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Synergistic Effect of Metformin and Fenugreek Extract in Improving Metabolic Disorders in a Rat Model of Type 2 Diabetes

Ahmed Mahdi Hassan

Department of Biology , College of Science , University of Kirkuk , Iraq

ahmedmahdi@uokirkuk.edu.iq

I. ABSTRACT

This study seeks to investigate the effects of fenugreek extract (Trigonella foenum-graecum) alone and in combination with metformin on type 2 diabetes-induced by highFat diet in rats. A total of 205 male Sprague Dawley rats were allocated into five groups, and diabetes was induced by streptozotocin (STZ). There were five groups including control, diabetic untreating group treated with metformin (200 mg/kg), fenugreek extract (100 mg/kg) and metformin + fenugreek extract. Fasting blood glucose, HbA1c and insulin resistance (HOMA-IR) were performed through biochemical analyses. Insulin signaling markers (PI3K, GLUT4, IRS-1) and inflammatory markers (STAT3, IL-6, Caspase-3) gene expression were assessed. Combination group (130.54 ± 7.23 mg/dL) have a significantly reduced fasting blood glucose (FBS) compare to the metformin (180.61 ± 10.34 mg/dL) and fenugreek (200 ± 9.21 mg/dL) groups. Also was HbA1c significantly changed, the combination group had a significantly lower value (5.50 ± 0.21%) than those receiving metformin (6.80 ± 0.30%) and fenugreek (7.24 ± 0.31%), respectively. The HOMA-IR index, which indicates sensitivity to insulin, was lower than in the combination group (3.01 ± 0.35). The expression of PI3K, GLUT4, and IRS-1 was the most increased in the combination group, implying better glucose uptake and insulin response than control and monotherapy compared to interaction data. Synergistic anti-inflammatory action was demonstrated by substantial reductions in the inflammatory markers such as STAT3 0.90 ± 0.08, IL-6 1.06 ± 0.03, and Caspase-3 0.93 ± 0.04 after combining metformin and fenugreek in the combination group..

Keywords : Trigonella foenum-graecum , Insulin Resistance , Insulin Signaling,Caspase,-3, PI3K,GLUT4,IRS-1.

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II. Introduction

One of the most common illnesses in the modern world is diabetes mellitus (DM), which has grown to be a significant public health concern [1]. The 2021 Diabetes Atlas published by the International Diabetes Federation (IDF) estimates that 537 million people worldwide suffer from diabetes. That figure is expected to rise to 643 million by 2030 and 783 million by 2045. In Bangladesh, an estimated 13.1 million persons had diabetes in 2021; by 2045, that number is expected to increase to 22.3 million [2]. One of the hallmarks of diabetes mellitus, a metabolic disorder, is chronic hyperglycemia brought on by a reduction in insulin action, synthesis, or both [3]. Type 1 and Type 2 diabetes are the two main forms of the disease. Type 1 diabetes is characterised by a complete loss of insulin production due to the autoimmune destruction of pancreatic β-cells. Type 2 diabetes, on the other hand, is brought on by insulin resistance and β-cell malfunction [4]. Kind 2 diabetes is the most common type of the disease, accounting for 90% of cases [5]. Nonetheless, long-term diabetes significantly worsens the microvascular and macrovascular effects, including nephropathy, retinopathy, neuropathy, cardiovascular disease, peripheral artery disease, and stroke [5]. Although the rate of recurrence is continually rising, strict blood glucose management reduces the frequency of these issues [6]. Since there is now no known cure for diabetes, developing innovative therapeutic approaches to manage the illness has become essential [5].

