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In-vitro antidiabetic, Hepatoprotective activities and HPTLC finger print profile of *Azadirachta indica flower*

Research Article

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Abstract

As per Siddha literature *Azadirachta indica* A.Juss relieves nausea, dry mouth, loss of taste, vomiting, prolonged pain and stomach worms; the infusion /decoction of neem flower improves the strength and cures ulcer. Objective: The core objective of the current evaluation is to study the antidiabetic activity against α-amylase and α-glucosidase, carbon tetrachloride induced hepato-protective activity from the ethanolic extract of *Azadirachta indica* and to study HPTLC of ethanolic extract as a quality regulatory parameter of the authentic drug. Methods: The *in-vitro* antidiabetic activity was assessed by measuring the inhibition of α-glucosidase and α-amylase. The 3,5-dinitrosalicylic acid method was used for the α-amylase inhibition assay, and α- glucosidase from *Saccharomyces cerevisiae* of enzyme inhibition assay, with minor modifications made to the standard drug acarbose of each activity. *In -vitro* experimental study involved administration of CCl_4 to goat liver homogenate in *in-vitro* to induce hepatotoxicity. Experimental protocol involved 6 experimental groups each containing goat liver homogenate. Results: The extract exhibited a substantial level of anti-diabetic activity when related with standards. As the concentration of neem flower increased, there was a concomitant increase in the α-amylase inhibition activity. The result was significant as the *A. indica* showed 39 % inhibitory activity with 200 µg concentration as compared to 36 % inhibitory activity exhibited by acarbose for the same concentration. Conclusion The extract revealed statistically significant decline in protein levels, liver biomarkers like alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase in extract exposed group as compared to control group.

Keywords: Antidiabetic, Hepato-protective, *Azadirachta indica*, α–glucosidase, α-amylase, HPTLC.

Introduction

The Siddha system of medicine (SSM), one of the oldest traditional medical systems in use today, has its roots in India and is mostly used in the country's south to treat a wide range of illnesses, including chronic ailments. (1) Diabetes mellitus (DM) is a long-lasting metabolic disorder, categorized by involving multisystem organ dysfunction like eye, heart, kidney, blood vessels, nerves, etc. with increased plasma glucose levels due to insufficient in insulin secretion and action or both. DM leads to hyperglycemia, carbohydrate, protein and fat metabolic disturbances. (2) Compared to type 1 diabetes, which affects 90% of diabetic patients, type 2 diabetes is more prevalent in low- and middle-income groups. An imbalance between insulin production and blood glucose absorption results in type 2 diabetes (T2DM). The development of type 2 diabetes is significantly influenced by postprandial hyperglycaemia. (3) One of the treatment alternatives for reducing postprandial hyperglycemia is the ability of a food or medication to inhibit the activity of carbohydrate hydrolysing enzymes such α-amylase and α-glucosidase, which slows the generation or absorption of glucose. (4) Currently, the mainstay of treatment is the use of insulin secretagogues and sensitisers; nevertheless, a dynamic aspect in managing hyperglycemia is the use of enzyme inhibitors that break down carbs and decrease intestinal glucose absorption. (5)

Nowadays, it is advised to use medicinal herbs to treat a number of illnesses, including diabetes. (6) Numerous phytoconstituents, some of which may have antidiabetic qualities, are present in these plants, including flavonoids, terpenoids, saponins, carotenoids, alkaloids, and glycosides. (7)

9.3% and 24.5%, respectively, of Indians had impaired fasting blood glucose (IFG) due to diabetes. Of individuals with DM, 15.7% had it under control, 36.1% were receiving treatment, and 45.8% were vigilant. For diabetes consultation (84.0%) and treatment (78.8%), over three-fourths of individuals seek out allopathic practitioners. (8) It is ranked as the seventh most common cause of death (9) and the second most common cause of renal failure, blindness. (10) The

