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A BIOLOGICAL EVALUATION OF TRIGONELLA FOENUM GRACEUM SEED EXTRACT OF ANTI DIABETIC ACTIVITY IN ALLOXAN INDUCED DIABETIC RATS, ANTI FUNGAL ACTIVITY AND ANTI BACTERIAL ACTIVITY

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INTRODUCTION

Diabetes mellitus

Diabetes mellitus (**DM**), commonly referred to as **diabetes**, is a group of metabolic diseases in which there are high blood sugar levels over a prolonged period. Symptoms of high blood sugar include frequent urination, increased thirst, and increased hunger. If left untreated, diabetes can cause many complications.

Acute complications can include diabetic keto acidosis, non ketotic hyper osmolar coma, or death. Serious long-term complications include heart disease, stroke, chronic kidney failure, foot ulcers, and damage to the eyes.

Diabetes is due to either the pancreas not producing enough insulin or the cells of the body not responding properly to the insulin produced.

There are three main types of diabetes mellitus:

- Type 1 DM results from the pancreas failure to produce enough insulin. This form was previously referred to as "insulin-dependent diabetes mellitus" (IDDM) or "juvenile diabetes". The cause is unknown.
- Type 2 DM begins with insulin resistance, a condition in which cells fail to respond to insulin properly. As the disease progresses a lack of insulin may also develop. This form was previously referred to as "non insulin-dependent diabetes mellitus" (NIDDM) or "adult-onset diabetes". The most common cause is excessive body weight and not enough exercise.
- Gestational diabetes is the third main form and occurs when pregnant women without a previous history of diabetes develop high blood sugar levels.

Prevention and treatment involve maintaining a healthy diet, regular physical exercise, a normal body weight, and avoiding use of tobacco. Control of blood pressure and maintaining proper foot care are important for people with the disease. Type 1 DM must be managed with insulin injections. Type 2 DM may be treated with medications with or without insulin. Insulin and some oral medications can cause low blood sugar. Weight loss surgery in those with obesity is sometimes an effective measure in those with type 2 DM. Gestational diabetes usually resolves after the birth of the baby.

As of 2015, an estimated 415 million people had diabetes worldwide, with type 2 DM making up about 90% of the cases. This represents 8.3% of the adult population, with equal rates in both women and men. As of 2014, trends suggested the rate would continue to rise.

BACTERIA

Bacteria constitute a large domain of prokaryotic microorganisms. Typically a few micrometres in length, bacteria have a number of shapes, ranging from spheres to rods and spirals. Bacteria were among the first life forms to appear on Earth, and are present in most of its habitats. Bacteria inhabit soil, water, acidic hot springs, radioactive waste, and the deep portions of Earth's crust. Bacteria also live in symbiotic and parasitic relationships with plants and animals. Most bacteria have not been characterised, and only about half of the bacterial phyla have species that can be grown in the laboratory. The study of bacteria is known as bacteriology, a branch of microbiology.

MATERIALS AND METHODS

Materials used

The designing of methodology involves a series of steps taken in a systematic way in order to achieve the set goal under the prescribed guidelines and recommendations. It, includes in it all the steps from field trip to the observation including selection and collection of the medicinal plant, selection of dose value, standardisation of protocol ,usage of instruments ,preparation of reagents, selection of specific solvents for extraction, formation of protocols and final execution of the standardized protocol. All this requires good build of mind and a good and soft technical hand to handle the materials and procedure in a true scientific manner.

Selection of animals

Healthy adult albino Wister rats weighing about 200-250gms of either sex were selected for the study. Animals were housed in appropriate cages in uniform hygienic conditions and fed with standard pellet diet and water.

They were fasted overnight before the day of experiment, after 72hrs of fasting from the day of alloxan induction. Animals were housed within the departmental animal house and the room temperature was maintained at 27^0 c. Animal studies was approved by IAEC.

Plant material collection

The seeds of trigonella foenum graceum were collected in any season from the market and was identified, authentificated from the authentification department.

