; however, the relative weight of the pancreas also showed significant decrease ($P < 0.05$) in both MetS+W50Tand MetS+E100T groups as compared to the MetS-induced group (Table 4).

Heart (absolute and relative) weights: There were highly significant increases ($P < 0.01$) in absolute and relative heart weights in the MetS-induced group compared with those in the control group. The absolute and relative heart weights were significantly decreased ($P < 0.01$) in all treated groups compared with those in the MetS-induced group.

Fat (absolute and relative) weights: There were highly significant increases ($P < 0.01$) in absolute and relative fat weights in the MetS-induced group compared to those in the control group, but absolute and relative fat weights were significantly decreased ($P < 0.01$) in all treated groups compared to those in the MetS-induced group (Table 5).

Biochemical parameters

Glucose homeostasis parameters

As illustrated in Table (6), levels of blood glucose, insulin, and HOMA-IR showed highly significant increases ($P < 0.01$) and HbA1c showed a significant increase ($P < 0.05$) in the MetS-induced group compared to those in the control group. Conversely, the levels of blood glucose and insulin were significantly decreased ($P < 0.05$) in both the MetS+E50T and MetS+MF groups, and HbA1c was significantly decreased ($P < 0.05$) in the MetS+W100T and MetS+MF groups compared to those in the MetS-induced group. The blood glucose level and HOMA-IR were significantly decreased ($P < 0.01$) in the MetS+W100T and MetS+MF groups compared to those in the MetS-induced group. In contrast, the insulin sensitivity index (ISI) was significantly decreased ($P < 0.01$) in the MetS-induced group compared to the normal group. ISI was enhanced in MetS- induced rats when treated with T. repens extracts or MF ($P < 0.01$) table (6).

Serum lipid profile

Table (7) depicted the results of the lipid profile; triglyceride (TG) showed a highly significant increase ($P < 0.01$) in the MetS-induced group compared to the control group. Meanwhile, TG was significantly decreased (P

< 0.05) in the MetS+W100T group and a highly significant decreased (P < 0.01) in the MetS+MF group compared to the MetS-induced group. The present study showed that total cholesterol (TC) had a significant increase (P < 0.05) in the MetS-induced group compared with that in the control group. TC was significantly decreased (P < 0.05) in MetS+E50T, MetS+W100T, and MetS+E100T groups, but the MetS+MF group had a highly significant decrease in TC (P < 0.01) as compared to the MetS-induced group. HDL-C level significantly decreased (P < 0.05) in the MetS-induced group compared to that in the control group. Among the MetS+E50 T, MetS+W100 T, and MetS+E100MetS+ T groups, HDL-C levels were significantly increased (P < 0.05) and a highly significant increase (P < 0.01) in the MetS+MF group compared to the MetS- induced group. There was also a significant decrease (P < 0.05) in LDL-C levels in the MetS+E100Tand MetS+MF groups compared with the MetS-induced group. VLDL-C in the MetS-induced group had a highly significant increase (P < 0.01) compared to the control group, but a highly significant decrease (P < 0.01) in the MetS+MF group compared to the MetS-induced group. Our results showed a significant decrease (P < 0.05) in TC/HDL (risk factor 1) and LDL-C/HDL-C (risk factor 2) in the MetS+E50 T, MetS+W100 T, and MetS+E100 T groups, but a highly significant decrease (P < 0.01) in the MetS+MF group compared to the MetS-induced group.

The findings related to kidney function, as presented in Table (8), indicate a highly significant decrease (P < 0.01) in urea levels in the MetS+W100 and MetS+MF groups. Additionally, the MetS+E100T group showed a significant decrease (P < 0.05) in urea levels compared with the MetS induction group. In contrast, the MetS-induced rat group exhibited a significant increase (P < 0.05) in creatinine levels compared with the control group. However, the MetS+MF group demonstrated a significant decrease (P < 0.05) in creatinine levels compared with the MetS-induced group.

Serum redox state: MDA level was significantly higher (P<0.01) in the MetS-induced group than in the normal group, as shown in Table (9). Glutathione Peroxidase Activity (GSHpx) was also measured Showing a highly significant decrease (P < 0.01) in the MetS induction group as compared to the normal group but a highly significant increase (P < 0.01) in all treated groups as compared to MetS induction group as illustrated in the table (9).