The biguanide medication class, which includes metformin, improves insulin sensitivity. Metformin is still a recommended medication both domestically and abroad for reducing blood glucose levels and enhancing insulin sensitivity in the treatment of Type 2 diabetes, despite the fact that its exact mechanisms of action are still unknown. It has been demonstrated that after taking metformin, patients with Type 2 diabetes experience a 75% decrease in their initial elevated hepatic glucose production. Metformin's main effects include lowering the synthesis of glucose in the liver, However, long-term research has indicated that its impact on insulin-mediated peripheral glucose absorption is negligible. Metformin's capacity to suppress ATP synthesis in mitochondria, which raises its intracellular concentration by 1000 times in comparison to the extracellular environment, is linked to its AMPK-associated effect. As a result, component 1 of the respiratory chain is inhibited [7]. The AMP-to-ATP and ADP-to-ATP ratios, which are important indicators for AMPK activation, rise as a result of these actions, which restrict ATP synthesis [8,9]. Since ancient times, plants have been utilised extensively to treat a variety of illnesses [10]. Plant extracts are a valuable source of novel medications because of the phytochemicals that give them their therapeutic benefits, including the control of diabetes [11]. Among its many pharmacological qualities, fenugreek (Trigonella foenum-graecum), a member of the Fabaceae family, has been shown to have anti-diabetic [12], antioxidant, antihyperlipidemic, anti-inflammatory [13], and anti-obesity actions [14]. Its seeds have long been used to treat diabetes in Asia and Africa [15].. Its anti-diabetic effects have been investigated in a number of preclinical and clinical investigations [16, 17].

Hyperglycemia has been demonstrated to be decreased by the bioactive substances found in fenugreek seeds, including galactomannan, (2S, 3R, 4S]-4-hydroxyisoleucine, saponins, diosgenin, trigonelline, quercetin, orientin, vitexin, and isovitexin [18]. However, fenugreek leaves are infrequently used, despite the fact that they contain soluble fibres, cinnamic acid, coumaric acid, saponins, quercetin, and catechin [19].

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III. Materials and Methods

2.1. Collection of Plant Seeds and Extract Preparation.

Fresh T. foenum-graecum seeds were collected from the local market. The sample was identified as T. foenum-graecum seeds by plant science professors from the Department of Life Sciences, College of Education, and College of Science at the University of Kirkuk.

The fresh T. foenum-graecum seeds were air-dried and ground into powder using a grinding machine. A total of 2 kg of the powder was extracted in glass flasks using 90% ethanol at room temperature. The maceration process was performed five times, each for 48 hours, with intermittent shaking and stirring. The complete extract was collected, filtered using Whatman No. 1 filter paper, and concentrated at 40°C under vacuum. Finally, it was freeze-dried to obtain 150 g of crude extract [20].

2.2. Animals Used in the Experiment

Sprague Dawley male rats, 1 to 1.5 months of age and weighing an average of 140 ± 10g, were acquired from Tikrit University's veterinary medicine college. A total of 205 rats were collected. The animals were placed in suitable-sized plastic cages with shredded wood litter as the bedding. The cages were cleaned and disinfected every two days. The animals were given a standard diet from the NRC [21] and maintained under proper conditions such as a temperature of 25°C, a light cycle of 12h on, 12h off, and proper airflow.

2.3. Effective Doses Used in the Study

The doses used in this study were those reported from previous studies conducted in animals and non-humans. A metformin dose of 200 mg/kg was selected based on studies that previously suggested this dose is effective [22]. The AS dosage of 100 mg/kg was selected as an optimal dose for fenugreek extract based on previous studies203. [23] and. [24] showed that the reduced glucose level has been improved by 28%. These doses were selected based on previous research to provide safe and reliable, but meaningful and relevant data. All doses were administered orally once daily at the same time using a gavage tube.

2.4. Experimental Grouping

This study employed five groups. The control group was CI, and the following four groups were STZ (Sigma-Aldrich) ethanol-induced type 2 diabetes. Citrate buffer (pH 4.5), in which the STZ had been dissolved, was injected intraperitoneally as a unique dose, according to the method described by [25].

Male rats (0.31321 kg body weight) were allocated per group (n = 41 animals), and the assay was carried out during three months with a daily free access to food and water. The groups were as follows:

- Control group (non-diabetic, untreated).
- Diabetic group (untreated).
- Group of diabetic rats treated with metformin (200 mg/kg).
- Fenugreek (100 mg/kg) ethanol extract predisease (Fenugreek-E).
- Metformin and fenugreek extract-treated diabetic group (200 mg/kg metformin + 100 mg/kg fenugreek extract)

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2.5. Blood Sample Collection

Animals were fasted for 12 hours and anaesthetized with chloroform. Blood samples were taken using cardiac puncture (4 mL rat−1) into Gell tubes. Incubation of the tubes was conducted at 37°C for 30 minutes for clotting. Serum was obtained by centrifugation at 3000 rpm for 15 minutes. Micropipettes were used to collect the serum, which were then stored at -20 °C for biochemical laboratory analysis.

