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Integrating *In Silico* and *In vitro* Approaches to Screen the Antidiabetic Properties from *Trachyspermum ammi* leaves

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

AIM: The purpose of this study was to explore the possible mechanisms through which *Trachyspermum ammi* may be beneficial in managing diabetes by *in vitro* methods and to predict the potential bioactive constituent/s responsible for its anti-diabetic activity through *in silico* docking study.
Study Design: This study engaged *in vitro* and *in silico* studies to investigate the enzyme inhibition assay and molecular docking studies of phytochemical constituents when compare with standard treatment.
Place and Duration of Study: Gokaraju Rangaraju college of pharmacy, Bachupally, Hyderabad, India. December 2021-2022.

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Methodology: Methanolic leaf extract of *Trachyspermum ammi* was prepared using maceration extraction. Plant extract was evaluated for its *in vitro* anti-diabetic activity. Further, docking screening was performed using Mcule software to predict potential moiety which may be responsible for its anti-diabetic activity.
Results: Maceration extraction resulted in extractive yield of 16.15%. The presence of phenolic and flavonoid components in extract (135.59 ± 8.87 GAE/gm and 62.1 ± 4.17 quercetin/gm) dried extract respectively. *In vitro* anti-diabetic assays revealed that anti-hyperglycaemic activity of this plant can be attributed to its high efficiency to inhibit α-amylase (69.7%) and glucosidase (62.23%) enzymatic activity and protein glycation assay (95.23%), which are well established targets for the management of diabetes. Further, through docking studies we predicted that Apigenin, luteolin, Germacrene D and γ-Tocopherol present in this plant might be responsible for the anti-diabetic properties exhibited by this plant. These results provide a scientific justification for the traditional anti-diabetic use of this plant
Conclusion: It may control diabetes through lowering dietary glucose uptake. Predicted anti-diabetic molecules need to be screened further for the management of hyperglycemia. It may conclude that *Trachyspermum ammi* is an important vegetable with a potent source of natural antioxidants and antidiabetic activity justifying its traditional use in green therapeutics.

Keywords: Trachyspermum ammi; antidiabetic activity; phenolics, flavonoids; Mcule; molecular docking.

1. INTRODUCTION

Diabetes is a chronic metabolic condition. disorder typified by excess amount of blood sugar, also known as glucose, which causes catastrophic collateral harm to the kidneys, eyes, blood arteries, heart, and nerves over time. Type 2 diabetes, which frequently affects adults and develops when the body becomes insulin resistant or is unable to produce enough insulin, is the most prevalent. The rate of type 2 diabetes has increased substantially across nations of any socioeconomic status over the last thirty years [1].

In the present research, we looked in to the anti-diabetic properties of crude extracts from *Trachyspermum ammi* leaves *in vitro*. The primary goal of this experimental investigation is to determine the anti-diabetic effectiveness of a Methanolic leaf extract using *in vitro*- amylase inhibition assays, assay regarding α- glucosidase inhibition, and assay regarding protein glycation inhibition. The natural compounds work by preventing glucose absorption by blocking hydrolysing enzymes of carbs such as pancreatic amylase. Inhibiting this enzyme's activity slows carbs digestion and lengthens overall digesting time for carbohydrate, resulting in a decrease in glucose absorption rate and, as a result, a dulling of the postprandial plasma glucose spike. Several indigenous medicinal plants have a strong potential for suppressing the activity of the α-amylase enzyme [2]. Alpha-glucosidases are digestive enzymes that metabolise carbs into glucose. Inhibiting the action of alpha-glucosidases with synthetic medicines is one technique for treating type 2 diabetes [3]. Chronically elevated glucose levels causes protein glycation and the slow generation of advanced glycation end products (AGEs) in numerous human tissues, which contributes to the onset of long-term diabetes problems. The production of AGEs occurs under normal biological settings, but it grows more rapid when blood sugar levels are raised. Depositions of AGEs are associated with a variety of illnesses. Diabetes-related problems such as diabetic retinopathy, nephropathy, cataract, and atherosclerosis are facilitated by chronic hyperglycemia resulting in AGEs [4].

