

Screening Indian Medicinal Plants to Control Diabetes – An *In silico* and *In vitro* Approach

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Abstract

Aim: The goal of this study is to analyze the compounds with high binding affinity to Peroxisome Proliferator-Activated Receptor Gamma (PPAR γ) by molecular docking and analyze the resulting medicinal plant by *in vitro* analysis.

Background: Diabetes Mellitus is a multifactorial disease leading to the several complications and thus, demanding for a therapeutic approach. Medicinal plants with several anti-diabetic compounds play an important role in treating diabetes. In this study, we report twenty eight compounds from fifteen Indian medicinal plants by targeting PPAR γ , using an *in silico* approach. PPAR γ is involved in adipocyte differentiation, glucose homeostasis, lipid storage and also improve insulin sensitivity and glucose tolerance and thus, helping in the treatment of diabetes. The goal of this study is to analyze the compounds with high binding affinity by molecular docking.

Results: The qualified herbal ligand Andrographolide has high binding potential against the target protein receptor, PPAR γ . The resulting ligand was also found to be fit with good druggable character, as per Lipinski's rule of five. Thus, this ligand could be a potential drug candidate for treating diabetes mellitus. The resulting medicinal plant was subjected to *in vitro* analysis where phytochemical evaluation, anti-oxidant activity, anti-diabetic activity, anti-inflammatory activity and the anti-glycation activity of the five different solvent extracts were determined. The results obtained indicated that the extracts possess a significant level of activity and are concentration dependent.

Keywords: Diabetes; PPAR γ ; Molecular docking; Andrographolide

Introduction

Diabetes Mellitus is a metabolic disorder of proteins, fats and carbohydrate metabolism which is characterized by high fasting and post prandial blood sugar levels. Diabetes mellitus results either from less production of insulin or insulin dysfunction [1].

The disease is estimated to increase from 4% in the year 1995 to 5.4% by the year 2025 [2]. Early diagnosis and adequate treatment is very important for management of the disease. Complications include diabetic ketoacidosis, coronary heart diseases, macro-angiopathy, micro-angiopathy, neuropathy, retinopathy, cataracts and renal failure [3].

Peroxisome proliferator-activated receptors (PPARs) belonging to the nuclear receptor family are ligand-activated transcription factors which bind to specific DNA response elements as heterodimers with the retinoid-X receptor (RXR) and control glucose and lipid metabolism, offering excellent therapeutic approach for treating the diabetes [4]. Specifically, PPAR γ , one of the isoforms, is highly sensitivity and glucose tolerance [5].

Considering the side effects and exorbitant cost of the many current medicines, in the past few years, herbal medicines are gaining momentum in treating various diseases because of their natural origin and less or no side effects.

Nearly 21,000 plants have been listed by the World Health Organization, which have numerous medicinal purposes around the world. Among these 2500 species are in India, out of which 150 species are used commercially on a fairly large scale [6].

For the current study, 15 Indian medicinal plants having anti-diabetic effect, that are used include: *Azadirachta indica*, *Andrographis paniculata*, *Swertia Chirata*, *Indigofera aspalathoides*, *Ocimum basilicum*, *Anethum graveolens*, *Momordica charantia*, *Trigonella foenum-graecum*, *Cinnamomum verum*, *Aegle marmelos*, *Emblica officinalis*, *Coccinia grandis*, *Ficus religiosa*, *Murraya koenigii*, *Punica granatum*.

PPAR γ is considered as legitimate target to design anti diabetic drugs and PPAR γ agonists are effective to control diabetes, however due to side effects of these existing drugs there is a pressing need for the retrieval of new ligands/agonists with antidiabetic activity with minimum or no side effects. Using bioinformatic approach, the novel ligands are identified from Indian herbal plants.

The goal of this present work is to use an *in silico* approach to predict the binding potential of these bioactive compounds with the target receptor (PPAR γ) by molecular docking. The best scored compounds identified from these herbal sources can act as potential drug candidates to control diabetes, and the resulting plant was further subjected to *in vitro* analysis where phytochemical analysis, anti-oxidant activity, anti-diabetic activity, anti-inflammatory activity and the anti-glycation activity of the five different solvent extracts were determined.

