RESEARCH ARTICLE

Phytochemical investigation and assessment of the hypoglycemic activity of two herbal extracts from selected Iraqi medicinal plants in alloxan-stimulated diabetic rats: a comparative study [version 1; peer review: 2 approved with reservations]

Amjed Haseeb Khamees¹, Hayder Adnan Fawzi², Hayder B. Sahib³

¹Department of Pharmacognosy, College of Pharmacy, University of Baghdad, Baghdad, 12221, Iraq

²Department of Pharmacy, Al-Rasheed University College, Baghdad, 12221, Iraq

³Department of Pharmacology, College of Pharmacy, Al-Nahrain University, Baghdad, 12221, Iraq

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Abstract

Background: *Cyperus rotundus* and *Trigonella foenum graecum* (fenugreek) have been utilized for many years as medical applications, mostly as traditional natural medications. The present research was designed to assess the hypoglycemic activity of the ethanol extract of Iraqi fenugreek seeds and tuberous roots of *C. rotundus* in alloxan-stimulated diabetic rats and to evaluate the diverse phytochemicals found in ethanol extracts that may have therapeutic activity.

Methods: Qualitative assessment of diverse secondary metabolites by corresponding chemical tests was been used to specify each group of compounds in the crude extracts of both plants. Male Wistar rats (n=40) were subdivided randomly into four main groups: group A, alloxan-stimulated diabetic rats with administered with distilled water (10mL/kg; control); group B-D, alloxan-stimulated diabetic rats administered with ethanol extract of *C. rotundus* (10 mg/kg; group B), glibenclamide (10 mg/kg; group C), and ethanol extract of fenugreek seeds (15mg/kg; group D). These medications were given orally for 6 weeks. Blood glucose of the rats was measured at baseline and after 1, 2, 4, and 6 weeks after administration of medication.

Results: Phytochemical analysis showed the presence of many secondary metabolites including alkaloids, flavonoids, anthraquinones, tannins, and steroids, in the crude extracts of *C. rotundus* and fenugreek seeds. After 6 weeks all groups of rats showed a significant decline in blood sugar level compared to the control group. Group C (glibenclamide) showed the lowest value, followed by group D (fenugreek seeds) and group B (*C. rotundus*).

Open Peer Review

Approval Status ? ?

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Any reports and responses or comments on the article can be found at the end of the article.

Conclusions: Ethanol extract of both plants possess considerable hypoglycemic activity in alloxan-stimulated diabetic rats, with superior efficacy shown by fenugreek seed extract, after 6 weeks of study.

Keywords

Trigonella foenum graecum, Cyperus rotundus, alloxan-stimulated diabetes rats, hyperglycemia effects

Corresponding author: Hayder Adnan Fawzi (hayder.adnan2010@gmail.com)

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Competing interests: No competing interests were disclosed.

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Introduction

Diabetes mellitus (DM) is a group of syndromes characterized by high blood sugar; modulated metabolism of lipids, carbohydrate and proteins; and an elevated risk from vascular diseases. It is associated with a metabolic defect with relative or absolute insulin deficiency and/or insulin resistance. Most patients can be classified clinically as either insulin-dependent DM or insulin-independent DM¹. Chronic complications include cardiovascular disorders, central nervous system damage, chronic kidney diseases, complication of the retina, and difficulties in wounds healing². These complications have a pronounced effect on the quality of life of the patients by increasing morbidity and mortality². Due to the multifactorial pathological nature of DM, it requires several therapeutic agents for optimal management. Most research on DM focuses on early diagnosis and preventive measure in highly at risk people.

Trigonella foenum graecum (fenugreek) has an extended range of applications (medical and non-medical), e.g. a flavoring agent of food³, use as a diuretic agent, ability to reduce blood pressure⁴ and blood cholesterol, cardiogenic and hypoglycemic properties, ability to enhance insulin production, antidiabetic effects, and antinociceptive and anti-inflammatory effects⁵. Fenugreek has been widely used as an ingredient of many blood glucose lowering substances, from different plant parts, including seeds and leaves and extracts in diverse solvents⁶. Its chemical component consists of different categories like saponins, sapogenins, alkaloids, and flavonoids. The hypoglycemic activity of fenugreek has been studied in different research; with the seeds of the plant as the main focus since its control over diabetes⁷. *Cyperus rotundus* (known as saed in Iraq) develops from a system of underground dark green tubers⁸. The tubers of the saed are utilized in many therapeutic indications, including as an antipyretic, anthelmintic, hypotensive, antihistaminic, and smooth muscle relaxant^{9,10}.