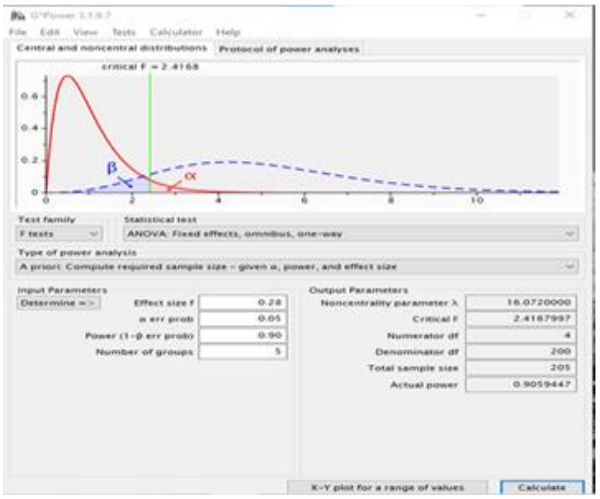
2.6. Measurement of Study Variables

Detection of percentage of glycated hemoglobin (HbA1c) using immunoassay detection method It is based on a nitrocellulose matrix where an antibody-antigen complex binds and is immobilized by a second fixed antibody on the strip of test.. The concentration of the antigen in the sample determines the strength of the signal, with the Ichroma™ device displaying the HbA1c percentage as part of the total hemoglobin [26]. The concentration of glucose in serum was measured using a Biolabo ready-to-use assay kit based on the [27] enzymatic method, which involves glucose oxidation. Insulin resistance (IR) was calculated using the HOMA-IR index, determined by multiplying the fasting blood glucose (mg/dL) by the fasting serum insulin level and dividing by 405, as per. [28]:

HOMA-IR = (Fasting Blood Glucose × Fasting Insulin) ÷ 405

2.7. Statistical Analysis

Sample size (205 rats) was determined using G*Power software based on a one-way ANOVA analysis, assuming a significance level of α = 0.05, a statistical power of 0.90, and an effect size (f) of 0.28, following. [24] to ensure accuracy and reliability.



Picture (1) Determining the Appropriate Sample Size for an ANOVA Test in G*Power

Statistical analysis was performed using SPSS version 19. Data normality was assessed using appropriate statistical tests. One-way ANOVA was conducted to compare group differences, followed by Tukey's post hoc test to determine significant differences between groups.Results were expressed as mean ± standard deviation (SD). Different superscripts in results indicated significant differences between means, while identical superscripts denoted no significant difference.

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IV. Results and Discussion

3.1. Biochemical Analysis

The results of the study presented in Table (1) indicate a significant increase in fasting blood glucose levels in the untreated group (290.45 ± 12.65 mg/dL), confirming the adverse effects of diabetes. In contrast, treatment with metformin alone resulted in a reduction in blood glucose levels to (180.61 ± 10.34) mg/dL, while fenugreek extract alone lowered it to (200 ± 9.21)mg/dL. When metformin and fenugreek were combined, glucose levels further decreased to (130.54 ± 7.23) mg/dL, compared to the control group, which recorded (90.23 ± 5.21 mg/dL).

Table (1) Results of Biochemical Variables

Parametetrs / Groups	Fasting Blood Glucose (mg/dL)	Hb1Ac (%)	HOMA-IR
Control Group	90.23 ± 5.21 e	4.52 ±0.22 e	2.11 ± 0.33 E
Diabetic Untreated Group	290.45 ±12.65 a	9.21 ±0.41 a	8.52 ± 0.51 A
Metformin Only	180.61 ± 10.34 c	6.80 ± 0.30 c	4.27 ± 0.48 c
Fenugreek Only	200. ± 9.21 b	7.24 ± 0.31 b	4.83 ± 0.42 B
Metformin + Fenugreek	130.54 ±7.23 d	5.50 ± 0.21 d	3.01 ± 0.35 D

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1.1. These findings suggest that metformin reduces blood glucose levels by decreasing hepatic glucose production and enhancing cellular insulin sensitivity—an established mechanism of action for this drug in the treatment of type 2 dia-betes [29]. On the other hand, previous studies have demonstrated that fenugreek extract contains bioactive compounds such as saponins and flavonoids, which contribute to carbohydrate metabolism regulation and stimulate pancreatic insulin secretion [30].The combination of metformin and fenugreek resulted in a greater improvement in blood glucose regulation compared to either treatment alone, supporting the hypothesis that fenugreek enhances the pharmacological effects of metformin. A recent study confirmed that the concomitant administration of fenugreek with metformin led to superior glucose control improvements compared to metformin alone, corroborating the findings of this study [31].

