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Study of hypoglycemic effect of Fenugreek seed extracts

Rand Ibrahim Bilal
Nouma Hasan
Dima Muhammad
Faculty of pharmacy || Tishreen University || Syria

Abstract: *Trigonella foenum graecum* (Fenugreek) is a popular herb widely used for culinary and pharmaceutical purposes. The seeds of the plant have been intensively studied, as they exhibit plenty of pharmacological properties. The objective of the current research was to investigate the hypoglycemic effect of fenugreek seeds *in vitro* and *in vivo*. Aqueous (Aq. ex) and ethyl acetate (ETAC. ex) extracts were prepared from the grinded seeds. The α-amylase inhibitory activity assay was used to evaluate the hypoglycemic effect *in vitro*. Acarbose (the standard drug) was used as a positive control. While the *in vivo* glucose tolerance test was assessed in normal and alloxan-induced diabetic mice. Glimepiride was used as a standard drug. Different concentrations from both extracts were used to evaluate the hypoglycemic effect both *in vitro* and *in vivo*. The *in vitro* results suggested that the ETAC. ex had significantly ($P< 0.05$) higher α-amylase inhibitory activity with IC_{50} of 106.39 µg/ml than the Aq. ex with IC_{50} = 159.55 µg/ml. However, it remained below the inhibitory activity of acarbose (IC_{50} = 68.92 µg/ml) dose-dependent. *In vivo*, the oral administration of ETAC. ex in alloxan-induced diabetic mice significantly decreased the rise of blood glucose levels ($p< 0.05$) after 30, 60, and 90 min of glucose administration compared to the diabetic mice which did not receive any treatment and sometimes similarly to diabetic mice which were treated with Glimepiride. Thus, this study concludes that fenugreek may have hypoglycemic activity, being able to moderately inhibit the intestinal absorption of glucose and possibly by other mechanisms.

Keywords: *Trigonella foenum graecum*, anti-diabetic effect, α- amylase IC50, Alloxan, diabetic mice.

دراسة الفعالية الخافضة للسكر لخلاصات بذور الحلبة

رند إبراهيم بلال
نعمى حسن
ديما محمد
كلية الصيدلة || جامعة تشرين || سوريا

المستخلص: نبات الحلبة *Trigonella foenum graecum* (Fenugreek) هو نبات شعبي شائع الاستخدام على نطاق واسع للطهي ولأغراض علاجية. تمت دراسة بذور هذا النبات بشكل مكثف كونها تبدي العديد من الخصائص العلاجية. كان الهدف من البحث الحالي هو دراسة الفعالية الخافضة لسكر الدم لبذور الحلبة في الزجاج وفي الجسم الحيّ. تمّ تحضير الخلاصتين المائية وخلات الايتيل لمسحوق بذور الحلبة. استخدِم اختبار الفعالية المثبطة لأنزيم ألفا أميلاز لتقييم الفعالية الخافضة للسكر في الزجاج باستخدام الأكاربوز (الدواء العياري) كشاهد إيجابي، بينما تمّ تقييم اختبار تحمّل الغلوكوز لدى الفئران الطبيعية والفئران المصابة بالسكري المُحدَث بالألوكسان، واستخدم الغليميبريد كدواء معياري. تمّ استخدام تراكيز مختلفة من كلا الخلاصتين لتقييم الفعالية الخافضة للسكر في الزجاج وفي الجسم الحي. بيّنت النتائج في الزجاج أنّ خلاصة خلات الايتيل أظهرت ارتفاعاً هامّاً إحصائيّاً ($p < 0.05$) في الفعالية المثبطة لأنزيم إلفا

أميلاز وقد بلغت قيمة التركيز التثبيطي النصفي 106.39 مكغ/مل بالمقارنة مع الخلاصة المائية التي بلغ التركيز التثبيطي النصفي لها 159.55 مكغ/مل، لكنّها بقيت أقل من الفعالية المثبطة للأكاربوز (التركيز التثبيطي النصفي يساوي 68.92 مكغ/مل)، وقد كان التأثير الخافض للسكر معتمد على الجرعة. في الجسم الحي، أدّى الإعطاء الفموي لخلاصة خلات الايتيل للفئران السكريين إلى انخفاض هامٍّ إحصائيٍ في الارتفاع بتراكيز السكر الدموية ($P < 0.05$) بعد 30، 60، 90 دقيقة من إعطاء الغلوكوز مقارنةً مع الفئران السكريين الذين لم يتلقّوا أيّ معالجة وأحياناً تماثل الانخفاض مع الفئران السكريين المعالجين بالغليمبيريد، وهكذا تمّ الاستنتاج أنّ الحلبة قد تملك فعالية خافضة للسكر، كونها قادرة على تثبيط امتصاص الغلوكوز المعوي بشكل معتدل وربما من خلال آليات أخرى.