Acarbose is a complicated oligosaccharide that inhibits alpha-amylase present in pancreas and membrane-bound intestinal alpha-glucosidase hydrolase in an effective, reversible manner. Acarbose reduces glucose absorption by delaying carbohydrate digestion, resulting in lower postprandial blood glucose concentrations [5]. In the Maillard's process, aminoguanidine acts as a scavenger of reactive carbonyl intermediates. Production of AGES is suppressed by Aminoguanidine which additionally decreases retinal, diabetic renal, vascular and neurological problems [6].

Trachyspermum ammi L., a member of the *Apiaceae* family, is a highly prized seed spice with significant therapeutic significance. Thymol,

the primary ingredient in this oil, has been utilised to treat bronchial issues, loss of appetite and gastrointestinal conditions [7]. The primary goal of the current investigation is to introduce different *in vitro* methodologies for doing diabetes research.

2. METHODOLOGY

2.1 Collection and Drying of Plants

In January, a collection of *Trachyspermum ammi* Linn leaves was made in Hyderabad, Telangana, and they were determined to be, verified and confirmed by a qualified botanist. The leaves are roughly blended in a mixer grinder after being shade-dried for about 1 week. The blended material was either saved or gathered for extraction.

2.2 Plant Extract Preparation

Maceration extraction principle This is an extraction technique where solvent for extraction is poured on top of coarsely powdered plant material, such as leaves, stem bark, or root bark, until the drug material is thoroughly covered. The solvent is then allowed to evaporate for at least 3 to 7 days after which the container is tightly closed [8]. The leaves of *Trachyspermum ammi* are extracted using methanol as a solvent.

2.3 Phytochemical Analysis

To determine the secondary metabolites present in *Trachyspermum ammi* leaves methanolic extract of the leaves were subject to preliminary phytochemical screening.

2.4 Quantitative Analysis

2.4.1 Total phenolic contents (TPC)

The Folin Ciocalteu (FC) reagent is used to calculate TPC. Add 5 µl of FC reagent to 5 µl of plant extract, and incubated at 22^{o}C. 50 µl of 20% sodium carbonate should be added. After 1hr 30 min 22°C, the absorbance at 765 nm should be adjusted. The dry weight of gallic acid is used as a reference to calculate the total phenolic content (mg/g). TPC is determined using a regression equation in Excel with the formula y=mx+c and various META concentrations.

2.4.2 Total flavonoid contents (TFC)

Aluminium chloride ($AlCl_3$) method is used for determining TFC. Mix two hundred microliters of Methanolic Extract *Trachyspermum ammi* with 5 µl of distilled water, seventy five microliters of 5% sodium nitrite, and 150 µL of 10% $AlCl_3$ after incubating at 25^{o}C for 5 minutes. The remaining 500 µl of the 1 mol/L NaOH solution were added to the reaction mixture. The absorbance at 510 nm was measured after 15 minutes of standing. Quercetin is used as a benchmark to assess the total flavonoid content (mg/g) of dry weight. TPC is determined using an Excel regression equation with the formula y=mx+c with varying META concentrations [9].

2.5 Acute Toxicity Studies

The OECD 425 recommendations were followed for conducting the acute toxicity studies. The current study was completed in the animal facility approved by CPCSEA Gokaraju Rangaraju College of Pharmacy's in Bachupally, Hyderabad, India (Registration Number 1175/PO/ERe/S/08/CPCSEA).

2.5.1 Experimental animals

We bought 200–250 g Wistar albino rats from Jeeva Life Sciences in Hyderabad. care and maintenance for animals were taken according to the guidelines approved by CPCSEA.

