



**International Journal of Biochemistry Research
& Review**

14(3): 1-10, 2016, Article no.IJBCRR.29875
ISSN: 2231-086X, NLM ID: 101654445



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Integrating *In-silico* and *In-vitro* Approaches to Screen the Antidiabetic Drug from *Trigonella foenum graecum* Linn.

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Authors' contributions

This work was carried out in collaboration between all authors. Authors TSB and BKS designed the study, wrote the protocol and supervised the work. Author TSB carried out all laboratories work and performed the statistical analysis. Authors TSB and BKS managed the analyses of the study. Author TSB wrote the first draft of the manuscript. Authors TSB, AG and SC managed the literature searches and edited the manuscript. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/IJBCRR/2016/29875

Editor(s):

(1) Mohamed Fawzy Ramadan Hassanien, Biochemistry Department, Zagazig University, Egypt.

Reviewers:

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(2) Mohamed Ahmed Mohamed Nagy Mohamed, El Minia Psychiatric Hospital, Egypt.

Complete Peer review History: <http://www.sciencedomain.org/review-history/16787>

Original Research Article

Received 1st October 2016
Accepted 25th October 2016
Published 4th November 2016

ABSTRACT

One of cardiovascular diseases, i.e. Diabetes is reported either through absence of insulin or resistance against insulin. Treatment for diabetic disease related to Type 1 and 2 which includes lots of expenditure every year in the form of capsules or preparing different types of formulation. Recently, more than 400 medicinal plants have been screened to fulfill the requirement of the potent antidiabetic drug. Isolation and purification of effective herbal medicine from these medicinal plants to cure diabetes disease are still challenging. In the present study, our group worked on different enzymes that played a crucial role in the carbohydrate metabolism. For these studies, number of secondary metabolites (19) and enzymes (5) were screened *in silico* for antidiabetic potential. The

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result of these studies which showed that alpha amylase formed best interaction with carpaine (an alkaloid) with the lesser binding energy -7.44 kcal/mol as compared with the standard drug Acarbose. Therefore, further *in-vitro* studies were included, the screening of alpha amylase inhibitory activity using different solvent extract. At the concentration of 1 mg/ml FGCE showed highest inhibitory activity i.e. 37.7%. Thin layer chromatography (TLC) was also performed to confirm the number of phytochemicals present in different extract. Overall, these studies claimed that medicinal plant posses a potential antidiabetic activity, but still investigation is required to find out and purify the bioactive compound which can be served as a potent antidiabetic drug.

Keywords: Fenugreek; diabetes; molecular docking; TLC; alpha amylase.

ABBREVIATIONS

TLC: Thin Layer Chromatography; **FGPE:** Fenugreek petroleum ether extracts; **FGCE:** Fenugreek chloroform extracts; **FGEE:** Fenugreek ethanol extract; **FGAE:** Fenugreek aqueous extract.

1. INTRODUCTION

Bioinformatics, Biochemistry and pharmacology techniques are more common and showed its significant impact on drug designing and also showed its demand for production of drugs against intracellular and extracellular diseases. These drugs should be designed and tested within a short period of time with low risk. Recently, Kirchmair and co-authors mentioned about *in silico* methods for the estimation of chemical interactions with drug metabolizing enzymes including prediction of possible metabolites [1]. In addition, drug basically targets specific molecules that are present in the cellular pathway specific for disease condition or specific against pathogenic microorganism, but remains ineffective to any other important metabolic pathway of the human body [2]. As human being constantly suffers from various life threatening diseases like diabetes, HIV, cancer, etc., it is still challenging to fulfill the demand of an efficient method of drug development to produce an ideal drug [3].