Methodologies

In silico studies

Our calculations are operated with SYBYL[®]-X 1.3 software package (<http://www.tripos.com>), which runs on OS - 32-bit Windows XP SP3 or 32-bit Windows 7 or 64-bit Windows 7.

Preparation of target protein structure and ligands

Protein Data Bank (PDB) is a repository of 3-D structural data of biomolecules. The crystal structure of human target protein PPAR γ (PDB ID: 4EM9) was retrieved from it. All the water molecules and crystallographic substructures from the target protein were eliminated and the necessary hydrogen atoms were added along with Gasteiger-Marsili charges. The minimization process was undergone and protein protocol was automatically generated and the final structure was visualized in PyMol (www.pymol.org) [7].

A total of 28 bioactive compounds from 15 Indian medicinal plants were selected by the literature survey for docking studies and the structures were downloaded from Pubchem.

Protein-ligand docking

Docking of the target protein PPAR Gamma and the twenty eight ligands was performed for finding their binding affinities. Both the receptor and the ligand were optimized for proper geometry and the compounds were ranked. The best five compounds were selected based on the docking score and number of residues [8-11].

In vitro Studies

Preparation of plant extracts

The resulting plant powder was successively extracted with different organic solvents (Hexane, Dichloromethane, Ethyl acetate, Ethanol and Methanol) in increasing polarity order according to Jeyaseelan et al. [12]. Briefly, 100 g of the powder was soaked in 300 ml hexane with intermittent shaking for 72 hours. It was then filtered through Whatman No.1 filter paper. The resulting residue was air dried and used for further extraction with dichloromethane and followed by ethyl acetate, ethanol and methanol. Finally, the solvents were removed from the extracts by keeping it in rotatory evaporator. After complete drying, yield of the each extraction was measured separately and the extracts were stored in refrigerator until used for further study.

Phytochemical screening

The extracts were subjected to various qualitative phytochemical tests to determine the active constituents present in the crude extracts using the procedures of Harborne, 1973 and Sofowora, 1982. The phytochemicals tested for were: tannins, flavanoids, terpenoids, cardiac glycosides, carbohydrates, saponins, resins, phenols, amino acids, alkaloids, glycosides and phytosterols.

Evaluation of antioxidant activity by DPPH radical scavenging method

Free radical scavenging activity of different extracts of the plant were measured by 1,1-diphenyl-2-picryl hydrazyl (DPPH). Plant extracts of different concentration ranging from 100-500 μ g were taken and the volume was made up to 1 ml with methanol. 4 ml of DPPH

solution was added to all the wells. Then the reaction mixture was kept for 30 min at dark. The optical density was measured at 490 nm against blank solution containing 5 ml of methanol and the same procedure was applied for ascorbic acid which acts as the standard [13].

The percentage of DPPH radical scavenging activity was determined in five concentrations using the equation,

$$\% \text{ scavenging activity} = \frac{A_0 - A_s}{A_0} \times 100$$

where, A_s is absorbance of the control sample, and A_0 is absorbance of the DPPH solution in the presence of plant extract.

Evaluation of anti-diabetic activity

Inhibition of alpha amylase assay: Alpha-amylase is an enzyme helpful in breaking down large insoluble starch molecules into absorbable molecules and is found in saliva and pancreatic juice [14]. Ethanol extract of different concentrations from 1–5 mg were taken into different test tubes and the volume was made up to 0.5 ml with phosphate buffer of pH 6.9. Blank was measured by taking 1 ml of phosphate buffer. The solution was then treated with 0.5 ml of alpha amylase (0.5 mg/ml). The solution was then incubated at 25°C for 10 minutes. Adding 0.5 ml of 1% starch solution in 0.02 M sodium phosphate buffer of pH 6.9 to all the tubes, they were incubated at 25°C for 10 minutes. The reaction was stopped by adding 1.0 ml of DNS and the reaction mixture was kept in boiling water bath for 5 minutes, and cooled to room temperature. The solution was made up to 8 ml with distilled water and the absorbance was read in the calorimeter at 540 nm against blank solution.

Standard maltose: 0.2-1 ml of standard maltose (1 mg/ml) was taken into different test tubes and the volume was made up to 1.0 ml with distilled water. Adding 1.0 ml of DNS reagent to the each tube, they were boiled for 15 minutes. The solution was then made up to 8 ml with distilled water. Then the absorbance of the solution was read spectrophotometrically at 540 nm against blank solution.

