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Evaluation of Antidiabetic activity of ficus benghalensis linn by in vitro pharmacological screening of prop root decoction with respect to a-amylase and a-glucosidase inhibitory activities

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ABSTRACT:
Diabetes mellitus 'the disease of modern civilization' is characterized by persistently elevated hyperglycemia leading to degenerative changes and Multi organ dysfunction if not intervened appropriately and Type II DM is the prevalent form and is multifactorial in etiology accounting for 80% of cases across the globe.. In this context this study was aimed to screen for potential α- amylase and α-glucosidase inhibitors from natural sources by *in–vitro* antidiabetic assays to determine their antidiabetic potential. The tender prop root decoction of *Ficus benghalnesis*,a sacred species widely known for its myriad health benefits was selected for our study. Various concentrations of FBPRD (50,100,200,400 and 800 µg/ml) were tested against fungal α- amylase and α-glucosidases as well for glucose uptake assay in yeast and glucose adsorption assay. The statistically (Graph pad prism version 8.2.3) processed results and IC 50 values indicated a prominent dose dependent inhibition of the enzymes (reference drug: acarbose)and increases in glucose uptake (sreference drug: Metronidazole) was evident. FBPRD also exhibited glucose adsorption capacity too. The results indicated that the polyphenolic compounds present in FBPRD might resulted in the utilization of glucose by yeast and servedas good inhibitors of key enzymes like α- amylase and α- glucosidase. Further rigorous pre- clinical assessment has to be carried out to establish the anti diabetic potential of FBPRD.

Keywords: Hyperglycemia, FBPRD, antidiabetic, α-amylase, α-glucosidase, glucose uptake assay, IC 50,.

INTRODUCTION:

In-vitro Pharmacological screening Preclinical safety testing of new drug candidates is a crucial step in pharmaceutical drug development and depends on a sequential series of,*in-vivo* and *in-silico* tests before administration to humans. Currently, *in-vivo* testing is a vital part of safety assessment, and is a regulatory requirement before a drug can progress into clinical trials[6]. The challenge for thepharmacologist always will be to correlate in vitro data with in vivo findings, bearing in mind the old saying:"*In- vitro simplicitas, in - vivo veritas"*[5] This chronic hyperglycemia is associated with long term damage, dysfunction and failure of various organs (Multi Organ Dysfunction Syndrome) leading to diabetic retinopathy, nephropathy, neuropathy and macro vascular complications[6]. Herbal drugs were appreciatively advantageous compared to synthetic and semisynthetics due to its less adverse effects such as gastric irritation. So the drugs which are traditionally used have to be reviewed for the activity in order to obtain a scientific confidence.

PLANT PROFILE Ficus *benghalensis Linn*:

Ficus benghalensis is a giant evergreen tree, belongs to the Family *Moraceae.* It is commonly known as "Banyan tree" and is considered as "India's National tree". It is considered to be sacred in many places like

India, Burma, South-East Asia, Pakistan, Thailand, and Malaysia and it is used in Traditional system of Medicines like Ayurveda, Siddha, Unani and Homeopathy.

Fig. 1 Banyan Tree **Fig 2. Prop roots of Banyan tree**

Table: 01 III.Taxonomical Classification:

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Super division:	Spermatophyta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Hamamelidae
Order:	Urticales
Family:	Moraceae
Genus:	Ficus
Species:	benghalensis

Habitat: The plant is an evergreen tree distributed all over India from sub-Himalayan regionto the deciduous forest of Deccan and south India. It is also grown in gardens and roadside for shade, it grows in the low altitudes up to 2000ft (610m) especially in the dry regions.