1.2. The current study results, as shown in Table (1), indicate a significant increase in HbA1c levels in the untreated group (9.21 ± 0.41%), suggesting poor long-term glycemic control. HbA1c levels decreased to (6.80 ± 0.30%) in the metformin group and to (7.24 ± 0.31%) in the fenugreek group. In the group that received both metformin and fenu-greek, HbA1c levels dropped to (5.50 ± 0.21%), indicating a greater improvement in glycemic control compared to the control group, which recorded (4.52 ± 0.22%). HbA1c is one of the most important indicators reflecting long-term blood sugar control, as its elevation signifies impaired glucose metabolism over a period of 8–12 weeks [32]. The present study demonstrated that the combination therapy group achieved the most significant reduction in HbA1c, aligning with previous studies that highlighted fenugreek's role in reducing HbA1c levels by 1–2% when regularly used in diabetic patients [33]. This suggests that incorporating fenugreek as an adjuvant to metformin may lead to better long-term glycemic control, potentially reducing diabetes-related complications such as cardiovascular diseases [34].

1.3. The results presented in Table (1) show that insulin resistance in the untreated group was (8.52 ± 0.51), which is a clear indicator of metabolic dysfunction. HOMA-IR decreased to (4.27 ± 0.48) in the metformin group and to (4.83 ± 0.42) in the fenugreek group. When both treatments were combined, the index dropped to (3.01 ± 0.35), indicating a greater improvement in insulin sensitivity compared to the control group, which recorded (2.11 ± 0.33). Increased insu-lin resistance is the primary cause of the development of type 2 diabetes, leading to decreased glucose uptake by cells despite adequate insulin availability. The present study found that the combination therapy improved insulin sensitivity more significantly than each treatment alone, suggesting that fenugreek may enhance the pharmacological effect of metformin through various mechanisms, such as regulating the expression of genes related to insulin signaling pathways [35]. Other studies have confirmed that fenugreek contains soluble fibers, which reduce carbohydrate absorption in the intestine, thereby improving the body's response to insulin [36]. These findings support the idea that fenugreek extract may be an effective adjunct in the treatment of insulin resistance [37].

1.4. 3.2. Molecular Analysis – Gene Expression (RT-PCR, Fold Change ± SD)

The results shown in Table (2) indicate a significant decrease ($p<0.05$) in Phosphoinositide 3-kinase (PI3K) levels in the untreated group (1.06 ± 0.09). When metformin treatment was administered, a significant rise in PI3K gene levels was detected (1.81 ± 0.11). Similarly, PI3K levels increased in the fenugreek group (1.58 ± 0.20). When compared to the other groups under study, the greatest significant increase was seen when fenugreek and metformin were taken together (2.25 ± 0.53).

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Table (1) Results of Genetic Variables

Parametetrs / Groups	PI3K	GLUT4	IRS-1
Diabetic Untreated Group	1.06 ± 0.09 d	1.10 ± 0.04 d	1.08 ± 0.07 d
Metformin Only	1.81 ±0.11 b	1.94 ± 0.40 b	1.77 ± 0.35 b
Fenugreek Only	1.58 ± 0.20 c	1.63 ± 0.64 c	1.49 ± 0.28 c
Met. + Fenug.	2.25 ± 0.53 a	2.58 ± 0.37 a	2.01 ± 0.36 a