2.6 *In-vitro* Methods for Antidiabetic Activity

2.6.1 α–Amylase inhibition assay

The colon's α-Amylase enzyme is inhibited, delaying the transformation of starch and sugars into monosaccharides prior to ingestion. As a result, it lowers blood sugar levels after a meal and glucose absorption. The sample concentrations (10–50 µg/mL), 10 mL of α-amylase which is (0.5 µg/mL) concentration, twenty mL of starch solution, are combined with hundred mL of 0.2 M phosphate buffer (pH – 6.9). After standing at 37°C for 5 minutes, the reaction was stopped by adding 2 ml of the 3, 5-dinitrosalicylic acid reagent. Using 10 mL of distilled water, a tray of ice is heated for fifteen minutes at 100 °C before the volume is changed. The activity of α-amylase, colour intensity at 540 nm is estimated using a spectrophotometer. The equation was used to calculate the % inhibition of

enzyme activity. Three separate runs of each test were completed. The standard used was acarbose as reference [10].

2.6.2 α-Glucosidase inhibition assay

Enzymes glucosidases are that increase conversion into simple sugars from starch. This facilitates digestion of carbohydrates from food, starch as well which increases blood glucose levels in people and causes sugar to be produced for intestinal absorption. Each sample concentration (10–50 mg/ml) receives a 10-min fifty microliters of α-glucosidase (Two U/ml) pre-incubated, and then fifty microliters of PB (pH 7.4) were incubated at thirty seven °C. The reaction was then provided with 50 µL of p NPG (20 mM) and at 37 degrees Celsius it was incubated for thirty min. Acarbose was used as a reference and the procedure was repeated three times. Utilising a spectrophotometer, the absorbance is constant at 540 nm [10].

2.6.3 Protein glycation inhibition assay

Reducing sugars and free amino groups are used in the non-enzymatic process of glycation, which results in reversible precursors that go through modifications to form AGEs. It was also acknowledged that the production of AGEs is influenced by a variety of metabolites, such as fructose, trioses, and dicarbonyl compounds, which participate much in the glycation reaction more quickly compared to glucose. Since glucose is most prevalent blood sugar and is present in high concentrations in diabetes, research on glucose and protein glycation has been extensive.

2.6.4 Procedure

Phosphate buffer is prepared in such a way that 0.1 M NaH_2PO_4 with pH 6 is combined with 0.2 M disodium hydrogen phosphate with the pH of 9, the overall pH is adjusted to 6.9 by addition of NaCl solution. Ten mL of PB (200 mM concentration) with 7.4 as pH, five mL of BSA twenty mg/mL in PB, 1 mL of plant extract 15.6-500 gm/mL, and 4 mL of 1000 mM fructose with PB are kept at room temperature for a week. Blank is made only with the extract of plant at the different concentrations, in contrast to blank control, that is made totally with BSA. Buffer is added to test tubes along with aminoguanidine at concentrations of 1.25, 0.75, and 0.25 mg/ml. A fluorescence spectrometer is used to detect the fluorescence emission of each pair at wavelengths of 355 nm and 440 nm, respectively [11].

2.7 *In silico*/Molecular Docking

From ligand design to drug development, molecular docking has been utilised to address a wide range of problems. By identifying the basic ingredient of the scoring functions for protein-protein docking between the two molecules, it models the interaction of protein-ligand complexes. The underlying principle of docking is that molecules with high affinity for protein-binding sites typically have molecular structures comparable to those of the binding sites. The form and spatial arrangement of the hydrophobic and hydrogen-bonding residues that surround the binding site are frequently used to describe the morphology of the binding site [12]. Proteins with PDB IDs 2QV4, 4W93 (for the amylase enzyme), 3L4J (for the glucosidase enzyme), and 5HY8 (for protein glycation) are docked against 12 botanical compounds acquired from GC-MS investigations for antidiabetic efficacy.

2.7.1 Docking simulations

Preparation of ligands: ligand is constructed and the chemical structures of molecules were both illustrated. Mcule's 2D ligands depicted docking on the ligand imported side.