Diabetes mellitus, a chronic metabolic disorder characterized through hyperglycemic condition resulting either through absence of insulin or resistance of available insulin in animal and human model [4]. In diabetes, hyperglycemic condition will appear only when blood glucose level is reported and enhanced in a significant amount and is associated with various abnormalities related to carbohydrate metabolism leading to various cardiovascular and neurological complications. Number of cases related to diabetes enhanced every year and roughly estimated more than 151 million people died per year and suggested that until 2030 the number of people with diabetes is likely to

exceed 553 million worldwide. Among diabetes types, Type 2 diabetes is more common and approximately more than 95% people suffering from this disease [5,6]. Type 1 diabetes is caused due to failure of insulin secretion by β -pancreatic cells while Type 2 diabetes occurs due to progressive insulin resistance [7]. As per the literature more than 400 traditional plants have been used for the treatment of diabetes disease [6]. The management of type 2 diabetes could be done by delaying the absorption of glucose through the inhibition of any one of the enzymes that are involved in the carbohydrate metabolism specially the one which hydrolyze or digesting it [8]. It could target inhibition of α -glucosidase, α -amylase [8], β -glucosidase [9], glycogen synthase kinase [10]. Activation of glucokinase is also a targeted therapy for the type 2 diabetes. *In vitro* studies play a crucial role to screen out potent inhibitors of these enzymes from various plants sources [11].

Trigonella foenum graecum Linn. or Fenugreek is most popular Indian medicinal plant because of its broad range applications. Fenugreek possesses various pharmacological properties such as, antioxidant activity, chemopreventive activity, anticancer activity, antidiabetic activity, immunomodulatory effect gastroprotective effect etc. [12]. Apart from its medicinal properties fenugreek also used as condiment, incense, as well as used to embalm mummies [13]. Major secondary metabolites present in the fenugreek are alkaloids, flavonoids, Saponins, tannins etc. as mentioned in Table 1 [14,15,16].

In the present study, *in-silico* as well as *in-vitro* attempt is made to understand the possible interaction of the target drugs that would enable to design the potent drug for the treatment of type 2 Diabetes mellitus.

2. MATERIALS AND METHODS

2.1 *In-silico* Approach (Molecular Docking)

The target proteins Human pancreatic alpha amylase (PDB ID: 1HNY), Human cytosolic beta glucosidase (PDB ID: 2JFE), Human glycogen synthase kinase-3 β (PDB ID: 4ACD), Human Glucokinase (PDB ID: 1V4T) Sugar beet alpha-glucosidase (PDB ID: 3W37), were retrieved from protein data bank (PDB) (<http://www.rcsb.org/pdb/>).

As shown in Table 1, secondary metabolites were identified from fenugreek [13,14]. Various compound structures were retrieved from PubChem database in the SDF format. Ligands were converted to pdbqt file using Open Babel software which is a freeware.

Energy minimization of these ligands and further docking analysis was performed using Autodock module available in PyRx Version 0.8 software (<http://pyrx.sourceforge.net/>). Total 10 conformers were analyzed for each compound to predict the most favorable interaction and final docking interaction between ligand and protein were analyzed using LigPlot software (<https://www.ebi.ac.uk/thornton-srv/software/LIGPLOT/>).

2.2 *In-vitro* Approach

2.2.1 Plant material and extract preparation

Fenugreek seeds were collected from a local market of India and authenticated by the Department of Botany, Padmashri Vikhe Patil College, Pravaranagar (Loni), Tal.Rahata, District Ahmednagar, (MS), India. Seeds were ground into fine powder. The powder was successively extracted by maceration in petroleum ether, chloroform, ethanol and water (increasing order of their polarity) and solvent was evaporated to obtain dry crude extracts. Percent yield of the extract in each solvent was calculated [17,18].

2.2.2 *In-vitro* Alpha amylase inhibitory activity assay

A total of 250 μ l of test samples and standard drug (100-1000 μ g/ml) were added to 250 μ l of 0.20 mM phosphate buffer (pH 6.9) containing α -amylase (0.5 mg/ml in 0.02 M phosphate

buffer, pH-6.9 with 0.006 M Sodium Chloride) solution. The reaction mixture was then pre-incubated at 25°C for 10 min. After incubation, 250 μ l of a 1% starch solution (in 0.02 M sodium phosphate buffer, pH 6.9) was added to each tube. The solution was further incubated at 25°C for 10 min. Finally, reaction was terminated with 500 μ l of DNSA (3, 5 dinitrosalicylic acid, a chromogen). The test tubes were incubated in a boiling water bath for 5 min and allowed to cool at room temperature. The solution was then diluted with 5 ml distilled water. Absorbance of the reaction mixture was measured at 540 nm. Control shows 100% enzyme activity and prepared using the same procedure replacing the extract with distilled water [19].