Inhibition of alpha glucosidase assay: The enzyme alpha-glucosidase is found in the mucosal brush border of the small intestine and helps in catalyzing the end step of digestion of starch and disaccharides that are abundant in human diet [15]. A solution of starch substrate (2% w/v maltose or sucrose) of 1 ml with 0.2 M Tris buffer pH 8.0 and various concentrations of plant extracts were incubated for 5 min at 37°C. The reaction was initiated by adding 1 ml of alpha- glucosidase enzyme (1 U/ml) to it followed by incubation for 40 min at 35°C. Then the reaction was terminated by the addition of 2 ml of 6N HCl. Then the intensity of the color was measured at 540 nm and the inhibitory activity was calculated [16].

Evaluation of anti-inflammatory activity by inhibition of albumin denaturation assay

The reaction mixture consists of test extracts of different concentrations and 1% aqueous solution of bovine albumin fraction, pH of the reaction mixture was adjusted using small amount at 37°C HCl. The sample extracts were incubated at 37°C for 20 min and then heated to 51°C for 20 min. After cooling the samples, the absorbance was measured at 660 nm and all the tests were performed in triplicates [17,18].

Determination of anti-glycation activity

Non-enzymatic reaction between free amino group of proteins and reducing sugar leads to the formation of glycated protein, which

further results in several advanced glycation end products (AGEs). These AGEs react with a free amino group nearby and form cross linking between proteins which are involved in diabetic conditions [19]. Thus, the compounds that inhibit the formation of AGEs have therapeutic potentials in patients with diabetes and age-related diseases [20].

The final reaction volume of the test includes 500 µl of Bovine Serum Albumin (1 mg/ml concentration) incubated with 400 µl of glucose (500 mM final concentration) and 100 µl of sample, 100 µl of phosphate buffer saline was used as the blank. The reaction was allowed to proceed for 24 hours at 60°C and terminated by adding 10 µl of 100% (W/V) trichloroacetic acid (TCA). The TCA added mixture was kept at 4°C for 10 minutes and centrifuged for 4 minutes at 13000 rpm. The precipitate was redissolved with alkaline phosphate buffer saline (pH 10) and the absorbance was read at 440 nm. Each sample

was taken in 1.5 ml eppendorf tubes and done in triplicates. Percentage of inhibition was calculated [21].

Results and Discussion

In silico Analysis

Docking Analysis

Molecular docking results were analyzed and the docking score of 28 compounds are listed in Table 1. Out of 28 bioactive compounds, top five compounds were more efficient in binding to the target receptor. Figure 1 and Table 2 illustrate these results. These compounds with highest docking score were further analyzed for the protein-ligand complex residues and the bond lengths.

S.No	Herb name	Compounds	Pubchem id	Docking score
1	<i>Ocimum basilicum</i>	Eugenol	3314	3.1297
		Ursolic acid	64945	4.21
		Linalool	6549	3.5936
		Caryophyllene	5281515	3.6543
2	<i>Anethum graveolens</i>	Carvone	7439	3.3694
		Limonene	22311	2.6638
3	<i>Momordica charantia</i>	Vicine	91446	5.6615
		Momordicine	57518366	3.5564
4	<i>Trigonella foerum-graecum</i>	4-hydroxy isoleucine	2773624	3.9645
		Trigonellin	5570	4.0694
5	<i>Azadirachta indica</i>	Nimbin	108058	3.2331
		Azadiradione	12308714	3.0943
		Gedunin	12004512	4.4097
6	<i>Cinnamomum verum</i>	Methyl hydroxyl chalcone(MHCP)	6440383	4.0383
		Proanthocyanidin	108065	3.3835
7	<i>Andrographis paniculata</i>	Andrographolide	5318517	5.4888
		Deoxy 11,12-didehydro andrographolide	6473762	4.109
8	<i>Aegle marmelos</i>	Marmarin	6450230	5.3997
9	<i>Emblica officinalis</i>	Ellagic acid	5281855	3.5157
10	<i>Coccinia grandis</i>	Beta amyryn acetate	92156	4.1748
		Beta cryptoxanthin	5281235	5.5196
11	<i>Ficus religiosa</i>	Beta sitosterol	222284	6.1005
12	<i>Murraya koenigii</i>	Murrayacine	5319962	3.4429
13	<i>Punica granatum</i>	Gallic acid	370	3.0459
14	<i>Swertia chirata</i>	Mangiferin	5281647	3.6686
		Swertiamarin	442435	3.8334

15	<i>Indigofera aspalathoides</i>	Dodecanoic acid	3893	5.9792
		Tetradecanoic acid	11005	5.3659

Table 1: List of selected medicinal plants, their compounds, pubchem id and the docking score.

