Correlation between the In-Vitro and In-Vivo Antihyperglycemic Effect of Ocimum Sanctum, Trigonella Foenum Graecum and Curcuma Longa

Inbaraj SD*, Muniappan M*

ABSTRACT

This study is carried out to investigate the correlation between the in-vitro and in-vivo studies which demonstrates the antihyperglycemic effect of Ocimum sanctum and Curcuma longa extracts. Methanolic seed extract of Trigonella foenum graecum, methanolic leaf extract of Ocimum sanctum, ethyl acetate rhizomes extract of Curcuma longa are prepared and supplied by Sami labs, Bangalore, India on request. In-vitro studies such as alpha glucosidase inhibitory and DPP-IV inhibitory activity were done for all the three extracts as per previous studies. After Institutional animal ethical committee clearance male albino rats (155–215 g) were divided into 5 groups. Each group consists of randomly assigned 6 albino rats. The placebo (Normal saline) control group, Standard (Vildagliptin) group and 3 above mentioned extract groups. Oral glucose tolerance test (OGTT) was done. Blood samples were collected for blood sugar estimation at -30 (before extract), 0, 15, 45 minutes and blood sugar levels were done by enzymatic assay. Results: The maximum alpha-glucosidase inhibitory activity at 100 μg/ml by Trigonella foenum graecum extract was 68% with IC 50 value of 57.25, Ocimum sanctum leaf extract was 65% with IC 50 value of 59.55, Curcuma longa was 72% with IC 50 value of 56.79 when compared to the Acarbose (STD) of 94% with IC 50 values of 42.78. The maximum % of DPP IV inhibition at 320 μg/ml of Trigonella foenum graecum extract was 72% with IC 50 value of 56.79 when compared to the Vildagliptin (STD) was 80.15% with IC 50 value of 22.98. The OGTT results showed significant reduction in blood glucose levels were done by enzymatic assay. Conclusion. Ocimum sanctum leaf extract, Trigonella foenum graecum seed extract shows significant alpha-glucosidase and DPP-IV inhibitory activity which correlates with the antihyperglycemic effects by in-vivo oral glucose tolerance test. Further clinical studies are necessary to establish the therapeutic potential of these extracts in the treatment of type 2 diabetes mellitus. Key words: Type 2 diabetes mellitus; Ocimum sanctum; Curcuma longa; Trigonella foenum graecum; GLP-1; Glucagon; Insulin.

INTRODUCTION

Diabetes mellitus is a chronic metabolic disease, characterized by hyperglycemia and derangement of carbohydrate, protein and fat metabolism due to relative or absolute insulin deficiency. International Diabetes federation data reveals currently 366 million people are suffering from diabetes and this rate will double by 2030. In India currently have 40 million people living with diabetes. India is predicted to have highest number of diabetic patients of 60 million by 2025. Indian type 2 diabetes commonly presents with high post prandial hyperglycemia mainly due to increased carbohydrate consumption in diet. This plays an important role in the progression of the disease resulting in microvascular and macrovascular complications. Apart from the dietary management of type 2 diabetes drugs which inhibit a-glucosidase enzyme responsible for absorption of glucose from gut plays a vital role in controlling post prandial hyperglycemia.

Indian diet constitutes more than 75% of carbohydrates, hence changing the dietary pattern exclusively is not possible. We can reduce the carbohydrate absorption from gut by using appropriate herbal products with less adverse effects and compliance of the patients. This is an interesting area of research which needs to be explored. The current study is designed to evaluate the correlation between in-vitro studies (alpha glucosidase inhibition and DPP-IV inhibition) and in-vivo study of antihyperglycemic effect of commonly used edible herbas like Curcuma longa, Ocimum sanctum and Trigonella foenum graecum in albino rat animal model. This study will pave the way for newer herbal drug development for type 2 diabetes mellitus in future.

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**Ocimum sanctum**

*Ocimum sanctum* (Family- Labiatae) known as Tulsi in Hindi and Holy basil in English is widely used in the Indian system of medicine. Every part of the plant has medicinal value. The extract of the stem and leaves has diaphoretic and expectorant effect. It relieves headache, earache and is used to treat dermatological disorders. The important effect of *Ocimum sanctum* leaves in decreasing blood glucose level and antidiabetic property has been reported in diabetic rats. *Ocimum sanctum* leaf powder supplementation at 1 to 2% dose levels showed significant hypolipidemic effect in rabbits.