Morphology:
Botanical description: A very large, spreading tree grows up to 30 meters in height with wide spreading branches sending down many aerial roots functioning as prop roots. Leaves are glossy, leathery and glabrous when mature, ovate, and mostly obtuse, base cordate or rounded, thickly coriaceous. Flowers are tiny, separate, male and female flowers. The male flowers crowded near themouth of the receptacle, whereas female flowers with the shorter perianth. Fruits are globose, sessile in axillary pairs with fleshy pericarp, they are dark red in colour whereas in the ripe condition they are dark purple in colour with 1.5-2.0 cm in diameter.
Chemical Constituents:

Claimed Medicinal Uses: The whole plant is astringent, , vulnerary, depurative, anti-inflammatory, ophthalmic, styptic, antiarthritic, diaphoretic, antidiarrhoeal, antiemetic and tonic. Leaves are good for ulcers, leprosy, allergic conditions of skin and applied hot aspoultice for abscesses.
AIM AND OBJECTIVE OF THE STUDY

AIM: The aim of this study was to carry out *in- vitro* pharmacological screening of prop root decoction of *Ficus benghalensis Linn*. for its antidiabetic activity with respect to α-amylase and α-glucosidase inhibitory activities as well as glucose uptake assay employing yeast as model organism.

OBJECTIVE:
1. Collection of tender prop roots of *Ficus benghalensis* and preparation of prop root decoction and measurement of quantity obtained.
2. Phytochemical screening of *Ficus benghalensis* prop root decoction.
3. Estimation of *in –vitro* α- amylase inhibitory potential of FBPRD by DNSA method.
4. Estimation of *in –vitro* α- glucosidase inhibitory potential of FBPRD.
5. Evaluation of FBPRD on Glucose uptake potential in yeast (Saccharomyces cerevicae).
6. Glucose adsorption capacity of FBPRD

MATERIALS AND METHODS:

Collection and authentification of tender aerial prop roots of *Ficus benghalensis* Linn:

The tender, aerial prop roots were collected from Sri Venkateswara Ayurvedic College premises and were authenticated by Dr.K.Madhava Chetty,Asst.Professor, Department of Botany, Sri Venkateswara University, Tirupati, A.P.,The roots were removed of extraneous matter, sundried for three days, cut into small pieces of approximately 5 cm length followed by preservation in an airtight container for further processing .

Preparation of Aerial root decoction:

The sundried, brittle prop roots (100 gm) were allowed for boiling in 100 ml of water along with a pinch of table salt for half an hour.The decoction is allowed to cool , settle followed by strained through muslin cloth. The decoction was prepared fresh for each experiment carried. Volume of the obtained decoction was measured with a measuring cylinder to calculate the percentage yield

Calculation of Percentage yield of FBPRD:

The percentage yield of FBPR decoction was calculated by using the formula.

$$\%\text{Yield of decoction} = \frac{\text{Quantity of decoction obtained (ml)}}{\text{Weight of dried Carica papaya leaves taken (gm)}} \text{X100}$$

Random determination of weight/volume of FBPRD:

one ml leaf decoction was transferred into a previously weighed clean specific gravity bottle (pycnometer) and the weight was noted as W1 and placed in hot air oven at a temeprature of around 40-45^{0}C until semisolid consistency was observed. Its weight was noted as W2. The difference in weights i.e,W1-W2 gives the weight/volume of the decoction.

Preparation of 50,100, 200,400 and 800µg/ml concentrations of FBPRD for further studies.

The wt/volume(ml) value of FBPRD obtained by the above method was employed to prepare the intended dilutions of the sample by using the formula

C1V1 = C2V2 where ; C1= concentration of stock solution

C2= Desired concentration of drug/test solution to be used in the assay. V1= volume of stock solution needed for obtaining desired concentration V2= Volume of drug/test solution to be employed in the assay.

α-Amylase inhibition assay by DNSA method:

Determination of α-Amylase inhibition carried by the quantification of the reducing sugar (maltose equivalent) liberated by the hydrolysis of starch under assay condition and was expressed as decrease in units of maltose liberated. Alpha-amylase activity can be measured *in-vitro* by hydrolysis of starch in presence of α-amylase. The reduced intensity of orange yellow colour indicates the inhibition enzyme-induced hydrolysis of starch into monosaccharides. In other words, the intensity of orange-yellow colour in test sample is directly proportional to α-amylase inhibitory activity.