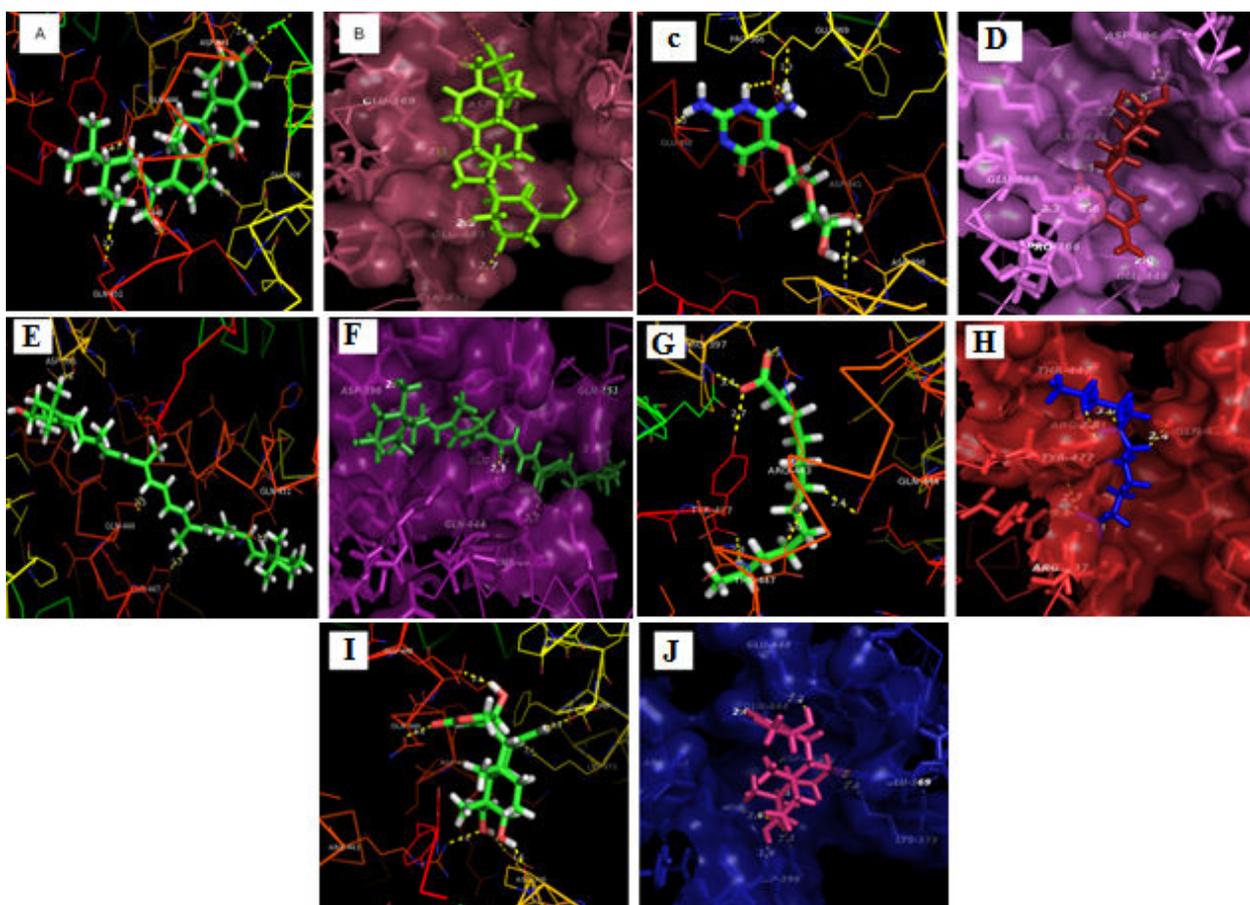


Figure 1: Interaction (A) and binding pocket (B) of PPAR γ with Beta sitosterol. Interaction (C) and binding pocket (D) of PPAR γ with Vicine. Interaction (E) and binding pocket (B) of PPAR γ with Beta cryptoxanthin. Interaction (F) and binding pocket (G) of PPAR γ with Dodecanoic acid. Interaction (I) and binding pocket (J) of PPAR γ with Andrographolide.

