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Antidiabetic potential of various traditional medicinal plants and i*n silico* Validation

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Abstract
A change in diet, lifestyle and exercise will help greatly in management of diabetes in the initial stages with lesser impact at later stages. Plant based products have been popular all over the world for centuries. In diabetics, some herbal alternatives are proven to provide symptomatic relief and assist in the prevention of the secondary complications of the disease. Some herbs have also been proven to the help in the regeneration of beta cells and in overcoming insulin resistance and to maintain normal blood sugar level. In the present study, an attempt has been made to investigate the antidiabetic activity of *Trigonella foenum-graecum* seeds, *Acorus calamus* rhizhome and *Citrullus colocynthis* fruit. Hexane, ethyl acetate and methanol extracts of three samples was assessed for phytochemical components qualitatively and quantitatively and for their antidiabetic activity. Methanol extract of *Citrullus colocynthis* shows the highest percentage of Glucose uptake activity and Alpha-amylase inhibition activity. The phytocomponents in the crude methanol extracts of the *Citrullus colocynthis fruits* were identified by GC-MS. The anti-diabetic compounds were sorted by the iGEMDOCK software docking. Then the compound binding was analysed at the active site of PPAR-gamma receptor using bioactive component.

Keywords: phytochemistry, extraction, glucose uptake activity, alpha-amylase inhibition activity, GC-MS, iGEMDOCK

1. Introduction

According to the fifth edition of the world diabetes atlas released by the International Diabetes Federation (IDF) as of 2011, the total adult population in the age group of 20-79 years stands at 4-3 billion, out of which millions live with diabetes, which is set to increase to 552 million by 2030. Today, a number of plants have been studied for their anti-diabetic potential. More than 800 plants have been studied for the antidiabetic activity. Due to hyperglycemia, these proteins are modified to form glycoproteins and deposit on the tissues such as lens, vascular walls and basement membranes which increases diabetic complications. Cataracts, microangiopathy, atherosclerosis and nephropathy arise due to diabetic complications [7].

Trigonella foenum-graecum is commonly known as fenugreek. It is a plant native to India and South Europe. It is an annual plant. The seeds are hard, yellowish brown and angular. The plant has been scientifically used for the treatment of wounds, inflammation, gastrointestinal ailments, as cholesterol, lowering agent on diabetes, bronchitis, chronic cough and liver disorder. Fenugreek helps attain hormonal balance in women and helps increase the lactation in breast feeding women [12]. Earlier studies indicate that the extract of fenugreek seeds notably decreased blood glucose level in diabetic rats. The plant should be considered as an excellent candidate for future studies on diabetes mellitus. In addition, further comprehensive pharmacologic investigations, including experimental chronic studies should be carried out [18].

Acorus calamus belongs to the family Acoroceae. The fruits are small and berry-like, containing few seeds. They are branched, cylindrical, knobby rhizomes and about a finger thick and they have numerous coarse fibrous below them. The scented leaves and rhizomes of sweet flag have traditionally been used as a medicine in Ayurveda in India. It is found that *Acorus calamus* has a beneficial effect on learning and memory process in mentally retarded children. Methanolic extracts of *Acorus calamus linn.* rhizomes exhibited significant role in medicinal chemistry for formulation of life saving drugs [25]. *Acorus calamus* rhizome extract showed concentration dependent reducing power activity. Thus an active molecule present in the acetone extract of *Acorus calamus* rhizome having both antioxidant and disease preventive property (anti-inflammatory activity, diabetes, CVD etc) and it may be useful in targeting free radical mediated diseases [15].

Citrullus colocynthis is native to the Mediterranean Basin and Asia. It is a desert vinyl plant that grows in sandy, arid soils. It is an annual plant in Indian arid zones. Fruits are extremely bitter and filled with a soft, dry and spongy pulp. Each plant produces 20-30 fruits. Fruit hydroalcoholic extract possess significant anti-arthritic properties. Many studies shows that the extract of the leaves have anticancer activity on human breast cancer cells and anti-inflammatory properties [17]. The

fruit extract possesses potent antimicrobial activity and proves the use of *Citrullus colocynthis* for the treatment of diseases caused by the test organisms. There is still an urgent need to identify novel substances that are active towards pathogens with high resistance [19]. The Petroleum ether extract of *Citrullus colocynthis* fruits are found to lower the blood glucose levels. Oral administration of two different doses of *Citrullus colocynthis* fruit extract exhibited a significant reduction in blood glucose level in diabetic rats [10].