The current work aimed to study the phytochemical properties, and the hypoglycemic effect of the ethanol extract of *C. rotundus* and fenugreek seeds in alloxan-stimulated diabetes rats. This is a preclinical study to assess these new compounds.

Methods

Study setting

The study conducted at the College of Pharmacy, Al-Nahrain University, Iraq, during the period from November 2019 to January 2020.

Ethical approval

All procedures performed in the study were approved by the Institutional Research Committee at the College of Pharmacy, Al-Nahrain University (Ref# 2019/0078) and in accordance with the animals Act 1986 (ASPA) for animals used in scientific research.

All their efforts were made to reduce animal suffering by provision of water and food regularly, changing the beds every other day, letting animals sleep during the night offering a calming and dark place, and checking for any inconvenience to the animals throughout the study.

Experimental animals

A total of 40 Wistar male rats (175 – 230 g weight) were used in the study. The animals were obtained from animal house at the Iraqi National Drug Quality Control Center and Drug Research (Baghdad, Iraq). The animals were individually caged and allowed free access to water and standard rodent chow diet (18% Protein, 5% fat, Envigo Teklad Global Rat Food Pellets 2018), food was provided in a standard stainless-steel hopper. The animals were maintained at room temperature (23 – 25°C), 30–40% humidity, and lights on from 7 AM to 5 PM. The rats were maintained in polypropylene cages with husk as the bedding material. The animals were kept for 7 days after transfer from their original housekeeping to allow accommodation before performing the study.

Sample size calculation

A resource equation approach was used (since it is not possible to assume the standard deviation and the effect size for this study) and the expected statistical analysis was ANOVA, based on the following equation:

$$n = \frac{DF}{k} + 1$$

DF: degree of freedom (range between 10 – 20¹¹), k: number of groups, n = number of subjects per group. Sample size between 4 – 6 was calculated, in order to increase the accuracy of the results a sample size of 10 was chosen for each of the four groups¹².

Study design

Animals were injected intraperitoneally with a single dose of alloxan monohydrate (Sigma Aldrich, USA) (120 mg/kg) in saline (0.9% NaCl) to induce diabetes¹³. The rats were fasted overnight for 12–13 hr but allowed access to water before blood glucose level was measured. After 72 h, glucose levels were recorded using a glucometer. Rats with blood glucose levels of 200 mg/dL and above were considered as diabetic and were selected for the study¹⁴.

The alloxan-induced diabetic animals were subdivided into four groups using a randomized block design (see below): group A, group A, alloxan-stimulated diabetic rats with administered with distilled water (10mL/kg; control); group B–D, alloxan-stimulated diabetic rats administered with ethanol extract of *C. rotundus* (10 mg/kg; group B), glibenclamide (10 mg/kg; group C), and ethanol extract of fenugreek seeds (15mg/kg; group D) (Table 1).

Randomized block design was selected as the experimental module. The rats were divided into four blocks: block one (group A) given distilled water at first week, block two (group B) started treatment the next week, block three (group C) started treatment the third week, and block four (group D) started treatment the 4th week. All the animals were kept in the same conditions. The previous order established when each block received their treatment, then in each block serum blood glucose was measure at baseline, 1 week, 2 weeks, 4 weeks, and 6 weeks after the initiation of treatment (no rat were sacrificed in the study). The allocation of rats was random in each block

Table 1. Animal group design and dosing details.

Group (n=10/group)	Dosing
Group A	Distilled water (10mL/kg) – negative control
Group B	<i>Cyperus rotundus</i> ethanol extract (10mg/kg)
Group C	Glibenclamide (Sigma-Aldrich, USA) (10 mg/kg) – positive control
Group D	Fenugreek seeds extract (15 mg/kg)

with the matching of between block in rats' weight, age, diets, and other fixed parameters. The treatment was considered a fixed effect.

Each of the groups contained 10 rats (total 40 rats).

All treatments were administered to the rats with 1.0 cc syringe using an oropharyngeal cannula. Doses were decided upon after review of the literature, taking into consideration of trying to provide the lowest dose possible.

Experimental outcome

Assessment of the change in blood glucose from baseline and after 1 week, 2 weeks, 4 weeks, and 6 weeks after the administration of the treatment.