Compounds	Receptor (PPAR γ) Residues	Atom name	Bond length
Beta sitosterol	GLN 444	H	1.7 A $^\circ$
	GLU 448	H	2.2 A $^\circ$
	ASP 441	H	2.4 A $^\circ$
	GLU 369	H	2.5 A $^\circ$
	GLN 451	H	2.7 A $^\circ$
Vicine	ASP 441	H	1.7 A $^\circ$
	ASP 396	H	1.7 A $^\circ$

	GLU 448	H	2.0 A°
	GLU 369	H	2.1 A°
	PRO 366	H	2.3 A°
Beta cryptoxanthin	GLN 451	H	1.8 A°
	GLN 444	H	2.3 A°
	ASP 396	H	2.4 A°
	THR 447	H	2.7 A°
Dodecanoic acid	GLN 444	H	2.4 A°
	TYR 477	O	2.7 A°
	THR 447	H	3.0 A°
Andrographolide	ASP 396	H	1.6 A°
	ASP 441	H	2.1 A°
	GLU 448	H	2.2 A°
	LYS 373	H	2.4 A°
	GLU 369	H	2.4 A°
	ARG 443	O	2.6 A°
	GLN 444	O	2.6 A°

Table 2: Residues and bond lengths of Ligands interacting with PPAR γ complex.

In vitro Study Analysis

The resulting ligand Andrographolide is from the traditional medicinal plant *Andrographis paniculata*, which is claimed to have anti-diabetic properties [22-24].

Phytochemical Analysis

This study on *Andrographis paniculata* powder revealed the presence of alkaloids, phenols, phytosterols and resins in the hexane

extract. Terpenoids, alkaloids, resins, amino acids and phytosterols in the dichloromethane extract. Terpenoids, saponins, resins, alkaloids and phytosterols in the ethyl acetate extract. Terpenoids, cardiac glycosides, phenols and phytosterols in the ethanol extract. Tannins and carbohydrates in the methanol extract and water extract did not show the presence of any of the phytochemicals. Flavanoids and glycosides were absent in all the extracts (Table 3).

S. No.	Phytochemical Constituents	Hexane	Dichloro-methane	Ethyl Acetate	Ethanol	Methanol	Water
1.	Tannin	-	-	-	-	+	-
2.	Flavanoid	-	-	-	-	-	-
3.	Terpenoid	-	+	+	+	-	-
4.	Cardiac glycosides	-	-	-	+	-	-
5.	Carbohydrates	-	-	-	-	+	-
6.	Saponin	-	-	+	-	-	-
7.	Resin	+	+	+	-	-	-
8.	Phenol	+	-	-	+	-	-
9.	Amino acids	-	+	-	-	-	-

10.	Alkaloids	+	+	+	-	-	-
11.	Glycosides	-	-	-	-	-	-
12.	Phytosterol	+	+	+	+	-	-

Table 3: Phytochemical screening of extracts of *Andrographis paniculata*. Note: + Present, - Absent

Anti-oxidant activity analysis

In Diabetes Mellitus, hyperglycemia causes the overproduction of mitochondrial reactive oxygen species (ROS) through the activation of five metabolic pathways namely the polyol pathway, protein Kinase C, hexoseamine pathway, formation of advanced glycation end products and expression of its receptors and ligands [25]. The activation of these pathways leads to lipid peroxidation, tissue damage, defective angiogenesis and expression of proinflammatory genes [26]. Thus generation of ROS accounts for the development of secondary complications in diabetes. Previous studies have shown that ROS are generated in diabetic beta cells, which are combated by the over expression of antioxidants enzymes including the Super Oxide Dismutase (SOD), Catalase and Peroxidase. Antioxidants protect the beta cells from oxidative damage by scavenging the free radicals generated thus protecting from the development of diabetes [27-29]. Similarly the over expression of SOD in transgenic mice, has shown to inhibit the development of diabetic complications. As oxidative stress

is directly related to the development of diabetic complications and inflammation, the current study aims to analyze the antioxidant activity of *Andrographis paniculata*.

