Original Research Article

In vitro study of Antioxidant, Anti-diabetic and Anti- atherosclerotic activity of Cold and Hot leaf extracts from Trigonella foenum gracecum. L

ABSTRACT

Background: Diabetes mellitus, a heterogeneous metabolic disease characterized by altered carbohydrate, protein and lipid metabolism. Increased glucose level has a causal correlation with atherosclerotic changes in diabetic patients. Regulating blood glucose may prevent diabetes-related tissue damage and secondary complications associated.

Methods: In the present study, an attempt has been made to investigate the Anti-diabetic and Anti-atherosclerotic effect exhibited by Cold and Hot extracts of *Trigonella foenum* leaves samples.

Results: It was deduced that the Cold extracts of fenugreek leaves had more of Phenolic compounds, while the Hot extracts had Increased amount of Carbohydrates. In the Anti Atherosclerotic studies, it was observed that the Cold extract (34%) possessed higher Cardioprotective activity than the Hot extract (14%) of *T. foenum* leaves when compared with the Ascorbic acid (80%) as Standard. Increased glucose level has a causal correlation with atherosclerotic changes in diabetic patients. The Antidiabetic studies of both the extracts Hot and Cold of *T. foenum* leaves were studied. In the Alpha-amylase inhibition assay, it was seen that both the Cold (70.1±3.5) and Hot extract (69.1±2.5) of *T. foenum* leaves showed significant results when compared with the Standard Acorbase (72.5±2.0). Similarly, in Glucose diffusion inhibition assay, it was seen that both the Cold (48.54%) and Hot extract (47.51%) of *T. foenum* leaves, when compared with the standard Metformin (70.84%) showed potent Anti-Diabetic activity.

Conclusion: From the above results, it can be concluded that *T. foenum* leaves in both Hot and Cold treated, possesses antioxidant activity, anti-atherosclerotic, anti-diabetic activity.

Keywords : Trigonella foenum; Antioxidant activity; Anti-Atherosclerotic; Anti Diabetic activity.

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1. INTRODUCTION

Medicinal plants have a long history of usage with low side effects [1-3]. Recent studies have shown potent results for these plants in prevention treatment of a wide variety of diseases such as diabetes, hypertension, atherosclerosis, cardiovascular disease, and cancer [4-18]. One of the medicinal plants that has been used since antiquity in the traditional medicine of India and for which significant therapeutic properties have been mentioned is Fenugreek.

Trigonella foenum-graecum is an angiosperm plant commonly called as Fenugreek, belongs to *Rosaceae* order, *Legouminosae* family, subfamily of *Papilonaceae* and *Trigonela* L. genus of the Trifolia group [19]. This plant is indigenous to the eastern coasts of the Mediterranean and North Africa. Fenugreek is widely grown in India, China, Africa, Saudi Arabia, Egypt, Turkey, Ukraine, Spain, and Italy. This plant is frequently exported from India, China, Turkey, and Morocco [20].

The seed and leaves of the fenugreek have broad range of pharmacological effects, including pain relief, anti-diabetes, anti-atherosclerosis, anti-inflammation, carminative, laxative, anti- spasmodic, anti-cancer, astringent, heart tonic, laxative, hypertension decreasing, triglyceride lowering, breast milk increasing, and oxytocic properties are reported for this plant19. Using this plant in the form of powder, infusion, decoction, and pomade has been very common in traditional medicine of Iran from ancient times [21]. This plant is locally used as an emollient in treatment of pellagra, loss of appetite, gastrointestinal disorders, and it is also used as a general tonic [22].

Free radicals are involved in the development of various diseases which include cellular aging, coronary heart disease, mutagenesis, diabetes, carcinogenesis and neurodegeneration [23]. Fenugreek also contains many important antioxidants and has the added benefit of protecting other dietary and internally produced antioxidants from free-radical damage. This has important cardio protective benefits, as well as helping to fortify the body against a range of other chronic heart conditions [24].

