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Physiological Activity and GC-Mass Analysis of *Trigonella strangulata*, *Trigonella filipes* and *Trigonella uncinata* Against Ethanol-Induced Hepatorenotoxicity in Rats

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ABSTRACT

Trigonella or fenugreek is one of the most important and the oldest known medicinal plants in the world which is used in the treatment of many diseases due to its various effective compounds. *Trigonella strangulata*, *Trigonella filipes* and *Trigonella uncinata* are three widely used species and folk medicine that grow well in the northern suburbs of Iraq. Therefore, the present study was designed to evaluate and identify the effective compounds of these species and to investigate their physiological and biochemical effects on biochemical, and hematological parameters in rats treated with ethanol. Phytochemical analysis of seeds and leaves of these taxa was performed by GC-MASs and their morphological evaluation was performed by SEM electron microscopy. Hematological and biochemical variables such as hepatic enzymes activity, glucose, lipid profile, ESR, CRP, renal function tests, and oxidative biomarkers were measured. Histological evaluation was also performed. Among the hematological parameters in the treated groups, only lymphocytes showed a significant change in the treated group with *T. uncinata*. The levels of liver enzymes, lipid profile and glucose in the ethanol group increased significantly compared to the control, while these parameters were significantly improved in the groups treated with these three taxa. In the ethanol group, renal function and oxidative indices along with ESR showed a significant increase, while treatment with these three taxa could improve these parameters. These results were also supported by histological findings. It can be concluded that these three plant species have high phytochemical compounds that play physiological roles such as improving the immune status, the function of liver and kidney and antioxidants status against ethanol-induced hepatotoxicity.

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Key words
***Trigonella strangulata, Trigonella filipes, Trigonella uncinata*, Ethanol, Liver**

INTRODUCTION

Lifestyle in today's world plays a huge role in determining the quality of life. Proper diet and physical activity, easy access to health centers, avoidance of sources of stress and lack of poverty are several important factors in determining the quality of life (Mustafa *et al*., 2021). With the urbanization of societies, many habits of people's lives changed. So that today, separating these facilities makes life somewhat difficult and impossible (Raeeszadeh *et al*., 2021; Fathi *et al*., 2021). Exposure to radio waves, drug and alcohol abuse, and high-calorie foods are some of the simplest things that are associated with life today and play a major role in many diseases (Jelodar *et al*., 2020; Akbari *et al*., 2016). Alcoholic liver disease (ALD) is one of the most common causes of chronic liver disease in the world caused by excessive alcohol consumption. Abuse of this popular drink is a major risk factor for dysfunction of many organs, including the kidney, brain, testis, and liver (Fathi *et al*., 2020; Akbari *et al*., 2016). The liver is one of the most dynamic and vital organs in the body that has several biological functions. Metabolism of nutrients, drugs, chemicals as well as detoxification are the main activities of this organ. The normal process of ethanol metabolism in the liver is carried out by alcohol metabolizing enzymes, namely alcohol dehydrogenase (present in the cytosol), CYP2E1 (present in microsomes), and catalase (CAT) (in peroxisomes), and the final product of this process is acetaldehyde (Choi *et al*., 2018). Acetaldehyde is then converted to acetic acid by aldehyde dehydrogenase in mitochondria. Chronic alcohol abuse increases nicotinamide adenine dinucleotide hydrate concentration and acetaldehyde dehydrogenase activity, leading to the release of free radicals and severe free fatty acid overload, triglyceride accumulation, and hepatic steatosis (Choi *et al*., 2018, 2019a). Hepatic steatosis is a metabolic complication also known as fatty liver and can

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lead to conditions such as cirrhosis and liver cancer if left untreated. Inflammation, oxidative stress and impaired hepatic metabolism are the most important causes of this complication (Akbari *et al*., 2019a; Nimrouzi *et al*., 2020). Efforts to improve this situation are primarily lifestyle changes and the use of drug therapy. One of the most common treatments for this complication, which can be both medication and lifestyle changes, is the use of complementary medicine, including herbal remedies (Mustafa *et al*., 2021; Fathi *et al*., 2020, 2021). *Trigonella* is a genus of the Fabaceae family. The most well-known member of the plant is fenugreek. There are about 135 species of this genus that are naturally found in the Canary Islands, southern Europe and subtropical Africa, western and central Asia, the Indian subcontinent and Australia (Chao *et al*., 2019; Wani and Kumar, 2018). Fenugreek seeds (*T. foenum-graecum*) have a number of medicinal properties and the seeds are used to treat various diseases from the past to the present (Syed *et al*., 2020; Pan *et al*., 2014). The leaves of this plant are used as a food. Research on different species of *Trigonella* in recent years demonstrated a number of health benefits and physiological characteristics in animal models and clinical trials (Wani and Kumar, 2018). These effects include anti-diabetic, hypocholesterolemic, anti-inflammatory, antioxidant, liver protection, antibacterial, antifungal and anti-cancer activities. In most of these studies, seed powder or different types of its extracts have been used (Wani and Kumar, 2018; Kaviarasan and Anuradha, 2007; Yadav and Baquer, 2014). Many of the medicinal activities of these species are due to the presence of active compounds (Yadav and Baquer, 2014). In addition, the seeds of these plant have a significant place in Ayurveda is an alternative medicine system (Pandey *et al*., 2013). *T. strangulata, T. filipes* and *T. uncinata* are species of *Trigonella* that have spread around the world and are the focus of most chemical and pharmaceutical research and are traditionally used by humans. Some studies showed that fenugreek (*Trigonella foenum graecum*) seed extract and its seed polyphenols prevent ethanol-induced toxicity (Kaviarasan *et al*., 2006; Kaviarasan and Anuradha, 2007). Despite studies on the role of liver protection of this popular plant, so far there has been no report on liver and kidney protection of this plant against toxicity caused by ethanol. Therefore, the aim of this study was to evaluate the effect of liver protection of three species of fenugreek against ethanol liver damage in rats.