Enzyme: (Fungal α-amylase, 0.5128 U/ml), stored at 2-8^{0}C

3.246 mg α-Amylase dissolved in 100 ml of Sodium phosphate buffer (0.02 M), pH

6.9 with 0.006 M sodium chloride

Sodium phosphate buffer (0.02 M), pH 6.9 with 0.006 M sodium chloride:

The following three solutions were prepared separately.

Preparation of 0.002 M of Na2HPO4:1.2 ml of distilled water was added to 1.582g of Di Sodium hydrogen phosphate.

Preparation of 0.002 M NaH2PO4 :2.2 ml of distilled water was added to 1.062g of NaH2PO4

Preparation of 0.006 M NaCl.

All the three solutions were mixed well followed by the addition of 400 ml distilled H2O as to obtain the desirable pH of 6.9. If the pH deviates from 6.9, the pH was adjusted by adding either Na2HPO4 as base or NaH2PO4 as acid. Finally the solution was brought up to the final volume of 1000 ml in volumetric flask. The buffer prepared was stored at 25°C and used within 2 weeks.

Substrate:1% Starch solution: .Soluble starch (1g) was dissolved in 100 ml of sodium phosphate buffer. Constant stirring at 90°C helped the dissolution of starch in buffer. The starch solution was then cooled and stored at 4°C. The starch solution was incubated at 25°C for 5 minutes prior to assay.

Positive Control: Acarbose (Glucobay ,Bayer pharma, India)

Stock solution of Acarbose:50 mg of Acarbose in 50 ml of 0.02 M Phosphate buffer **Dinitrosalicyclic acid (DNSA) reagent**:n1 gm of Dinitro salicyclic acid is

dissolved in 50 ml of distilled water followed by the addition of 28.2 gm of Rochelle salt. Then 20 ml of 2N NaOH was added ,made upto 100 ml with distilled water and stored in light resistant container. The reagent was prepared freshly prior to assay.

α –amylase inhibition assay procedure:

500 µL of sample of FBPRD /Acarbose(positive control)

↓

200 µL of fungal α- amylase prepared in 0.02M Sodium phosphate buffer(pH 6.9 with 0.006 M NaCl)

↓

Pre incubation at 37 °C for about 10min

↓

500 µL of 1% Starch solution in in 0.02M Sodium phosphate buffer(pH 6.9 with 0.006 M NaCl

↓

Re incubation at 37 °C for about 10min

↓

Arrest of the reaction by the addition of 500 µL of DNSA reagent

↓

Incubation in boiling water bath for 5 minutes

↓

Cooled to Room Temperature , diluted with 5 ml of distilled water

↓

Measurement of OD at 540 nm.

(Suitable reagent blank and inhibitor controls were carried simultaneously and subtracted)

All the experiments were carried in triplicate. % Inhibition =(Absorbance of Control- Absorbance of Test)x100/Absorbance of Control

S3.7∝-Glucosidase inhibition assay

∝-Glucosidase activity can be measured in-vitro by the determination of the reducing sugar (glucose) arising from hydrolysis of sucrose by α-glucosidase enzyme100.

MATERIALS:

Substrate: Sucrose

Positive control: Acarbose (Glucobay, Bayer Pharma, India)

Glucose reagent: Span diagnostics, India

Lyophilized powder of ∝-Glucosidase from saccharomyces cerevisiae (Sigma- Aldrich.(10 units/mg of protein).

Preparation of 0.1M potassium phosphate buffer (pH 6.9) : 9.11 g of K2HPO4 was dissolved in 200 ml distilled H2O and 6.49 gm ofKH2PO4 was also dissolved in another 200 ml distilled H2OBoth the two solutions were then mixed well followed by the addition of 400ml distilled H2O as to obtain the desirable pH of 6.90. If the pH deviates from 6.9, the pH was adjusted by adding either K2HPO4 . Finally the solution was brought up to a final volume of 1000 ml in volumetric flask. The buffer prepared was Stored at 25°C and used within 2 weeks.

Substrate (37 mM): 316 mg of sucrose dissolved in 25 ml 0.1M phosphate buffer pH 6.9).