In this present study, the main objective was to determine the antidiabetic activity of three plant parts extracts with different solvents such as hexane, ethyl acetate and methanol. Also, their constituents are analyzed by gas chromatography and mass spectroscopy and the results were used for docking studies by iGEMDOCK software.

2. Materials and Methods

2.1 Collection of plant sample

Trigonella foenum-graecum seeds, *Acorus calamus* rhizome and *Citrullus colocynthis* fruit powder were collected and allowed to dry in shade and pulverized to powder using a mechanical grinder. The powder was stored in air tight packet.

2.2 Sequential extraction

Dried powdered materials (50 g) (*Trigonella foenum-graecum* seeds, *Acorus calamus* rhizome and *Citrullus colocynthis* fruit) were soaked separately in 300 ml of each solvents (Hexane, Ethyl Acetate and Methanol) in Soxhlet apparatus for 72 hrs [23]. Extracts were evaporated under vacuum and the residues were separately dissolved in the same solvent.

2.3 Phytochemical screening

2.3.1 Qualitative Analysis

Phytochemical screening of the crude extract was carried out using standard procedures to reveal the presence of chemical constituents such as Carbohydrates, Tannins, Saponins, Flavonoids, Alkaloids, Quinones, Glycosides, Cardiac glycosides, Terpenoids, Phenols, Coumarins, Steroids, Phytosteroids, Phlobatannins, Anthraquinones. This was done with the standard procedure described in Phytochemical methods [8], Medicinal plants and traditional medicine in Africa [22], Pharmacognosy [24].

2.3.2 Quantitative Analysis

Estimation of total phenols

The total phenolic compounds present in the extracts were estimated with folin-ciocalteau phenol reagent as suggested by Slinkard and Singleton [21].

Estimation of total flavanoids

The total flavanoid content was estimated using the method described by Ghasemi *et al.,* [6].

Estimation of total tannins

The total tannin content in the extract was measured by Folin-Denis method as suggested by Schanderi [20].

2.4 Evaluation of anti-diabetic activity

2.4.1 Glucose uptake by Yeast Assay

Yeast cells were prepared as per Cirillo's method [5]. *Saccharomyces cerevisiae* was suspended in distilled water and was washed by repeated centrifugation (4,200 rpm, 5 min) in distilled water until supernatant appeared clear. A 5% (w/w) suspension of washed yeast was prepared in distilled water.

The glucose uptake by yeast cells was assessed. Four concentrations (250, 500, 750, 1000 µg/ml) were prepared out of each extract. A standard glucose solution of concentration 100 mM/L was prepared. Various concentrations of the extract was added to 1 ml of the glucose solution and incubated for 10 mins at 37ºC. The reaction was started by adding 100 µL of yeast suspension, vortexed, and further incubated at 37º C for 60 mins. After incubation, the tubes were centrifuged (3800 rpm, 5 min) and glucose was estimated in the supernatant using Anthrone method [26].

$$\text{Increase in glucose uptake (\%)} = \frac{\text{Abs control - Abs sample}}{\text{Abs control}}$$

Where, Abs control is the absorbance of the control reaction (containing all reagents except the test sample), and Abs sample is the absorbance of the test sample.