The α -amylase inhibitory activity was calculated by using the following formula:

$$\% \text{ Inhibition} = \frac{[(\text{Abs}_{\text{control}} - \text{Abs}_{\text{extracts}}) / \text{Abs}_{\text{control}}] \times 100}{}$$

IC₅₀ value was determined graphically.

2.2.3 Chromatographic separation

TLC was performed to confirm the presence of different bioactive compound. Chromatoplates were prepared by mixing silica gel and distilled water in 1:2 ratios and applied on a microscope slide at a uniform thickness of 0.5 mm. After drying, the plates were activated in an oven at 110°C 1 hr. 10 μ l of the extract were spotted on a chromatoplate, dried and allowed to develop a chromatogram using an appropriate solvent as shown in Table 3. Developed chromatogram was observed with exposure of the plates to iodine vapor [20,21]. Rf value was calculated using the following formula:

$$R_f = \frac{\text{Distance travelled by solute (cm)}}{\text{Distance travelled by solvent (cm)}}$$

3. RESULTS AND DISCUSSION

3.1 Molecular Docking Analysis

The molecular docking technique is used to estimate the binding affinities and energies of ligand which played a crucial role in the structure based drug design process [22,23]. In our study, total 19 secondary metabolites reported and isolated from fenugreek which was docked with key regulatory enzymes that are important in the diabetes management. The docking result of

Table 1. Binding energies (kcal/mol) obtain during docking analysis of the compounds with their target proteins / enzymes

Sr. no.	Compounds	Alpha-amylase	Beta-glucosidase	Glycogen synthase kinase-3 β	Glucokinase	Alpha – glucosidase
1	Coumarine	-4.62	-5.36	-4.29	-4.31	-4.68
2	Diosgenin	-7.12	-8.5	-7.29	-6.55	-6.45
3	Galactomannans	1.74	0.86	1.43	0.89	1.34
4	Gentianin	-4.54	-4.51	-3.79	-3.97	-4.17
5	Gitogenin	-7.08	-8.32	-7.67	-5.8	-5.91
6	Nicotinic acid	-2.94	-3.34	-4.66	-3.92	-2.84
7	Arabinoside isoorientin	-2.25	-1.23	-0.79	-1.13	-1.43
8	Greacunin	-1.86	0.67	-0.72	1.78	-0.29
9	Phytic acid	4.15	5.04	0.79	5.2	4.66
10	Quercetin	-3.6	-4.64	-4.2	-3.16	-3.41
11	Sarsapogenin	-7.06	-6.93	-6.58	-6.56	-6.1
12	Scopoletin	-3.79	-4.93	-4.87	-3.86	-3.97
13	Trigogenin	-7.28	-8.34	-7.83	-6.71	-6.59
14	Trigonelline	-3.95	-4.01	-4.41	-4.33	-3.69
15	Triterpenoids	-5.04	-4.63	-6.53	-4.64	-5.01
16	Vitexins	-2.38	-2.35	-1.99	-2.73	-2.28
17	Yamogenin	-6.86	-7.27	-6.97	-6.41	-6.32
18	Yuccagenin	-6.63	-7.42	-7.46	-6.16	-6.29
19	Carpaine	-7.44	-8.17	-5.97	-7.43	-7.33
20	Acarbose	-0.46	-2.72	-2.66	-1.87	-2.05

these studies is shown in Table 1. The best docked structure of the ligands was confirmed on the basis of the binding affinity predicted by PyRx, hydrogen bond and hydrophobic interactions [24]. The protein-ligand complex that gives a favorable interaction and was selected for further *in-vitro* analysis. Lesser binding energy indicates higher binding affinity [25]. Out of 19 secondary metabolites, it is found that carpaine shows favorable interaction with three enzymes, i.e. alpha amylase, glucokinase and alpha glucosidase showing relatively lesser binding energy i.e. -7.44, -7.43 and -7.33 kcal/mol respectively.