The glucose level in the blood was measured with a glucometer (ACCU-CHEK Active, Mannheim, Germany), and values > 200 mg/dL were considered diabetic¹⁵. The animals were kept on fasting status for 12 – 13 hrs on days of sampling, and blood samples were obtained from tail vein by tail nipping procedure (no anesthesia was required). Blood glucose was measured early in the morning after overnight fasting¹⁴.

Experimental diabetes in rats was induced by injecting alloxan monohydrate (Sigma Aldrich, USA) at a single dose of 150 mg/kg, i.p. in ice-cold normal saline. After 72 h, glucose levels were recorded using a glucometer. Rats with blood glucose levels of 200 mg/dL and above were considered as diabetic and were selected for the study.

Plant materials

The Iraqi plants were collected from Baghdad, in November 2019 and the plants were specified and authenticated by the Biology Department, College of the Sciences, Baghdad University, Iraq. The rhizomes of *C. rotundus*, and seeds of *T. foenum graecum* were obtained.

Preparation of extract

The preparation of extract was performed using the method described by Ali *et al*¹⁶. In total, 500 g of coarse powdered *C. rotundus* (rhizomes were coarsely grounded and sieved) was loaded into a Soxhlet apparatus for the extraction process. The powder was first defatted with petroleum ether solvent (60–80°C) before extraction with ethanol. The ethanolic extract of 500 g *T. foenum graecum* seeds was obtained from the

reflex apparatus that was prepared two days previously (defatted extract). The ethanol extracts were evaporated using a rotatory evaporator and dried in a water bath.

Preliminary qualitative analysis of phytochemicals

Chemical tests to assess active constituents in the plants were done using plant crude extracts and standard procedures¹⁷ (Table 2).

Statistical analysis

Two-way ANOVA test was used to analyze the difference in blood glucose throughout the study. All analysis carried out using SPSS version 22.1 (Chicago, IL). P-value was considered to be significant if <0.05.

Results

Phytochemical analysis

The phytochemical analysis revealed the presence of many secondary metabolites, including alkaloids, flavonoids, anthraquinones, tannins, and steroids (Table 3).

Effect of plants extracts on blood glucose levels in alloxan-stimulated diabetic rats (Table 4)

At baseline, there was no significant difference in blood glucose levels between rats as they had typical blood glucose levels (range, 73.4 ± 5.2 to 78.8 ± 5 mg/dl) at the beginning of the experiment.

At 1 week, group D (fenugreek seeds) showed a similar reduction in glucose levels to animals in group B (*C. rotundus*) (p-value = 0.99). This reduction was significantly more than group A (negative control) (p-value < 0.001).

At 2 weeks, there were no significant differences in values between groups D and B, and both groups showed no significant differences compared to group C (glibenclamide).

At 4 weeks, the results showed a significant decline in all groups' blood glucose readings from baseline. There was no significant difference between groups C and D.

At 6 weeks, the final readings showed that all groups had a significant decline in blood glucose level compared to the group A. Group C had the lowest value, followed by group D and group B. Figure 1 shows the assessment of glucose levels during the whole study period.

Table 2. Chemical tests used for identification of active constituents. Reproduced with permission from 18.

Constituent	Test
Alkaloids	<i>Mayer's Test:</i> 10 ml of crude extract was mixed with 5 ml of 1% HCL on a steam bath then added the reagent; the result is white precipitate indicating +ve test for alkaloids. <i>Wagner's test:</i> 10 ml of ethanol extract was mixed with 5 ml of 1% HCL on a steam bath then add the reagent; the result is reddish-brown color precipitate indicating +ve test for alkaloids.
Flavonoids	<i>Lead acetate:</i> 5ml of ethanol extract mix with 1 ml of 10% Lead acetate to yield yellowish-white precipitate indicating the presence of flavonoids.
Steroids	<i>Liebermann-Burchard:</i> 3 ml of extract stirred with chloroform, acetic anhydride and few drops of sulphuric acid to yield dark pink or red color indicate the presence of steroids. <i>H₂SO₄:</i> 2 ml of extract were treated with sulphuric and acetic acids.to develop a greenish color to indicate the presence of steroids
Anthraquinones	<i>Borntrager's test:</i> 3ml of the extract was shaken with 3 ml of benzene. Then, filtration was performed and 5 ml of 10% ammonia solution was added to the filtrate. The mixture was shaken well, the development of a pink, red or violet color in the ammonia (lower) phase indicates the presence of anthraquinones.
Tannins	<i>FeCl₃ solution (5%w/v):</i> 3 ml of distilled leaves extract (in water) mixed with 3ml of FeCl ₃ solution (5%w/v) to produce dark green or blue-black precipitate indicate the presence of tannins.
Terpenoids	<i>Keller-Kiliani:</i> Ethanol extract (2ml) was dissolved in chloroform (2ml) and evaporated to dryness. Concentrated sulphuric acid (2ml) was then added and heated for about 2 min. A grayish color was considered an indication of the presence of terpenoids.