Preparation of plant extract

The trigonella foenum graceum seeds were crushed to a powder by using a blender. This was done to increase the surface area of the simplisia so that contact point between the trigonella and solvent became larger. The method used in

this study was soxhlet extraction since it had one advantage which the solvent could be recovered after the extraction process was completed. The technique was that the solvent had to be volatile and should only be used for compounds with high boiling point. One hundred gram samples of trigonella seed powder extracted with 350ml of 98% ethanol yielded in 99.9ml of ethanol extract of trigonella seeds. This has then concentrated by rotary evaporator to produce 5.68mg of trigonella seed extract. The extract was in the form of a dark brown viscous liquid, pungent, and slightly bitter taste.

SCREENING OF ANTI DIABETIC ACTIVITY

Experimental Rats and Induction Diabetes

Albino male wistar rats 7-8 weeks old and weighing 150-200g was used for the present study. The individuals which were obtained from a private animal husbandary, Trichy, brought to the laboratory and were maintained under controlled environment. The rats were randomized into control and experimental groups and housed 4-5 cages. Standard pellets obtained from sai durga feeds and foods, Vijayawada were used as a basal diet during the experiment. The Control and experimental individuals were provided food and drinking water.

Diabetes mellitus was induced by single intra-peritoneal injection of alloxan(150mg/kg of body weight) dissolved in 0.1ml citrate buffer(pH4.5) to over night fasted albino rats.The diabetes was assessed in alloxan-induced rats sby determining the blood glucose level ,48hrs after injection of alloxan.The rats with blood glucose level above 250mg/dlwere selected for the experimental studies.

Drug administration

The ethanolic extract of fenugreek seed was suspended in phosphate buffer and administered through paediatric cathedral tube at doses of 100mg/kg body weight. The volume of administered extract was 1ml for each animal.

Experimental work

The rats were divided into five groups of six animals each

Group-1: Served as normal control rats and received distilled water.

Group-2: Served as Disease control group, received alloxan 150mg/kg body weight.

Group-3: Served as test-1(100mg/kg) treated diabetic rats received ethanolic seed extract for 2 weeks.

Group-4: Served as test-2(200mg/kg) treated diabetic rats received ethanolic seed extract for 2 weeks.

Group-5: Served as standard drug treated group received glibenclamide for 2 weeks.

The drug treatment was carried out every day morning with the help of oral cavage needle for 2 weeks.

(1) Fasting blood glucose level

The all animals in the group has undergoes fasting over night before administration of extract/glibenclamide/vehicle. Then the blood samples were collected by retro orbital puncture at 0,1,2,4 and 8 hours after the administration of the drug. Again the blood samples were collected on 0^{th}, 7th, 14th, and 21^{st} day after 1 hour administration for sub acute study. Fasting Blood glucose levels were measured by using glucometre apparatus.

(2) Oral glucose tolerance test (ogtt) in alloxan induced diabetic rats

On the 8^{th}, 15th and 22^{nd} day OGTT was carried out on the same alloxan induced diabetic animals used for assessment of anti-diabetic activity studies.

PROCEDURE

Animals were divided randomly into 5 groups of six each and were fasted to overnight. All the animals in each group were administered 2g/kg of glucose for one hour after the administration of extract/glibenclamide/vehicle. The blood samples were collected by retro-orbital puncture at 0hour, 0.5hour, 1 hour, 1.5hour and 2hours after the administration of the glucose load. The Blood samples were collected by tail vein and its blood glucose levels were measured by using a glucometre apparatus.

STATISTICAL ANALYSIS

1. The values were expressed as mean ±SEM data was analyzed using one- way ANOVA followed by T-test.
2. Diabetic control Vs All Treated groups.

RESULTS

Effect of ethanolic extract on fasting blood glucose level (fbgl) in alloxan induced diabetic rats

The animals treated with ethanolic extract shows decrease of glucose levels in 7^{th}, 14^{th}, 21^{st} day of treatment when compared to other group of animals. The ethanolic extract have reduced more (%) in FBGL. The detailed results are summarized in the below table.