الكلمات المفتاحية: نبات الحلبة، الفعالية المضادة للسكري، إلفا أميلاز، التركيز التثبيطي النصفي IC50، الألوكسان، الفئران السكريين

Introduction:

T2DM is a chronic, multiplex disease characterized by insulin resistance and β-cell dysfunction in the pancreas. It is the most common type of diabetes, accounting for 90% of all cases of the disease[1]. T2DM is expected to affect more than 350 million individuals worldwide by 2025, according to WHO predictions[2]. It's a long-term metabolic disorder that's related to many of the comorbidities, such as thyroid disorder[3] and several pulmonary abnormalities[4], as well as microvascular and macrovascular complications such as retinopathy, nephropathy, and neuropathy[5]. In addition, it is known to increase cardiovascular risk factors[6]. Drugs like metformin, thiazolidinediones, sulphonylureas, Meglitinide, α-glucosidase inhibitors, sodium glucose co-transporter-2 inhibitors, and others are currently used to manage type 2 diabetes[7]. However, the adverse side effects of oral anti-hyperglycemic agents, as well as an increasing trend in treatment costs, increase challenges in achieving targeted glycemic control. In addition to drug therapy, the American Diabetes Association (ADA) and the European Association for the Study of Diabetes (EASD) recommend focusing on lifestyle modification in T2DM patients to ensure successful control and long-term maintenance of blood glucose levels [2]. To satisfy an unmet medical need, natural products can be taken as an "add-on therapy" to medicine in the long-term treatment of Type 2 diabetes. A wide range of phytoconstituents derived from herbal sources has been reported for the treatment of T2DM with demonstrated clinical safety and efficacy.

Traditional medicines that have been recognized as hypoglycemic agents include fenugreek (*Trigonella foenum-graecum Linn.* (The family is Fabaceae). Fenugreek has a wide range of pharmacological and therapeutic effects. It has cardioprotective and hypolipidemic, anti-arthritic, antioxidant, anti-inflammatory, and antibacterial effects [8]. In many animal [1, 9, 10] and human [5, 11]studies, fenugreek has been revealed to have anti-diabetic properties. Many active elements are included in fenugreek seeds, such as the alkaloid (Trigonelline), amino acid (4-Hydroxyisoleucine), galactomannan, and flavonoids, which are suspected to be responsible for its hypoglycemic effects [12-14]. More exhaustive mechanistic studies have suggested that 4-hydroxyisoleucin improves insulin secretion, galactomannan reduces insulin resistance and glucose resorption from the gut, trigonelline stimulates β-cell regeneration [15], and inhibitory activity against α-amylase and α-glucosidase might well be due to the existence of polyphenols and flavonoids (vitexin, tricin, naringenin, quercetin, and tricin-7-O-beta-D-glucopyranoside)

in fenugreek [16]. The importance of this study comes in shedding light on the role of fenugreek seeds, which are rich in many active elements, due to the effectiveness of these ingredients in lowering blood glucose with fewer side effects, and presenting fenugreek seeds as a nutritional and functional component in the control and prevention of diabetes in pre-diabetic patients. Therefore, we tested the hypoglycemic potency of fenugreek seeds *in vitro* using the α-amylase inhibitory activity test as well as *in vivo* in alloxan-induced diabetic mice.