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liver is one of the most important organs for metabolism, excretion and storage. It is situated in the upper right quadrant of the abdominal cavity, beneath the diaphragm, cone shaped, dark reddish brown in colour. It helps in the metabolism of carbohydrates, fats, in the secretion of bile and storage of vitamins. (11) It has the capacity to detoxify the metabolic toxic waste products and synthesis useful principles. The organ helps in maintaining and regulating the homeostasis of the body. It helps in excreting most of the chemicals from the blood. Helps in carrying out waste from the blood, bile production which excretion of waste and breakdown fats in the small intestine through digestion. The process of gluconeogenesis happens here. Haemoglobin processing and protein degradation occurs in this organ. The bile juice secreted by the liver helps in digesting the food particles. As health effects are increasing with the liver problems particularly cirrhosis, inflammatory conditions, hepatitis. Currently, herbal medicines are gaining a significant role in improving liver functions and preventing the hazards caused by liver dysfunction. Neem, or *Azadirachta indica* A.Juss., is one of the important medicinal plants in human history. *A. indica* has been used from prehistoric times to the present. Siddha medicine (10,000 B.C. to 4000 B.C.), utilised in south India, is the oldest medicinal system. Neem, also known as margosa, is said to have been the first medicinal plant to be included in the Siddha medical system, according to Tamil literature. (12) The majority of neem compounds' biological and pharmacological properties, including their antitumor, hypoglycemic, anthelminthic, antioxidant, anti-inflammatory, antiarthritic, antipyretic, antiviral, and spermicidal properties, have been discovered. (13) Many medications, including emetics, anti-cancer, and antimicrobials, are derived from plants. Because they have these characteristics, phytochemicals are essential. (14)

The present study was carried out to evaluate the HPTLC, *In-vitro* antidiabetic, hepatoprotective activities of flower extract of *A. indica*. *In-vitro* antidiabetic, hepatoprotective activities of ethanolic extract of *A.indica* were examined α-amylase Inhibition and α- Glucosidase Inhibition assays also *in-vitro* hepatoprotective study was done against carbon tetrachloride induced liver damage using silymarin as control.

Materials and Methods

In-vitro Antidiabetic activity:

Chemicals Required

Chemicals as soluble starch, Porcine Pancreatic α-Amylase [PPA], Sodium Phosphate Monobasic, Sodium Phosphate dibasic, sodium chloride, sodium hydroxide, 3,5-dinitrosalicylic acid, sodium potassium tartrate, acarbose, α- glucosidase, para-nitrophenyl and sodium carbonate. These were the chemicals used for investigating the anti-diabetic activity of neem flowers. Sodium phosphate dibasic dihydrate, potassium phosphate monobasic, silymarin, carbon tetrachloride, sodium bi-carbonate, calcium chloride, magnesium sulphate heptahydrate, magnesium chloride heptahydrate, sodium phosphate monobasic, dextrose, and sodium phosphate monobasic monohydrate. These substances were employed in the study of neem flowers hepatoprotective properties. Every chemical and reagent used was analytical grade, and it was bought from SRL Chemicals in Chennai.

Plant authentication

Dried flowers of *A. indica* (neem flower) were procured from Sai medicals at Arumbakkam in Chennai, Tamil Nadu and authenticated by the Research Officer (Pharmacognosy) of this Institute.

Preparation of *A. indica* Extract

The neem flower (250 g) was powered, packed in a Soxhlet apparatus and extracted with ethanol for 8 hours, the filtrate was collected and ethanol was evaporated through a rotary evaporator at 45ºC and stored at 25ºC until further use.

Procedure for *In-vitro* Ant diabetic Assay

α-Amylase Inhibition Assay

The 3,5-dinitrosalicylic acid technique was utilised to assess the inhibitory activity of α-amylase. (15) At pH 6.9, a 0.02M sodium phosphate buffer containing 350 mg of sodium chloride was used to prepare the 1% starch solution. To make the α-amylase solution, 5 mg of the enzyme were added to 5 millilitres of buffer. DMSO was used to dissolve *A. indica*, yielding a concentration of 200–1000µg/ml. The colour reagent was made by mixing 0.500 mg of DNS with 250 ml of H_2O and 18.2 g of sodium potassium tartrate in 10 ml of 2.14% NaOH. Methodically mixed medication and 0.05 units of α-amylase solution was placed in a tube and 20 minutes incubation. After that, each tube was filled with 500 µL of starch solution, and it was incubated for 15 minutes. After adding 250 µL of DNSA reagent and heating it for ten minutes, the reaction was finished. The UV Visible spectrophotometer was then used to measure the absorbance at 540 nm. A tube containing PPA but no drug was utilized as a control with 100% enzyme activity.The positive control was a 50 mg acarbose solution diluted in 10 ml of buffer. The equation was utilized to compute the percentage inhibition of the α-amylase inhibitory experiment.