Treatment	Dose Mg/Kg	Blood Glucose Level		
		7^{th} Day	14^{th} Day	21^{st} Day
DIABETIC CONTROLL	10	360.19±4.9	398.2±12.4	412.8±13.4
GLIBENCLAMIDE	10	314.15±6.04	287.32±9.02	221.30±4.69
ETFG1	100	286.66±3.64	184.52±6.67	168.49±9.25
ETFG2	200	240.25±6.02	224.60±4.62	196.31±6.64

Values are expressed as mean +/-S.E.M.n=6.Significant values were compared with $p<0.05$.Normal control Vs all groups. Paranthesis indicates %reduction in BGL(Blood Glucose Level).

ORAL GLUCOSE TOLERANCE TEST(OGTT)ON 8^{th}, 15^{th} and 22^{nd} DAY

The ethanolic extract of trigonella foenum graceum was significantly suppresses the rise in FBGL after glucose load (2g/kg) in rats, at first half-an-hour and upto 2hrs time period as compare with other groups extract with glibenclamide on 8^{th},15^{th} and 22^{nd} day. While ethanolic extract produced significant reduction in FBGL. Glibenclamide (10mg/kg) showed ($p<0.05$) significant suppression in FBGL rise at first half-an-hour, 1hr and normalized FBGL within 2hrs. The detailed results are tabulated here:

EFFECT OF ETHANOLIC EXTRACT ON 8^{th}, 15^{th} and 22^{nd} DAY IN DIABETIC RATS

Treatment	Dose Mg/Kg	Blood Glucose Level		
		8^{th} Day	15^{th} Day	22^{nd} Day
DIABETIC CONTROLL	10	356.2±16.9	374.0±14.8	382.8±6.03
GLIBENCLAMIDE	10	258.6±18.49	154.4±5.21	141.06±4.62
ETFG1	100	257.3±13.6	168.4±11.0	145.61±5.12
ETFG2	200	328.9±12.04	309.5±9.07	265.6±8.57

Values are expressed as mean +/-S.E.M. n=6. Significant values were compared with $p<0.05$. Normal control Vs all groups. Paranthesis indicates % reduction in BGL (Blood Glucose Level).

Oral administration of fenugreek seed extract by oral cathatral tube

Measuring of blood glucose level after administration of fenugreek seed extract

Decreased blood glucose level after administering the fenugreek seed extract orally by using glucometre

Measuring of blood glucose level in 21st day

SCREENING OF ANTI-FUNGAL ACTIVITY

(A) Selection of fungal strains

Depending on the availability, we have been selected two fungal strains. They are:

1. Aspergillus flavus
2. Aspergillus niger

(B) Preparation of Sub-cultures

The fungal strains in the microbiological laboratory was taken to prepare sub-cultures by using fungal medium i.e. SABOURAUD DEXTROSE MEDIUM.(LIQUID)

Sabouraud dextrose medium composition

Ingredients

1. Peptones----------10.0gms
2. Dextrose Monohydrate----------40.0gms
3. Purified Water----------1000ml

- Adjust the PH so that after sterilization it is 5.6+_0.2 sterilize immediately before use,add 0.1gm of benzyl pencillin sodium and 0.1gm of tetracycline or alternatively add 50mg of chloramphenicol per litre of medium as sterile solutions.
- 35ml of Sabouraud dextrose medium was taken in a conical flask and then incubateby using autoclave15lb pressure at 20min. After 20 min the Saboraud dextrose medium was taken into the test tubes under laminar air flow. The fungal strains were innoculated and incubated in an incubator at room temperature for 24hrs.
- Sterilize all the glass ware before starting the experiment Procedures**.**

SABOURAUD DEXTROSE AGAR MEDIUM COMPOSITION

Ingredients	**Quantity**
Peptones	10.0gms
Dextrose Monohydrate	40.0gms
Agar	15.0gms
Purified Water	1000ml

250ml of Sabouraud dextrose agar medium was prepared and incubated at 15lb pressure at 20min by using auto clave. The petri dishes are sterilized and the Sabouraud dextrose agar medium has to transfer into the petri dishes under laminar air flow and allow it to solidify.