MATERIALS AND METHODS

Plant material and extraction procedure

The healthy and mature plants of *T. strangulata*, *T. filipe* and *T. uncinata* were collected from north of Iraq. They were identified and recorded with 5992 reference number at the Harran University Herbarium. For GAS chromatography analysis, the plants were collected in Erbil Mountains in the First week of March 2021. They were stored in the refrigerator at + 4 °C until the extracts. N-hexane was added to a round bottom flask into a Soxhlet extractor and condenser on an isomantle. The solvent was heated to reflux and evaporated. Then, the solvent vapor traveled up to distillation arm that insulated with glass wood, and flooded into the chamber housing the thimble containing dried and grinded plant. Once the level of solvent reached the siphon it poured back into the flask and the cycle began again. The process should run for a total of 3 h. Finally, hexane was evaporated by a rotary evaporator, and a small yield of extracted plant material (2 to 3ml) was left in the glass bottom flask. Afterwards, the extract was collected in tightened vials and stored in a refrigerator for later analysis. For study with experimental animals, we used water as a solvent because people use tap water for boiling plants in traditional use. Before being applied to rats, boiled extracts were retained at + 4°C refrigerator for a week.

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analysis of n-hexane extracts of *T. strangulata*, *T. filipes* and *T. uncinata* performed using thermo GCTrace ultra version 5.0 gas chromatography interfaced to Thermo MS DSQ II mass spectrometer instrument employing the following conditions: DB5–MS Capillary standard non polar column (30 X 0.25 mm X 0.25 µm) and helium gas were used as a carrier gas at a constant flow rate of 1 ml/min. The oven temperature kept at 70˚C and programmed to reach 260 ºC at a rate of 6˚C/min. Mass ranged m/z 50–650. The total running time completed in 43 min. The chromatogram obtained from gas chromatography then analyzed in mass spectrometry to get the mass of all fractions. The identification of phytochemicals components achieved through retention time and mass spectrometry by comparing the mass spectra of unknown peaks with those stores in the Wiley 9 GC-MS library (Dąbrowski, 2020).

Animals and their maintenance

The research has been approved by the Animal Research Ethic Committee, Salahaddin University with approval number A19REC214. Forty albino male rats (230-250 g), *Rattus norvegicus*, free of complications used for this study were procured from the Laboratory Animal Center (College of Science, University of Zakho, Duhok Province, Iraq). The animals were kept for a minimum of one week before the onset of the experiment,

under observation to exclude any undercurrent infection and to acclimatize the laboratory conditions. They were preserved in polypropylene cages with wire mesh covers and maintained in a controlled environment of light (12-h day/night cycles, lights on 6:00 a.m. to 6:00 p.m. by using electronic programmable timer switches) and normal atmospheric temperature (22°±2°C) in an air-conditioned and well-ventilated room. Wood saw freshly spread the cages to absorb the urine of animals and were changed twice every week. All the experimental animals were observed daily for clinical signs and mortality, if any, during the entire period of study.

Animals were fed on certified pelleted feed supplied by Top Feeds Company in Erbil city (1 kg of pellet contains 311 g wheat, 301 g soybean meal, 345 g corn, 10.4 g soybean oil, 10 g vitamin-mineral premix, 9 g limestone, 1.3 g lysine, 3.3 g methionine, and 9 g mono-dicalcium phosphate) and water *ad libitum* all over the experimental period. The rats were marked by tail marking. The care and maintenance of the experimental animals was carried out in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals approved by Harran University.

Experimental design

Forty rats were used for the experiment. During the experimental period, body weights were measured weekly using an EKS weighing balance (EKS 842/0558 (D), P.R.C). Dosing solutions were fixed for changes in rats' body weights throughout the administration period. The animals' body weight ranged from 230 to 250 g (10-12 weeks old). The age and body weight variables of these animals had to be similar because the reactivity of some inflammatory factors depends on individual traits such as sex, age, or strains under invariable environmental factors (Zablocki-Thomas *et al*., 2018). After being weighed on an electronic balance, the animals divided randomly into five equal groups (n=7 in each group), and all exposures were performed in the forenoon, between 09:00–10:30 for 60 consecutive days. All rats in the groups survived. The dosing route in all experimental groups was given by oral gavage:

Group I: Control group, standard diet was given. Group II: patient group, rats in addition to normal diet received 20% alcohol solution (v/v, 0.5ml/100g body weight) for 60 consecutive days. Group III: *T. strangulata* extraction (TSE) group, in addition of normal diet and 20% alcohol, the rats daily were treated orally with the TSE for 30 days; the dose was 200 mg/kg body weight. Group IV: *T. filipes* extraction (TFE) group, in addition of normal diet and 20% alcohol, the rats daily were treated orally with the TFE for 30 days; the dose was 200 mg/kg body weight. Group V: *T.uncinata* extraction (TUE) group, in addition of normal diet and 20% alcohol, the rats daily were treated orally with the TFE for 30 days; the dose was 200 mg/kg body weight.