2.4.2 Alpha amylase assay

Extraction of wheat alpha amylase

The alpha amylase from wheat was extracted according to the method followed by Nair *et al.,* (2013) [14]. 500 g of malted whole wheat flour was added slowly with stirring to 1 liter of 0.2 % calcium acetate solution at room temperature and continuously stirred for 2 hrs on a stirrer. The suspension was then centrifuged at 4° C at 12000 g for 10 mins. The resultant clear brown supernatant was stored at 2° C to 3° C prior to heat treatment. Since beta amylase interferes with the enzymatic determination of alpha amylase, it was inactivated by heating the extract at 70° C for 15 minutes. Alpha amylase is resistant to inactivation by this treatment at pH between 6.5 and 8.0. The pH of the extract was first adjusted to 6.6 with cold 4 % ammonium hydroxide. Heat treatment was carried out at 85° C to 90° C and other at 72° C to 74° C using a water bath with continuous stirring. The extract was then cooled to 2° C to 3° C until use.

Determination of Wheat alpha amylase inhibitor activity

The alpha amylase inhibition activity of the extract was determined by the method of Jung *et al.* [11]. The assay mixture containing 200 µl of 0.02M sodium phosphate buffer, 1 ml of enzyme and the plant extracts in concentration 5000 µg/ml were incubated for 10 mins at room temperature followed by addition of 3 ml of starch in all test tubes. The reaction was terminated with the addition of 3 ml 3, 5-Dinitrosalicylic acid reagent and placed in boiling water bath for five minutes, cooled and diluted with 15 ml of distilled water and absorbance was measured at 540 nm. The control sample was prepared without any plant extracts. The percentage inhibition was calculated according to the formula.

$$\text{Inhibition (\%)} = \frac{\text{Abs control - Abs sample}}{\text{Abs control}} \times 100$$

2.5 GC-MS

GC-MS analysis of the *Citrullus colocynthis* fruits extracts in the methanol was performed using the Hewlett-Packard (now Agilent) trade name "Mass Selective Detector" (MSD). Experimental conditions were as follows: HP-5 MS Ultra Inert (30 m x 250 µm x 0.25 µm) was used and the flow rate of

mobile phase (carrier gas: He) was set at 1.0 ml/min. In the gas chromatography part, temperature programme was setup in the oven from 50 °C and raised to 240 °C after 10 mins and injection volume was 1 milli litre. Samples which dissolved in methanol were run fully at a range of 50-6650 m/z.

2.6 In-silico docking

2.6.1 Protein preparation

The three dimensional structure of the receptors and ligands are the initial need for the in-silico docking. Mostly, the three dimensional structure of receptors are available in RCSB-PDB database (www.rcsb.org/pdb). The three dimensional structure of receptors such as PPAR-Gamma was retrieved from RCSB protein data bank (PDB Code: 2p4y). The whole structure of the receptors was targeted for our molecular docking study.

2.6.2 Ligand preparation

The structure of the compounds present in the plant was determined through gas chromatography. According to the hit value provided in GC-MS result the active compound for docking was selected. Then the information and structure of those compound ligands were collected from various databases such as PUBCHEM (www.ncbi.nlm.nih.gov/ pubchem), chemicalbook, chemspider database. The 2D structure of those ligands was converted to 3D structure using Discovery Studio Visualizer software. The obtained 3D structure was used for docking study.

2.6.3 Molecular docking

For docking, screening and post-analysis program, iGEMDOCK was used to gain the docking results of the listed compounds with the target. Initially both protein and ligand molecules were prepared by assigning bonds, bond orders, explicit hydrogen's, charges, flexible torsions. The contribution of hydrogen bond energy to the docking score is assigned a penalty based on the deviations from the ideal bonding angle. The molecular docking was done by iGEMDOCKV2.1 software. It has been decided to select the following parameters for docking such as Population size: 200, Number of generations: 70 and Number of solutions: 3. The Standard Docking was selected in default setting. In the present study three compounds were selected for docking. The anti-diabetic compounds were sorted at the end of docking process based on their interaction energies and fitness value produced by the iGEMDOCK software docking. Then the compound binding was analysed at the active site of PPAR-gamma and CEBP-alpha receptor by discovery studio software. Finally, iGEMDOCK ranked and visualized the screening compounds by combining the pharmacological interactions and energy-based scoring function of iGEMDOCK [3].