**Curcuma longa**

*Curcuma longa* (Turmeric) (Family Zingeberaceae), commonly known as Haldi in Hindi has been used as spice and coloring agent. It is a well-known household herbal remedy used for healing of wounds, allergy, digestive disorders, hepatic disorders, sinusitis, rheumatism and cosmetics etc., Many experimental studies showed its anti-inflammatory, antioxidant, anticarcinogenic effect, antibacterial, anti-fungal, antiviral and anticoagulant effects. Curcumin is known for its therapeutic potential as anti-oxidant, anti-inflammatory and diabetes related liver disorders, adipocyte dysfunction, neuropathy, nephropathy, vascular diseases, pancreatic disorders. *C. longa* rhizomes have been reported to possess antidiabetic properties in alloxa-induced diabetic rats. Antidiabetic effect was established by many animal studies. Curcumin reduced advanced glycation end products and its complications.

**Trigonella foenum graecum** (Fenugreek)

*Trigonella foenum graecum* (also known as fenugreek, locally called as methi), is widely used as spice and household medicine in India. Experimental studies showed it has got hypolipidemic, anti-inflammatory, and reducing gastrointestinal diseases, anti-inflammatory activity as local application in the form of gel. Earlier animal studies suggested hypoglycemic and anti-hyperglycemic action of oral fenugreek seed powder in diabetic rat models.

Most of the studies done previously showed antidiabetic activity and prevention of complications but exact mechanism of action is yet to be explored. Hence this study is conducted to evaluate and correlate the *in-vitro* and *in-vivo* studies in establishing the antihyperglycemic effect of the above herbs. This may significantly contribute for the new drug development of type 2 diabetes mellitus.

**MATERIALS AND METHODS**

**In-vitro studies**

Determination of alpha – glucosidase inhibitory activity:

**Materials required**

- Phosphate buffer: 50 mM, pH 6.8
- Sodium carbonate: (0.1 M).
- PNPG: 1 mM
- Sample: extract with range of concentrations 0-100 μg /ml
- Alpha- glucosidase: 1 u/ml-SRL

**Procedure**

Alpha-glucosidase inhibitory activity of extracts was carried out according to method of Bachhawat *et al*. with slight modification. Reaction mixture containing 50 μl phosphate buffer, 10 μl alpha-glucosidase and 20 μl of varying concentrations of extracts was pre-incubated at 37°C for 15 min. Then 20 μl p-nitrophenyl-α-D-Glucopyranoside (PNPG) was added as a substrate and incubated further at 37°C for 30 min. The reaction was stopped by adding 50 μl sodium carbonate. The yellow color produced was read at 405 nm. Each experiment was performed along with appropriate blanks. Acarbose at various concentrations (0-100 μg/ml) was included as a standard. Negative control without extracts was set up in parallel. The result is expressed as percentage inhibition.

**Calculation**

Inhibition (%) = Abs.control – Abs.sample/Abs.control X 100,

**DPP4 inhibitory in-vitro assay**

Dipeptidyl peptidase-4 (DPP4), also known as CD26 and adenosine deaminase complexing protein 2, is a serine exopeptidase that cleaves X-Proline and X-Alanine residues from the N-termini of polypeptides. DPP4 is a transmembrane glycoprotein whose activity regulates the bioactivity of multiple peptides such as growth factors, chemokines, and neuropeptides. DPP4 plays a major role in glucose metabolism via the regulation of glucagon-like peptide-1 and inhibitors of DPP4 are commonly used for the treatment of type 2 diabetes. DPP4 also plays an important role in immune regulation and may play a role in tumor suppression. In this assay, DPP4 cleaves an non-fluorescent substrate, H-Gly-Pro-AMC, to release a fluorescent product, 7-Amino-4-Methyl Coumarin (AMC) (lex = 360/lem = 460 nm). One unit of DPP4 is the amount of enzyme that will hydrolyze the DPP4 substrate to yield 1.0 pmole of AMC per minute at 37°C.