PREPARATION OF DILUTIONS

1. ETHANOLIC EXTRACT DILUTION

0.1 gm ethanolic extract +100ml water----------STOCK SOLUTION

1mlstock+ 9ml H2O (100µg/ml)	**2ml stock+ 8ml H2O (200 µg/ml)**	**3ml stock+ 7ml H2O (300 µg/ml)**	**4ml stock+ 6ml H2O (400 µg/ml)**	**5mlstock+ 5ml H2O (500µg/ml)**

2. PETROLEUM ETHER EXTRACT DILUTION

0.1gm petroleum ether extract +1ooml water-----STOCK SOLUTION

1mlstock+ 9ml H2O (100µg/ml)	**2ml stock+ 8ml H2O (200 µg/ml)**	**3ml stock+ 7ml H2O (300 µg/ml)**	**4ml stock+ 6ml H2O (400 µg/ml)**	**5mlstock+ 5ml H2O (500µg/ml)**

3. CHLOROFORM EXTRACT DILUTION

0.1gm chloroform +100ml water--------STOCK SOLUTION

1mlstock+ 9ml H2O (100µg/ml)	**2ml stock+ 8ml H2O (200 µg/ml)**	**3ml stock+ 7ml H2O (300 µg/ml)**	**4ml stock+ 6ml H2O (400 µg/ml)**	**5mlstock+ 5ml H2O (500µg/ml)**

These dilutions are taken in a watch glass separately &paper disks are allow to soak for 2-3 minutes. These paper disks get transfer into the solidify sabouraud dextrose agar mediumby using laminar air flow to avoid outside contamination.

Now incubate them in an incubator for 36-48hrs and compare the zone of inhibition with standardized flucanazole drug.

OBSERVATION

Aspergillus Niger Petri Dishes

Ethanol extract **Petroleum ether extract** **Chloroform extract** **Standard flucanazole**

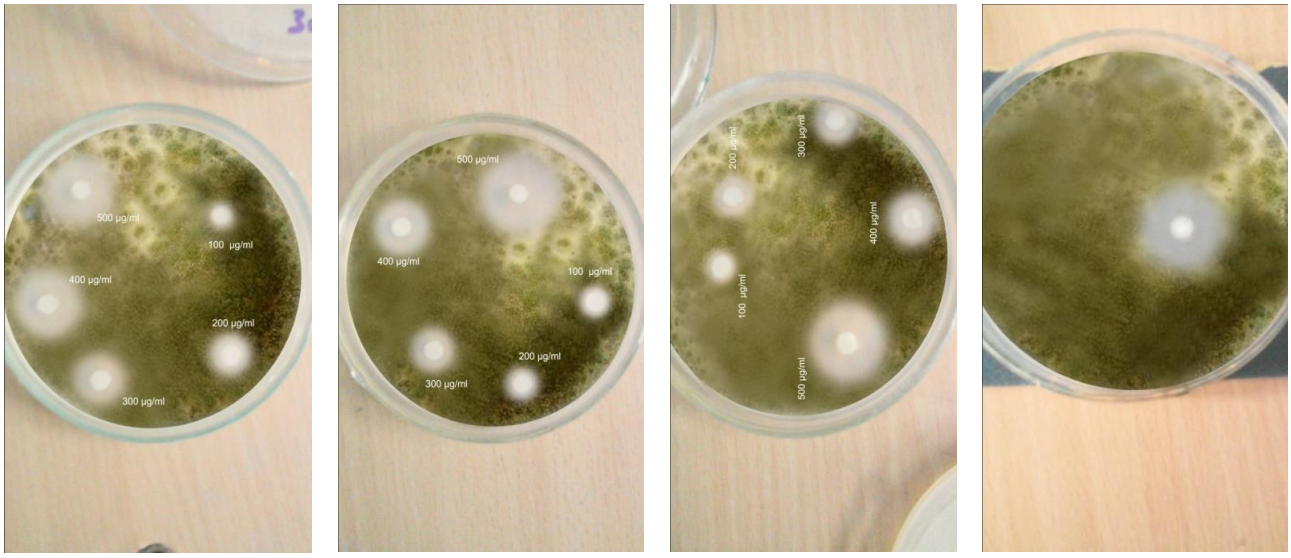
DRUG TAKEN	ZONE OF INHIBITION(cm)				
	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml	500 µg/ml
Ethanolic extract	1cm	1.7cm	2.1cm	2.8cm	3.9cm
Petroleum ether extract	1.0cm	1.4cm	2.0cm	2.2cm	3.0cm
Chloroform Extract	0.5cm	1.2cm	1.8cm	2.2cm	2.9cm