1.5. Since PI3K plays a major role in regulating intracellular insulin signalling, type 2 diabetes patients suffer from a reduction in PI3K [24]. It is an important part of the PI3K/AKT pathway that controls glucose uptake and energy storage. Binding of insulin to its receptors on the cell surface activates the PI3K/AKT pathway, which regulates glucose transport into the cells via the predominant glucose transporter (GLUT4) of skeletal muscle and adipose tissues. PI3K also plays an important role in regulating fat and glycogen metabolism [39], thereby maintaining energy homeostasis. [38] PI3K are increased when metformin is given since metformin activates AMP-activated protein kinase (AMPK), which is an energy regulator in cells [40]. Moreover, metformin stimulates PI3K activity to enhance its sensitivity to insulin signalling, which in turn improves glucose uptake by stimulating GLUT4 functions [41]. Like wise, fenugreek also elevates PI3K35 due to its active ingredient that includes saponins and soluble fibers. These compounds are insulin receptor agonists and act by activation of PI3K and lowering blood glucose level [42]. The study shows that combining metformin and fenugreek gives the most efficient treatment with the best potential to raise PI3K levels. This may be due to the dual effect of metformin and sorafenib, where metformin is activating the PI3K/AKT pathway but through activation of AMPK [43]. Both substances are also anti-inflammatory agents and metformin lowers levels of TNF-α and IL-6 and these changes improve insulin sensitivity [44]. Fenugreek is also a natural source of antioxidants that combat oxidative stress and what is related to pro-inflammatory cytokines. This increased inhibitory effect of inflammatory inhibitors on PI3K [45] and relays this hypothesis at the level of functional interventions.

Results found in the Table (2), show a statistically significant difference ($p<0.05$), with the lowest levels of GLUT4 (Glucose Transporter Type 4), found in the untreated group (1.10 ± 0.04). Conversely, GLUT4 was observed to significantly increase with metformin (1.94 ± 0.40) as well as fenugreek (1.63 ± 0.64). The highest of significant increase was the combination of metformin with fenugreek group compared to the other groups (2.58 ± 0.37). GLUT4 An explanation of how regulation of glucose translocation affects the development of type 2 diabetes through its role in glucose uptake from the circulation into cells for production or storage of energy.. Thus, a reduction in GLUT4 ex-

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pression or its impaired transport to the plasma membrane hinders glucose uptake in insulin-sensitive tissues [46]. Metformin does not directly increase insulin secretion but enhances the cellular response to insulin through several mechanisms. For instance, it affects the liver by inhibiting the gluconeogenesis pathway, thereby reducing glucose production and, in turn, insulin resistance [47]. Metformin also stimulates the PI3K/AKT pathway, which is responsible for transporting GLUT4 in skeletal muscles, thereby enhancing glucose uptake [36]. Fenugreek positively impacts GLUT4 levels, likely due to its soluble fiber content, such as Galactomannan. These fibers slow carbohydrate absorption in the digestive system, reducing postprandial blood glucose levels, which lowers insulin secretion and promotes more efficient GLUT4-mediated glucose transport [48]. Studies suggest that fenugreek may increase both GLUT4 mRNA and protein levels, while also reducing inflammation, which further improves glucose uptake [49]. When metformin and fenugreek were combined, GLUT4 levels were higher than when either was used alone. This could be due to the combined effects of both substances, as they activate AMPK through distinct pathways, stimulating the gene expression of SLC2A4, the gene responsible for GLUT4. This leads to greater GLUT4 availability and activity in cells, enhancing glucose uptake [50].

V. 3.3.Inflammatory Markers

In rats with type 2 diabetes, the study findings in Table (3) showed a definite synergistic impact of fenugreek extract and metformin in lowering inflammatory markers. The levels of the inflammatory proteins STAT3, IL-6, and Caspase-3 were measured in order to evaluate inflammatory indicators. The results showed that these markers decreased more in the combination treatment group (metformin + fenugreek) than in the other groups. In terms of STAT3, the untreated diabetic rat group had the greatest level (1.50 ± 0.06), whereas the metformin-only group and the fenugreek-only group had lower levels (1.1 ± 0.04) and (1.2 ± 0.05), respectively. With a statistically significant decrease ($P < 0.05$), the combined treatment group displayed the lowest level at (0.9 ± 0.04).

Table (3) Results of Inflammatory Variables

Parametetrs / Groups	STAT 3	IL-6	Caspase-3
Diabetic Untreated Group	1.50 ± 0.06 a	2.05 ± 0.09 a	1.80 ± 0.16 a
Metformin Only	1.10 ± 0.14 b	1.35 ± 0.08 b	1.20 ± 0.05 b
Fenugreek Only	1.13 ± 0.11 b	1.42 ± 0.06 b	1.38 ± 0.07 B
Met. + Fenug.	0.90 ± 0.08 c	1.06 ± 0.03 c	0.93 ± 0.04 C

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The untreated group's levels of IL-6, a crucial cytokine involved in the inflammatory response, were higher at(2.05 ± 0.09), whereas metformin and fenugreek alone reduced them to 1.35 ± 0.08 and 1.42 ± 0.06, respectively.. However, when metformin and fenugreek were combined, IL-6 levels dropped to (1.06 ± 0.03), indicating the effectiveness of the combination therapy in suppressing the inflammatory response. For Caspase-3, which is critically involved in the regulation of apoptotic pathways, the level in the untreated group was significantly elevated (1.80 ± 0.16) Metformin alone treatment decreased this level to (1.20 ± 0.05) as compared to fenugreek alone treatment (1.38 ± 0.07) and the combination therapy (0.9 ± 0.04) had a higher decrease. The role of combined therapy for protection of chronic inflammation induced cellular damage will be discussed below.