Preparation of protein: The proteins with the PDB IDs 2QV4, 4W93 (for the amylase enzyme), 3L4J (for the glucosidase enzyme), and 5HY8 (for protein glycation) were initially retrieved in PDB format from the RCSB protein bank website. The SBD site sphere's attributes are prepared and recorded using the discovery studio visualizer.

2.7.2 Protein-ligand interactions

Through docking simulations, the binding position of drug candidates to their protein targets is predicted. Mcule was used to develop docking simulation experiments.

2.7.3 Ligand docking and scoring

3L4J (X= 43.922180, Y= 100.994820, Z= 32.862640), 5HY8 (X= -17.511549, Y= 15.878669, Z= 66.503238), and 2QV4 (X=

13.758000, Y= 58.583058, Z= 22.465406) proteins were uploaded with sphere attributes, and the ligand structures had been docked against the corresponding proteins. According to docking, few of the compounds have strong affinity for the proteins 3L4J, 2QV4, 4W93, and 5HY8. With the use of Mcule Docking, flexible glide-ligand docking was able to enhance protein ligand interactions. The docked compounds show a docking score. The proteins 2QV4, 4W93, 3L4J, and 5HY8 interact with chemicals found in *Trachyspermum ammi* in the ways shown below.

2.7.4 Visualization and analysis

The final docking places were pictured using the discovery studio visualizer. The ligand interactions were visualized in order to comprehend the binding interactions between ligands and proteins. The best docked forms were chosen using the glide score technique. The binding will be increasingly beneficial as the value decreases. Additionally, the docked ligand locations and various ligand receptor interactions were examined [12].

2.8 Statistical Analysis

Values are presented as the Mean SEM, with n = 6. ANOVA was used for the statistical analysis, which was followed by Dunnett's test. Comparisons were made between each group and the standard groups, control and disease-control, significance is denoted by the following symbols: standard (A=p<0.0001), control group (*=p<0.001), a=p<0.0001 and b = p<0.001), and ns- non significant.

3. RESULTS AND DISCUSSION

Antioxidant and antidepressant properties for Methanolic extract of *Trachyspermum ammi* leaves were investigated. The complete set of research's findings is shown below.

3.1 Preparation of Methanolic Extract of *Trachyspermum ammi* Leaves

Trachyspermum ammi leaves were macerated to create a methanolic extract. The equation below is used to calculate the extract's percent yield.

% yield of extract = Amount of extract obtained/ Amount of powder used x 100

= 32.3/200×100
= 16.15 % w/w.

Quantity obtained was 16.15 % w/w.

3.2 Acute toxicity studies

2000 mg/Kg bd.wt, per oral dose methanolic extract of *Trachyspermum ammi* leaf was administered to female albino mice. At 2000 mg/Kg body weight, the plant extract exhibited no symptoms of mortality or morbidity. All of the animals were safe even after fourteen days of observation. The biological analysis was performed at doses of 200 and 400 mg/kg body weight, orally.

3.3 Quantitative Analysis

3.3.1 Total phenolic content (TPC)

The calibration curve's linear regression equation and coefficient of linear regression are, respectively, y = 0.00090x-0.04190 and R2 = 0.98710. Where x = absorbance, y = concentration for gallic acid solution in (g/ml). META has a TPC of 135.59 ±8.87 mg GAE/gm given in Table 1.

3.3.2 Total Flavonoid content (TFC)

The calibration curve's equation for linear regression and linear regression coefficient were, respectively, y = 0.0015x+0.0517 and R2 = 0.9442, where x=absorbance, y=concentration of the quercetin solution (µg/mL). Methanolic extract of *Trachyspermum ammi* had a TFC of 62.1±4.17 mg quercetin/gm represented in Table 2.