Blood collection

At the end of the experimental period, rats were fasted for 12 h and then anaesthetized by intramuscular injection of a single dose of xylazine-ketamine combination (1:9) in the same syringe. Blood samples were collected by withdrawing the blood from the right ventricle of the heart for hematological, biochemical and hormonal assays. About 2 ml of blood was collected in tubes containing EDTA as an anticoagulant for hematological analysis. However, about 5 ml of blood was collected in a gel tube then allowed to stand at room temperature for 10-15 min. The serum was then separated by centrifuging at 3500 rpm for 15 min and was stored at -20°C until used for biochemical and hormonal assays.

Hematological analysis

Hematological variables were assessed on the day of the collection of blood. Complete blood counts including a total count of white blood cells (WBC), differential count, the total red blood cells count (RBC), hemoglobin (Hb), hematocrit (HCT), RBC indices such as mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC), and platelets count were analyzed by a fully automated 3-part differential hematology analyzer. The glucose concentration of serum was tested by the method described by Barham and Trinder (1972). Barham and Trinder (1972), using a commercial kit (CENTRONIC GmbH). The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP) activities, and total serum bilirubin level were determined using kits and enzymatic-colorimetric method with an autoanalyzer (COBAS INTEGRA 400 plus system automated biochemical analyzer, Germany). The hepatic enzyme activity of serum was assayed by using a commercial kit (BIOLABO SAS) as outlined by the manufacturer. The serum levels of urea, creatinine, and uric acid were determined using kits and enzymatic-colorimetric method with an auto-analyzer (COBAS INTEGRA 400 plus system automated biochemical analyzer, Germany). The renal function biomarkers concentration of serum was assayed by using a commercial kit (N.S.BIO-TEC) according to the manufacture's instruction. Serum total cholesterol (TC), triglyceride, and HDL-C were estimated by using a commercial kit (Centronic GmbH-Germany). Low-density lipoprotein cholesterol (LDL-C) was calculated using the Friedewald equation (Knopfholz *et*

al., 2014).

Erythrocyte sedimentation rate (ESR) was estimated with the standard Westergren method.

The concentration of serum sodium (Na^+), potassium (K^+), chloride (Cl^-) and gamma-glutamyl transferase (GGT) were estimated by using Cobas C311. The C-reactive protein (CRP) levels in blood serum were quantified using commercially available kit from BioVendor (Rat hsCRP ELISA kitBioVendor, Laboratory Medicina). The whole process was maintained according to the manufacturer's recommendations. Serum levels of oxidative biomarkers of total oxidant capacity, malondialdehyde, catalase and total antioxidant capacity were measured by diagnostic kits prepared by Kooshan Zist Iran Company.

Methodology for electron microscope

For scanning electron microscope, the seed and leaves of plants washed with sterilized distiled water in Eppendorf tubes were dehydrated with alcohol series 50%, 70%, 80%, 85%, 90%, 95% and three times 100%, followed by three times of 100% acetone for 30 min each time. The plant material was now dried to eliminate the large number of impurities that might hinder vision of the structural characteristic of the wall. Finally, the seed and leaves mounted on metal stub with double-side cellophane tape and then coated with a film of gold palladium by the aid of sputtering chamber. The coated samples were viewed under scanning electron microscope (INSPECT S50) in College of Science, Soran University, Soran, Iraq.

Histopathological assessment

Median lobe and right kidney were removed and fixed in a 10% formalin solution to evaluate histopathological assessment. After 48 h, the 10% formalin was replaced with a 4% formalin solution. After two weeks, all samples were embedded in block paraffin. Sections 5µm thick were cut and stained with hematoxylin eosin.

Statistical analysis

In this study, GraphPad Prism 6 version 7.01 was used for statistical analyses. A comparison of data was conducted using the one-way ANOVA Tukey test. Group averages were communicated as ± standard deviation, and between-group comparisons were given as ± standard error. For parametric data analysis, one-way ANOVA was used. The statistical importance of between-group differences was examined through the Tukey HSD test. In graphics, while the groups were being compared, $p< 0.05$ was regarded to be the value of statistical meaningfulness. Any statistically meaningful difference was communicated via letters on the data in the tables.

RESULTS

Phytochemical compounds of Trigonella

The results of seed composition analysis of three *Trigonella* taxa are shown in Table I. The results of showed that the content of 2, 4-Di-tert-butylphenol. Ester compounds, fatty acids, phytosterol and amides in *T. strangulata* is higher than the other two species. In general, phytochemical findings indicate that the seeds and leaves of *T. strangulata* are rich in phenol, esters, amides, acids, etc.

Ultrastructure of leaves and seeds of Trigonella *plant species*

Based on the electron microscopy observations, the examined *Trigonella filipes*, *T. strangulata* and *T. uncinata* showed variation in seed and leaf characteristics (Figs. 1 and 2). The characteristics seeds of these three species shown in Figure 1, the species of the genus *Trigonella* have variable seed characters that can be used in the subscription of them. The *T. filipes* seeds are oblong in shape with truncate poles in surface, seed length of this species is about 1.5-3.1 mm and its width is 05.-0.8mm. The *T. stragulata* seeds are elliptic in shape with round poles in surface, seed length of this species is about 0.8-0.9 mm and its width is 0.5-0.6 mm, seed coat pattern is mounded with papillae. *T. uncinata* seeds have elliptic shape and are 1.8-2.5 mm in length and 1-1.25 mm in width they are mounded with papillae too. The upper epidermis layer of the *T. filipes* covered by long unicellular hairs with present of ordinary stomata among the epidermal cells. In *T. strangulata* the upper layer of epidermis covered by many short trichomes which are same as *T. filipes* unicellular and stomata apparatus also are present. *T. uncinata* leaf hairs are long and few in numbers and stoma are distributed as regular in epidermal cells.