Table (3): Initial and final body weights (g) of control, METS, and different treatment groups.

parameter	control	MetS	MetS+W50T	MetS+E50T	MetS+W100T	MetS+E100 T	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++

Values represent the mean ±SE of six rats in each group. *p< 0.05, **P<0.01 in comparison with the control group while, +p<0.05, ++P <0.01, in comparison with the MetS group using *the t-test*.

Table (4): Absolute and relative weights of liver and pancreas in control, METS, and different treated groups.

parameter	control	MetS	MetS+W50 T	MetS+E50 T	MetS+W100 T	MetS+E100 T	MetS+MF
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 pancreas	2.67±0.07	2.92±0.13	2.20±0.06++	2.58±0.12	2.02±0.07++	1.93±0.06+ +	2.32±0.29
The Relative weight of the pancreas	0.80±0.17	1.06±0.16	0.70±0.01+	0.82±0.01	0.80±.01	0.72±0.01+	0.80±0. 01

Values represent the mean ±SE of six rats in each group. *p< 0.05, **P<0.01 in comparison with the control group while, +p<0.05, ++P <0.01, in comparison with the MetS group using *the t-test*.

Table (5): Absolute and relative weights of heart and fat in control, METS, and different treatment groups.

parameter	**control**	**MetS**	**MetS+W50 T**	**MetS+E50 T**	**MetS+W100 T**	**MetS+E100 T**	**MetS+MF**
Absolute heart	1.25±0.06	2.40±0.10**	1.25±0.06++	1.00±0.00++	1.17±.03++	1.27±0.05+ +	1.22±0.04 ++
The Relative weight of the heart	0.46 ±0.02	0.81±0.05**	0.48±0.04++	0.38±0.01++	0.49±0.02++	0.50±0.02+ +	0.56±0.01 ++
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 ++

Values represent the mean ±SE of six rats in each group. *p< 0.05, **P<0.01 in comparison with the control group while, +p<0.05, ++P <0.01, in comparison with the MetS group using *the t-test*.

Table (6): Percentage of glucose, insulin, HOMA-IR, and HbAc1 in the control, METS, and different treatment groups.

parameter	control	MetS	MetS+W50T	MetS+E50T	MetS+W100T	MetS+E100 T	MetS+MF
Glucose(mg/dl)	85.40±2.04	118.20±5.39**	113.2±4.9	99±3.57^{+}	92.6±2.26^{++}	95±1.86^{++}	88±2.4 $^{++}$
Insulin(mu/ml)	1.94±0.06	3.75±0.17**	3.86±0.17	3.85±0.11	3.42±0.19	2.94±0.16	3.06±0.18^{+}
HOMA-IR	0.41±0.02	1.07±0.03**	1.07±0.05	0.94±0.05	0.78±0.05^{++}	0.69±0.05^{++}	0.67±0.042^{++}
HbA1c	3.54±0.17	4.60±0.183*	4.34±0.12	4.28±0.26	3.98±0.17^{+}	4.14±0.11	3.82±0.1^{+}
ISI	0.18±0.006	0.16±0.002*	0.16±0.002	0.17±0.002^{++}	0.17±0.002^{++}	0.18±0.003^{++}	0.18±0.003^{++}

Values are means ±SE of six rats in each group. *p< 0.05, **P<0.01 in comparison to the control group while,

+p<0.05, ++P <0.01, comparison to the MetS group using *the t-test*.

Table (7): Changes in lipid profile in control, METS, and different treatment groups.

parameter	control	MetS	MetS+W50T	MetS+E50T	MetS+W100T	MetS+E100 T	MetS+MF
Triglyceride(mg/dl)	77.40±1.67	136.00±2.21**	134.20±1.8	133.20±5.1	122.00±3.8^{+}	131.00±2.9	102.20±2.9^{++}
Total cholesterol(mg/dl)	95.60±2.5	110.20±1.2*	95.20±3.2	87.00±2.5^{+}	90.40±1.9^{+}	89.80±1.1^{+}	83.80±1.8^{++}
HDL-C(mg/dl)	33.00±0.70	21.00±0.20*	24.40±1.2	26.80±1.2^{+}	29.20±1.05^{+}	28.60±1.7^{+}	32.40±1.87^{++}
LDL-C(mg/dl)	48.00±3.2	60.12±2.9	44.36±2.39	35.68±2.7	36.80±1.89	35.9±1.2^{+}	31.96±2.8^{+}