$$\text{Inhibition in \%} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Control absorbance}} \times 100$$

α-Glucosidase Inhibition Assay

The assay was executed with slight changes using a glucosidase from *Saccharomyces cerevisiae*. (16) The assay mixture contained of 200 µL of 0.1M (pH 6.9), 167 units of α-glucosidase, and a concentration of 1 mg /ml (w/v). The assay was pre incubated at 37ºC for 10 minutes. Then 500 µL of 0.02 M para- nitrophenyl α-D-glucopyranoside in 0.1 M sodium phosphate buffer was added and incubated at 37ºC for 25 minutes. The reaction was terminated by using 500 µL of 0.02 M sodium carbonate (Na_2CO_3). The yellow color was developed and measured at 405 nm. The tube with α-

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glucosidase but without drug served as the control with 100% enzyme activity and acarbose served as positive control.

$$\text{Inhibition in \%} = \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Control absorbance}} \times 100$$

Procedure for *In-vitro* Hepatoprotective Assay:

***In-Vitro* Hepatoprotective activity:**

Extract Preparation

250g of *A. indica* was powered and extracted with 150 ml of ethanol for 8 hours using Soxhlet apparatus. Then filtrate was collected, condensate using a rotary evaporated at 45°C and kept at room temperature until further use.

Chemical required

Sodium chloride, Potassium chloride, Calcium chloride, Magnesium sulfate heptahydrate, Magnesium chloride heptahydrate, Sodium phosphate dibasic dihydrate, Potassium phosphate monobasic, Dextrose, Sodium bi-carbonate, Sodium phosphate monobasic monohydrate, Silymarin, Carbon tetra chloride. All the above chemicals of analytical grade were purchased from (SRL Company) in Mumbai.

Collection of Liver

The local abattoir provided fresh goat (*Capra aegagrus*) liver, which was subsequently cleaned in regular saline, submerged in cold, sterile Hank's Balanced Salt Solution (HBSS), and kept at 4°C.

Preparation of Liver Slices

Using a sterile surgical blade (10 g each), the liver sample was thinly sliced before being submerged in 2.5 ml of cool, sterile HBBS solution. The entire procedure was conducted within a laminar airflow bench to ensure the tests were sterile and aseptic.

Homogenization and Centrifugation

Using a mortar and pestle method, 100ml of (0.1M) phosphate buffer (pH 7) was used to homogenise the liver sample. The liver homogenates were centrifuged (REMI manufacture) for 10 minutes at 10,000 rpm after homogenization. Subsequently, supernatants were gathered and refrigerated for additional study.

Preparation of biochemical models

Hepatotoxicity induced using carbon tetrachloride (CCl4) with 20 µl per 10g of liver sample. All sterile test tubes containing 10 g of liver sample was taken for each group and totally 6 groups (in triplicate) were divided for hepatotoxicity study (Table 1).

Biochemical analysis

Using the BA 400 Analyzer system and lab test kits, the liver parameters such as bilirubin (BIL), Serum Glutamic Pyruvic Transaminase (SGPT), Alkaline phosphate, Total protein, and serum Albumin were measured.

HPTLC Procedure

The extract (100 mg) was dissolved in 10 ml methanol, filtered and taken in a sample vial for HPTLC study. Extract (5, 10µl) was applied on aluminium plate precoated with silica gel $60F_{254}$ of 0.2 mm thickness (Merck) using fully automated ATS4 (CAMAG, Muttenz, Switzerland) applicator as a of 8 mm width band leaving 6 mm distance between tracks. The plate was developed using the mobile phase Toluene: Ethyl Acetate: Formic Acid (9.2:0.7:0.1, v/v/v) in a twin trough chamber of 10 x10 cm size. After development, the plate was visualized under UV 254 nm, 366 nm and their images were captured using Camag's Visualizer. The plate sprayed with vanillin-sulphuric acid reagent, heated for a few minutes till the development of coloured spots, immediately an image documented and scanned the plate in white light at 520 nm using scanner 030618 attached with WINCATS software. (17)

Results

***In-vitro* Antidiabetic Assay**

α –Amylase Inhibition Activity

In Table 2, Fig. 1, it shows that 200 µg of neem flower had a 36% inhibitory effect on α-amylase, while the common drug acarbose had a 39% inhibitory effect.