Effect of ethanol and fenugreek on hematological parameters

Evaluation of hematological parameters in different groups of this study showed that ethanol consumption compared to other groups had no effect on these parameters (Table II). In addition, the results showed that the consumption of these plant species in response to ethanol poisoning in comparison with the ethanol and control group had no effect on these parameters and did not cause a significant difference.

Effect on liver enzymes and lipid profile

The results of measurements of serum levels of liver enzymes and lipid profiles are shown in Table III. Serum content of liver enzymes was measured in this study

Table I. GC-MS phytochemical constituents of Trigo.

No	Organic class	Name	Molecular formula	Molecular mass (g/mol)	Sample (Relative abundance %)		
					T. uncinata	*T. filipes*	*T. strangulata*
1	Phenol	2,4-Di-tert-butylphenol	$C_{14}H_{22}$	206.32	0.4	0.23	0.58
2	Ester	Methyl 9,12,15-octadecatrienoate	$C_{19}H_{32}O_2$	292.5	-	-	0.37
3		Methyl linoleate	$C_{19}H_{34}O_2$	294.5	-	0.01	-
4		Methyl stearate	$C_{19}H_{38}O_2$	298.5	-	0.24	0.40
5		1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	390.55	-	0.1	-
6		Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	270.45	0.16	-	-
7	Fatty Acid	Palmitic acid	$C_{16}H_{32}O_2$	256.43	1.24	0.33	1.33
8		Linolenic acid	$C_{18}H_{30}O_2$	278.43	0.44	-	-
9		9-Octadecenoic acid (Z)-, methyl ester	$C_{19}H_{36}O_2$	296.49	-	-	0.42
10	Phytosterol	Stigmasterol	$C_{29}H_{48}O$	412.7	-	-	0.38
11		Campesterol	$C_{28}H_{48}O$	400.69	-	-	-
12	Alcohol	Phytol	$C_{20}H_{40}O$	128.17	0.29	-	0.16
13		1-Eicosanol	$C_{20}H_{42}O$	298.5	-	-	0.86
14		Hexadecanol	$C_{16}H_{34}O$	242.44	0.42	-	-
15		2 Ethyl Hexanol	$C_8H_{18}O$	130.23	11.08	8.47	9.83
16		2,4,7,14-Tetramethyl-4-vinyl-tricyclo [5.4.3.0(1,8)]tetradecan-6-ol	$C_{20}H_{34}O$	290.5	-	0.2	-
17		Neophytadiene	$C_{20}H_{38}$	278.5	-	0.46	1.83
18		Phytol	$C_{20}H_{40}O$	128.17	0.29	0.33	0.43
19	Hydrocarbon	Squalene	$C_{30}H_{50}$	410.73	0.14	0.11	0.23
20		Eicosane	$C_{20}H_{42}$	282.5	0.28	0.25	0.37
21		Terpinene	$C_{10}H_{16}$	136.23	0.19	-	-
22		Amyrin	$C_{30}H_{50}O$	426.72	0.32	0.27	0.67
23		Gamma.-Sitosterol	$C_{29}H_{50}O$	414.71	0.23	0.42	0.38
24		1,2-Epoxyoctadecane	$C_{18}H_{36}O$	268.5	-	0.27	-
25		Tetradecene	$C_{14}H_{28}$	196.37	0.19	0.5	-
26		Tetradecene	$C_{14}H_{30}$	198.39	-	0.24	0.41
27		Cyclodecane	$C_{10}H_{20}$	140.27	0.20	0.12	0.15
28		Cyclododecane	$C_{12}H_{24}$	168.32	-	0.14	-
29		Cyclohexane, (2-methylpropyl)-	$C_{10}H_{20}$	140.26	-	2.12	0.75
30		Cyclohexane, 1,1,2-trimethyl-	C_9H_{18}	126.24	-	0.17	-
31		Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	667.4	0.81	-	-
32		Cyclopentane, 1-butyl-2-propyl-	$C_{12}H_{24}$	168.32	1.61	1.39	0.99
33		Cyclotetracosane	$C_{24}H_{48}$	336.6	-	0.12	-
34		Decane	$C_{10}H_{22}$	142.28	0.21	0.29	0.45
35		Docosane	$C_{22}H_{46}$	310.6	0.17	0.15	0.25
36		Dodecane	$C_{12}H_{26}$	170.34	7.64	9.71	10.27
37		Hexadecane	$C_{16}H_{34}$	226.44	2.38	2.47	2.46
38		Nonacosane	$C_{29}H_{60}$	408.79	0.32	0.42	1.64
39		Octadecane	$C_{18}H_{38}$	25.49	0.7	0.69	0.77
40		Tetracosane	$C_{24}H_{50}$	338.66	5.82	6.03	6.18
41	Lactam	2-Piperidinone	C_5H_9O	99.13	0.21	0.14	0.42
42	Amide	Benzamide	C_7H_7NO	121.13	0.61	0.52	0.79
43		Erucylamide	$C_{22}H_{43}NO$	337.6	0.43	0.33	0.48
44		Coumarin	$C_9H_6O_2$	146.14	1.03	0.89	1.52
45		Lupeol	$C_{30}H_{50}O$	426.72	0.78	0.98	1.8
46		Methylundecane	$C_{12}H_{26}$	170.33	0.36	0.42	0.52