**Methods and materials**

- DPP4 Assay Buffer 25 mL (Catalog Number MAK088A)
- DPP4 Substrate, H-Gly-Pro-AMC 0.2 mL (Catalog Number MAK088B)
- DPP4 Positive Control 20 mL (Catalog Number MAK088C)
- AMC Standard, 1 mM 0.1 mL Catalog Number MAK088D
- DPP4 Inhibitor, Vildagliptin 1 mL

**Procedure**

All samples and standards should be run in duplicate Standards for fluorometric detection. Dilute 10 mL of the 1 mM AMC Standard solution with990 mL of water to prepare a 10 mM (10 pmole/mL) standard solution. Add 0, 2, 4, 6, 8, and 10 mL of the10 mM standard solution into a 96 well plate, generating 0 (blank), 20, 40, 60, 80, and 100 pmole/well standards. Add DPP4 Assay Buffer to each well to bring the volume to 100 mL.

**Sample preparation**

Samples can be directly added to the wells. A sample blank is required for each test sample. Prepare a duplicate well for each sample to be used as the sample blank. Bring test samples and sample blanks to a final volume of 50 mL with DPP4 Assay Buffer. For the positive control, add 1–2 mL of the DPP4 positive control solution to wells and adjust to 50 mL with the DPP4 assay buffer.

**Assay reaction**

1. Add 10 mL of the DPP4 Assay Buffer to each of the sample wells. Add 10 mL of the DPP4 inhibitor to each of the sample blank wells. Mix well by pipetting, and incubate for10 minutes at 37°C.

2. Set up the Master Reaction Mix according to the scheme in Table 1. 40 mL of the Master Reaction Mix is required for each sample and sample blank well. Do not add the Master Reaction Mix to the Standard Curve wells.
Reagent volume

<table>
<thead>
<tr>
<th>DPP4 Assay Buffer 38 mL</th>
<th>DPP4 Substrate 2 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>3. Add 40 mL of the Master Reaction Mix to each of the wells. Mix well by pipetting. Cover the plate and protect from light during the incubation.</td>
<td></td>
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<tr>
<td>4. Incubate the plate at 37°C. After 5 minutes, take the initial measurement (T initial). Measure the fluorescence intensity (FLU) initial, lex = 360/lemax = 460 nm.</td>
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<tr>
<td>Note: It is essential (FLU) initial is in the linear range of the standard curve.</td>
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<tr>
<td>5. Continue to incubate the plate at 37°C taking measurements (FLU) every 5 minutes. Protect the plate from light during the incubation.</td>
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<tr>
<td>6. Continue taking measurements until the value of the most active sample is greater than the value of the highest standard (100 pmole/well). At this time the most active sample is near or exceeds the end of the linear range of the standard curve.</td>
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<tr>
<td>7. The final measurement ([FLU]final) for calculating the enzyme activity would be penultimate reading or the value before the most active sample is near or exceeds the end of the linear range of the standard curve, see step 5. The time of the penultimate reading is T final.</td>
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</table>

Calculations

Correct for the background by subtracting the final measurement ([FLU]final) obtained for the 0 (blank) AMC standard from the final measurement ([FLU]final) of the standards and samples. Background values can be significant and must be subtracted from all readings. Plot the standard curve.

Note: A new standard curve must be set up each time the assay is run.

Calculate the change in measurement from T initial to T final for the samples.

DFLU = ([FLU] final – (FLU) initial)

Also, subtract the Sample Blank D measurement value from the sample D measurement values. Compare the DFLU of each sample to the standard curve to determine the amount of AMC released by the DPP4 assay between T initial and T final (B).

The DPP4 activity of a sample may be determined by the following equation:

DPP4 Activity = B×Sample Dilution Factor/(Reaction Time)×V

B = Amount (pmole) of AMC released between T initial and T final.

Reaction Time = T final – T initial (minutes)

V = sample volume (mL) added to well

DPP4 activity is reported as pmole/min/mL = microunit/mL

One unit of DPP4 is the amount of enzyme that will hydrolyze the DPP4 substrate to yield 1.0 mmole of AMC per minute at 37°C.

Statistical analysis

The collected data from the observations were assessed for descriptive statistics such as mean, Standard deviation, standard error and % of inhibition is calculated accordingly. The statistical analysis was done using Microsoft Excel program 2013.