Best binding poses for alpha amylase, alpha glucosidase and that of glucokinase in complex with carpaine are shown in the Figs. 3A, 3B and 3C respectively. Beta glucosidase-diosgenin complex have a binding energy of -8.5 kcal / mol, while that of glycogen synthase kinase 3 β – tigogenin complex have binding energy -7.83 kcal / mol (Table 1). The best binding pose for beta glucosidase with diosgenin is shown in Fig. 1b, while best binding pose for glycogen synthase kinase is shown in Fig. 3D. In comparison with standard drug all ligands show lesser binding energy. Moving towards the hydrogen bonds it is clear from the complex that among the five complexes only two complexes form hydrogen bond with their ligands

(Figs. 2 and 1a). Carpaine forms a hydrogen bond with the Gln63 residue of the A chain of the alpha amylase and the bond distance is 3.28Å° (Fig. 2A).

Alpha glucosidase does not form any hydrogen bond, but it does interact with carpaine by hydrophobic interaction (Fig. 2B). Similarly Glycogen synthase kinase 3 β does not form any hydrogen bond with tigogenin but interact with hydrophobic non-covalent interactions (Fig. 2D). With the Glucokinase carpaine interact by forming hydrogen bond with Asn204 and Asn 231 with bond distance 3.00Å° and 2.77Å° respectively (Fig. 2C). The hydrogen bond is very much important for the directionality and specificity, which is required for the selective molecular interaction [26]. Trp58, Tr59, Tyr62, Leu162, Thr163, Asp300, His305 are the residue of the A chain of the alpha amylase those interact with carpaine by hydrophobic interactions (Fig. 2A). Hydrophobic interaction of carpaine with glucokinase clears that Asn204, Asp205, Thr206, Gly229, Cys230, Asn231, Glu256, Gln287, Glu290 amino acid residues of glucokinase are involved (Fig. 2C). As both alpha amylase and glucokinase showed best docking interaction the two enzymes were the topic of interest for further *in-vitro* analysis. In drug designing, alpha amylase inhibition while glucokinase activation is the important aspect.

3.2 In-vitro Alpha Amylase Inhibitory Activity Assay Analysis

Various carbohydrates hydrolyzing enzymes are targeted to decrease the hyperglycemia. These drugs are very much effective in the regulation of glucose metabolism without stimulating or activating the insulin / insulin secretion [27]. Acarbose and Vaglibose are the examples which are considered to be inhibited alpha amylase activity [28]. Alpha amylase inhibitors are a kind of anti-nutrients which suppress hydrolysis of starch [29]. In the present study Acarbose was taken as a control to compare our results. Acarbose showed the highest percent inhibition 65% at the concentration of 1 mg/ml (Table 2), while at the same concentration FGCE showed the highest percent inhibition value at 37.7% (Table 3). The other three plants extract i.e FGPE, FGEE and FGAE showed the percent inhibition of 26.62%, 27.72%, and 19.4% respectively at 1 mg/ml concentration (Table 3). IC_{50} of the control Acarbose was 52.21 ($\mu\text{g} / \text{ml}$) while FGCE was 1622 ($\mu\text{g} / \text{ml}$). Our values were well matched with literature [30]. It can be thought that the potent antidiabetic activity shown by the different extract may be due to the presence of various phytochemicals like phenols, flavonoids, saponins, steroids, alkaloids, terpenoids etc. as a bioactive compound [31].

To detect the bioactive compounds present in the fenugreek extracts we proceeded further for the separation of these phytochemicals by TLC method.

Table 2. Alpha amylase inhibition by standard drug Acarbose. Tests were carried out in triplicate manner and values are expressed as the mean \pm standard deviation. The IC_{50} value is nothing but the concentration of inhibitor which inhibits 50% of its activity under the assayed conditions

Standard drug	Concentration ($\mu\text{g} / \text{ml}$)	% Inhibition	IC_{50} values ($\mu\text{g} / \text{ml}$)
Acarbose	100	42.15 \pm 0.68	52.21
	200	57.88 \pm 2.42	
	400	62.24 \pm 0.53	
	600	63.76 \pm 0.96	
	800	64.66 \pm 1.26	
	1000	65.99 \pm 1.76	

3.3 TLC Fingerprinting

TLC fingerprinting of fenugreek plant extract using different solvent confirmed the presence of diverse phytochemicals while their R_f values reveal us about their polarity, high R_f value indicates the low polarity and vice versa [32,19]. Investigation of phytochemicals that are present in fenugreek, found that petroleum