Materials and Methods

Chemicals and equipment

The following chemicals were obtained from Titan Biotech (India): alloxan, DNSA (3, 5-Dinitrosalicylic acid), α-amylase, starch, and glucose. Ether petroleum, ethyl acetate, and butanol were purchased from Doummar & Sons Co (Syria) (in cooperation with the German company i-Fischer Technology). Sodium carbonate ($NaCO_3$) was procured from BDH (England). Sodium Dihydrogen phosphate ($NaH_2PO_4.H_2O$) and Di-Sodium hydrogen phosphate ($Na_2HPO4._7H_2O$) were purchased from Merck (Germany). HCL was acquired from SHAM Lab (Syria). Sodium hydroxide (NaOH) was obtained from Avonchem Ltd. Sodium potassium tartrate tetrahydrate was from SCP Ltd. Acarbose and Glimepiride were purchased from Syrian Oushar Pharmaceutical and Syrian Asia Pharmaceutical respectively. Glucolab (Blood Glucose Monitoring System) was procured from infopia Co.,Ltd (Korea).

Extraction

Dried ripe fenugreek seeds (200g) were grinded into powder and defatted with petroleum ether (2L). Thereafter, the extraction was processed by ultrasound-assisted extraction at room temperature successively with ethyl acetate (2L) and water (2L) to obtain the corresponding extracts (ETAC.ex, Aq.ex). The aqueous extract (Aq.ex) was desaponified with n-butanol in a separatory funnel by liquid-liquid extraction. Finally, the organic extracts were dried by rotary evaporation, and the aqueous extract (Aq.ex) was lyophilized and stored in a dry place protected from light at room temperature.

α-Amylase Inhibitory activity assay:

The α-amylase inhibitory activity was measured according to Miller's method[17]. The complete assay mixture contained 200 µl of 0.02M sodium phosphate buffer, 20 µl of α- amylase enzyme, and 200 µl of plant extracts with a concentration of 120 µg/ml. The mixture was maintained for 10 minutes at room temperature before adding 200 µl of 1% starch to all test tubes. The reaction was stopped by adding 400 µl of DNSA color reagent. Then, the reaction was kept in a boiling water bath for 5 minutes, cooled to room temperature, and diluted with 15 ml of distilled water, then the absorbance was measured at 540 nm (Schimadzu-UV-VIS Spectrophotometer). Acarbose samples were prepared in the same way as the

plant extract. The blank included neither the enzyme nor the standard drug or extract, and the negative control samples included the enzyme without any plant extracts or standard drug. All assays were performed in triplicate and the mean value was used to indicate the inhibitory activity. The results were calculated as percent inhibition using the following formula:

Inhibition activity (%) = [Abs (neg. control) - Abs (extract)] × 100/ Abs (neg. control)].

The assay was performed using different concentrations of plant extracts ranging from 20–120 µg/ml (as well for Acarbose) to obtain the IC50 values (the inhibitor concentration at which 50% inhibition of enzyme activity occurs).

Miller's method: G. Miller first introduced the DNSA method for calculating the concentration of reducing sugars in a sample in 1959. Many reagents can be reduced by reducing sugars. A reducing sugar is one that generates an aldehyde or ketone in a basic solution that includes a free carbonyl group. The only reduced product of 3, 5-dinitrosalicylic acid (DNSA-yellow) is 3-amino-5-nitrosalicylic acid (ANSA-orange), which is reduced in an alkaline solution by the oxidation of the aldehyde group of all monosaccharides such as glucose. During the reaction, oxygen gas is emitted, and water is used as a reactant. Changes in the amount of light absorbed in maximum wavelengths are caused by the production of 3-amino-5-nitrosalicylic acid. The quantity of decreasing sugar is clearly relative to the absorbance as determined by a spectrophotometer [18].

3, 5-dinitrosalicylic acid (Aldehyde group) → 3amino, 5nitro salicylic acid (carbonyl group)

3,5-dinitrosalicylic acid (yellow)

3 amino, 5-nitro salicylic acid (orange-red)

Animals and study design:

Animals:

Male albino mice weighing 20–30g were used for the experiment. They were housed in a climate-controlled environment (25 °C, humidity 65 ± 10%, and a 12-hour dark/12-hour light cycle) and fed a standard diet. To induce T2DM, Alloxan was injected intraperitoneally in a single dose of (150 mg/kg) after the mice have fasted for 16-hour Fasting blood glucose (FBG) was measured 72 hours after Alloxan administration to test for the development of hyperglycemia. Mice with FBG levels ≥ 200 mg/dl were considered diabetic in our study.