DPPH is a stable free radical helpful in accepting an electron or hydrogen radical to become a stable molecule. The capability of the natural antioxidants to reduce the DPPH free radical is measured by the decrease in absorbance at 490 nm. The extracts showed minimum scavenging activity of 24% and maximum was 96% (Figure 2A and 2B). Decreasing of the absorbance indicates that scavenging activity was more. The results revealed that the three extracts–hexane, ethanol and methanol of the plant *Andrographis paniculata* have best scavenging activity than the other two extracts. Thus, the bioactive compounds of this plant can act as antioxidants and play an important role in the treatment of various diseases including diabetes mellitus. The natural compounds having good antioxidant property are said to correlate with the anti-diabetic property as well or vice versa.

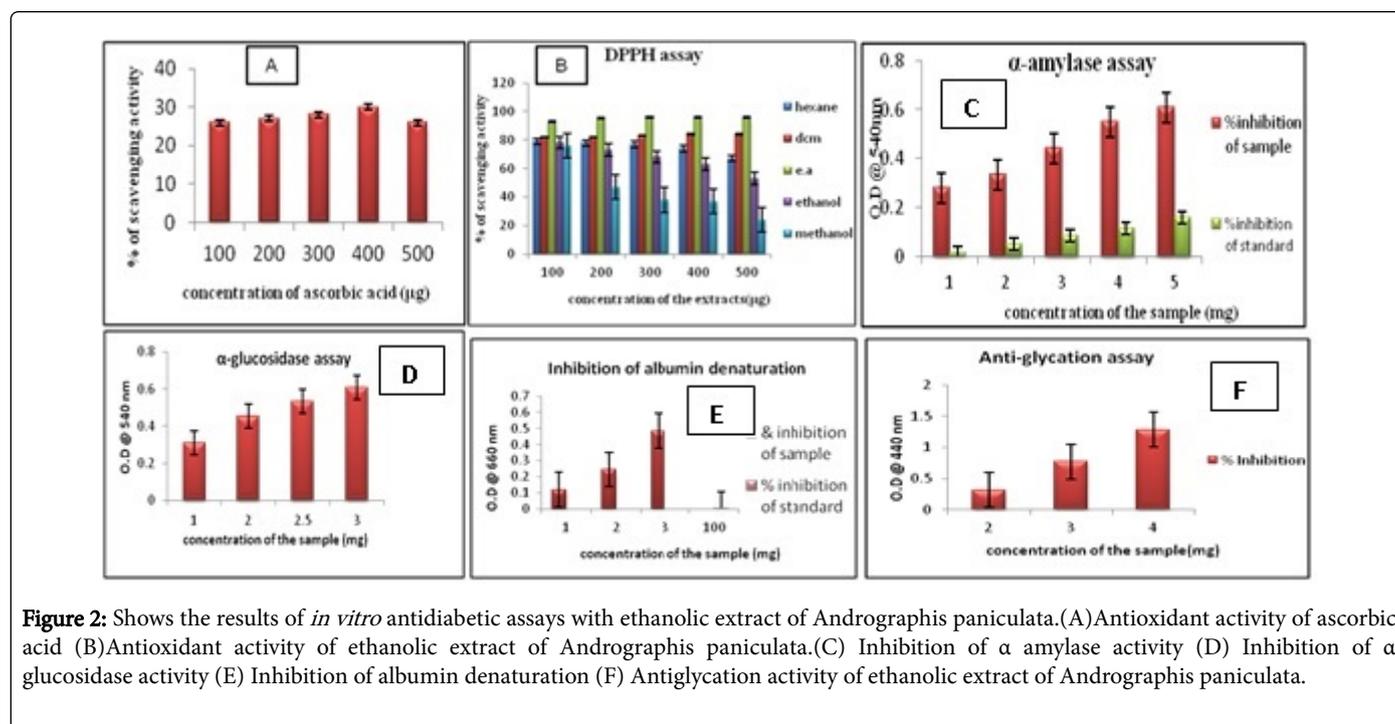


Figure 2: Shows the results of *in vitro* antidiabetic assays with ethanolic extract of *Andrographis paniculata*. (A) Antioxidant activity of ascorbic acid (B) Antioxidant activity of ethanolic extract of *Andrographis paniculata*. (C) Inhibition of α amylase activity (D) Inhibition of α glucosidase activity (E) Inhibition of albumin denaturation (F) Antiglycation activity of ethanolic extract of *Andrographis paniculata*.