*Flucanazole standard-------2.5cm(zone of inhibition)

Depending on occurrence of zone of inhibition it tells that the extracts of trigonella foenum graceum has Anti-fungal activity.

Aspergillus flavus Petri plates

Ethanol extract **Petroleum ether** **Chloroform extract** **Standard flucanazole**



DRUG TAKEN	ZONE OF INHIBITION(cm)				
	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml	500 µg/ml
Ethanolic Extract	0.6cm	1.2cm	2.0cm	2.5cm	3.1cm
Petroleum ether Extract	0.5cm	1.0cm	1.5cm	2.0cm	2.9cm
Chloroform Extract	2.0cm	2.2cm	2.4cm	2.5cm	3.0cm

*Flucanazole standard-------2.9cm(zone of inhibition)

Depending on occurrence of zone of inhibition it tells that the extracts of trigonella foenum graceum has Anti-fungal activity.

SCREENING OF ANTI BACTERIAL ACTIVITY

Depending on availability, we have selected E.COLI bacterial strain for our work. E.Coli can be grown on nutrient agar medium also. Nutrient agar is a general purpose medium supporting growth of a wide range of non-fastidious organisms. It typically contains (mass/volume):

- 0.5% Peptone - this provides organic nitrogen
- 0.3% beef extract/yeast extract - the water-soluble content of these contribute vitamins, carbohydrates, nitrogen, and salts
- 1.5% agar - this gives the mixture solidity
- 0.5% Sodium Chloride - this gives the mixture proportions similar to those found in the cytoplasm of most organisms
- distilled water - water serves as a transport medium for the agar's various substances
- pH adjusted to neutral (6.8) at 25 °C.

These ingredients are combined and boiled for approximately one minute to ensure they are mixed and to sterilize them. Then they are cooled to around 50 °C (122 °F) and poured into Petri dishes which are covered immediately. Once the dishes hold solidified agar, they are stored upside down and are often refrigerated until used. Inoculation takes place on warm dishes rather than cool ones: if refrigerated for storage, the dishes must be rewarmed to room temperature prior to inoculation.

COMPOSITION OF NUTRIENT AGAR MEDIUM

INGREDIENTS	QUANTITY
Beef Extract	5.0gms
Peptone	10.0gms
Agar	15.0gms
Sodium Chloride	5gms
Distilled water	**1000ml**

Final PH 6.8 +/- 0.2

80ml of nutrient agar medium was taken in a conical flask and sterilize by using autoclave & boil the medium on 15 lb pressure at 20min. After, 20min cool the medium and transfer the medium into sterilized petri plates in a laminar air flow chamber. Allow them to solidify.

PREPARATION OF DILUTIONS

1. ETHANOLIC EXTRACT DILUTION

0.1 gm ethanolic extract +100ml water----------STOCK SOLUTION

↓

1mlstock+ 9ml H2O (100µg/ml)	2ml stock+ 8ml H2O (200 µg/ml)	3ml stock+ 7ml H2O (300 µg/ml)	4ml stock+ 6ml H2O (400 µg/ml)	5mlstock+ 5ml H2O (500µg/ml)

2. PETROLEUM ETHER EXTRACT DILUTION

0.1gm petroleum ether extract +1ooml water-----STOCK SOLUTION

↓

1mlstock+ 9ml H2O (100µg/ml)	2ml stock+ 8ml H2O (200 µg/ml)	3ml stock+ 7ml H2O (300 µg/ml)	4ml stock+ 6ml H2O (400 µg/ml)	5mlstock+ 5ml H2O (500µg/ml)