Study design and evaluation of extracts on glucose tolerance:

Thirty-six mice were divided into 6 groups (n = 6) as follows:

I. Group NC (Normal control): received 0.5 ml of saline 0.9% intraperitoneally.
II. Group DC (Diabetic control): alloxanized mice did not receive any treatment.
III. Group PC (Positive control): alloxanized mice received the standard drug (Glimepiride 4 mg/kg) by oral gavage.
IV. Groups DC1, DC2, and DC3: alloxanized mice received ETAC.ex (1ml) of Fenugreek by oral gavage in concentrations of (12.5 mg/ml, 25 mg/ml, and 50 mg/ml, respectively).

After an overnight fast, the mice were administered glucose (2 g/kg) orally before being given the standard drug (glimepiride) or the extract 15 minutes later. Blood samples were collected before (time point 0) and after glucose administration, at 30, 60, 90, and 120 minutes, and blood glucose levels were measured.

Statistical analysis:

The results are expressed as mean ± standard error of mean (SEM). One-way ANOVA, followed by the LSD (least significant differences) test was assessed using the statistical analysis software program SPSS version 20. The significance level was set at 0.05. Means followed by common letters are not significant while means with the different letters are statistically different.

Results:

***In virto* α-amylase inhibition activity:**

The α-amylase inhibitory activity calculated as percentage according to the former equation for Aq.ex, ETAC.ex and Acarbose are presented in table 1 and plotted in figure 1.

Table 1: The mean of α-amylase inhibition activity % of each concentration (µg/ml) of fenugreek seeds extracts and acarbose

	Concentrations µg/ml					
α-amylase inhibition activity % Mean	20	40	60	80	100	120
ETAC.ex	12.85	B 25.31	C 29.96	C 36.02	C 43.33	C 60
Aq.ex	9.38	B 11.77	B 16.86	B 26.62	B 30.70	B 39.02
Acarbose	23.40	A 29.69	A 45.50	A 50.24	A 72.68	A 81.82
P-value	0.079 n.s	*0.024	**0	**0	**0	**0
LSD	-	11.89	3.85	5.52	11.36	3.63

The results show significant statistical differences between the means of the inhibition activity of each of acarbose, Aq.ex and ETAC.ex of Fenugreek at all concentrations (except for 20 µg/ml) compared to the negative control (p-value < 0.05), suggesting an inhibitory activity of Fenugreek on alpha-amylase. However, the IC50 values for Aq.ex and ETAC.ex (159.55 µg/ml versus 106.39 µg/ml) were higher than the IC50 of acarbose (68.92 µg/ml) by (131.5% and 54.37%), respectively figure 2. The IC50 for Aq.ex was higher than that for ETAC.ex by 49.97%. These results suggest that fenugreek extracts are less potent to inhibit the activity of α-amylase than Acarbose. As well as, the ETAC.ex showed significantly higher potency on α-amylase inhibition compared to Aq.ex (except for the concentration of 40 µg/ml).

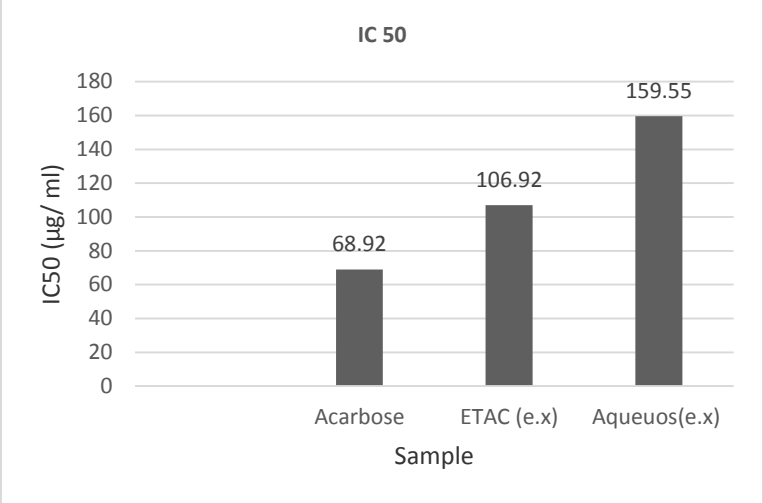

Figure 1: Inhibitory activity of *Trigonella foenum ¯graecum* seeds extract against α¯ amylase