Anti-diabetic activity analysis

Inhibition of alpha amylase assay: The alpha amylase inhibitory studies demonstrated that *Andrographis paniculata* possesses anti-diabetic activity. There was a dose-dependent increase in the percentage inhibitory activity of crude plant ethanolic extract against alpha amylase enzyme. A concentration of 1 mg of plant extract showed a percentage inhibition of 28% and for 5 mg, it showed an

inhibition of 61%. From the results, the inhibitory activity of the extract was greater than the standard maltose as shown in Figure 2C.

The extract showed 50% inhibition of the alpha-amylase enzyme in the high concentration of 4 mg. Thus, from the current inhibitory studies, it is revealed that the plant extract of *Andrographis paniculata* is effective in inhibiting the alpha amylase enzyme, which is further

helpful in delaying the breakdown of starch into glucose and thus, the glucose levels can be maintained in the diabetic patients.

Inhibition of alpha glucosidase assay: The *Andrographis paniculata* ethanol extract also revealed a significant inhibitory action of alpha-glucosidase enzyme. The percentage inhibition showed a dose dependent increase at 1–3 mg concentrations of *Andrographis paniculata* extract. The percentage inhibition varied from 60%-31% for highest and lowest concentrations respectively (Figure 2D). Thus, by the inhibition of alpha glucosidase enzyme, the extract of *Andrographis paniculata* containing numerous natural bioactive compounds play a great role in reducing the rate of digestion of carbohydrates which then decrease the levels of blood glucose and thus, diabetic conditions are maintained.

Anti-inflammatory activity analysis by inhibition of albumin denaturation assay

Protein denaturation is a well-known cause of inflammation. In diabetes, the activation of pro inflammatory genes triggers the inflammation of membranes and tissue proteins. The extent of protein denaturation is a measure of the degree of inflammation. The inhibition of albumin denaturation was analysed to measure the anti-inflammatory activity [30] of *Andrographis paniculata*.

Our results indicate that the ethanolic extract was effective in inhibiting the heat induced albumin denaturation. A maximum inhibition of 48% was observed at the concentration of 3 mg of the extract and minimum inhibition 12% at the concentration of 1 mg. Aspirin, a standard anti-inflammation drug showed the inhibition 0.5% at the concentration of 100 µg /ml. The inhibitory activity of the extract was greater than the standard drug (Figure 2E).

The results suggest that the extract of *Andrographis paniculata* helps in inhibiting the protein denaturation process which further leads to the decrease in inflammation process in the diabetic patients.

Anti-glycation activity

In the present study, the ethanolic extract of *Andrographis paniculata* was demonstrated to possess anti-glycation activity when tested by glucose-BSA assay. The anti-glycation activity was dose-dependent. At the concentration of 2 mg, the percentage inhibition was 32% and at the concentration of 4 mg, the percentage inhibition was 128% (Figure 2F). Thus, by inhibiting the glycation process of proteins, the extract plays a significant role in normal functioning of the cells, i.e., there is no disruption of molecular conformation and receptor function and no altering of enzyme activity. Hence, the anti-glycation activity of the extract interrupts the development of diabetic complications.

Conclusion

- As Diabetes Mellitus is epidemic throughout the world, effective drugs with less/no toxicity need to be developed. And one such way is the use of herbal medicines which will have no side effects.
- PPAR γ plays a great role in treating the diabetes and related complications.
- From the *in silico* study, it is suggested that the herbal ligands, chief components of ordinarily available Indian medicinal plants, demonstrated potential binding property towards the specific target protein, PPAR γ , and thus, can be a potential target to develop drugs that can interact and control diabetes.

- *In vitro* analyses proved that the extract of the plant *Andrographis paniculata* (nila vembu) can act as a potential anti-diabetic agent.

Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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