The amount of α-amylase inhibitory action rose in tandem with the concentration of neem flower. The α-amylase inhibitory activity of neem flower was significantly higher than that of a conventional medication.

Table 1: Represents the experimental groups

S.No	Particulars	Procedure	Solvent
1	Group-I	Normal control	Hank's Balanced Salt Solution (HBSS)
2	Group-II	Inducer control (CCl_4)	CCl_4 2ml/kg, - 20µl / 10g
3	Group-III	Standard treatment (CCl_4)	CCl_4-20µl Silymarin 500mg/kg
4	Group-IV	Extract treatment Dose-I	CCl_4-20µl Ethanolic extract of *A. indica* (Ex-Ai) -10mg/kg
5	Group-V	Extract treatment Dose-II	CCl_4-20µl Ethanolic extract of *A.indica* (Ex-Ai) -25mg/kg
6	Group-VI	Extract treatment Dose-III	CCl_4-20µl Ethanolic extract of *A.indica* (Ex-Ai)– 50mg/kg

Table 2: Results of α-Amylase inhibition activity

Concentration (µg)	Control	Acarbose	*A. indica*	% Inhibition activity neem flower	%Inhibition activity acarbose
200	0.6542	0.3985	0.4146	36	39
400	0.6542	0.3132	0.3532	46	52
600	0.6542	0.2543	0.2650	59	61
800	0.6542	0.1642	0.2231	65	74
1000	0.6542	0.1243	0.1435	78	80

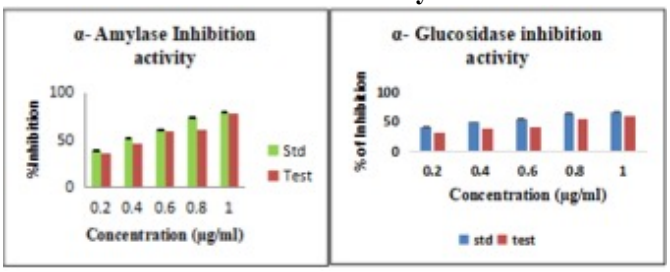
α-Glucosidase Inhibition Assay

A. indica α-glucosidase inhibitory assay was measured and contrasted (Table 3) with that of the common medication acarbose. *A. indica* α-glucosidase inhibitory activity was nearly identical to that of acarbose in this regard. The outcome was noteworthy since *A. indica* demonstrated 39% inhibitory activity at a 200 µg concentration, while acarbose only displayed 36% inhibitory activity at the same concentration.

Table 3: Results of α- Glucosidase inhibition activity

Concentration (µg/ml)	Control	Acarbose	*A. indica*	%Inhibition activity neem flower	% of Inhibition activity acarbose
200	0.9409	0.554	0.6292	33	41
400	0.9409	0.4655	0.5648	39	50
600	0.9409	0.4211	0.5544	41	55
800	0.9409	0.3211	0.4223	55	65
1000	0.9409	0.3023	0.3644	61	67

Figure 1: Graph for activity of α-Amylase and α-Glucosidase enzymes



***In-Vitro* Hepatoprotective activity**

The ethanolic extract of *Azadirachta indica* (Ex-Ai) has a hepatoprotective effect against hepatotoxicity caused by carbon tetrachloride (CCl_4) in an experimental model of fresh Goat (*Capra aegagrus*) liver was collected from local slaughter house. Table 4 and Fig. 2 display the results of the liver parameters, which included bilirubin, SGPT, ALP, total protein, and albumin.