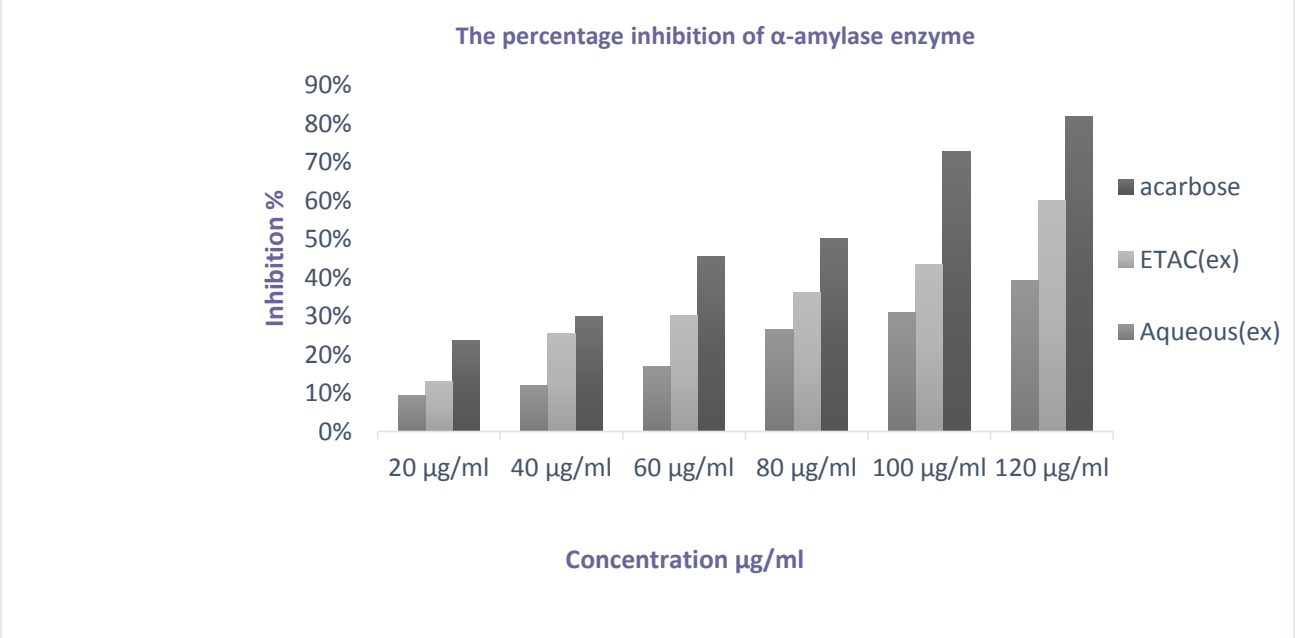

Figure 2: IC50 values for aqueous, ethyl acetate of Trigonella foenum-graecum seed extracts, and acarbose (µg/ml).

***In vivo* hypoglycemic activity of Fenugreek seed extracts:**

Results of the effect of ETAC extract in concentrations of (12.5- 25 and 50 mg/ml) on glucose tolerance are shown in Table 2. Half an hour after glucose administration, the blood glucose level increased by 78.55%, 65.89%, ($p < 0.05$) and 7.20% ($p > 0.05$) in normal controls, diabetic controls, and in mice receiving Glimepiride, respectively, while the rise in glucose level was only 24.81%, 20.63%, and 8.69% ($p < 0.05$) in diabetic mice administered with 12.5, 25, and 50 mg/ml (groups DC1, DC2, and DC3) of ETAC extract. Furthermore, treatment with fenugreek extract significantly reduced the increase in blood glucose levels after 30, 60, and 90 minutes of glucose administration ($p < 0.05$) (groups DC1, DC2, and DC3) compared to the mice that did not receive any treatment (group DC), and this decrease in blood glucose levels caused by ETAC.ex with two concentrations (50 and 25 mg/ml) (groups DC3, and DC2) after 30, 60, and 90 minutes was statistically similar to the decrease caused by Glimepiride (group PC). Thus, fenugreek extract may be effective in stimulating insulin secretion. The decrease in blood glucose levels was not dose-dependent on fenugreek extract, as the two concentrations of 50 and 25 mg/ml of ETAC.ex (groups DC3, and DC2) were similar in hypoglycemic activity. However, the activity was better than that caused by 12.5 mg/ml (group DC1) after 60 and 90 minutes of glucose administration ($p < 0.05$). (Fig 4)