VLDL-C(mg/dl)	15.50±0.33a	28.20. ±0.44**	26.80±0.36	26.60±1.03	24.40 ±0.77	26.30±0.58	20.40±0.33^{++}
TC/HDL	2.90±0.1	5.20±0.2	3.98±0.2	3.20±0.5^{+}	3.10±0.12^{+}	3.11±0.28^{+}	2.56±0.17^{++}
LDL/HDL	1.45±0.1	2.86±0.18	1.82±0.15	1.33±0.14^{+}	1.26±0.09^{+}	1.26±0.13^{+}	0.99±0.14^{++}

Values represent the mean ±SE of six rats in each group. *p< 0.05, **P<0.01 in comparison with the control group while, +p<0.05, ++P <0.01, in comparison with the MetS group using *the t-test*.

Table (8): Serum urea and creatinine levels in control, METS, and different treatment groups.

parameter	**control**	**MetS**	**MetS+W50T**	**MetS+E50T**	**MetS+W100T**	**MetS+E100 T**	**MetS+MF**
Urea	43.00±1.29	43.60±2.09	40.8±1.68	40.00±1.29	32.20±1.5^{++}	36.40±1.16^{+}	30.40±1.38^{++}
Creatinine	0.68±0.026	0.8±0.02*	0.72±0.031	0.71±0.053	0.72±0.040	0.71±0.020	0.68±0.031^{+}

Values represent the mean ±SE of six rats in each group. *p< 0.05, **P<0.01 in comparison with the control group while, +p<0.05, ++P <0.01, in comparison with the MetS group using *the t-test*.

Table (9): Malonaldehyde, glutathione, glutathione peroxidase activity in control, MetS, and different treatment groups.

parameter	**control**	**MetS**	**MetS+W50T**	**MetS+E50T**	**MetS+W100T**	**MetS+E100 T**	**MetS+MF**
MDA (mmol/ml)	2.33±0.15	3.05±0.18*	3.03±0.16	2.88±0.19	3.00±0.18	2.87±0.17	2.78±0.20
GSH Red (U/L)	20.06±1.72	24.00±2.02	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++	24.00±0.13++	29.20±0.22++	29.20±0.22++	26.92±0.11++

Values represent the mean ±SE of six rats in each group. *p< 0.05, **P<0.01 in comparison with the control group while, +p<0.05, ++P <0.01, in comparison with the MetS group using *the t-test*.

Discussion

High fat diet and high fructose in drinking water induced many metabolic disorders in rats. This is a valuable approach for studying the complex interactions among diet, metabolism, and disease [32]. Metabolic syndrome is

a constellation of common metabolic disorders associated with cardiovascular diseases and type 2 diabetes. Dyslipidemia and Insulin resistance play central roles in the pathophysiology of this syndrome [33]. This study aimed to examine the risks associated with MetS and provide effective strategies to reduce them. Medicinal plants are a rich repository of biologically active molecules that have various pharmacological effects in mammals. The majority of the world's population (approximately 60–80%) still relies on traditional medicinal methods to treat common illnesses. In addition, finding plant-derived antioxidants that scavenge reactive oxygen species (ROS) has become a central focus of drug development research. Plants are rich sources of biochemical compounds such as fatty acids, saponins and phenols [34].

Phytochemicals have numerous health benefits. It can normalize body weight and fat in mouse and rat models of diet-induced MetS [13] and humans [14]. Trifolium (*Fabaceae*) is an important plant species. All Trifolium species are known to act as traditional medicines [35]. In this study, *HPLC fractionation of T. repens flower extracts* showed many active biochemical compounds, such as phenols (e.g., pyrocatechol and vanillin), phenolic acids (e.g., gallic acid, chlorogenic acid,caffeic acid, coumaric acid, ferulic acid, rosmarinic acid, ellagic acid, and methyl gallate), flavones (e.g., naringenin), flavonols (e.g., kaempferol and rutin), and 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), as depicted in Table 2. These important phytochemicals may be used as therapeutic agents for the treatment of side effects of MetS. Previous studies have used these phytochemicals to protect against oxidative damage and reduce the metabolic and cardiovascular complications associated with MetS [36].