Positive control: Stock- 50 mg of Acarbose in 50 ml of 0.1M phosphate buffer pH 6.9).

Procedure for α-glucosidase inhibition assay:

500 µL of sample of FBPRD / Acarbose (positive control)

↓

100 µL of alpha-glucosidase prepared in 0.1M Sodium phosphate buffer (pH 7)

↓

Pre- incubation at 37°C for about 10min

↓

500 µL 37mM of Sucrose solution

↓

Re incubation at 37°C for about 10min

↓

Kept in boiling water bath for 2 mins, cooled to RT

↓

Addition of 250 µL Glucose reagent

↓

Incubate for 10 mins at RT and measure absorbance at 510 nm.

(Suitable reagent blank and inhibitor controls were carried simultaneously and subtracted) All the experiments were carried in triplicate. The ∝-glucosidase inhibitory activity was expressed as inhibition %

Inhibition percentage = (Absorbance of control – Absorbance of extract) × 100

Absorbance of control:

Glucose uptake studies by Yeast cells Materials: Substrate: Glucose 25 mM

Positive control: Metronidazole (Flagyl, Pfizer)

DNSA reagent, Yeast suspension

Yeast was washed by repeated centrifugation at 3000 rpm for 5 minutes in distilled water until the supernatant fluids were clear**.** A 10% (v/v) of yeast suspension was prepared in distilled water. Various concentrations of FBPRD (25,50,100,200,400 and 800 µg/mL)) were added to 1mL of glucose solution (25 mM) and incubated together for 10 min at 37 °C. Reaction was started by adding 100 µL of yeast suspension followed by vortexing and further incubation at 37 °C for 60 min. After 60 min, the tubes were centrifuged (2,500 × g, 5 min) and amount of glucose was measured spectrophotometrically (540 nm) by DNSA method in the supernatant (Cirillo, 1962). Metronidazole was used as standard drug.

Increase in glucose uptake (%) = absorbance (Sample) – Absorbance(Control) f X 100
Absorbance (Sample**)**

Glucose Adsorption Assay:

Glucose adsorption capacity of the extract was determined by the method of Ou *et al.* . Approximately, 10 ml of FBPRD was added to 100 mL of glucose solution of five diferent concentrations (5, 10, 15, 20, and 25 mM). Each of these mixtures was mixed well, stirred, and incubated in a shaker water bath at 37∘ C for 6 hours, respectively. Afer incubation, the mixture was centrifuged at 4800 rpm for 20 minutes and fnally the glucose content was determined in the supernatant by using glucose oxidase peroxidase diagnostic kit. The amount of bound glucose was determined by the given formula:G1-G6, where Here, G1 represents the glucose concentration of the original solution, while G6 represents the glucose concentration afer 6 hours.

Glucose adsorbed = Initial Glucose concentration- Glucose concentration after 6 Hours

Calculation of IC50 for enzyme inhibitory activity:

According to the FDA, IC50 represents the concentration of a drug that is required for 50% inhibition *in -vitro*.[102]

IC50 was calculated by linear interpolation method using the formula

A =Percentage of inhibition, that is immediately less than50%

$$IC_{50} = \frac{50 - A}{B - A} \times (D\text{-}C) + C$$

B = Percentage of inhibition, that is immediately greater than or equal to 50%C = The concentration of inhibitor that gives A% inhibition

D = The concentration of inhibitor that gives B % inhibition

Statistical analysis:

The experimental data obtained were statistically analyzed by employing the trial version of Graph Pad Prism, San Diego version (Prism graph pad version 8.2.3 (263, GraphPad Software, Inc. La Jolla, CA USA).

RESULTS:

Table : 02 The Percentage yield of *Ficus benghalensis Linn.* prop root decoction:.

S. No.	Test article	% Yield (ml/100 gm)
1.	Ficus benghalensis Linn. prop root decoction	35.75 ± 2.13

Values are expressed as Mean ± SEM of three observations.