Fenugreek seeds, leaves and extracts had been used as an anti-diabetic in many model systems [25-27]. Fenugreek has been reported to markedly suppress the clinical symptoms of diabetes such as polyuria, polydipsia, weakness and weight losses largely due to its high content of soluble fiber which acts to decrease the rate of gastric emptying, thereby delaying absorption of glucose from the small intestine [28,29]. Fenugreek proved to be effective in reducing the extent of myocardial damage and significantly counteracted the oxidative stress during isoproterenol-induced myocardial infarction in rats has strong modulating effect on blood lipid levels, ability to lower cholesterol, triglycerides and LDL levels while raising HDL levels [30,31], so can substantially reduce the risk of atherosclerosis. Another property of fenugreek is the reduction of platelet aggregation due to its coumarin and other constituents which, in turn, dramatically reduces the risk of abnormal blood clotting associated with heart attacks and strokes [32].

2. MATERIAL AND METHODS

2.1 Preparation of Plant Extract

Separate 10% cold and hot extracts of the *T. foenum* leaves were prepared and this was used to quantify the phytochemical constituents present in the leaf. Phytochemical screening was carried out using 10% cold and hot extract of leaves. For quantitative analysis of

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bioactive molecule and *in vitro* studies 1% and 5% aqueous cold and hot extracts were used. Cold extract was prepared by homogenizing the leaves using pestle and mortar followed by filtration and centrifugation at high speed. Hot extract was prepared by homogenizing the leaves using a pestle and mortar followed by boiling in water (water is taken according to extract percentage to be prepared). Boiling was carried out for 10min and was cooled. This was followed by filtration and centrifugation at high speed.

2.2 Qualitative Analysis of bioactive molecules

Chemical tests were carried out using cold and hot extracts to identify the presence of phytochemicals. Standard procedure was used to identify the constituents as described by experiment carried out previously.

2.2.1Test for Alkaloids

1ml of the sample extract was taken to which few drops of concentrated Hydrochloric acid were added stirred followed by the addition of Dragendroff's Reagent. Appearance of reddish brown colour indicates the presence of alkaloids [33].

2.2.2 Test for Coumarin Glucosides

1ml of the sample extract was made alkaline with 10% Sodium Hydroxide. Appearance of blue green fluorescence shows the presence of Coumarin Glucosides [34].

2.2.3 Test for Saponin Glycosides

1ml of the sample extract was separately boiled with 10ml distilled water in a water bath for 10 minutes. The mixture was filtered while hot and allowed to cool. Demonstration of frothing was carried out 2.5ml of the filtrate was diluted to 10ml with distilled water and shaken vigorously for 2 minutes. Frothing indicated the presence of saponins in the filtrate [35].

2.2.4 Test for Flavonoids

1 ml of the sample extract was taken and few drops of 20% Sodium Hydroxide solution was added. A change to yellow colour which on the addition of acid changed to colourless solution depicted the presence of flavonoids [35].

2.2.5 Test for Phenols

Ferric chloride test: 1ml of the sample extract was taken to which 5% Ferric Chloride was added. Appearance of violet shows the presence of phenols [36,37].

2.2.6 Test for proteins

1ml of the sample extract was taken and 5% Sodium Hydroxide and 1% Copper Sulphate was added. Appearance of purple colour shows the presence of proteins and free amino acid [36,37].

2.2.7 Test for Terpenoids

5ml of the sample extract was taken and few drops of chloroform was added and 3ml of concentrated Sulphuric Acid was added. Appearance of reddish brown colour shows the presence of terpenoids [36,37].

2.2.8 Detection of carbohydrates

The extracts were dissolved individually in 5ml distilled water and filtered. The filtrate was used to test for the presence of carbohydrates.

2.2.8.1 Molisch's test

The filtrates were treated with 2 drops of alcoholic α-napthol solution in a test tube and shaken well. Concentrated sulphuric acid was added from the sides of the test tube.

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Development of a violet ring at the junction of two liquids confirmed the presence of carbohydrate [36].