In table 2, our study revealed the presence of a large group of polyphenolic compounds, Flavonoids constitute, flavanones(e.g. naringenin, hesperetin) and flavonols (e.g.quercetin).The presence of unique isoflavonoids (e.g., daidzein) in clover plants has effects on various chronic diseases. Several studies have been conducted on the bioavailability of daidzein in human and rat abdominal obesity and hypertension [37,38]. So, isoflavone intake may affect individual conditions that cause metabolic syndrome and the process of disease development [39]. Quercetin was the major phenolic compound detected in our plant and found to be responsible for several bioactivities (e.g., antioxidant, antimicrobial activity, etc.) [40].

The results of our study are similar to those of other authors who demonstrated that HPLC can be used to determine the content of polyphenols in plant extracts. This study confirmed the significant effect of using alcohol as the extraction medium compared to water. Ethanol increased the antioxidant properties of clover extracts. Jakubczyk *etal*. [41] also found that ethanolic extracts had better antioxidant properties than aqueous extracts.

Our findings showed an increase in body weight gain in MetS-induced ratsrats compared to that in the control group (Table 3). This finding was supported by Marques *et al.* [42] and Moreno-Fernández *et al.* [43], who found significant increases in body weights in Wistar male rats after feeding HFD (high fat/high sucrose and high-fat/high fructose). These results might be due to an imbalance between energy uptake and expenditure, leading to fat accumulation in white adipose tissue. The Final body weights of MetS-induced rats treated (one month) with *T. repens* flower extracts (W and E)as well as MF drug showed significant decreases in final body weights as follows: MetS+W50T, MetS+E50T, MetS+W100T, MetS+E100Tand MetS+MF, respectively, compared to the MetS group. Rufino *et al.* [44] found that body weight significantly decreased in *T. repens*, suggesting the potential anti-obesity effects of flavonoids. These effects include appetite control, reduced food intake, and decreased intestinal fat absorption.

The results of this study revealed that feeding rats 30% HFD and 10% fructose in drinking water produced many symptoms of MetS. After 12 weeks of the experiment, absolute and relative liver weights were higher in rats with MetS than in those without MetS. These results agree with those of Perumpail *et al.* [44], who showed that nonalcoholic fatty liver disease (NAFLD) is strongly associated with the features of MetS. Increased absolute and relative liver weights are associated with steatotic liver disease. The term 'hepatic steatosis' reflects the lipid infiltration of hepatocytes, typically resulting from an imbalance between lipid acquisition (uptake and

synthesis) and lipid disposal (oxidation and export). Suppression of lipolysis is impaired in the milieu of insulin resistance, leading to an increased influx of free fatty acids into the liver [45]. Relative liver weight decreased in all MetS-induced groups treated with *T. repens* (W and E). These effects may be due to the flowers extracts phenolic compound content, which can be used as a source of beneficial bioactive compounds with hepatoprotective function [46].

In this study, both the absolute and relative weights of the pancreas increased in MetS-induced rats compared to those in the control group. Karunakaran and Park [47] demonstrated that oxidative stress (MetS components) is linked to β-cell dysfunction in diabetic conditions (metabolic disorders) because of their inadequate antioxidant defense mechanisms. Maintaining a delicate balance between oxidants and antioxidants is crucial for cell survival in both health and disease conditions. Using antioxidants from *T. repens* flower extracts reduced the weight of the pancreas in all treated groups (W and E). This may be due to the elimination of oxidative stress and effective treatment of β-cell failure during MetS [48]. Moreover, phenolic acids (eg; gallic acid (GA) affect glucose uptake in an insulin-resistant cell culture model and hepatic carbohydrate metabolism in rats with a high-fructose diet (HFD)–induced diabetes. These results hypothesized that GA ameliorated hyperglycemia via alleviating hepatic insulin resistance by suppressing hepatic inflammation and improved abnormal hepatic carbohydrate metabolism by suppressing hepatic gluconeogenesis and enhancing the hepatic glycogenesis and glycolysis pathways in HFD-induced diabetic rats[49] .