3. Results and Discussions

3.1 Qualitative analysis

Table 1: Qualitative analysis of *Trigonella foecum-graecum, Acorus calamus, Citrullus colocynthis*

	Trigonella foecum-graecum			***Acorus calamus***			***Citrullus colocynthis***		
Test	**HE**	**EAE**	**ME**	**HE**	**EAE**	**ME**	**HE**	**EAE**	**ME**
Carbohydrate	-	+	+	-	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	+
Saponins	+	-	-	-	+	+	+	+	-
Flavonoids	+	+	-	+	+	-	-	+	+
Alkaloids	-	-	+	-	+	-	-	+	-
Quinones	+	+	+	+	+	+	+	+	+
Glycosides	-	-	-	-	+	-	-	-	-
Cardiac glycosides	+	+	-	+	-	-	+	-	+
Terpenoids	+	-	+	+	+	+	+	+	+
Phenols	+	-	+	+	+	+	+	+	+
Coumarins	+	+	+	+	-	+	-	+	+
Steroids	-	+	-	+	+	+	+	+	+
Phytosteroids	+	-	-	-	-	-	-	-	-
Phlobatannins	-	-	-	-	-	-	-	-	-
Anthraquinones	-	-	-	-	-	-	-	-	-

HE-Hexane Extract + Present
EAE-Ethyl acetate Extract **-** Absent
ME-Methanol Extract

3.2 Estimation of Flavonoid, Phenol and Tannin

Trigonella foenum-graecum

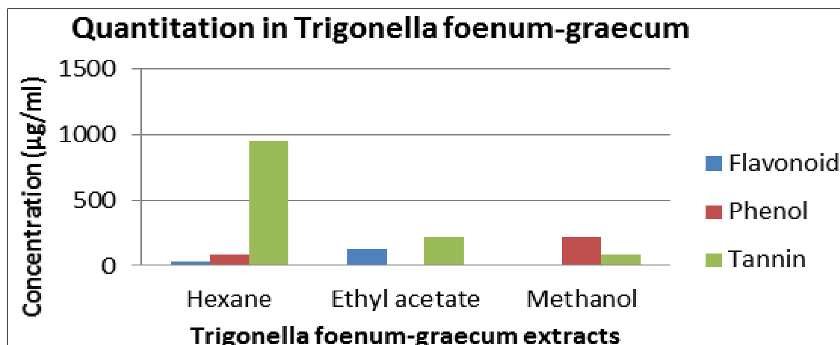

Fig 1: Estimation of Flavonoid, Phenol and Tannin in *Trigonella foenum-graecum*

From Figure 1 the extract has shown a dose – dependent increase in flavonoid, phenol, tannin content with Hexane, Ethyl acetate and Methanol extract of *Trigonella foenum-graecum.* Among the three extracts, Ethyl acetate extract have high quantity of flavonoids (12.51811898 mg QE/g). Phenol content was high in Methanol extract (22.37807 mg GAE/g) than in HE and EAE. When compared with the earlier study [4], the content of flavonoid is less in Ethyl acetate extract and high in methanol extract. The obtained estimation shows that the tannin content is high in hexane (95.27453725 mg TAE/g) than in EAE, ME but the quantitation of tannin is high when compared with Abdouli *et al.,* [1].

Acorus calamus

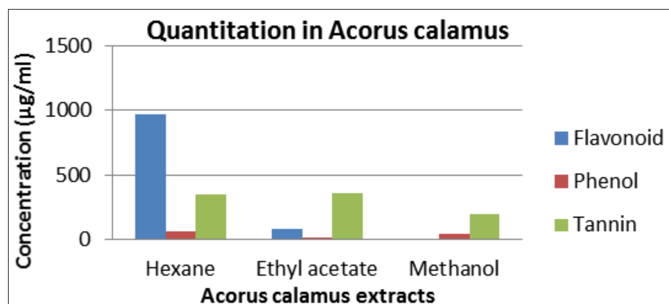

Fig 2: Estimation of Flavonoid, Phenol and Tannin in *Acorus calamus*

The quantitation of flavonoid, phenol, tannin content in hexane, ethyl acetate and methanol extract of *Acorus calamus* are shown in Figure 2. In this, Hexane extract has a high quantity of flavonoids (96.83206306 mg QE/g), phenol (6.471684 mg GAE/g) and tannin (35.17187743 mg TAE/g) than ethyl acetate and methanol extract. When comparing with earlier studies, the Hexane extract of *A. calamus* has higher flavonoid content than ethanolic extract [16] and high tannin content was found in acetone extracts [15].