3. CHLOROFORM EXTRACT DILUTION

0.1gm chloroform +100ml water--------STOCK SOLUTION

↓

1mlstock+ 9ml H2O (100µg/ml)	2ml stock+ 8ml H2O (200 µg/ml)	3ml stock+ 7ml H2O (300 µg/ml)	4ml stock+ 6ml H2O (400 µg/ml)	5mlstock+ 5ml H2O (500µg/ml)

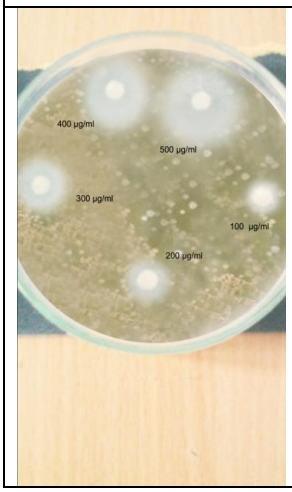
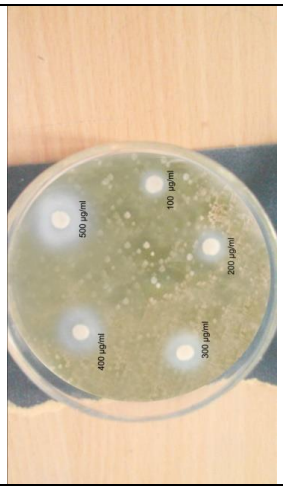
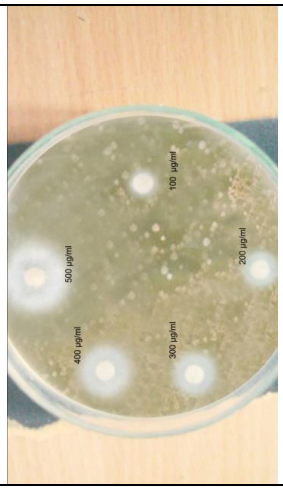
These dilutions are taken in a watch glass separately &paper disks are allow to soak for 2-3 minutes. These paper disks get transfer into the solidify nutrient agar mediumby using laminar air flow to avoid outside contamination.

Now incubate them in an incubator for 36-48hrs and compare the zone of inhibition with standardized cipro floxacins drug.

OBSERVATION

Anti Bacterial Results

E.coli petri plates

Ethanol Extract	Petroleum Ether Extract	Chloroform Extract	Standard Ciprofloxacin
			

Drug Taken	Zone of Inhibition(cm)				
	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml	500 µg/ml
Ethanolic extract	0.5cm	1.0cm	2.1cm	2.6cm	3.2cm
Petroleum ether extract	0.6cm	1.1cm	2.3cm	2.6cm	2.9cm
Chloroform Extract	0.4cm	0.8cm	1.4cm	2.1cm	3.0cm

*Ciprofloxacin standard-------2.9cm(zone of inhibition)

Depending on occurrence of zone of inhibition it tells that the extracts of trigonella foenum graceum has Anti-Bacterial activity.

DISCUSSION

Despite the fact that diabetes has high prevalence, morbidity and mortality globally, it is regarded as non curable but controllable disease. Different synthetic drugs, plant remedies and dietary modification play an effective role in the reduction of the suffering that it causes. In order to identify the plants with anti-diabetic properties various plants have been tested in-vivo using animal models, for example rats, against the complications caused by inducers of diabetes, and it has been established that many plants possesses the potential to lower the fasting blood glucose levels and besides help in improving other diabetic complications. The sustained reduction in hyper glycaemia automatically decreases the risk of other major complications of diabetes. Effective glucose control is the key for preventing or reversing the diabetic complications and improving the quality of life of the diabetes.