Tablet 2: The effect of various concentrations of *Trigonella foenum-graecum* seed extract on blood glucose level of diabetic mice compared with controls:

Treatment group	0 min	30 min	60 min	90 min	120 min
NC	A 91.67±5.58	A 163.67±4.84*	A 134.17±4.02*(18.02)	A 120.17±2.93*(10.43)	A 108±3.12*(10.12)
DC	B 320.5±6.59	D531.57±18.01*	D 555.83±16.04*	D 430±11.83*(22.64)	B 345.83±13.69*(19.57)
PC	B 340.5±18.04	B 365±15.60	B 355±14.42*(2.74)	B 340±13.26*(4.23)	B 334±12.90*(1.76)
DC1	B 322.5±10.55	C 402.5±7.5*	C 389±6.06*(3.35)	C 370.67±5.33*(4.71)	B 333.33±6.28*(10.07)
DC2	B 307.83±5.38	BC 371.33±5.49*	B 359.33±3.99*(3.23)	B 345.83±3.05*(3.76)	B 332.83±3.23*(3.76)
DC3	B 322.17±11.1	B 350.17±8.49*	B 341.5±9.14*(2.48)	B 332.5±8.98*(2.64)	B 323.33±9.64*(2.76)

Values are shown as a mean ±S.E. for groups of six mice each. The value in parentheses indicates the percent decrease of plasma glucose levels in comparison to the previous reading. NC, normal control; DC, diabetic control; PC, positive control; DC1; DC2; DC3, ethyl acetate extract of fenugreek seed (12.5, 25, and 50 mg/ml)

*Values are statistically significant at $p \leq 0.05$.

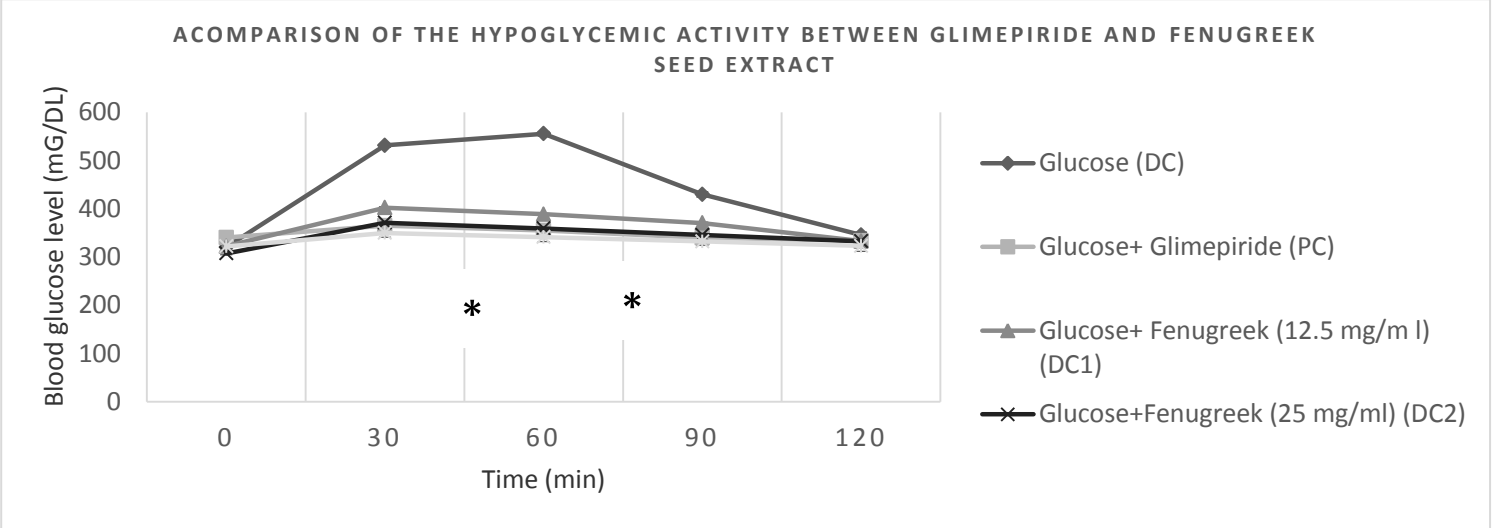

Figure 3: Effects of ETAC extract on blood glucose levels in diabetic mice following glucose administration. The mice were fasted for 16 hours before receiving glucose (2 g/kg) and ETAC extract concentrations (12.5, 25, and 50 mg/ml) via oral gavage (groups DC1, DC2 and DC3). Glucose alone (group DC) or glucose plus 4 mg/kg body weight of glimepiride (group PC). Blood glucose levels were measured before administration, 30, 60, and 120 minutes after glucose administration. The results were expressed as mean ± SEM, n = 6, $^{*}P < 0.05$.