Citrullus colocynthis

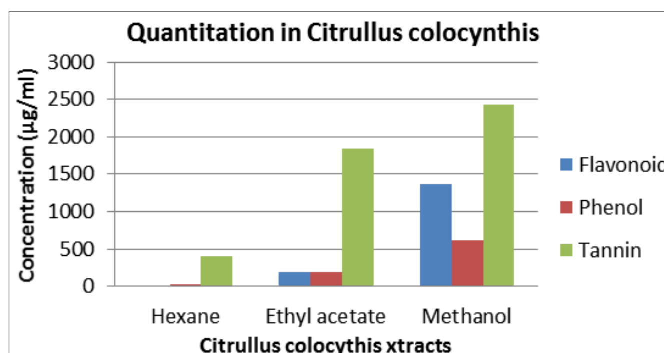

Fig 3: Estimation of Flavonoid, Phenol and Tannin in *Citrullus colocynthis*

From this estimation shown in Figure 3, Methanol extracts show high quantity of flavonoids (136.58 mg QE/g), phenol (61.81666667 mg GAE/g) and tannin (242.26111 mg TAE/g) than ethyl acetate and Hexane extract. The phenol and flavonoid content is much higher than in methanol extract of *Citrullus colocynthis* pulp [13]. When compared to the tannin present in seeds, fruits have high tannin content [9].

3.3 Glucose uptake by Yeast Assay

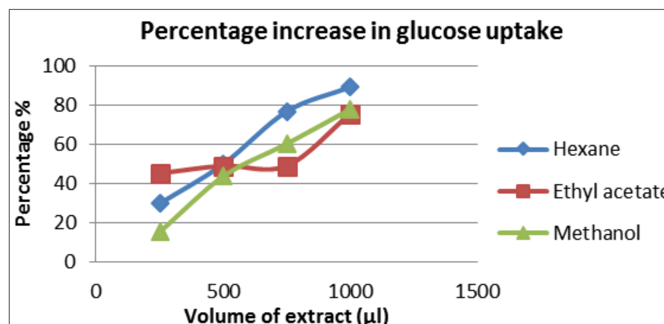

Fig 4: Percentage increase in glucose uptake with *Trigonella foenum-graecum* extracts

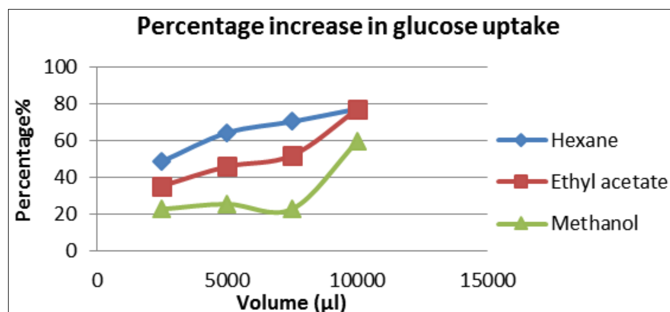

Fig 5: Percentage increase in glucose uptake with *Acorus calamus* extracts

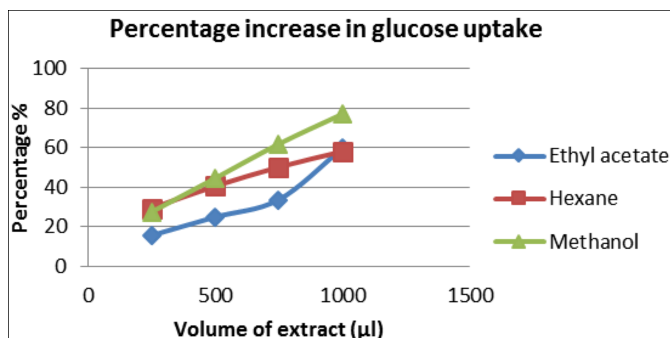

Fig 6: Percentage increase in glucose uptake with *Citrullus colocynthis* extracts