Table No 03: Random determination of weight/ millilitre of Ficus benghalensis Linn. Prop root decoction

S.No.	Name of the leafy vegetable	Wt/ml (mg/ml)
1.	Ficus benghalensis Linn. prop root decoction	400

In- vitro assay of α-amylase inhibition by FBPRD (3,5-Di nitro salicylic acid method)

Table No 04: Weight/millilitre of *Ficus benghalensis Linn.* prop root decoction

S. No.	Concentration of FBPRD (µg/ml)	% α-amylase inhibition
1	50	23.67±0.260
2	100	31.45±0.125
3	200	39.73±0.170
4	400	49.17±0.210
5	800	56.71±0.029

The values expressed were the Mean ± SEM of 3 observations (n=3)

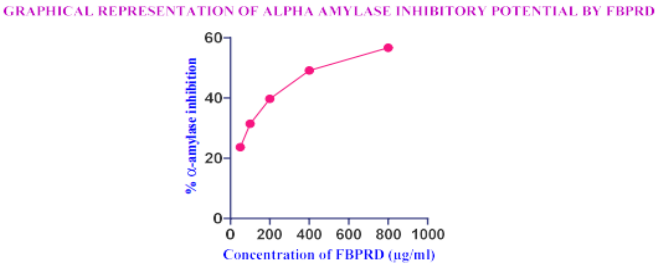

Fig:03 Graphical representation of α-amylase inhibitory activity by FBPRD

The IC_{50} i.e., half maximal inhibitory concentration value of FBPRD on α- amylase was determined by Non-linear regression analysis by plotting log (inhibitor) vs. normalized response -variable slope (GRAPH PAD PRISM VERSION 8.2.3) was found to be 553.9µg/ml.

In-vitro assay of α-amylase inhibition by Acarbose (3,5-Dinitro salicylic acid method)

Table No. 5 Percentage inhibition of α-amylase activity by Acarbose.

S. No.	Concentration of Acarbose (µg/ml)	% α-amylase inhibition
1	50	31.43±0.11
2	100	39.27±0.61
3	200	49.18±0.18
4	400	54.12±0.27
5	800	59.75±0.06

The values expressed were the Mean ± SEM of 3 observations (n=3).

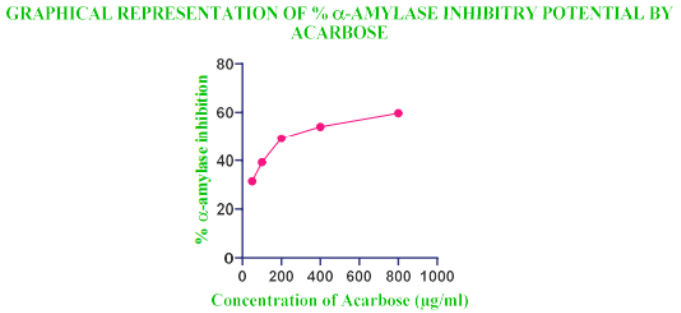

Fig: 04 Graphical representation of α-amylase inhibitory activity by Acarbose (ReferenceStandard drug)

IC_{50} value of Acarbose on α- amylase inhibition:

The IC_{50} i.e, half maximal inhibitory concentration value of Acarbose on α- amylase was determined by Non-linear regression analysis by plotting log (inhibitor) vs. normalized response -variable slope (GRAPH PAD PRISM VERSION 8.2.3) was found to be 274.4µg/ml.

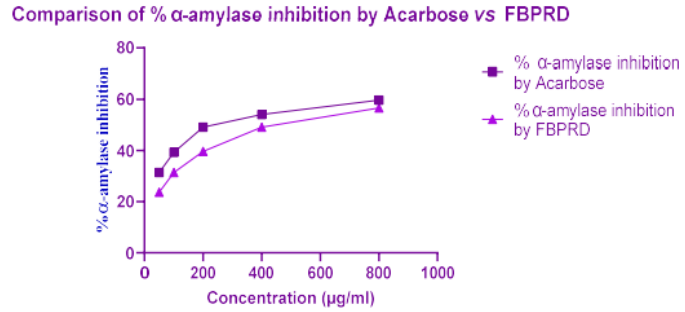

Fig:04 Graphical comparison of % α-amylase inhibition by FBPRD *vs* Acarbose

***Table 06 In- vitro* assay of α-glucosidase inhibition by FBPRD**

S. No.	Concentration of FBPRD (µg/ml)	% α-glucosidase inhibition
1	50	15.24±0.031
2	100	21.61±0.026
3	200	37.12±0.091
4	400	42.76±0.076
5	800	55.16±0.12