1.6. The results presented in Table (2) show a significant decrease ($p<0.05$) in Insulin Receptor Substrate (IRS-1) levels in the untreated group (1.08 ± 0.07). When metformin treatment was applied, a significant increase in IRS-1 levels was observed (1.77 ± 0.35). Similarly, IRS-1 levels increased in the fenugreek group (1.49 ± 0.28). The highest significant increase was observed when both metformin and fenugreek were used together (2.01 ± 0.36), compared to the other studied groups. IRS-1 is one of the key proteins involved in insulin signaling, as it forms part of the signaling pathway associated with the insulin receptor, which is essential for regulating glucose metabolism [51]. When metformin is used, IRS-1 levels improve and increase due to its role in stimulating and activating AMPK, which in turn reduces ATP levels in cells, thus enhancing the cells' response to insulin signaling [52]. Additionally, metformin improves the proper phosphorylation of IRS-1, a chemical process in which a phosphate group is added to the protein, thereby enhancing its response to insulin signals [44]. Fenugreek, when used as a treatment for insulin resistance, has a positive effect on increasing IRS-1 levels. Fenugreek contains antioxidants that help reduce oxidative stress and chronic inflammation, both of which are significant factors contributing to insulin resistance, thus improving IRS-1 function [53]. Highest increase in IRS-1 levels were seen when metformin and fenugreek together were used, as shown in our results. This could be due to the multiple mechanisms involved when both substances are used and potentially works synergistically. An important mechanism is increasing adiponectin levels, which can improve insulin sensitivity and at the same time is able to increase IRS-1 efficiency in transmitting the insulin signal [54]. In addition, the two compounds act synergistically on the AMPK pathway, leading to enhanced glucose control and decreased insulin resistance [55].

These results demonstrate the anti-inflammatory effect of the metformin–fenugreek extract combination, as at 0, 24, and 48 h, a significant decrease in inflammation markers was shown with the combination compared with metformin or fenugreek extract alone. This effect has been linked to a variety of biological processes (56). Metformin attenuates the inflammation primarily through the blockade of the activation of inflammatory signalling pathways such as STAT3 and reducing levels or production of inflammatory cytokines such as IL-6. The higher levels of STAT3 in the combination therapy group suggest both drugs in conjunction had a more potent inhibitory activity on these pathways [57]. IL-6 — a potent driver of chronic inflammation — is associated with increased insulin resistance. The more significant lowering of IL-6 levels observed with the combination treatment indicates a synergistic anti-inflammatory effect of fenugreek and metformin may occur [58]. In DM, the increased levels of Caspase-3 are responsible for high apoptosis and thus further contribute to cellular damage. Results of the study, where combination therapy exhibited substantially lower levels of Caspase-3 than either treatment alone [59], can be confirmed with the theory that fenugreek may potentiate metformin against cellular damage. This synergistic effect may be due to the combination of the anti-inflammatory effects of metformin with the antioxidant mechanisms of fenugreek. The bioactive ingredients present in fenugreek extract are believed to reduce oxidative stress and improve cellular insulin sensitivity, which can augment the effect of the metabolic drug metformin in reducing inflammation and improving metabolic function. [60] [61].

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VI. 4.Conclusions

The findings of the study show that the treatment achieved with the combination of fenugreek extract and metformin therapy is superior to monotherapy with either of them in the management of type 2 diabetes. It resulted in improved HbA1c, enhanced insulin sensitivity and a 97% reduction in blood glucose levels. The insulin signalling gene expression of insulin response related molecules was induced and inflammatory markers were suppressed in the presence of the combined therapy, resulting in improved insulin signalling at this level [84]. These results support the potential validity of combination therapy as one of reasonable potential type 2 diabetes management strategies..

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