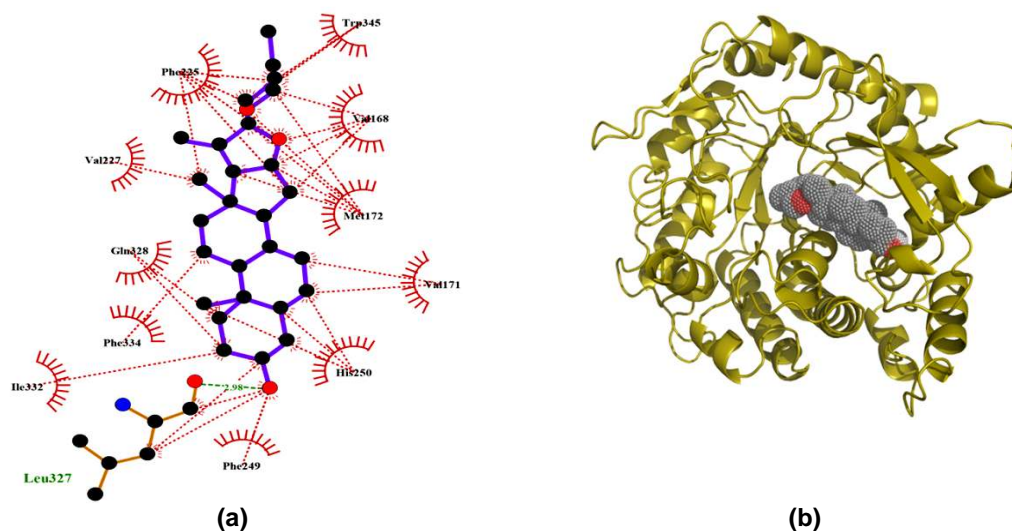


Fig. 1. (a) Hydrophobic and hydrophilic interactions of beta glucosidase with diosgenin showing the binding site pocket for diosgenin. These binding site residues of beta glucosidase – diosgenin complex is similar to that of an actual active site with substrate specificity [34] Brown colored half circle indicates the hydrophobic reactions of diosgenin with the target enzymes beta glucosidase. Green dotted lines indicate the hydrogen bond while a green colored value indicates their bond length. (b) Best binding pose of diosgenin with beta glucosidase