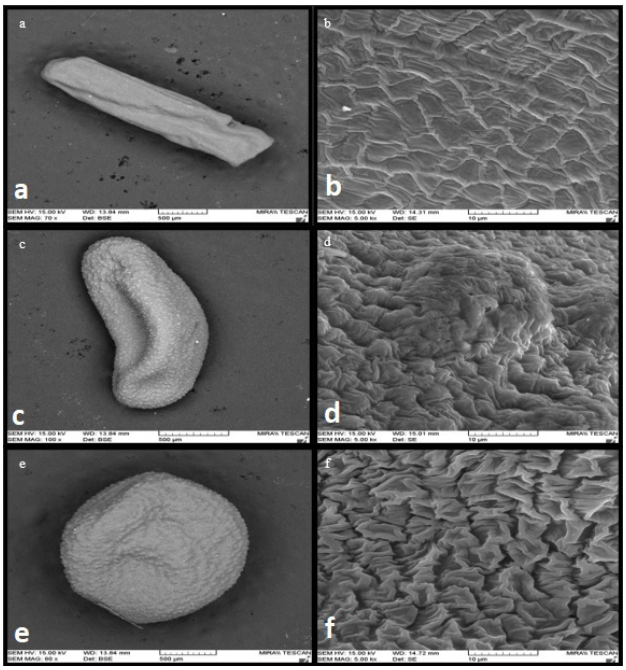

Fig. 1. SEM microphotographs of *Trigonella* L. seeds. a-b, *T. filipes*; c-d, *T. strangulata*; e-f, *T. uncinata.*

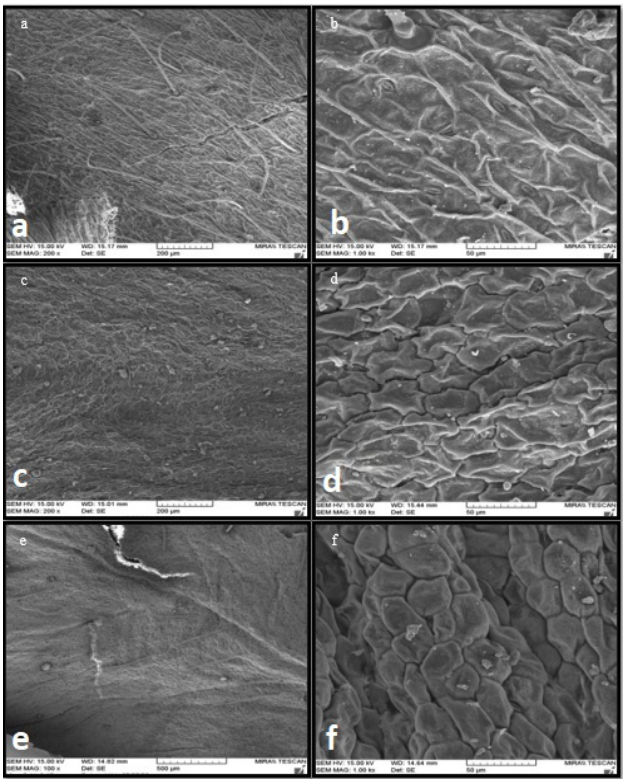

Fig. 2. SEM microphotographs of *Trigonella* L. leaves. a-b, *T. filipes*; c-d, *T. strangulata*; e-f, *T. uncinata.*

to evaluate liver activity. Lipid profile levels were also measured along with fasting glucose levels as metabolic factors that can be altered by liver activity. The results showed that the serum content of AST, ALP, ALT, GGT and TSB enzymes in this study was significantly increased in the ethanol group compared with the other groups. However, the use of fenugreek species could significantly improve these changes. The results also showed that the serum content of cholesterol, triglyceride and LDL significantly increased in the ethanol group compared to the control group and the serum HDL content in this group compared to the control group showed a significant decrease. In addition, fasting serum glucose level significantly increased in the ethanol group and fenugreek species could reduce these metabolic parameters compared to the ethanol group.

Effect on kidney function and serum inflammatory factors

In this study, we also measured the content of serum electrolytes, uric acid, urea and creatinine as parameters to assess kidney function. The results showed that the levels of these parameters in the ethanol group increased significantly compared to the other groups (Table IV). Despite the different results in the species of this plant, they did not have any significant differences and were able to reduce the levels of these parameters to a considerable extent. Serum levels of CRP and ESR are inflammatory factors that have been evaluated in many studies and clinical settings. In this study, the ESR showed a significant change in the ethanol group compared to the control group in contrast to the CRP level. However, treatment with different species of this plant could moderate this effect and bring it closer to normal.

Effect on serum antioxidant enzymes activity and MDA

The results of this study showed that ethanol increased the level of MDA and decreased the level of antioxidant enzymes in serum and consumption of different species of this plant could significantly improve the level of these factors and oxidative damage caused by ethanol (Table V).

Histological effects

Histological findings of the control and ethanol groups are shown in Figure 3. Liver and kidney section from the control group showing normal hepatic architecture with anintact nucleus and normal sinusoids (Fig. 3a, b); ethanol group showing highly deformed hepatic architecture with fatty lesion due to fatty infiltration and necrosis in liver and highly deformed architecture in renal tissue (Fig. 3c, d); Liver and kidney sections of ethanol + *T. strangulata* (e and f), ethanol + *T. uncinata* (g and h)

Table II. Effect of ethanol and fenugreek on hematological parameters or rats.