Table 4: Biochemical parameters in CCl_4 induced hepatotoxicity of the ethanolic extract of *A. indica*

Parameter	Inducer Control	Standard	10 mg/ml	25 mg/ml	50 mg/ml
Bilirubin	2.167±0.039	0.177±0.072	0.023±0.003	0.040±0.008	0.047±0.005
SGPT	128± 0.258	92.000±2.113	86.67 ±0.298	102.0±0.258	92.00±0.258
ALP	356.33± 0.394	239.00±1.862	261.3±0.830	291.6±0.53	280.0±4.155
T.Protein	2.567±0.039	1.067±0.015	1.133±0.015	1.233±0.030	1.100±0.026
Albumin	0.332±0.258	0.400±0.000	0.400±0.0	0.433±0.030	0.400±0.0

Figure 2: Graph for Bilirubin, SGPT, Alkaline Phosphatases, Total protein and Albumin of In-*Vitro* Hepatoprotective activity

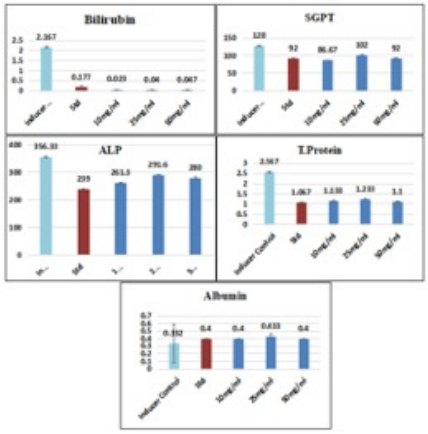
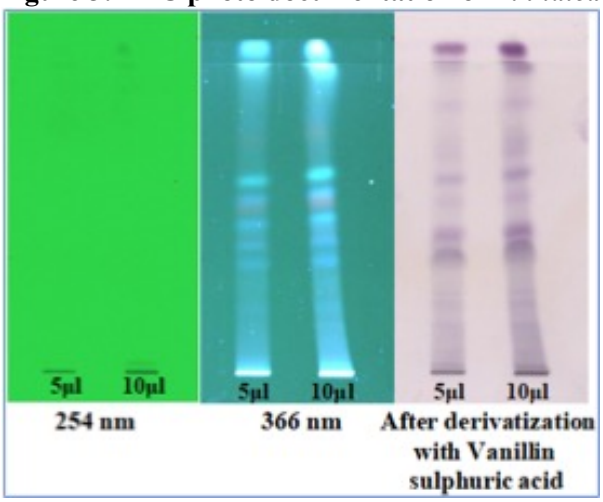


Figure 3: TLC photo documentation of *A. indica*



TLC/HPTLC

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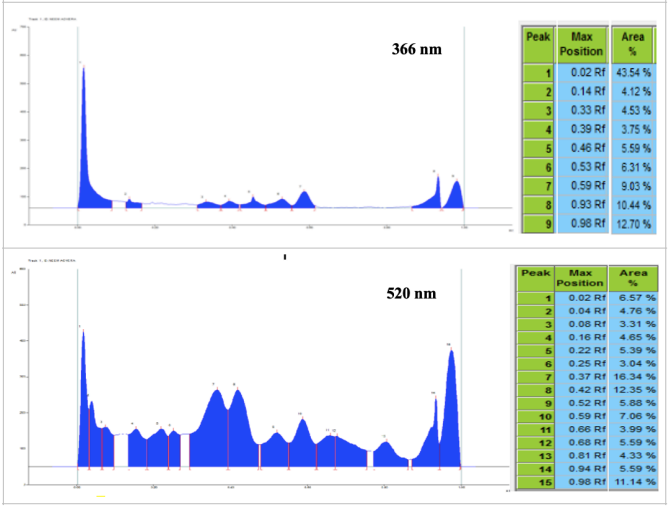
The thin layer chromatography development of *A. indica* flower are shown in Fig.3 and the R_f of the separated compounds are presented in Table 5. The TLC plate showed 13 spots in white light after derivatization with vanillin sulphuric acid reagent and 9 fluorescent spots under 366 nm. Under UV 254 nm, only few spots were viewable near the application position and near the solvent front.