Table 2 has been reproduced with permission from [Khomees AH, 2017. *In vitro Analysis of Antioxidant and Antimicrobial Activity of Iraqi Bryonia dioica*]¹⁸

Table 3. Qualitative phytochemical analysis of *Cyperus rotundus* and fenugreek seed ethanol extracts.

Tested metabolite	Test	<i>Cyperus rotundus</i>	Fenugreek seeds
Alkaloids	Mayer	+++	++
	Wagner	+++	++
Flavonoids	Lead acetate	+++	Nil
Steroids	Liebermann-Burchard	+	+
	H ₂ SO ₄	+	+
Tannins	FeCl ₃	+++	++
Anthraquinones	Borntrager	Nil	+
Terpinoids	Keller-Kiliani	++	+

Table 4. Assessment of blood glucose (mean ± SD; mg/dl) in various groups of alloxan-stimulated diabetic rats during the study period.

Group	Baseline	Week 1	Week 2	Week 4	Week 6
A (distilled water; 10mL/kg)	73.4 ± 5.2	406.4 ± 20.1	414.6 ± 21.2	412.3 ± 29.8	412.0 ± 22.0
B (<i>Cyperus rotundus</i> ethanol extract; 10mg/kg)	78.8 ± 5.0	397.3 ± 7.6	298.4 ± 13.1	240.2 ± 37.5	155.4 ± 26.8
C (glibenclamide; 10mg/kg)	73.2 ± 5.2	315.8 ± 44.7	265.7 ± 44.4	170.9 ± 31.3	81.6 ± 14.2
D (fenugreek seeds ethanol extract; 15mg/kg)	75.8 ± 5.2	388.1 ± 36.9	293.3 ± 29.7	178.5 ± 34.2	129.9 ± 10.8

Figure 1. Assessment of blood glucose (mean $\pm$ SD; mg/dl) in various groups of alloxan-stimulated diabetic rats during the study period. Group A, distilled water (10mL/kg); B, *Cyperus rotundus* ethanol extract (10mg/kg); Group C, glibenclamide (10mg/kg); Group D, fenugreek seeds ethanol extract (15mg/kg).

No adverse effects were reported during the study period.

Discussion

Phytochemical analysis

The existence of these groups of compounds provides scientific evidence for the previously presumed medical applications of *C. rotundus* in the treatment of specific disorders. The existence of flavonoids, alkaloids, tannins and terpenoids appears to be considerable. *T. foenum graecum* also showed positive results for alkaloids, anthraquinones, tannins, and steroids, while the extract exhibited negative results towards flavonoids.

Effect of plants extracts on blood glucose levels in alloxan-stimulated diabetic rats

The results indicate that both ethanol extracts of fenugreek seeds and *C. rotundus* reduced the blood glucose level in diabetic rats. This hypoglycemic effect shows that these extracts have medicinal activity. The bioactive constituents of both extracts may play a role in increasing insulin secretion from β -cells¹⁹. Other supposed mechanisms may include this insulin-like effect or other modes of action, such as initiation of peripheral tissue glucose uptake, inhibition of endogenous glucose output, or enhancement of gluconeogenesis process in corresponding tissues, for example the liver and muscle tissue. A similar mode of action has been shown for other plant extracts with hypoglycemic activity²⁰. In addition, it is established that flavonoids control aldose reductase enzyme²¹. Many researchers report that there are several flavanols that can control diabetes,

by bringing about restoration of pancreatic islet cells and increasing insulin production in streptozotocin-induced diabetes. In addition, it may also enhance Ca^{2+} uptake from isolated islet cells thus giving an indication that is very effective in type II DM²². Further analyses are needed to detect the complete mode of hypoglycemic action of these plants.

Conclusion

The current study observed that fenugreek seeds and *C. rotundus* ethanol extracts possess considerable hypoglycemic activity in alloxan-stimulated diabetic rats with considerable higher activity for fenugreek seeds in contrast to *C. rotundus* tuberos roots extract. The hypoglycemic activity of both plant extracts was compared with standard oral hypoglycemic treatment glibenclamide. These plants are considered as a perfect candidate for further investigations for justifying the modes of hypoglycemic effect, as well as for isolation and determination of phytochemicals that possess or are responsible for the hypoglycemic and anti-diabetic activity of the plant. Further extensive pharmaceutical surveys, including long term studies, could be of value to estimate the possible toxic activity of these plants.