The values expressed were the Mean ± SEM of 3 observations (n=3)

Graphical representation of α-glucosidase inhibitory activity by FBPRD

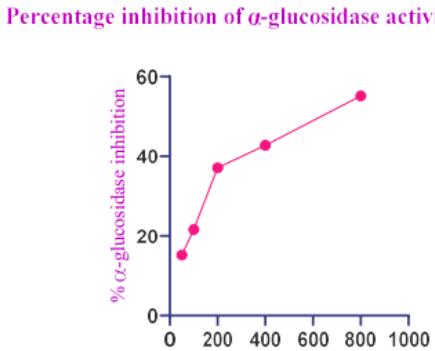

Fig 05 FBPRDIC50 value of FBPRD on α- glucosidase inhibition

The IC50 i.e., half maximal inhibitory concentration value of FBPRD on α-glucosidase was determined by Non-linear regression analysis by plotting log (inhibitor) vs. normalized response -variable slope (GRAPH PAD PRISM VERSION 8.2.3) was found to be 570.0µg/ml.

Table 07 *In- vitro* assay of α-Glucosidase inhibition by Acarbose

S. No.	Concentration of Acarbose (µg/ml)	% α-glucosidase inhibition
1	50	17.81±0.25
2	100	31.57±0.390
3	200	53.85±0.215
4	400	69.21±0.123
5	800	80.31±0.14

The values expressed were the Mean ± SEM of 3 observations (n=3)

Graphical representation of α-Glucosidase inhibitory activity by Acarbose.

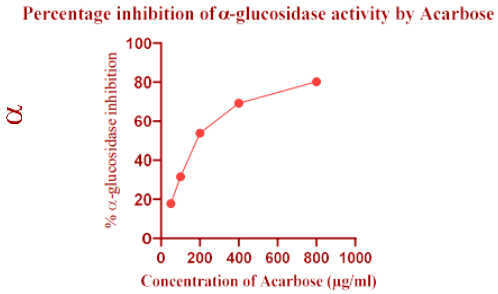

Fig no 6 Percentage inhibition of α-glucosidase activity by Acarbose IC50 value of Acarbose on α- glucosidase inhibition

The IC50 i.e., half maximal inhibitory concentration value of Acarbose on α- glucosidase was determined by Non-linear regression analysis by plotting log (inhibitor) vs. normalized response-variable slope (GRAPH PAD PRISM VERSION 8.2.3) and was found to be 192.5 µg/ ml.

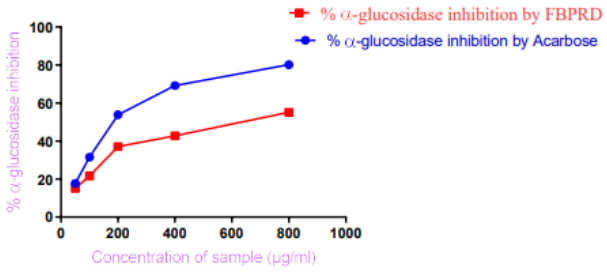

Figure No.7 Comparison of % α-glucosidase inhibition by FBPRD *vs* Acarbose

Table 09 Effect of LPJA treatment on glucose uptake in yeast

S. No.	Concentration of FBPRD(µg/ml)	% increase in glucose uptake by yeast cells
1	50	15.52±0.460
2	100	24.45±0.125
3	200	30.73±0.370
4	400	36.17±0.410
5	800	42.71±0.22

The values expressed were the Mean ± SEM of 3 observations (n=3)