Many natural active compounds have been isolated from plants of different species. These active principles are complex carbohydrates, alkaloids, Flavonoids, saponins, amino acids, steroids, peptides, terpenoids and others. These compounds have been shown to produce potent hypoglycaemic, anti-hyperglycaemic and glucose suppressive activities. These effects might be achieved by facilitating insulin release from pancreatic β-cells, inhibiting glucose absorption in gut, stimulating glycogenesis in liver and/or increasing glucose utilization by the body. These compounds may also exhibit anti-oxidant, hypo-lipidemic and anti-cataract activities, and restore enzymatic functions, repair and regeneration of pancreatic islets and alleviation of liver and renal damage.

Crude ethanolic extract of trigonella foenum graceum seeds at a dose of 100 and 2000mg/kg showed significant effect on the glucose tolerance of rats and it also showed reduction in the fasting blood glucose levels of the normal glycemic rats, thus revealing the hypo glycemic nature of the extract. These findings indicate that the extract might be producing hypo glycemic effect by a mechanism independent from the insulin secretion.
Eg: By the inhibition of intestinal glucose absorption.

Alloxan mono hydrate is one of the chemical agents used to induce diabetes mellitus in animals. It induces diabetes by dose dependent destruction of β-cells of islets of langerhans. It is a generator of free radicals of oxygen which causes extensive DNA damage. It was observed that single intra venous dose of alloxan exhibited significant hyperglycaemia. Excessive hepatic glycogenolysis and gluconeogenesis associated with decreased utilization of glucose by tissues is the fundamental mechanism underlying hyperglycemia in the diabetic state. As the hyperglycemia induced by alloxan falls under category of mild diabetes and may reverse after 2 weeks, the hypoglycaemic effect of the plant in hyperglycaemic rats was studied during 22 days treatment. The difference observed between the initial and final fasting serum glucose levels of extract treated hyperglycaemic rats revealed anti-hyperglycaemic effect of seeds of trigonella foenum graceum throughout the period of study .The effect of the extract was compared to that of reference standard, glibenclamide and was found to be significant.

Phyto chemical analysis of extracts of seeds of trigonella foenum graceum revealed the presence of secondary metabolites that have been shown to possess anti diabetic effect in other plants. Saponins, alkaloids and Flavonoids which were responsible for the anti diabetic effect in other plants were also detected in the extracts of this plant. The presence of phenols in the plant could also be responsible for the anti diabetic effect have been shown to prevent the destruction of β-cells by inhibiting the peroxidation chain reaction and thus they may provide protection against the development of diabetes. Extract of seeds of trigonella foenum graceum appear to be attractive materials for further studies leading to possible drug development for diabetes. Development of Phyto medicines is relatively in expensive and less time consuming, it is more suited to our economic conditions than allopathic drug development which is more expensive and spread over several years.

Anti bacterial activity and anti fungal activity also studied in this work, it reveals that the extracts of trigonella foenum graceum inhibits the growth of bacteria and fungi.

CONCLUSION

1. The dried seeds of trigonella foenum graceum were used for this project work were procured locally.
2. The dried seeds of trigonella foenum graceum was successively extracted with ethanol.
3. Therapeutic dose of the extract was calculated after carrying acute oral toxicity studies in rats.
4. Extract was tested for their anti-diabetic activity in alloxan induced diabetic rats.
5. The, following parameters were assessed:

- Fasting blood glucose levels at 7^{th}, 14^{th} and 21^{st}day in alloxan induced rats.
- Oral glucose tolerance test at 8^{th}, 15^{th} and 22^{nd} day in alloxan induced rats.

6. Ethanolic extract of trigonella foenum graceum seeds(20 and 30 mg/kg) showed significant effect in blood glucose lowering activity and improved oral glucose tolerance test(OGTT) in short term (7^{th} day) and long term (14^{th} day and 21^{st} day) repeated administration in alloxan induced diabetic rats.
7. The above studies showed that ethanolic extract of trigonella foenum graceum seeds had potent on anti-diabetic activity on repeated administration.
8. Not only anti-diabetic activity the extracts of trigonella foenum graceum shows anti bacterial and anti fungal activities the by inhibiting the growth of E.coli & Aspergillus flavus, Niger.

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