2.2.8.2 Fehling's test

The filtrate was acidified with dilute HCl, neutralized with alkali and heated with Fehling's A and B solution. Formation of red precipitate indicated the presence of reducing sugars [36].

2.3 Quantitative analysis of bioactive molecules

2.3.1 Total phenolic content

Colorimetric estimation method was employed for the determination of total phenolics present in the different samples of fenugreek leaves. The test was performed in duplicates to obtain concordant readings. The phenol content in the extract was expressed in terms of catechol equivalent (mg/ml) of extract [38].

2.4 Evaluation of Antioxidant activity by DPPH assay

Antioxidant activity of leaf extracts was assessed by DPPH free radical scavenging capacity. This assay was performed according to the methodology of Braca *et al.* 2002 [39]. A solution of Ascorbic acid (10mg in 100mL) was used as positive standard. The percentage of DPPH by the sample was calculated as follows:

Scavenging effect (%) = [(OD of control – OD of the sample)/OD of control] *100

2.5 Evaluation of anti-atherosclerotic activity using Blood serum

The anti-atherosclerotic property of the leaves of *T. foenum* was studied using 1% of both cold and boiled extracts for its anti-oxidant property. In the control group, the serum was exposed 3ppm lead acetate for the specific period of time in order to evaluate the lead induced oxidation of lipoproteins which poses the major cause for atherosclerosis. In the test group, serum was administered with the plant aqueous extract of *T. foenum* and lead acetate for specific time duration to investigate a protective effect of the antidote against lead toxicity.

2.5.1 Extraction of serum

The serum 5ml was obtained and separated from freshly drawn blood under low-speed centrifugation at 2330 rpm at 4˚ C for 10 min.

2.5.2 Inhibitory effect on lead-induced LDL oxidation:

The blood serum was extracted from freshly drawn blood by centrifugation. The serum samples were exposed to aqueous solutions of lead-acetate (3ppm) and plant extract of *T. foenum* (1% extract) for 120min time duration. The control and exposed serum were maintained at same condition in shaker incubator at 37˚ C and was subjected to LPO assay for investigating protective effect of the antidote against lead induced oxidation.

2.5.3 Biochemical analysis:

To measure the effect of the leaf extract on lead acetate induced oxidation of serum lipoproteins, 0.4ml serum was first exposed to 1% *T. foenum* cold and aqueous leaf extract separately. Ascorbic acid (25mg/ml) was used as the standard antioxidant. Oxidation reaction of lipoproteins was then initiated by adding freshly prepared 3ppm lead acetate solution and incubated for 120min time duration. The control and exposed serum were maintained at the same conditions in shaker incubator at 37˚ C. Serum along with 3ppm lead acetate was used as control.

2.6 Evaluation of anti-diabetic activity

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2.6.1 Alpha amylase inhibition assay

For this assay 200µl of the porcine pancreatic α-amylase solution (0.5mg/mL) was used. The enzyme was pre-incubated at room temperature for 15min with different concentrations (200-1000µg/ml) of the plant sample. 2ml of 1% starch solution (substrate) prepared in sodium phosphate buffer was added to the reaction mixture which was further incubated at 37˚ C for 5min. The reaction was stopped by the addition of 1000µl of 3, 5- dinitrosalicyclic acid and the reaction mixture was then incubated in a boiling water bath for 10 min. The absorbance was measured at 540nm against the blank.

Activity (%) = [(OD of control – OD of the sample)/OD of control]*100

2.6.2 Glucose Inhibition assay

The potential of fenugreek leaves extracts to inhibit glucose diffusion into external solution was investigated at set time interval. For this assay 1ml of sample was placed in a dialysis membrane. To the sample, 1ml of glucose solution (22mM in 0.15M NaCl) was added. The membrane was knotted at both ends using a thread and was dipped in a beaker containing 10mL of distilled water and 40mL of 0.15M NaCl. The control was prepared by adding 1mL of distilled water and the same volume of 0.15 NaCl containing 22mM glucose solution to the dialysis membrane. The beakers were kept on a magnetic stirrer at room temperature for 3 hours. 1ml of solution from the dialysis membrane and the beakers were for glucose estimation. The volume was made up to 2ml with distilled water. 2ml of 3, 5- dinitrosalicyclic acid was added to the test and control which was then incubated in boiling water bath for 15min. The total volume in each case was made up to 20ml with distilled water. The absorbance was measured at 540nm.