In-vivo study

This is a randomized controlled study using animals (albino rats) to the correlation between the in-vitro and in-vivo studies demonstrating the antihyperglycemic effect of Curcuma longa, Ocimum sanctum and Trigonella foenum graecum extracts. Institutional research committee and animal ethical committee approval (Protocol No.002/09/2015/IAEC/SBMCH) obtained through proper channel. All the three extracts were prepared and supplied by Sami Labs Limited, Bangalore on request. Product Code 2045, Batch No. C170698EM. Date of manufacture April 2017. Methanolic leaf done extract of Ocimum sanctum containing Ursolic acid by HPLC 2.53% w/w, Oleanolic acid by HPLC 1.95% w/w, Curcumin C3 Complex (Curcuma longa extract) Product Code 0330, Batch No. C161833. Date of manufacture Nov 2016. Ethyl acetate rhizomes extract consists of total curcurminoids by HPLC 95.78%. Fenugreek (Trigonella foenum graecum) seed methanolic extract (Fensteroids) Product Code 0566, Batch No. H170111. Date of manufacture January 2017. Content of steroidal saponins by gravimetry 52.04% w/w, Alkaloids 0.63% and Diosgenin by HPLC 1.51% w/w.

Physical, Chemical and Microbiological testing for all the above extracts and certificate of analysis was issued by the Sami Labs Limited (www.samilabs.com). These extracts were used for the study.

T. Vildagliptin 50 mg is procured from the local pharmacy. Manufactured by Novartis (LOT no. KA 395, MFD: 05-2017, EXD: 10 2018). Powdered and made into a suspension (10 mg/ml) and orally administered to rats by oral feeding tube. The dose of Vildagliptin 50 mg/kg was arrived based on the research article.15

Male & female albino rats (weighing 150-250 grams) total 70 numbers were purchased from King Institute, Guindy, Chennai. The rats were provided with a commercial diet and water, and they were kept under conventional conditions of controlled temperature, humidity, and lighting (22 ± 2°C, 55 ± 5%) and a 12-hr light/dark cycle with lights on at 7:00 AM. All procedures were conducted according to the CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines & Animal ethical Committee's guideline.

Preliminary acute toxicity study

Done with single oral dose of 2000 mg/kg for 3 extract groups of 6 albino rats (3 males and 3 females in each group) weighing 155-215 gm.14 Similarly, one control group of 6 rats administered orally distilled water (1 ml/100 g).

The animals were monitored for clinical symptoms and mortality intensively for first 6 hours and next 14 days. The results showed that all the three extracts at a single oral dose of 2000 mg/kg did not cause death or any abnormal clinical symptoms in male or female rats.

The effective dose is found out by referring to previous studies carried out in this direction. The following doses were used for the in-vivo study.

Ocimum sanctum extract 200 mg/kg.

Trigonella foenum graecum extract 2 gm/kg, Curcuma longa 200 mg/kg, Vildagliptin (Standard drug) (50/50). The doses were determined by previous similar research studies.17

Oral glucose tolerance test (OGTT)

Oral glucose tolerance test method is considered as the best physiological test to evaluate the effect of any substance on the gut secretary mechanism.18 It matches the correct route of (i.e., orally) of administration of carbohydrates by human beings. The ingested glucose or starch administered into the stomach is absorbed in the intestinal tract and absorbed into the splanchnic circulation and then enters the systemic circulation. The high blood glucose concentration stimulates the pancreatic beta cells to release insulin, which in turn activates the glucose uptake by peripheral tissues and organs and controls blood sugar. The passage of the carbohydrates and nutrients through the early
part of the intestine stimulates the release of the gut hormones (e.g., Glucose dependent insulin tropic polypeptide - GIP, and Glucagon like peptide-1 GLP-1), which in turn physiologically stimulate the beta cells to synthesis and release insulin and thereby control the blood sugar.

**Grouping of animals**

Male albino rats (155–215 g) were randomly divided into 5 groups each with 6 albino rats.

- **Group 1**: Placebo control group (N=6)
- **Group 2**: Standard (Vildagliptin 50 mg/kg) group (N=6)
- **Group 3**: *Ocimum sanctum* (dose 200 mg/kg) (N=6)
- **Group 4**: *Trigonella foenum graecum* (dose 2 gm/kg) (N=6)
- **Group 5**: *Curcuma longa* (dose 200 mg/kg) (N=6)

**Procedure**

The baseline fasting blood glucose (-30 minutes) was measured for all the above groups of animals.