The Figures 4, 5, 6 are showing the percentage increase in glucose uptake after the addition of plant extracts to the bacterial cell which uptake the glucose. *Sacchoromyces cerveciae* acts similar to those of human cells in glucose uptake. Therefore, various dose of extracts showed dosage dependent increment in glucose uptake. Among the various extracts, highest percentage of glucose uptake was shown in hexane extract of *Trigonella foenum-graecum* (89.14%), methanol extract of *Citrullus colocynthis* (77.07%) and hexane extract of *Acorus calamus* (77.24%). All the extracts showed equivalent increase in glucose uptake with various extracts. Thus comparing with the three plant extracts *Trigonella foenum-graecum* showed better result than *Acorus calamus* and *Citrullus colocynthis.*

3.4 Alpha amylase inhibitor activity

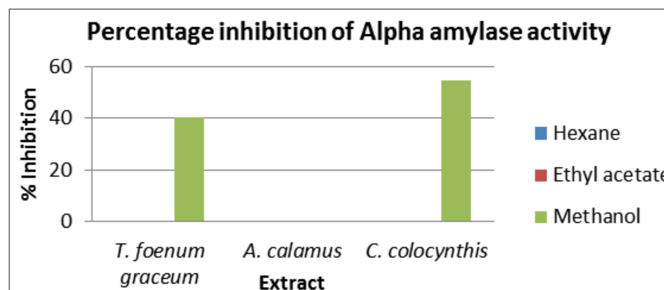

Fig 7: Percentage inhibition of Alpha amylase activity

The percentage of Alpha-amylase inhibition activity with the extracts of *T. foenum-graecum, A. calamus and C. colocynthis was shown in.* In the present study, the highest percentage was shown in methanol extract of *C. colocynthis* (IC_{50}= 916.6675833 µg/ml). *Trigonella foenum-graecum* showed less alpha-amylase inhibition activity but this inhibition activity was not found in *Acorus calamus.* However, the IC_{50} value of methanol extract of showed less inhibition activity when compared to the methanol extract of *Citrullus lanatus* leaves [2]. Anti-diabetic activity was high in methanolic extract of *Citrullus colocynthis* fruit. Therefore, the active compound identification was carried out in the methanol extract of *C. colocynthis.*

3.5 Identification of bioactive compound

The phytocomponents in the crude methanol extracts of the *Citrullus colocynthis fruits* were identified based on the retention time and area covered by the peaks on HP-5 MS Ultra Inert (30 m x 250 µm x 0.25 µm) column, Mass spectrum were interpreted using the database of National Institute Standard and Technology (NIST), Gaitherburg, Maryland. Different parameters were identified such as

Table 2

S. No	Compound Name	Formula	RT	Molecular Weight
1	p-Toluidine, N-methyl-N-nitroso-	$C_8H_{10}N_2O$	4.649	150
2	Phenol, 4-(methoxymethyl)-	$C_8H_{10}O_2$	5.039	138
3	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	5.175	150
4	Benzenemethanol, 3-hydroxy-	$C_7H_8O_2$	5.274	124
5	3-Oxo-androsta-1,4-dien-17β-spiro-2'-3'-oxo-oxetane	$C_{21}H_{26}O_3$	5.347	326
6	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	6.222	206
7	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	6.343	206
8	Ethyl α-d-glucopyranoside	$C_8H_{16}O_6$	7.375	208
9	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O_2$	10.834	270
10	Methyl 10-trans,12-cis-octadecadienoate	$C_{19}H_{34}O_2$	13.358	294
11	9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	$C_{21}H_{38}O_4$	22.259	354
12	Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-	$C_{12}H_{38}O_5Si_6$	27.5	290