Data availability

Zenodo: Hypoglycemic Activity of Two Herbal Extracts, <https://doi.org/10.5281/zenodo.3701362>²³

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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Open Peer Review

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Reviewer Report 28 September 2020

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? **Supriti Sarkar**

¹ Department of Zoology, City College (Affiliated to University of Calcutta), Kolkata, West Bengal, India

² City College, Kolkata, India

1. *Is the work clearly and accurately presented and does it cite the current literature?*

Answer: Partly. The work done here though was not complicated rather was over-simplified but presented clearly. However the review of literature was insufficient and not put accurately. That is explained as follows: 1, In the first Para, where general description of diabetic complications are stated, the reference no 1 is not suitable. There are many works to refer to for such generalised diabetic complications e.g. Lin and Sun (2010¹).

Again, the hypoglycaemic activity of Fenugreek seeds has been studied by a number of works, few of such didn't mention here, which need to be cited e.g. El-Wakf *et al.* (2015²). One of the main and important components of fenugreek seed is 4-hydroxyisoleucine that was not mentioned here (Singh *et al.*, 2010³).

The hypoglycaemic effects of *Cyperus rotundus* was not reviewed here, though other functional properties were mentioned. The authors can consult at least these references: 1) Singh *et al.* (2015⁴); 2) Al-Snafi (2016⁵) and 3) The Medical Journal Of Tikrit University (2003);9(2).

2. *Is the study design appropriate and is the work technically sound?* Answer: Partly. The study design followed here was appropriate but could add some more parameters to strengthen the hypoglycaemic effects of fenugreek seed as well as tubers of *Cyperus rotundus*. Many previous workers already experimentally evidenced the hypoglycaemic effects of both fenugreek seed and *Cyperus rotundus*. In respect of that the current study didn't show any novel findings. The blood glucose could be estimated by any standard biochemical methods instead of instant glucometer.

3. *Are sufficient details of methods and analysis provided to allow replication by others?* Answer: Partly. The way of administration of both fenugreek seed extract as well as *Cyperus rotundus* should need to be mentioned, whether these were gavaged or mixed with food? Dose of

alloxan seems to be little high. Was a single dose of alloxan sufficient to maintain diabetic blood sugar till the end of the experiment up to the 6th week? Why is the dose of alloxan as described in Experimental outcome and Study design different?

A little description of phytochemical analysis need to be described for each specimen. Did the extraction of phytochemicals from fenugreek seed follow the same reference method? If possible names of different phytochemicals as extracted from both plants can be put here, because types of alkaloids etc. present in these plants are already available.

4. *If applicable, is the statistical analysis and its interpretation appropriate?* Answer: Yes.

5. *Are all the source data underlying the results available to ensure full reproducibility?* Answer: Partly. Few more parameters could be estimated if possible.

6. *Are the conclusions drawn adequately supported by the results?* Answer: Partly. It would be better if the authors could do some other parameters like estimation of insulin from the serum samples of all studied groups. At least any enzymes of glucose metabolic pathway from liver like hexokinase could be measured to improve the findings more scientifically. The obtained results then could support the hypoglycaemic effects of both plants more strongly. As most of the results described here are already known.

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Is the work clearly and accurately presented and does it cite the current literature?

Partly

Is the study design appropriate and is the work technically sound?

Partly

Are sufficient details of methods and analysis provided to allow replication by others?

Partly

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Partly

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Endocrinology. Reproductive endocrinology, Effects of Phytochemicals on Diabetes.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 17 July 2020

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Talasila Eswara Gopala Krishna Murthy

Bapatla College of Pharmacy, Affiliated to JNTUK (Jawaharlal Nehru Technological University, Kakinada), Kakinada, Andhra Pradesh, India

The article may be considered for publication after major revision.

1. Table 2 has been reproduced with permission from 'Khamees AH, 2017. In vitro Analysis of Antioxidant and Antimicrobial Activity of Iraqi Bryonia dioica.' The original references for these tests shall be specified.
2. The basis for selection of alcoholic extract shall be specified.
3. The criterion for dose selection shall be specified.
4. The percent reduction in blood glucose levels shall be specified.
5. The statistical interpretation of the data is required for better understanding.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

No

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Pharmaceutical product development and Isolation of active principles from plant materials.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

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