Table 1. Calibration data for gallic acid

S.no	Concentration (µg/mL)	Absorbance
1.	0.100	0.0650
2.	0.200	0.1240
3.	0.300	0.1940
4.	0.400	0.2970
5.	0.500	0.3720
6.	0.600	0.4950

Table 2. Calibration data of quercetin

S.no	Concentration (µg/mL)	Absorbance
1.	0.100	0.1250
2.	0.200	0.3570
3.	0.300	0.6130
4.	0.400	0.7240
5.	0.500	0.7950
6.	0.600	0.9120

3.4 Antidiabetic Activity

In-vitro models

3.4.1 α-Amylase inhibition assay

To calculate the IC_{50} values of the test extract and acarbose, the α-amylase inhibition assay is used. Increasing concentration lead to a rise in the percent inhibition given in Table 3. Test extract and acarbose have respective IC_{50} values of 33.950 µg/mL and 24.830 µg/mL.

α-Amylase is a crucial digestion enzyme that is mostly found in saliva and pancreatic juice [13]. Inhibit and target this enzyme to lower elevated blood sugar levels during the night. Since the quantity of the extract affects the amount of enzyme inhibition, the amount of enzyme blocked appears to be dose-dependent. Amylase and glucosidase activities are strongly inhibited by β-pinene [14]. The pancreatic α-amylase enzyme of humans is inhibited by fucosterol [15].

3.4.2 α-Glucosidase inhibition assay

The 50% inhibition concentration values of the acarbose and test extract are determined using the α-glucosidase inhibition assay. Test extract and acarbose have IC_{50} values of 32.040 µg/mL and 25.620 µg/mL, respectively given in Table 4.

Table 3. *In-vitro* α-amylase inhibition assay of *Trachyspermum ammi*

S. no	Compounds	Concentrations (µg/mL)	% Inhibition	IC_{50} (µg/mL)
1.	**Test Extract**	10.0	17.84 ± 0.510	33.95
		20.0	30.19 ± 0.400	
		30.0	44.18 ± 0.670	
		40.0	55.28 ± 0.620	
		50.0	69.7 ± 0.880	
2.	**Acarbose**	10.0	27.33 ± 0.950	24.83
		20.0	40.26 ± 0.310	
		30.0	65.46 ± 0.490	
		40.0	71.43 ± 0.660	
		50.0	80.92 ± 0.820	

Experiment was performed for 3 times, and Mean±SEM was used to represent the percentage of inhibition

Table 4. *In-vitro* antidiabetic activity of META using α-glucosidase inhibition assay

S. no	Samples	Concentration (µg/mL)	% Inhibition	IC_{50} (µg/mL)
1.	**Test Extract**	10.0	15.630±0.670	32.04
		20.0	31.980±0.650	
		30.0	46.810±0.750	
		40.0	57.400±0.530	
		50.0	62.230±0.420	
2.	**Acarbose**	10.0	17.060±0.360	25.62
		20.0	39.020±0660	
		30.0	58.070±0.420	
		40.0	67.640±0.180	
		50.0	72.340±0.130	

Experiment was performed for 3 times, and Mean±SEM was used to represent the percentage of inhibition

The α-Glucosidase enzyme, which is found on the mucosal brush border of the small intestine, also functions as a crucial digestive enzyme. Its function is to convert and disintegrate complicated carbohydrates into tiny, intuitive, and easily absorbed ones. Its inhibition is a useful method for postponing glucose uptake and lowering elevated postprandial blood glucose levels, and this may slow the course of diabetes [16]. The greatest quantities of thymol exhibit the highest inhibitory action for α -glucosidase rather than α-amylase [17].

3.4.3 Protein glycation inhibition assay

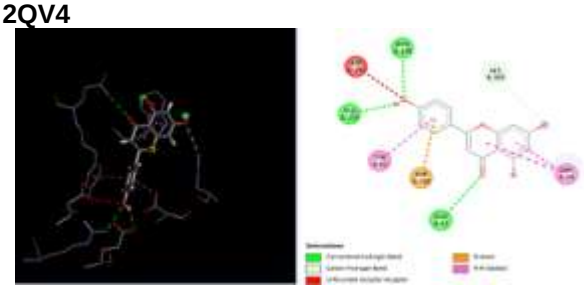
The rate of inhibition was determined to be 92.85±0.87 and 85.71±0.87, respectively, in the protein glycation inhibitory assay of the extract and aminoguanidine at 250µg/mL. We found that concentration affects the % inhibition of the extract and standard given in Table 5.