The identified compound were namely, p-Toluidine, N-methyl-N-nitroso- (9.45%), Phenol, 4-(methoxymethyl)- (74.6%), 2-Methoxy-4-vinylphenol (35.3%), Benzenemethanol, 3-hydroxy- (38.0%), 3-Oxo-androsta-1,4-dien-17β-spiro-2'-3'-oxo-oxetane (7.60%), Phenol, 2,4-bis(1,1-dimethylethyl)- (56.5%), Phenol, 2,4-bis(1,1-dimethylethyl)- (50.9%), Ethyl α-d-glucopyranoside (27.8%), Hexadecanoic acid, methyl ester (31.5%), Methyl 10-trans,12-cis-octadecadienoate (8.61%), 9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester (6.87%), Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl- (21.7%). The GC-MS spectrum confirmed the presence of these 12 compounds with the retention time 4.649, 5.039, 5.175, 5.274, 5.347, 6.222, 6.343, 7.375, 10.834, 13.358, 22.259, 27.5.

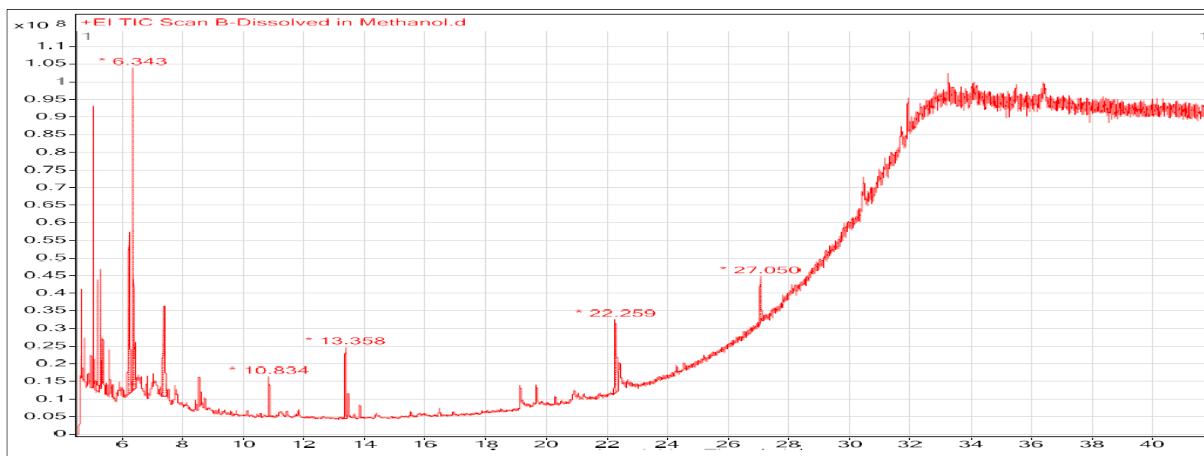

Fig 8

The GC-MS spectrum confirmed the presence of these 12 compounds with the retention time 4.649, 5.039, 5.175, 5.274, 5.347, 6.222, 6.343, 7.375, 10.834, 13.358, 22.259, 27.5.

3.6 Molecular docking

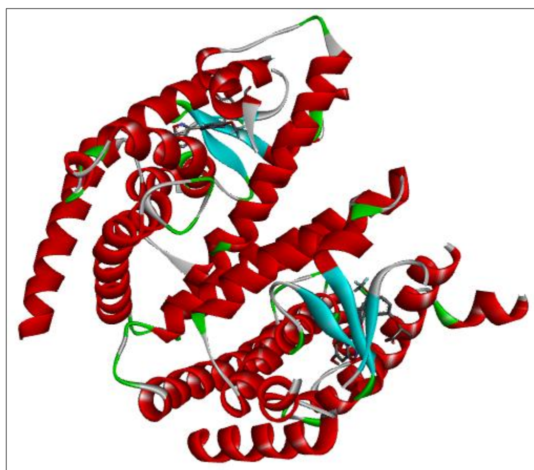

Fig 9: Structure of PPAR-Gamma receptor (PDB ID: 2p4y)