Parameters (M±SD)	Sham control	Experimental groups			
		Ethanol	*T. strangulata*	*T. uncinata*	*T. filipes*
Hb (g/dl)	12.61±1.04	12.35±0.39	12.03±0.72	12.22±0.72	12.37±0.48
HTC (%)	35.63±2.15	36.87±1.69	35.97±0.70	36.12±1.24	36.32±1.16
Total RBC ($XL10^{12}$/L)	5.90±0.57	5.91±0.28	5.76±0.28	5.74±0.16	5.71±0.51
Total WBC ($x10^9$/L)	6.01±1.81	8.05±2.42	6.11±1.70	6.18± 1.76	6.55±1.82
Lymphocyte (%)	82.70±1.83	72.50±10.23	77.28±2.35	77.60±2.75**	78.12±3.20
Platelets ($X10^9$/L)	799.7±48.00	658.7±47.02	813.3±56.12	812.3±61.39	814.7±70
MCV (fl)	61.32±0.91	61.18±2.16	61.92±1.81	61.67±1.08	61.90±1.25
MCH (pg)	21.23±0.28	21.35±0.58	21.27±0.25	21.02±0.96	21.23±0.76
MCHC (g/dl)	34.45±0.23	34.10±0.86	34.03±0.64	33.07±1.18	34.07±1.83
MPV (fl)	7.71±0.19	7.86±0.32	7.73±0.15	7.63±0.21	7.66±0.24

WBC, white blood cells; RBC, red blood cells count; HB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MPV, mean platelet volume. Asterisk shows significant difference with other groups ($P < 0.01$).

Table III. Effect of ethanol and fenugreek on liver enzymes and lipid profile of rat blood sample.

Parameters (M±SD)	Control	Experimental groups			
		Ethanol	Ethanol+ *T. strangulata*	Ethanol+ *T. uncinata*	Ethanol+ *T. filipes*
AST (U/L)	96.08±14.71	157.0±22.39*	93.1±106.5	106.5±8.30	109.3±7.47
ALT (U/L)	50.77±23.57	87.34±7.49*	44.13±13.85	47.08±13.44	50.75±10.72
ALP (U/L)	125.8±30.61	188.0±44.53*	123.2±23.57	132.3±24.20	139.7±19.75
TSB (mg/dl)	0.51±0.12	0.68±0.11*	0.48±0.04	0.51±0.11	0.53±0.10
Gamma-glutamyl transferase (IU/L)	2.16±0.98	6.50±1.04*	2.50±0.83	2.83±0.98	2.87±1.16
Cholesterol (mg/ dl)	57.00±4.29	75.50±3.98*	52.17±4.70	53.67±2.73	56.00±2.82
Triglyceride (mg/ dl)	71.00±11.05	122.8±8.37*	74.67±12.24	82.17±5.41	85.83±5.26
LDL-C (mg/ dl)	22.40±1.95	27.33±1.63*	18.50±3.45	20.67±1.86	22.50±2.73
HDL-C (mg/ dl)	26.67±2.73	14.33±1.21*	22.67±2.73	21.83±2.13	21.67±1.75
Fasting blood sugar (mg/dl)	92.0±63.43	218.8±28.35*	121.0±57.64	124.2±65.83	133.3±61.85

ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase: HDL-C, Low-density lipoprotein cholesterol; LDL-C, Low-density lipoprotein cholesterol; TSB, total serum bilirubin. Asterisk shows significant difference with other groups ($P < 0.05$).

Table IV. Renal function test, electrolytes, C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) of rat blood.

Parameters (M±SD)	Sham control	Experimental groups			
		Ethanol	*T. strangulata*	*T. uncinata*	*T. filipes*
Serum creatinine (mg/dl)	0.52±0.05	0.82±0.08*	0.50±0.04	0.54±0.02	0.53±0.3
Serum urea (mg/dl)	28.17±4.53	43.50±2.25*	27.17±2.63	29.83±3.06	28.67±2.58
Serum uric acid (mg/dl)	1.16±0.17	1.50±0.15*	1.05±0.12	1.23±0.13	1.10±0.14
Sodium (mmol/L)	142.0±2.0	148.7±1.86	143.5±1.04	144.0±1.41	146.4±1.64
Potassium (mmol/L)	3.92±0.39	5.35±1.18*	4.32±0.48	4.76±0.32	4.73±0.39
Chloride (mmol/L)	97.85±1.49	102.9±2.93	98.68±0.41	99.85±1.39	100.7±1.60
ESR (mm/1hr)	0.16±0.40	1.83±0.75*	0.50±0.54	0.83±0.75	1.00±0.89
CRP (mg/dl)	0.04±0.01	0.06±0.01	0.05±0.01	0.05±0.009	0.06±0.007

ESR, Erythrocyte Sedimentation Rate; CRP, Serum C-reactive protein. Asterisk shows significant difference with other groups ($P < 0.05$).

Table V. Effect of ethanol and fenugreek on antioxidant enzymes activity and MDA of rat blood serum.