2.7 Data Analysis:

The tests were performed in duplicates for the all the in vitro assays. The statistical analysis was performed using the MS-EXCEL 2019. The data were presented as Mean ± SD for each group.

3. RESULTS AND DISCUSSION

3.1 Qualitative analysis of bioactive molecules

The qualitative analysis of leaf extracts of *T. foenum* carried out showed the following results as tabulated in ~~the~~ Table 1. Alkaloid, flavonoids and phenols were found to be present in both Cold and Hot aqueous extracts of the leaves, where terpenoids were found to be extracted only in Cold aqueous extracts.

Table 1. Qualitative Analysis of Phytochemicals

Parameters	Cold extract	Hot extract
Carbohydrates	+	+
Proteins	-	-
Coumarin Glucosides	+	+
Saponins	+	+
Flavonoids	+	+

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Phenols	+	+
Alkaloids	+	+
Terpenoids	+	-

+ indicates presence, - indicates the absence

3.2 Quantitative analysis of Bioactive molecules

3.2.1 Total phenolic content

The phytochemical phenol was quantitatively determined is present in Table 2. The total phenolic content in Cold extract was 0.212±0.040 mg/ml and in Hot extract it was found to be 0.167±0.005 mg/ml. The Cold extract contained more phenol compared to that of the Hot extract.

Table 2. Quantitative analysis of bioactive molecules

Parameters	Cold extract	Hot extract
Total phenols (mg/ml)	0.212±0.040	0.167±0.005

Experiment was performed in duplicates and the results are presented as mean±SD

3.3 Evaluation of antioxidant activity by DPPH assay

The antioxidant activity of both Cold and Hot extract was compared with Ascorbic acid where the percentage of anti-oxidant property of Ascorbic acid (20µg/ml) was found to be 90% and the percentage of Anti-oxidant property of Cold and Hot extract was found to be 80% and 52% respectively (Fig. 1.).

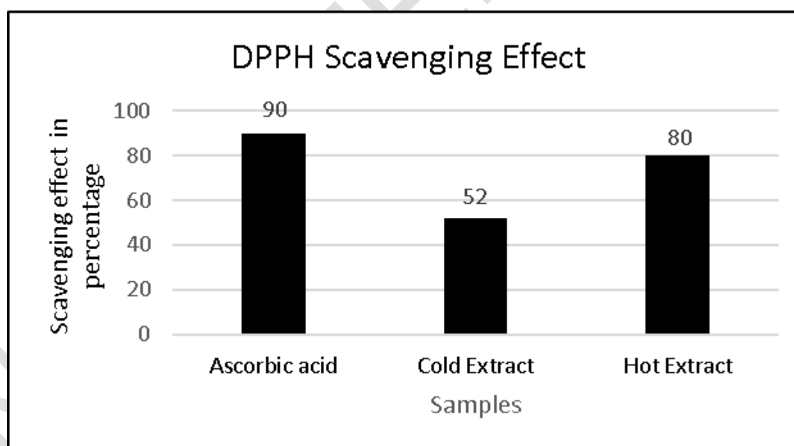

Fig. 1. Evaluation of percentage Free radical Scavenging activity of Cold and Hot extracts of plant

3.4 Evaluation of anti-atherosclerotic activity using Blood serum

3.4.1 Inhibitory effect on lead induced LDL oxidation

The percentage of inhibition of TBARS formation as a result of the LPO in the serum sample exposed to 3ppm concentration of lead acetate. The oxidation of human LDL is compared with Ascorbic acid (20µg/ml) as Standard. The percentage inhibition of Ascorbic acid was found to be 80% while the inhibitory effect of Cold extract was 34% and that of Hot extract was 14% (Fig. 2.). The result showed that Cold and Hot extract of *T. foenum L* had less inhibitory effect on TBA reacting species when compared to Ascorbic acid. It Infers to have

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mild Anti- atherosclerotic effect. Addition of Cold and Hot extract of *T. foenum L* (1% extract) to serum sample did not cause any significant effect on LPO level.