Glycation is a physiological cycle that takes place at a very slow rate in the human body and is a major cause of ageing. Haemoglobin A1c (HbA1c), a minor species of haemoglobin that is present in humans naturally, was shown to be present in higher proportions in diabetic persons, and it is currently being thought as a biomarker of diabetes mellitus. The Amadori product synthesis reaction, which is the protein glycation process, can be accelerated by high blood sugar levels [18]. By lowering *in vitro* lipid peroxidation, -tocopherol inhibits the glycation of haemoglobin in erythrocytes. *In vitro* glycation assays showed that a number of polyphenols exerted inhibitory effects on the glycation reaction. Polyphenols are the most abundant antioxidants in our diets. The main classes of polyphenols are phenolic acids (mainly caffeic acid) and flavonoids (the most abundant in the diet are flavanols, especially catechins plus proanthocyanidins), anthocyanins and their oxidation products), which account for one- and two-thirds of dietary polyphenols, respectively. Polyphenols are reducing agents, and together with other dietary antioxidants, such as vitamin C, vitamin E and carotenoids, protect the body's tissues against oxidative stress and associated pathologies such as cancers, coronary heart disease as well as inflammation [19].

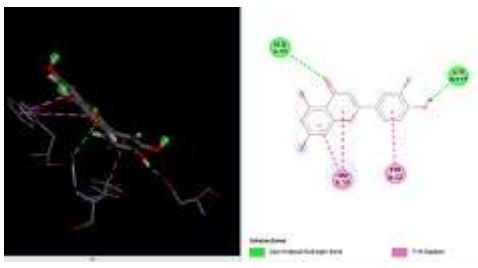
3.5 Molecular Docking

To predict the affinity and behavior of small molecules and drug candidates, computational molecular docking is commonly used to predict the binding orientation to their protein targets [20, 21]. We used molecular docking to model various bioactive compounds isolated from the Jasmine plant that is known to inhibit glucosidase. Hydrogen bonds play a crucial role in the structure and interaction of protein-protein or ligand-receptor complexes. Hydrogen bonds are important in drug design to ensure that the drug is unique to the protein target. The presence of such associated hydrogen and hydrophobic bonds indicates a positive response to the inhibition of the *α*-amylase enzyme. As a result, phytochemicals found in various plant extracts may be responsible for these extracts' antidiabetic properties.