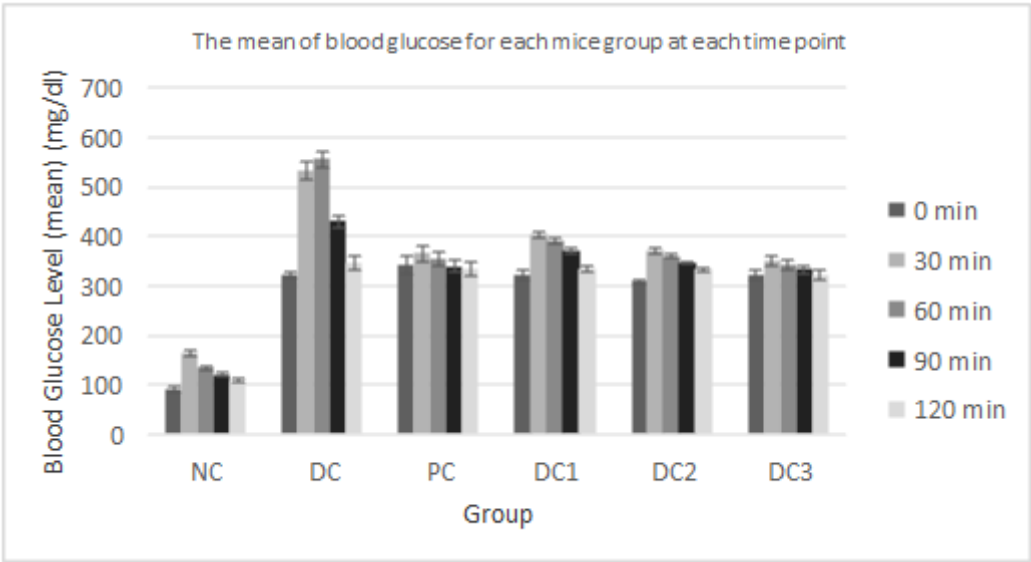

Figure 4: Effects of fenugreek seed ethyl acetate extract on blood glucose levels in diabetic mice following glucose administration. The mice were fasted for 16 hours before receiving glucose (2 g/kg) and ethyl acetate extract of fenugreek seed concentrations via oral gavage. NC, normal mice were given glucose alone (n = 6); DC, diabetic mice were given glucose alone (n = 6); PC, diabetic mice were given glucose plus 4 mg/kg body weight of Glimepiride (n = 6); DC1 group, diabetic mice

were given glucose plus 12.5 mg/ml of ethyl acetate extract (n = 6); DC2 group, diabetic mice were given glucose plus 25 mg/ml of ethyl acetate extract (n=6); DC3, diabetic mice were given glucose plus 50 mg/ml of ethyl acetate extract (n=6); (Blood glucose levels (mg/dl) were measured prior administration, 30, 60, 90, and 120 minutes after glucose administration. The results were expressed as mean ± SEM.

Discussion:

This study aimed to investigate if fenugreek seed extract had anti-diabetic activity and if it might be used as an alternative or additive treatment for T2DM. T2DM is characterized by a rapid increase in blood glucose levels caused by pancreatic α-amylase hydrolysis of complex starch and α-glycosidase hydrolysis of oligosaccharides and disaccharides into glucose, followed by glucose absorption in the small intestine [16]. This could be regulated by inhibiting the enzymes involved in carbohydrate assimilation. In contrast to typical treatment with drugs like acarbose, consuming inhibitors naturally derived from dietary ingredients could be a viable therapy for treating postprandial hyperglycemia with minimal adverse effects. Therefore, the α-amylase inhibitory activity of fenugreek seed extracts (ethyl acetate and aqueous) was tested *in vitro*. The comparative analysis demonstrates that the ethyl acetate extract of fenugreek seeds has high inhibitory activity, suggesting that the inhibitory ingredient is more dissolved in ethyl acetate. Perhaps flavonoids and polyphenols present in the extract are responsible for this activity. Moreover, because fenugreek contains a wide range of active factors, studies suggest that it may play a role in improving T2DM as an amino acid. 4-Hydroxyisoleusine [19] has also been shown to stimulate insulin secretion [12], as well as alkaloids, trigonelline, which has anti-diabetic activity by improving the insulin-signaling pathway[15], and flavonoids, which have antioxidant properties that protect the pancreas from the damages of free radicals [1] Therefore, this research was conducted to study the organic extract (ETAC e.x) with three different concentrations (12.5, 25 and 50 mg/ml) (groups PC1, PC2, and PC3) on alloxan-induced diabetic mice compared to normal, diabetic, and Glimepiride controls (groups NC, DC, and PC). Alloxan, a urea-derived chemical, is the most commonly used chemical agent for inducing DM in trial animals such as mice. Alloxan causes experimental DM by harming pancreatic cells selectively [20]. The Alloxan's structural resemblance to glucose allows for selective uptake into pancreatic cells, causing a vicious cycle of sulphydryl (-SH) group oxidation, inhibition of the glucokinase enzyme, and the generation of free radicals, all of which impair intracellular calcium homeostasis [21]. In diabetic mice, the ETAC.ex showed an obvious hypoglycemic activity and it was sometimes close to the effect of Glimepiride at tested doses. This indicates that the extract has other mechanisms that aren't limited to the intestines, but also that it is effective in increasing insulin secretion, as well as antioxidant activity, which may protect the pancreas against the disruptive effects of alloxan by generating reactive oxygen species (ROS) and superoxide radicals. Many previous authors, including Aditya Genshpurkar and others, have evaluated the

anti- hyperglycemic properties of fenugreek extracts *in vitro*[16], who investigated two fenugreek leaf extracts (aqueous and ethyl acetate) at five concentrations (50, 100, 150, 200, and 250 µg/ml) and discovered that only the ethyl acetate extract had a significant α-amylase inhibitory potential (64, 55% at 250 µg/ml) and that polyphenols in the extract could be responsible. While, Asmena Mowla and others [22] evaluated the effects of different doses (2, 1, 0.5, and 0.1 g/kg) of fenugreek seeds (ethanolic extract) on blood glucose in alloxan-diabetic rats. The extract's hypoglycemic outcome was compared to a single dose of 4 mg/kg of glimepiride (standard drug), knowing that the doses of 2, 1, and 0.5 g/kg in this study were simulated at 50, 25, and 12.5 mg/ml in our study. Fasting blood glucose was evaluated two hours after the extract or drug administration, and the extract exhibited significant activity ($p < 0.05$) against the alloxan-induced diabetic rats. However, the strength of the hypoglycemic action changed depending on the dose. The best active dose reported was 1g/kg (39.32%), but this is still less than the standard antidiabetic drug (57.19%), which has been gradually followed by 2 mg/kg (33.92%), 0.5 mg/kg (12.40%), and 0.1 mg/kg (8.72%), respectively. The authors attributed this effect to the fact that the ethanolic extract of fenugreek seeds contains active components such as alkaloids, as shown in the phytochemical analysis. Furthermore, Xiao-Yan Li and colleagues confirmed the role of the anti-oxidant activity of fenugreek seeds (ethyl acetate extract) in hypoglycemic activity in mice with streptozotocin (STZ)-induced diabetes [1].

Conclusion:

Despite the differences in hypoglycemic efficacy and the unique mode of action, *Trigonella foenum-graecum* seed extracts can be considered an effective option for diabetes. The traditional usage of locally produced herbs for the treatment of diabetes mellitus was also supported by this investigation. Clinical trials are still required to demonstrate the effectiveness of this homegrown medicine. To identify pure active compounds from extracts, activity-directed phytochemical studies are additionally necessary. Such research could support the discovery of novel chemical components as well as the dietary usage of *Trigonella foenum-graecum* seeds as a modulator in the treatment of diabetes mellitus and ultimately as a therapy in its management.

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