This study found that MetS is a risk factor for heart failure (HF) since absolute and relative heart weights increased in comparison with those in the control group. MetS increased the risk for cardiovascular disease (CVD) and type 2 diabetes mellitus (T2DM) in both men (RR=2.88, 95% CI 1.99 to 4.16) and women (RR=2.25, 95% CI 1.31 to 3.88). Specifically, MetS is associated with the occurrence of more than one-third of CVD in men [50]. The absolute and relative weight of the heart decreased in all treated groups with *T.repens* (W and E) groups and MF drug. The presence of a large group of polyphenolic compounds, flavonoids constitute, flavanones (e.g. naringenin, hesperetin) and flavonols (e.g.quercetin) in flower extractions of *T.repens* explained the beneficial effects of these compounds as protectors for heart against the bad effect of MetS. Dietary isoflavone's effects, including a reduction in LDL cholesterol, inhibition of platelet aggregation, and an improvement in vascular reactivity, have been attributed to several cardioprotective benefits [51].

Absolute and relative fat weights recorded highly significant increases in MetS-induced rats as compared to those in the control group agreeing with Buettner *et al*. [52]. They said that HFD feeding can induce obesity, adipose tissue and metabolic disorders in rodents that resemble the human MetS. On the other hand, Absolute and relative fat weights highly significantly decreased in all treated groups of *T.repens* flower extracts and MF drug compared to the MetS induction group. These results are supported by Oliveira *et al*. [53]. The anti-obesity effects of flavonoids in *T.repens* occur through the modulation of proteins, genes, and transcription factors that contribute to reduced lipogenesis, increased lipolysis, and energy expenditure. Previous results in this paper indicated that water and ethanolic extraction of *T. repens* positively affected body and organ weights, as well as MF, according to Jakubczyk *et al*. [54].

According to our research, consuming a diet high in fat (30%) and fructose in drinking water (10%) led to elevated blood glucose levels, insulin concentration, HOMA-IR, and HbA1c (Table 6). Hyperglycemia and HFD are key risk factors for the development of MetS [55]. According to Stohs *et al*. [56] and Prasetya *et al*. [57], they said that carrying excess fat, especially around the central areas of the body, is a well-known risk factor for developing type 2 diabetes. There is increasing evidence that consuming sugar-sweetened beverages is linked with an elevated risk of diabetes due to its effects on adiposity, independent of other metabolic factors [71]. This can cause elevated HbA1c levels due to reduced insulin sensitivity and poor blood sugar control [58]. *T.repens* is used for therapeutic purposes as traditional medicine[59]. It contains important phytochemicals, which are the potential source of health-beneficial bioactive components (isoflavones eg; Daidzein) that improve human health and decrease the risk of diabetes. It has little or no side effects. These results agree with Unuofin and Lebelo [60] who reported bioactive molecules isolated from natural sources have been proven to lower blood glucose levels *via* regulating one or more of the following mechanisms: improvement of beta cell function,

insulin resistance and glucose (re)absorption. Early evidence highlighted the liver as the major organ involved in the effect of metformin on reducing blood levels of glucose [61], so it was used as a reference drug. In our study with rats, MF led to a decrease in glucose levels, insulin levels, HOMA IR, and a decrease in HbA1c compared to MetS-induced rats. Based on the findings above, *T.repens* showed better results as well as MF drug, confirming that *T.repens* contains antioxidant-rich phytochemical found in it like chlorogenic acid (CGA) which is a powerful antioxidant. CGA has been touted as having the ability to lower blood sugar levels and perhaps reverse or decrease Type 2 Diabetes symptoms. It has also been linked to weight reduction and the prevention of obesity [62]. Caffeic acid (CA) is a natural polyphenol obtained from various plants. CA found in *T.repens* exerts anti-diabetic effects by modulation of inflammatory cytokines and transcription factors[63]. Naringenin found in *T.repens* decreased insulin resistance and blood sugar levels. By raising high-density lipoprotein (HDL) cholesterol and decreasing triglycerides and low-density lipoprotein (LDL) cholesterol, naringenin's capacity to regulate lipid metabolism contributes to diabetes control. This control of lipids aids in preventing cardiovascular complications that are frequently linked to diabetes. Naringenin also affects the activity of enzymes involved in the metabolism of carbohydrates, which helps stabilize blood glucose levels [64]. In general, bioactive compounds in *T.repens* have benefits for fasting blood sugar (FBS) and fasting insulin by decreasing IR, and HbA1c, as indicated by Mahdavi *et al*[65].