2QV4

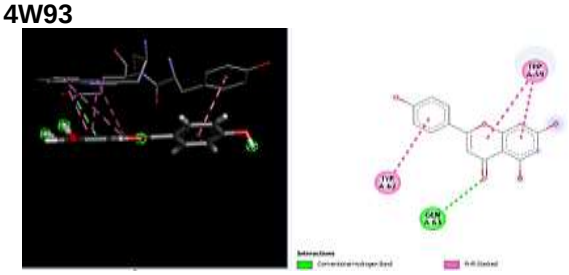


Apigenin – 8.2

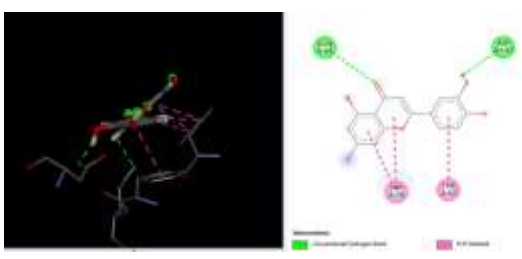


Luteolin – 8.7

4W93

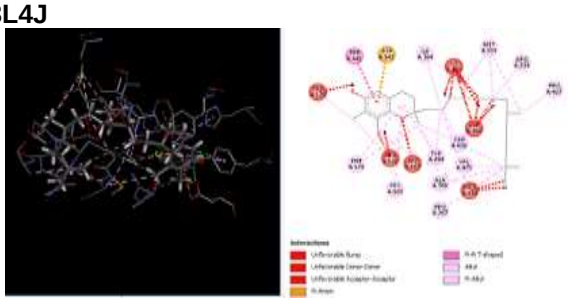


Apigenin – 8.0

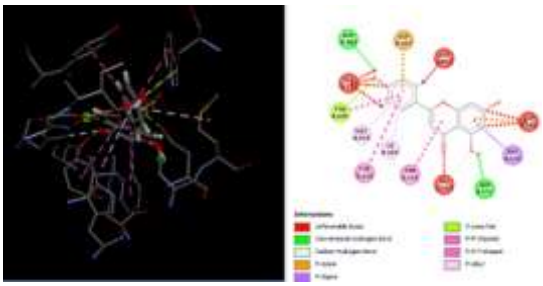


Luteolin – 8.2

3L4J

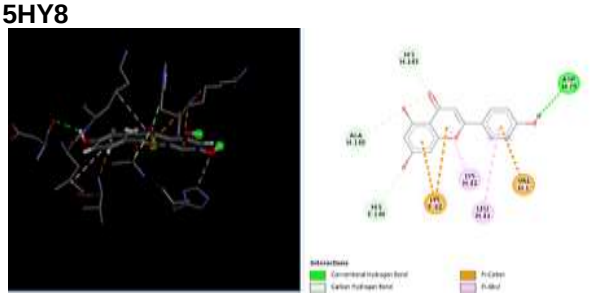


γ-Tocopherol – 18.7

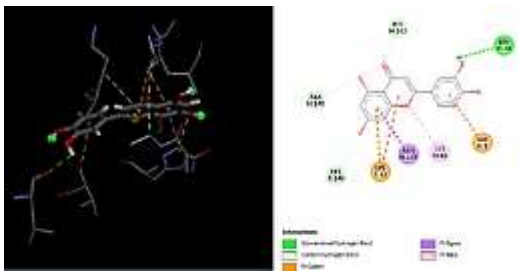


Apigenin – 21.2

5HY8



Apigenin –-6.9



Luteolin – -7.4

Fig. 1. Hydrogen bonding interactions of few compounds with highest score against the proteins

Table 5. *In-vitro* antidiabetic activity of *Trachyspermum ammi* using protein glycation inhibition assay

S. no	Samples	Concentration (µg/mL)	
1.	**Test Extract**	0.15	21.420±0.670
		0.30	41.260±0.840
		0.60	53.380±0.540
		0.120	75.390±0.570
		0.250	92.850±0.870
		0.500	95.230±0.850
2.	**Aminoguanidine**	0.250	85.710±0.870
		0.750	099±0.630
		0.1250	1090±0.740

Experiment was performed for 3 times, and MEAN±SEM was used to represent the percentage of inhibition

Table 6. Molecular docking studies of isolated compounds from *Trachyspermum ammi* against respective proteins

Compounds	Proteins			
	2QV4	**4W93**	**3L4J**	**5HY8**
Thymol	5.9	5.9	-5.2	-4.9
β-pinene	5.7	5.5	-5.2	-4.6
Sabinene	-5.9	5.9	-5.4	-4.6
terpinene-4-ol	5.8	5.7	-6.2	-4.9
Germacrene D	7.2	7.0	5.2	-6.0
γ-terpinene	6.0	5.8	-5.5	-4.8
Terpinolene	5.7	5.5	-3.3	-4.8
β-phellandrene	5.6	5.5	-6.0	-4.9
δ-3-caren	5.7	5.5	-5.2	-4.8
γ-Tocopherol	7.3	7.8	18.7	-5.6
Apigenin	8.2	8.0	21.2	-6.9
Luteolin	8.7	8.8	0.1	-7.4

4. CONCLUSION

The results of this investigation suggest that *Trachyspermum ammi* leaf extract has strong anti-diabetic effect. Further studies are necessary to elucidate *Trachyspermum ammi's* mode of action.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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