Parameters (M±SD)	Sham control	Experimental groups			
		Ethanol	*T. strangulata*	*T. uncinata*	*T. filipes*
SOD (nmol/L)	1.35±0.21	0.80±0.12*	1.24±0.05	1.20±0.22	1.16±0.24
CAT (nmol/L)	76.96±6.82	54.68±14.38*	72.97±9.45	73.14±9.27	74.30±9.03
MDA (nmol/L)	0.051±0.01	0.317±0.008*	0.067±0.015	0.059±0.022	0.058±0.24

CAR, Catalase; MDA, Malondialdehyde; SOD, superoxide dismutase. Asterisk shows significant difference with other groups ($P < 0.05$).

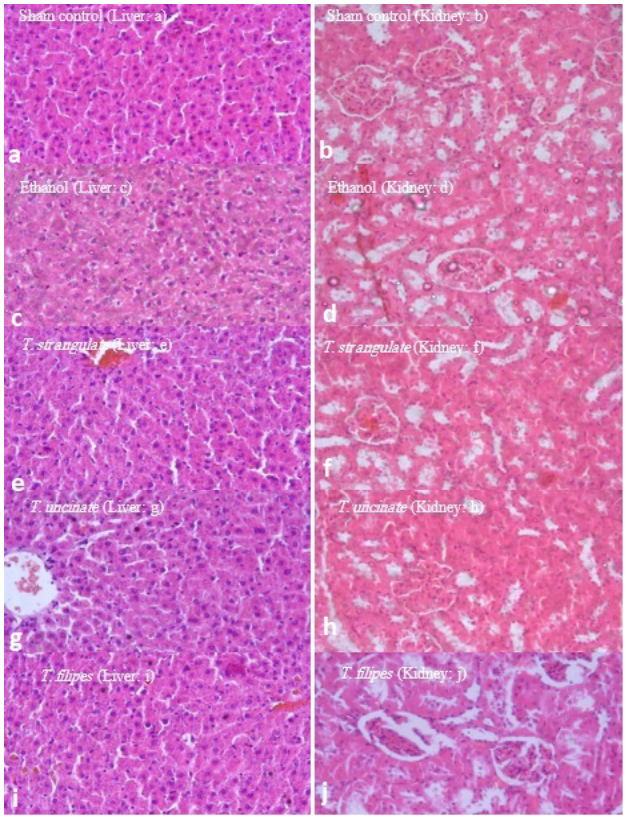

Fig. 3. Effects of oral administration of *Trigonella* on histopathological changes in the liver and kidney tissues of treated and control rats.
Hematoxylin/Eosin staining (H and E), 40×. Liver and kidney sections from the control group demonstrating normal hepatic architecture with an intact nucleus and normal sinusoids (a and b); Ethanol showing highly deformed hepatic architecture with fatty lesion due to fatty infiltration and necrosis (c and d); Section of liver and kidney from ethanol + *T. strangulata* (e and f), ethanol + *T. uncinata* (g and h) and ethanol + *T. filipes* (i and j) groups showing improved hepatocellular architecture with the intact nucleus and normal sinusoids.

and ethanol + *T. filipes* (i and j) groups showing improved hepatorenocellular architecture with the intact nucleus and normal sinusoids (Fig. 3e-j).

DISCUSSION

The results of GC-MASS analysis showed that seeds and leaves of different species of *Trigonella* have rich phytochemical compounds such as carbohydrates, phenol, esters, amides, acids, etc. Trigonella seeds have long been known as a plant material. Today, this plant is widely cultivated as a medicinal plant around the world. In traditional Iranian medicine, *Trigonella* seeds were used for invigoration as well as lowering blood sugar. *Trigonella* seeds generally contain 43-27% carbohydrates (usually mucilage fibers), 57% -27% protein (tryptophan and lysine), 5-10% lipids, and small percentage of pyridine alkaloids (mainly trigonelline), choline and isoleucine (Mehrafarin *et al*., 2010; Hashjin *et al*., 2019). Examining the results of the analysis of the study data, it can be concluded that different species of this plant, as species that grow in northern Iraq and other parts of Asia, are important in terms of effective compounds and they could potentially be cultivated for medicinal purposes. The results of electron microscopy showed that the shape and cross section of these three species have significant morphological differences.

The results of this study showed that ethanol significantly causes liver and kidney damage. Liver enzymes levels, lipid profiles, and renal indices increased in rats receiving ethanol. These data, in agreement with previous findings suggested that ethanol abuse cause kidney and liver damage (Fathi *et al*., 2020, 2021; Akbari *et al*., 2017, 2019b, 2020). Manifestations of this damage are increase in lipid profile, liver enzymes and renal indices. The results also showed that treatment of alcoholic rats with different species of fenugreek could reduce the damage caused by ethanol and in some cases completely improve these damages. Many studies have closely examined the pathogenesis of ethanol abuse on liver and kidney damage (Osna *et al*., 2017; Stickel *et al*., 2017). Most of these studies agree that oxidative damage and inflammation are the main pathogenesis involved in ethanol toxicity (Akbari *et al*., 2017, 2019b; Fathi *et al*., 2020, 2021; Stickel *et al*., 2017). The source of oxidative damage is mitochondrial activity defect and electron