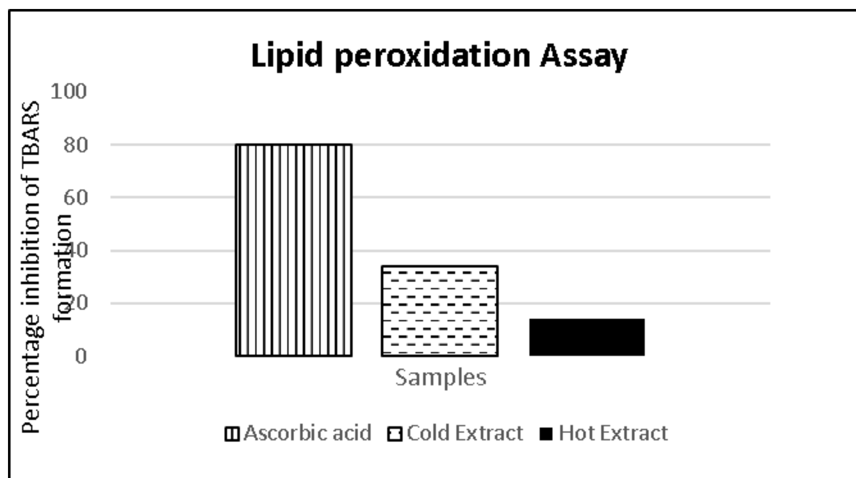

Fig. 2. Percentage Inhibition of TBARS formation of Cold and Hot extracts of *T. foenum*

3.5 Evaluation of Anti-diabetic activity

3.5.1 Alpha amylase inhibition assay

In vitro Alpha amylase inhibition activity of the individual Cold and Hot sample extracts of *T. foenum L* along with Acorbase as positive control was investigated. The plant samples were found to Inhibit the enzyme Alpha amylase. The plant extracts were compared with a positive control Acorbase (500 µg/ml) which showed an inhibition of (72.5±2.0). Of the Cold and Hot sample extracts of *T. foenum L* the Cold extract showed maximum inhibition at (70.1±3.5). Suitable inhibition was also shown by the Hot extract of *T. foenum L.* (69.1±2.5). The percentage inhibition of the plant samples is clearly represented in Fig. 3.

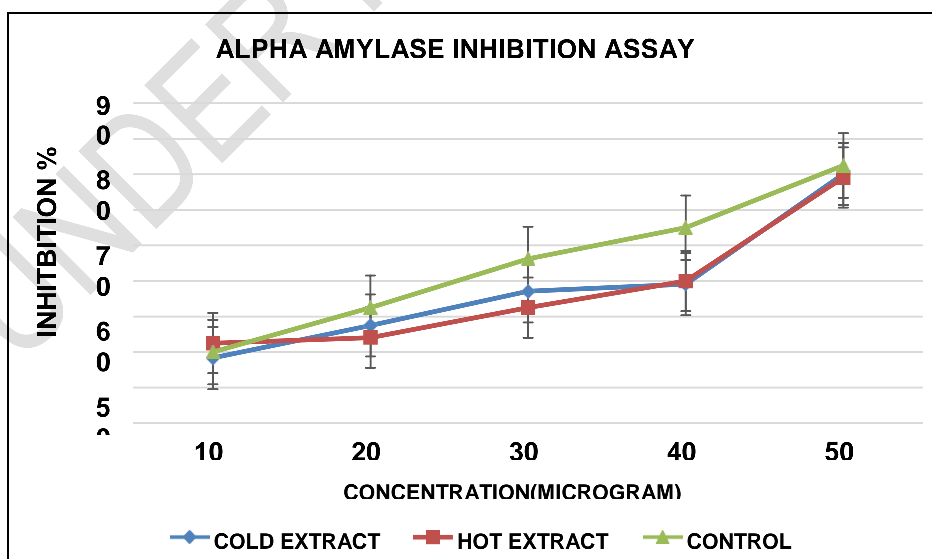

Fig. 3. Inhibition of Alpha amylase activity at different concentration

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3.5.2 Glucose diffusion inhibition assay

From results shown in Fig. 4., it is very evident that both cold and hot extracts of *T. foenum L.* helps in delaying the glucose diffusion for upto 90min thereby providing sufficient time for the system to eliminate the glucose and prevent its uptake into the cells of the body [40].