**Oral glucose tolerance test**

After overnight fasting oral administration of the following extracts to 5 groups of rats were done separately using rat feeding tube. *Ocimum sanctum* (dose 200 mg/dl), *Trigonella foenum graecum* (dose 2 gm/kg), *Curcuma longa* (dose 200 mg/kg), Vildagliptin 50 mg/kg, and control (Normal saline). 30 minutes later oral glucose load (dose 2.2 gm/kg) is administered in few seconds by oral feeding tube. Blood samples were collected at 0, 15, 45 minutes after test drugs and standard drug and mixed with 140 µl of 0.6 M perchloric acid. After centrifugation, the supernatants were assayed for glucose using an enzymatic assay kit.

**Blood glucose determination**

Blood samples (0.2-0.25 ml) were collected from the rat tail vein at 0.15, and 45 mts after test drugs and standard drug and mixed with 140 µl of 0.6 M perchloric acid. After centrifugation, the supernatants were assayed for glucose using an enzymatic assay kit.

**Statistical analysis**

Data are expressed as the mean ± S.E.M. Differences in the values of blood glucose in the Oral glucose tolerance test between the groups treated with vehicle, standard drug, and 3 extracts were determined by one-way ANOVA, followed by Dunnett’s multiple comparison test. *p* value of < 0.05 (two-sided) was considered statistically significant. Statistical analyses were performed using Graph Pad software (Prism Windows 5).^21^

**RESULTS**

The following are the results of *in-vitro* and *in-vivo* tests

**Alpha glucosidase enzyme inhibitory action**

This *in-vitro* study showed maximum alpha glucosidase inhibition of *Trigonella foenum graecum* 68%, *Ocimum sanctum* 65% and *Curcuma longa* 72% at 100 µg/ml, where as the standard drug Acarbose at 100 µg/ml was 94% (Table 1 and Figure 1).

**Dipeptidyl peptidase -IV enzyme inhibitory action**

The DPP-IV inhibition of *Ocimum sanctum* extract is 86.97%, *Trigonella foenum graecum* extract is 77.84% and *Curcuma longa* 76.47% at 320 µg/ml of concentration whereas the standard drug Vildagliptin at same concentration shows 80.15% inhibition at 320 µg/ml of concentration (Table 2 and Figure 2).

**Results of *in-vivo* test**

**Oral glucose Tolerance test**

Graph shows effect of Vildagliptin 50 mg/kg, *Ocimum sanctum* 200 mg/kg, *Trigonella foenum graecum* 2 g/kg, *Curcuma longa* 200 mg/kg on blood glucose (Tables 3 and 4).

VG (STD 50 mg/kg) and FG (2 gm/kg) shows significant reduction in blood glucose (*p*=<0.001) at 15 minutes when compared to control. OS (200 mg/kg) shows reduction in blood glucose (*p*=<0.05) at 15 minutes when compared to control (Table 5 and Figure 3).

Vildagliptin shows significant reduction in plasma glucose at 15 minutes of OGTT

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**Figure 1:** Percentage inhibitory activity of *Trigonella foenum graecum*, *Ocimum sanctum*, *Curcuma longa* on alpha-glucosidase enzyme activity in comparison with acarbose.

TFG: *Trigonella foenum graecum*; OS: *Ocimum sanctum*; CL: *Curcuma longa*
Inbaraj, et al.: Correlation between the In-Vitro and In-Vivo Antihyperglycemic Effect of Ocimum Sanctum, Trigonella Foenum Graecum and Curcuma Longa

Figure 2: Percentage inhibitory activity of Curcuma longa, Trigonella foenum graecum and Curcuma longa on Dipeptidyl Peptidase-IV enzyme in comparison with vildagliptin.
TFG: Trigonella foenum graecum; OS: Ocimum sanctum; CL: Curcuma longa

Figure 3: Effect of Trigonella foenum graecum, Ocimum sanctum and Curcuma longa on glucose tolerance test in albino rats.
OS: Ocimum sanctum; TFG: Trigonella foenum graecum; CL: Curcuma longa

Table 1: Percentage inhibitory activity of Trigonella foenum graecum, Ocimum sanctum, Curcuma longa on alpha-glucosidase enzyme activity in comparison with Acarbose (n=6).