leakage from the electron transfer chain in the mitochondria and endoplasmic reticulum (Akbari *et al*., 2019a; Guo *et al*., 2013; Akbari and Jelodar, 2013). Increased levels of free radicals exert adverse effects through changes in cellular signaling, alterations in the metabolism of certain nutrients, and damage to organelles and cell membranes (Akbari *et al*., 2016). Apoptosis, changes in cholesterol metabolism, dehydration, increase in malondialdehyde, and decrease in activity of antioxidant enzymes are some of the major cellular damages that have been well documented by ethanol in previous studies (athi *et al*., 2020, 2021; Akbari *et al*., 2020). In fact, the main mediator of all these events is the pathophysiological level of free radicals. Therefore, the use of compounds and agents that can control free radical levels and return to normal is a logical solution to control and treat the damage caused by ethanol. In this study, we investigated the protective effects of *Trigonella* of different species on hematological parameters, liver and kidney protection in alcoholic rats. The results of GC-MASS analysis showed that seeds and leaves of different species of *Trigonella* plant have rich phytochemical compounds such as carbohydrates, phenol, esters, amides, acids, etc. Trigonline is the main ingredient of this plant which has many pharmacological properties (Hashjin *et al*., 2019). *Trigonella* seeds have many medicinal properties that have been used to treat various diseases from the past to the present (Khorshidian *et al*., 2016). The green parts of this plant are used as food. Its seeds are used as whole spices or ground in a mixture of spices in Asian cuisine (Wani and Kumar, 2018; Khorshidian *et al*., 2016). Species of this plant could improve ethanol toxicity by inhibiting oxidative damage and inflammation. The hematological findings of this study showed that the consumption of different species of this plant had no effect on hematological parameters such as hemoglobin level, blood indicators and platelet level except leukocyte content. This in fact indicated the weak role of this plant in modulating the status of the immune system. Improvement of biochemical parameters indicated improving the function of liver and kidney in the treated groups. Moreover, various studies in addition to ethanol have reported the protective effects of this plant against diethylnitrosoamine (Abdelgawad *et al*., 2012), cyclophosphamide (Bhatia *et al*., 2006) and 1,2-dimethylhydrazine (Devasena and Menon, 2007). The seeds contain fixed oil (mainly polyunsaturated fatty acids such as oleic, linoleic, and linolenic acids), flavonoids, alkaloids, saponins (diosgenin, gitogenin, and yamogenin), mucilage, coumarins, amino acids, protein, dietary fibers (soluble and insoluble fibers), volatile oil, vitamins (A, B1, B2, C, D), and various other functional elements. Modern pharmacological studies showed that fenugreek has many medicinal qualities, such as anti-inflammatory, antiulcer, hypocholestrolaemic, analgesic, antidiabetic, antipyretic, wound healing, CNS-stimulant, immunomodulatory, antifertility, anti-cancer, antimicrobial, antioxidative, and gastroprotective properties. Nowadays, production and marketing of fenugreek are becoming increasingly important because of medicinal and nutraceutical properties. The results of this study showed that the content of 2-ethyl hexanol, dodecane and hexadecane is the highest amount among other compounds in these species. Among these species, *T. strangulata* had the highest content of measured compounds. Other studies showed that the seeds of fenugreek contain several coumarin compounds as well as a number of alkaloids (e.g., trigonelline, gentianine, carpaine) and diosgenin which are commercially extracted (Wani and Kumar, 2018). The large amount of trigonelline is degraded to nicotinic acid and related pyridines during roasting (Acharya *et al*., 2006). The major bioactive compounds in fenugreek seeds are believed to be polyphenol compounds, such as rhaponticin and isovitexin (He *et al*., 2015). Trigonline is an alkaloid compound that has a hormonal role in plants. This metabolite is made through the methylation of nicotinic acid and has been identified in many plant species including coffee, fenugreek, soybeans, peas, alfalfa and others. This alkaloid has important medicinal properties such as anti-cancer, anti-migraine, antiseptic, lipid-lowering and anti-diabetic. Several studies have shown that trigonelline decreased blood glucose level by inhibiting the activity of key enzymes in glucose metabolism. Accordingly, the metabolite trigonelline could lead to the development of a new drug to control and treat diabetes (Mehrafarin *et al*., 2012). Due to the presence of these compounds, this plant has extensive pharmacological properties that have attracted the attention of many researchers (Kaviarasan and Anuradha, 2007). In this study, in addition to its antioxidant and anti-inflammatory roles, we showed that the consumption of species of this plant improves hematologic characteristics in alcoholic rats. Kaviarasan and Anuradha (2007) showed that fenugreek (*Trigonella foenum graecum*) seed polyphenols protect the liver from alcohol toxicity by inhibition of apoptosis (Kaviarasan and Anuradha, 2007). Improving the levels of lipid profiles and glucose can actually be due to modulating the activity of their metabolic pathways. Fathi *et al*. (2020, 2021) showed that ethanol cause lipid accumulation and disrupt the hepatic metabolism of cholesterol. They also showed that ethanol activated genes involved in apoptosis (Fathi *et al*., 2020, 2021). Kaviarasan and Anuradha (2007) also showed that fenugreek seed polyphenolic extract modulated the activity of alcohol dehydrogenase and aldehyde dehydrogenase and improved liver damage

induced by ethanol (Kaviarasan and Anuradha, 2007).

CONCLUSIONS

It can be concluded that these three species that grow in northern Iraq have high phytochemical compounds and have physiological effects such as improving the immune status, the function of the liver and kidney and antioxidants activity against ethanol-induced hepatorenotoxicity. This research can also be the basis for further work to extract medicinal compounds from other fenugreek these species, especially native these species of Iraq such as *T. strangulate, T. uncinata* and *T. filipes*.

Statement of conflict of interest

The authors have declared no conflict of interest.

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