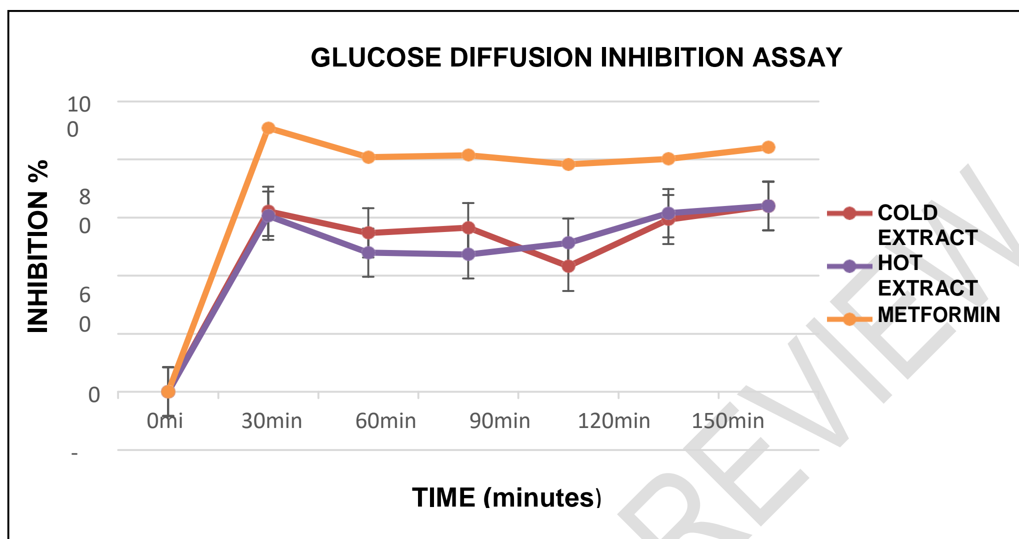

Fig. 4. Effect of Cold and Hot extracts on the movement of glucose out of dialysis tube for every 30 min of Interval Incubation period

4. CONCLUSION

It was deduced that the Cold and Hot extracts of *T. foenum* owed positive results for Antioxidant, Antidiabetic and Anti Atherosclerotic activity. The phytochemical analysis confirmed the presence of the primary metabolites such as Carbohydrates and secondary metabolites such as Phenol and Flavonoids as major constituents present in both Cold and Hot extracts in *T. foenum* leaves.

Antioxidant property of Fenugreek leaves evaluated can be correlated between the polyphenolic components present in the extract and its antioxidant activity. Free radicals have shown to Increase oxidative stress and are implicated in a variety of diseases, including diabetes, cardiovascular diseases and neurological disorders [41,42].

Fenugreek has a considerable antidiabetic effect from the studies of both the extracts Hot and Cold of *T. foenum* leaves by the Glucose diffusion inhibition assay and Alpha amylase inhibition assay when compared with the standard Metformin. It can slow down the absorption of sugar in the gastrointestinal tract and stimulate insulin release, resulting in lowering the blood sugar level in diabetic patients [43,44]. The hypoglycemic effect by enhancing the peripheral utilization of glucose, correcting the impaired hepatic glycolysis and limiting its gluconeogenic formation similar to insulin. Hence fenugreek can be used as a potent drug. *T. foenum* leaves has the ability to maintain homeostasis and cure artherosclerosis, and it might be a good lead candidate for a herbal drugs and dietary supplement preparations.

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