<table>
<thead>
<tr>
<th>Conc. Of sample (μg/ml)</th>
<th>% Inhibition of Trigonella foenum graecum</th>
<th>% Inhibition of Ocimum sanctum</th>
<th>% Inhibition of Curcuma longa</th>
<th>% Inhibition of Acarbose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.21</td>
<td>0.22</td>
<td>0.20</td>
<td>0.22</td>
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</tr>
<tr>
<td>100</td>
<td>68*</td>
<td>65*</td>
<td>72*</td>
<td>94</td>
</tr>
</tbody>
</table>

Maximum % of inhibition by Trigonella foenum graecum, Ocimum sanctum, Curcuma longa *P<0.05 when compared to Acarbose (n=6)

Table 2: Inhibitory concentration IC 50 of Trigonella foenum graecum, Ocimum sanctum, Curcuma longa on alpha-glucosidase enzyme activity in comparison with Acarbose.

<table>
<thead>
<tr>
<th>IC50 (in-vitro Alpha glucosidase inhibition)</th>
<th>Microgram/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcuma longa</td>
<td>56.79</td>
</tr>
<tr>
<td>Trigonella foenum graecum</td>
<td>57.25</td>
</tr>
<tr>
<td>Ocimum sanctum</td>
<td>59.55</td>
</tr>
<tr>
<td>Acarbose</td>
<td>42.78</td>
</tr>
</tbody>
</table>
**Ocimum sanctum** (200 mg/kg) shows reduction in blood sugar level (p=<0.01) at 15 minutes of OGTT.

**Trigonella foenum graecum** (2 gm/kg) showed significant (p=<0.001) reduction in blood glucose level at 15 minutes of OGTT.

**DISCUSSION**

This study evaluated the correlation between the *in-vitro* that is alpha glucosidase inhibition, DPP IV inhibition and the *in-vivo* Oral glucose tolerance test in albino rats. Similar studies were done to review the *in-vitro* and *in-vivo* studies. There are many separate *in-vitro* and *in-vivo* studies done to establish the different mechanisms of antidiabetic drugs and antidiabetic phytochemicals. However, most of them are not having correlation when compared to the *in-vivo* studies hence this study is done to evaluate the correlation that will help in the development of new drug for the treatment of diabetes mellitus.

**Trigonella foenum graecum** shows significant alpha glucosidase inhibition (p<0.05) when compared to Acarbose and significant DPP IV inhibition (p<0.05) when compared to Vildagliptin. This finding confirms the similar study results done with the plants in Indonesia. These findings correlate with the results of Oral glucose tolerance test at 15 minutes with significant reduction in blood glucose (p<0.001) when compared to the control. An *in-vivo* alloxan induced diabetes in rats showed hypoglycaemic effect. *Ocimum sanctum* shows significant alpha glucosidase inhibition (p<0.05) when compared to Acarbose and very significant DPP IV inhibition (p<0.001) when compared to the Vildagliptin. Though the *in-vivo* studies show significant results the OGTT study shows less significance (p<0.05) in blood sugar reduction.

**ACKNOWLEDGEMENT**

I thank Prof. M. Muniappan, Professor of Pharmacology, Sree Balaji Medical college and Hospital, BIHER for guiding me to undertake the research. I gratefully acknowledge Sami labs limited, Bangalore for supplying the extracts free of cost and encouraged me to do research.
AUTHORS CONTRIBUTIONS

Dr. S. D. Inbaraj conducted the experiment and prepared the manuscript. Prof. M. Muniappan designed and supervised the experiment.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

REFERENCES

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**GRAPHICAL ABSTRACT**

**EXTRACTS OF O. SANCTUM, T. FOENUM GRAECUM, C. LONGA**

**IN-VITRO STUDY IN-VIVO STUDY**

**ABOUT AUTHORS**

Dr. S. D. Inbaraj, MBBS, MD (Pharmacology) is a Research Scholar and Professor in the department of Pharmacology, Sree Balaji Medical college and Hospital, Bharath Institute of Higher Education and Research [BIHER]. No.7 works road, Chromepet, Chennai- 600 044. Having more than 10 years of teaching experience in Pharmacology for undergraduate and post graduate medical students. Having vast experience in treating general as well as Diabetic patients. His research interest is in the field of edible herbs and their effect on incretins and diabetes mellitus.

Dr. M. Muniappan, MSc, PhD., retired Professor of Pharmacology, Sree Balaji Medical college and Hospital, Bharath Institute of Higher Education and Research [BIHER]. No.7 works road, Chromepet, Chennai- 600 044. Having more than 25 years of teaching experience in Pharmacology for undergraduate and post graduate medical students. He guided many PhD scholars. His field of research is herbal medicines.

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