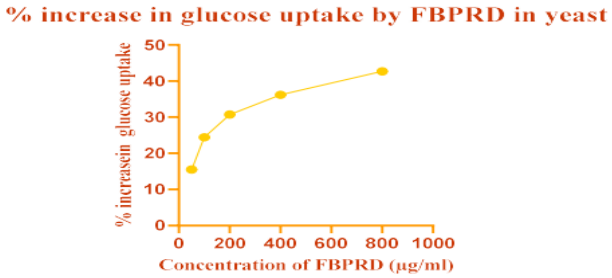

Fig No 8 Graphical representation of effect of FBPRD treatment on glucose uptake in yeast

Table 10 Effect of Metronidazole treatment on glucose uptake in yeast

S. No.	**Concentration of Metronidazole(µg/ml)**	**% increase glucose uptake by Yeast cells**
1	50	16.67±0.25
2	100	31.65±0.524
3	200	49.73±0.237
4	400	54.17±0.810
5	800	56.71±0.029

% increase in glucose uptake by yeast cells upon treatment withMetronidazole

The values expressed were the Mean ± SEM of 3 observations (n=3)

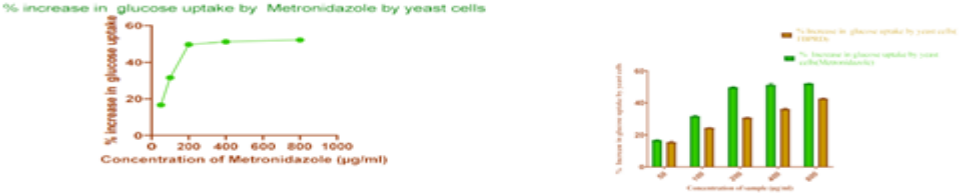

Fig No 9 Comparison of % increase in glucose uptake by Metronidazole *vs.* FBPRD

Table 11 Effect of FBPRD incubation on *in -vitro* glucose adsorption capacity

S. No.	**Concentration of Glucose(Mm/L)**	Glucose Adsorption Capacity(mg/dL)
1	5	6
2	10	8
3	15	16
4	20	19
5	25	19

Graphical representation of glucose adsorption capacity by FBPRD:

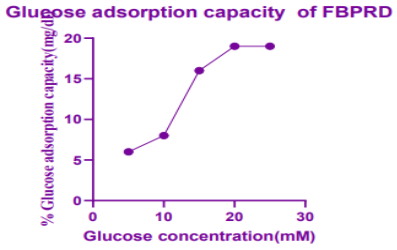

Fig No 10 Graphical representation of glucose adsorption capacity by FBPRD

Statistical Analysis:
The experimental data obtained was statistically analyzed by employing the trail version of Graph Pad Prism,(8.2.3) San Diego version .The IC 50 values aere derived by transforming the concentrations of test and standard samples to the logarithm to the base 10 followed by non linear regression analysis(variable slope) method.

DISCUSSION:
Ficus is one of the largest genera in the plant kingdom that belongs to the *Moraceae* family. Thespecies contain a range of flavonoids, phenolics, terpenes and terpenoids, fatty acids, sterols, organic acids, proteins, and some long-chained hydrocarbon compounds..
Hence the present *in -vitro* antidiabetic study employed the decoction of *Ficus benghalensis* tender prop roots so that this species if found promising, can be utilized as functional foods and pharmaceutical ingredients with respect to its pharmacological potentials and its availability in nature.

CONCLUSION:
In line with the *in-vitro* anti-diabetic assays on aerial prop roots of *Ficus benghalensis Linn.*. which revealed statistically significant results with respect to *in-vitro* anti diabetic assays.. However these studies are not sufficient to claim and hence rigorous, stringent battery of pharmacological ,photochemical and bio analytical studies followed by observational studies in humans are to be carried so as to fortify the claimed statement of Hippocrates 400 BC (TheFather of medicine) i.e, “Let food be your medicine and medicine be your food” to accord functional food status for this herb i.e,*Ficus benghalensis Linn.*.

FURTHER SCOPE OF RESEARCH:
Future studies may involve some more broad scale in vitro ,in vivo and in silico methods and to specifically identify and isolate the molecule of significance in treating Diabetes mellitus.

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