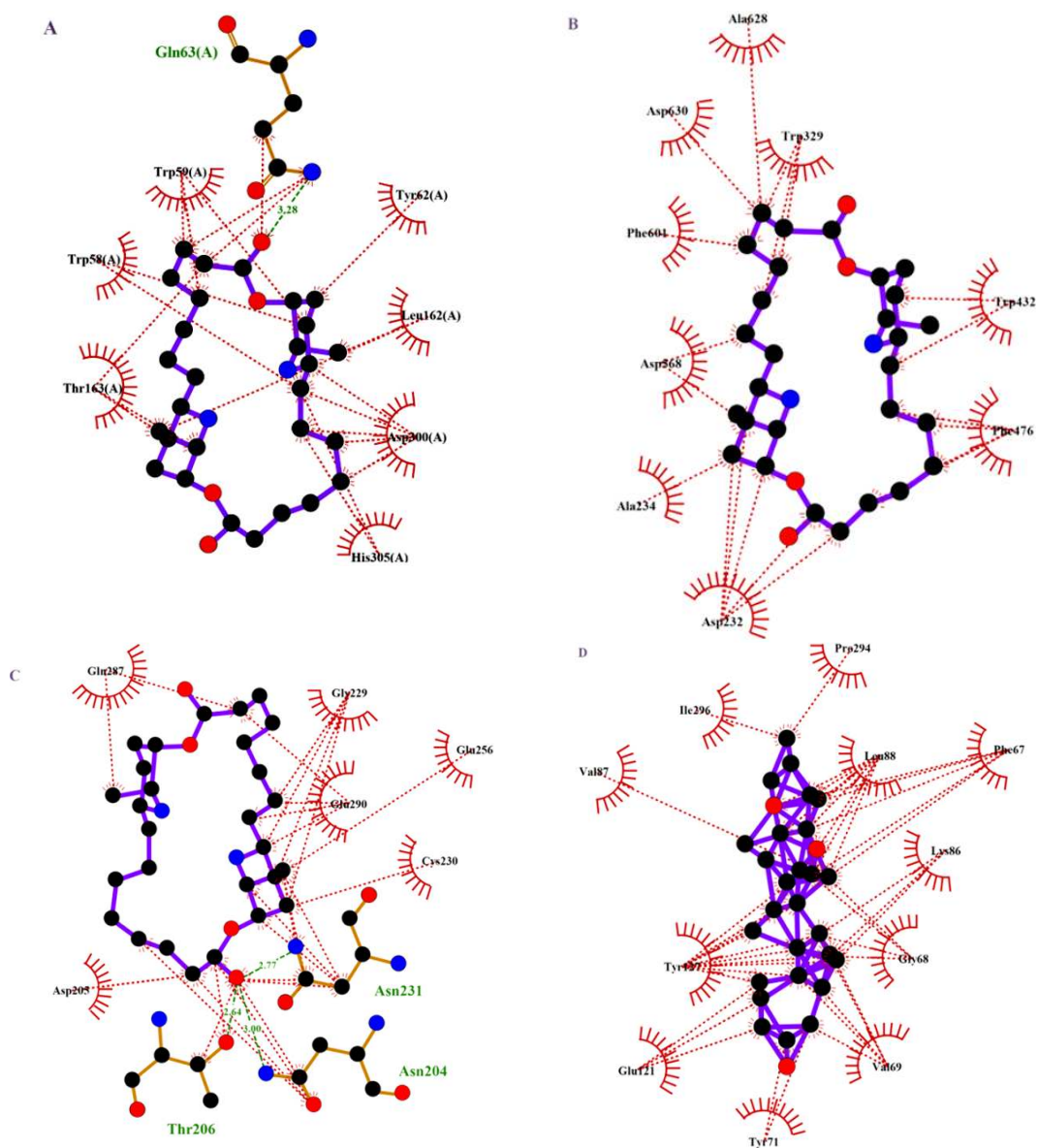


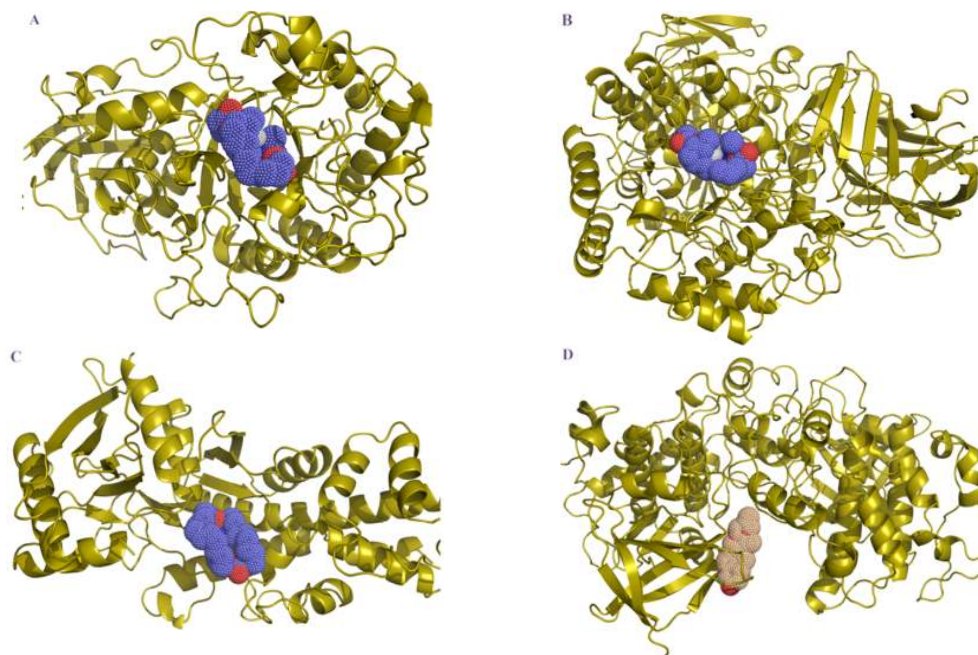
Fig. 2. Hydrophobic interactions and hydrogen bonds between the compounds and target proteins show the hydrophobic residues involved in the interactions. A. Carpaine in complex with alpha amylase B. Carpaine in complex with Alpha glucosidase C. Carpaine in complex with Glucokinase and D. Tigogenin in complex with glycogen synthase kinase 3β. Hydrogen bonds between the target proteins (cartoon) and the compounds (sticks) are shown by the green dotted lines. Figure (green) represents the hydrogen bond distance between the enzyme- ligand complexes