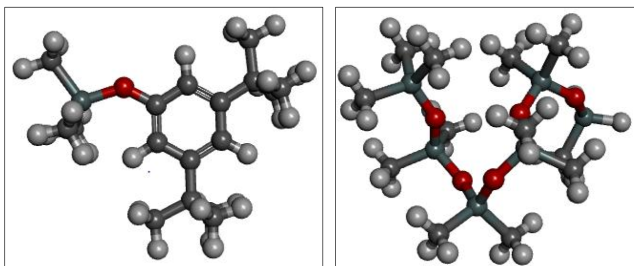

Fig 10: Structure of Phenol, 2,4-bis(1,1-dimethylethyl)- and Hexasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11-dodecamethyl-

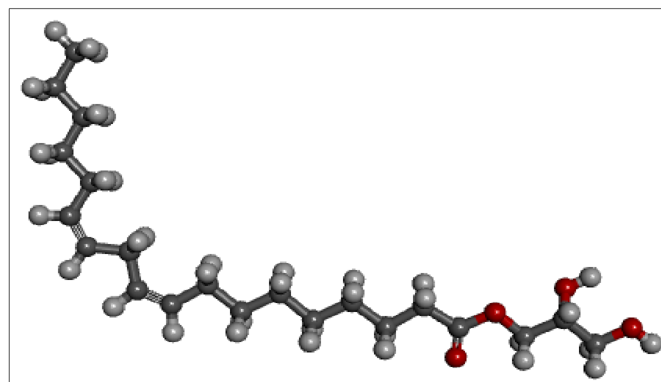

Fig 11: Structure of 9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester

3.7 Docked pose

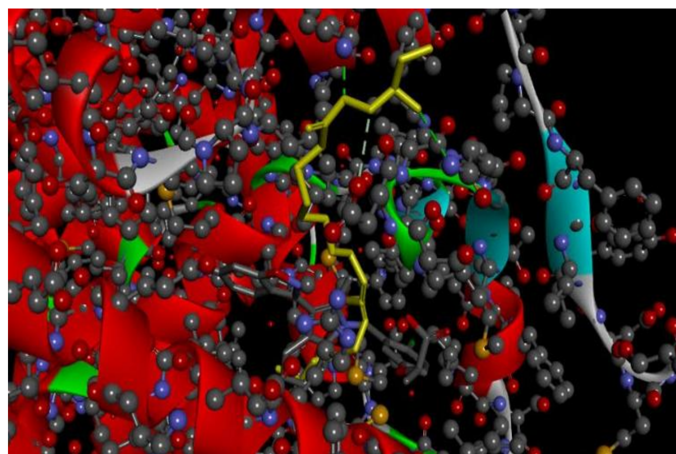

Fig 12: Interaction of 9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester with PPAR-gamma

Ligand	Total energy	VDW	H-Bond Distance	Electron
Hexadecanoic acid, methyl ester	-76.4573	-74.287	-2.17024	0
Phenol, 2,4-bis(1,1-dimethylethyl)-	-77.9834	-75.4834	-2.5	0
9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester	-97.0347	-91.2011	-5.83359	0

The ligand molecules with least binding energy selected as the best inhibitors. The binding energy of Hexadecanoic acid, methyl ester, Phenol, 2,4-bis(1,1-dimethylethyl)-, 9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester are -76.4573 Kcal/mol, -77.9834 Kcal/mol, -97.0347 Kcal/mol, -4.23Kcal/mol and -5.01Kcal/mol respectively. 9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester shows the highest ligand binding energy, hence this molecule is

considered as the lead compound from this plant.

4. Conclusion

Mostly plants play a major role in traditional medicinal system to combat several diseases. Generally plants have many phytochemicals like alkaloid, tannin, saponin, phlobatannis, flavonoids, terpenoids with specialized properties. Present study had some evidence for their antidiabetic effect which was proved by Cirillo and Nair, Kavrekar, Mishra's method. It can be concluded that the Anti-Diabetic activity of *C. colocynthis* could be due to activation of PPAR Gamma by 9,12-Octadecadienoic acid (Z,Z)-, 2,3-dihydroxypropyl ester present in its extract. Therefore, it seems that physicians can rely on these herbs, at least as complementary therapeutics, along with current hypoglycemic drugs to improve management of diabetic patients.

5. References

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