Dyslipidemias are common in MetS [66]. The study found a significant increase in total cholesterol, triglycerides, LDL-C and VLDL but a decrease in HDL-C (-16%) in MetS as compared to the control group these results were supported by Vladu *et al*.[67] due to insulin resistance leading to decreased lipoprotein enzyme activity, resulting in increased triglycerides and contributing to elevated LDL-C and VLDL-C levels. *T.repens* improved lipid profile disorders by decreasing levels of TC, TG, LDL-C, and atherogenic index, as well as increasing levels of HDL-C as Kosmas *et al*.[68] suggested that the *T.repens* contained isoflavones phenolic compounds namely (quercetin and kaempferol) with a chemical structure similar to that of estradiol. They are present mainly in white clover (*T.repens*) which can improve lipid metabolism, as well as antioxidant activity that was determined by DPPH (table 1). Presence of these antioxidants may be the cause of oxidative stress reduction and positively impact lipid profiles accordingly, *T.repens* reduced lipid profile similar to MF drug table (7).

The kidney plays an important role that keeping the chemical role of the body fluid by urine acidification and serum osmolality and electrolyte concentrations, as well as the production of hormones and also plays a role in the excretion of waste products and toxins such as urea and creatinine [69]. In this study, it was found that an increase in creatinine levels was significantly linked to MetS and its components, which suggests a potential kidney dysfunction table (8). This is in line with Garcia *et al*. [70] who demonstrated that obesity, a common factor of MetS, imposes a hemodynamic overload on the kidneys. Zeni *et al* [71] showed the concentration of creatinine becomes elevated in the blood when renal disease and Diabetes Mellitus (DM) occur. Diabetic hyperglycemia leads to increased levels of blood urea and creatinine, which are considered indicators of renal dysfunction [72]. Creatinine is the most commonly used endogenous marker for assessing glomerular function. Creatinine is the by-product of creatine phosphate in muscle, and it is produced at a constant rate by the body. For the most part, creatinine is cleared from the blood entirely by the kidney [73] Elevation of blood creatinine levels shows that more creatinine was retained in the blood [74].

HFD increases the risk of chronic kidney dysfunction, independent of insulin resistance and adipose tissue [75]. *T. repens* reduces urea levels due to its contents of flavonoid components (quercetin, kaempferol, naringin and diazein) that help neutralize free radicals, improve urea and creatinine levels, and protect the kidneys. These compounds can prevent renal dysfunction and improve renal function by blocking or suppressing deleterious pathways such as oxidative stress and inflammation as demonstrated by Cao *et al* [76]. *T. repens* flower extracts improved kidney function tests similar to the MF drug. The most effective orders were MF drug next MetS+W100T then MetS+E100T (table 8).

A high-fat diet increases the production of reactive oxygen species (ROS), leading to oxidative stress. MDA is a byproduct of lipid peroxidation caused by ROS, and elevated levels of MDA indicate increased oxidative

damage [77] This process damages cell membranes and other structures, contributing to various metabolic disorders [78]. GSH-Px is an enzyme that helps neutralize reactive oxygen species (ROS) by converting hydrogen peroxide and lipid peroxides into water and lipid alcohols, respectively. A high-fat diet increases oxidative stress, prompting the body to upregulate GSH-Px activity as a protective response. The impact of a high-fat diet on GSH-Px activity can vary between different [79]. While some tissues may show a decrease in activity due to overwhelming oxidative stress, others might exhibit an increase as part of the body's adaptive response to counteract the elevated ROS [80].

The research revealed that levels of GSH-Px decreased in *T. repens* as a result of caffeic acid (one of the components of *T. repens)*. It is a polyphenol compound known to improve redox status. According to Pavlíková [81], it contains antioxidants that can help prevent cardiovascular damage by inhibiting inflammation associated with obesity [82]. Caffeic acid can positively influence gut microbiota, which in turn can reduce oxidative stress and inflammation. A healthy gut microbiota can improve overall metabolic health and reduce the negative effects of a high-fat diet [83]. The simultaneous increase of both MDA and GSH-px reflects a biological response where the body attempts to balance the oxidative damage (indicated by MDA) with an enhanced antioxidant defense (indicated by GSH-px) in induced MetS [84].

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