ether extract contains two compounds, chloroform plant extract contains one compound, ethanol plant extract contains total four compounds while aqueous extract contains one compound (Table 4). These compounds can be considered as bioactive compounds. TLC study at least gives an idea about the possibilities of the compounds those might be present in the

different extract and responsible for antidiabetic activity. Petroleum ether generally extracts the alkaloids, terpenoids, coumarins etc. whereas chloroform extracts for terpenoids and flavonoids; ethanol extracts for tannins, polyphenols, polyphenols, polyacetylenes, flavonol, terpenoids and sterols while aqueous extract for anthocyanins, saponins, etc. [33].

Table 3. Alpha amylase inhibition by plant extracts of different solvents. Tests were carried out in triplicate manner and values are expressed as the mean \pm standard deviations. The IC₅₀ value is nothing but the concentration of inhibitor which inhibits 50% of activity under the assayed conditions

Standard drug	Concentration ($\mu\text{g/ml}$)	% Inhibition	IC ₅₀ values ($\mu\text{g/ml}$)
FGPE	100	14.43 \pm 1.44	1051
	200	16.44 \pm 0.13	
	400	18.76 \pm 3.01	
	600	19.96 \pm 3.30	
	800	22.23 \pm 3.27	
	1000	26.62 \pm 2.29	
FGCE	100	13.50 \pm 1.66	1622
	200	14.47 \pm 0.75	
	400	16.48 \pm 0.18	
	600	22.01 \pm 1.17	
	800	26.77 \pm 1.11	
	1000	37.72 \pm 1.00	
FGEE	100	14.69 \pm 1.78	3063
	200	17.58 \pm 1.25	
	400	18.8 \pm 0.43	
	600	19.62 \pm 0.27	
	800	21.36 \pm 2.46	
	1000	27.72 \pm 2.35	
FGAE	100	10.93 \pm 1.52	4547
	200	12.93 \pm 0.75	
	400	16.79 \pm 0.06	
	600	16.65 \pm 0.70	
	800	17.67 \pm 0.31	
	1000	19.4 \pm 2.00	



**Fig. 3. Best binding poses for the enzyme ligand complexes. Cartoon represents the different enzymes while colored dotted surface represents the compounds showing the energetically favorable position that fit into the target protein. A. Carpaine in complex with alpha amylase
B. Carpaine in complex with Alpha glucosidase C. Carpaine in complex with Glucokinase
D. Trigogenine in complex with glycogen synthase kinase 3 β**

Table 4. TLC result of different extracts of *Trigonella foenum graecum* Linn visualized by iodine chamber

Sr no	Extract	Solvent system used	Rf value
1	Petroleum ether	Chloroform: Ethyl acetate (3:1)	0.8, 0.2
2	Chloroform	Chloroform: Ethyl acetate (4:6)	0.94
3	Ethanol	Ethyl acetate: Methanol: Water (5:1.1:1)	0.97, 0.54, 0.35, 0.10
4	Aqueous	Toluene: Ethyl acetate (4:1)	0.25

4. CONCLUSION

By analyzing the result we have concluded that secondary metabolites isolated from fenugreek possess and showed potent antidiabetic activity. *In-silico* studies hypothesized that among the four enzymes studied alpha amylase enzyme in complex with the carpaine alkaloid would prove the best for further regulation of the antidiabetic activity. *In vitro* assay confirmed that the phytochemicals present in the different fenugreek extract are responsible for antidiabetic activities. These phytochemicals extracted in different plant extracts includes alkaloids, terpenoids, flavonoids, tannins, polyphenols, polyacetylenes, sterols, saponins are responsible for this activity. Further investigation is required to find out and purify the bioactive compound which can be served as a potent antidiabetic drug.

ACKNOWLEDGEMENTS

All authors are highly grateful to Pravara Institute of Medical Sciences (Deemed University) and VSBT Baramati, for providing the support to conduct research work. Reviewers and Editor of this article are also greatly acknowledged for their tremendous effort in reviewing the manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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