Table 5: R_f value and Color of TLC spots

λ = 254 nm		λ = 366 nm		λ = 520 nm	
Color	**R_f**	**Color**	**R_f**	**Color**	**R_f**
Dark	0.03	Blue	0.06	Violet	0.03
Dark	0.08	Blue	0.34	Violet	0.08
Dark	0.91	Blue	0.40	Violet	0.15
Dark	0.96	Blue	0.44	Violet	0.21
-	-	Red	0.51	Violet	0.24
-	-	Blue	0.52	Violet	0.38
-	-	Blue	0.58	Violet	0.42
-	-	Blue	0.90	Violet	0.52
-	-	Blue	0.97	Violet	0.59
-	-	-	-	Violet	0.67
-	-	-	-	Violet	0.80
-	-	-	-	Violet	0.91
-	-	-	-	Violet	0.96

The HPTLC finger print profile of *A. indica* flower showed nine peaks under 366 nm as seen in the TLC and 15 peaks including the peaks near application position (R_f 0.02) and at solvent front (R_f 0.98).

Figure 4: HPTLC chromatogram with peak R_f and peak area at 366 nm and 520 nm



Discussion

Several studies have been done with traditional medicines in an attempt to develop new drugs for anti-diabetic and hepatotoxicity activity. α-Amylase (EC 3.2.1.1) randomly cleaves the α - (1-4) glycosidic linkages of amylase to yield dextrin, maltose, or maltotriose,(18) whereas α-glucosidase (EC 3.2.1.20) hydrolyzes the terminal nonreducing 1-4 linked α -glucose to release glucose molecules. (19) Maltose is produced when a-amylase is incubated with the substrate; however, 200 µg of neem flower had a 36% inhibitory effect on α-amylase, whereas the common medication acarbose had a 39% inhibitory effect (Table 2). The result was significant because, at a 200 µg dose, *A. indica* showed 39% inhibitory action, whereas acarbose only showed 36% inhibitory activity (Table 3). The present research shows the ethanolic extract of *A. indica* has significant anti-diabetic and hepato protective activity. In the present study, the neem flower shows the inhibition of pancreatic α-amylase and α-glucosidase enzyme activity which causes antihyperglycemic activity and helps in treating diabetes.

The liver is more vulnerable to damage than other organs because so many chemicals must travel through it in order to enter the general circulation. (20) Hepatotoxicity, which results in acute liver failure, is a serious issue for individuals who take paracetamol on purpose or by mistake. (21) When liver damage occurs, ALT, AST, ALP, GGT, and bilirubin are the primary enzyme values that are raised. The explanation is that when the liver is injured, these enzymes, which are mostly contained in the liver, are released into the blood. Additionally, the disruption and dissociation of polyribosomes on the endoplasmic reticulum results in a decrease in protein production, which lowers the amounts of albumin and total protein. (22) The normalization of the above enzyme and parameter levels in goat liver with the plant drug establishes the hepatoprotective effect of *A. indica*. Decrease in the biochemical parameters was observed after treatment with *A. indica*. The effectiveness of the extract in the normal functional status of the liver is comparable to that of silymarin.

Therefore, the ethanolic extract of *A. indica* possesses hepatoprotective activity. n-Hentriacontane, *n*-nonacosane, n-pentacosane, 2-methoxy-5,40-dimethyl-benzenebutanal, methyl octadecanoate acid, etc. are some of the compounds identified by GC-MS in the neem flower. (23) Compounds like nimbidin, azadirachtin, nimbin, nimbolide, gedunin, mahmoodin have been reported in seed oil of *A. indica.* Their presence in flowed are yet to be ascertained. The flavonoids and other chemicals reported from the flower include quercetin, myricetin, kaempferol, astragalin, melicitrin, hyperin, nimbin, nimbidin, rutin, β-sitosterol, azadirachtin and gallic acid.(24) However, the TLC studies showed the presence of 13 violet colored spots which are not viewable under 254 nm. Most of the separated compounds were of terpene type. (25-27) In HPTLC, totally 15 peaks were documented out of which three peaks with R_f 0.37 (16.24 %), 0.42 (12.35 %), 0.98 (11.14 %) were the major contributing phytochemicals.

Conclusion

This ethanolic extract of *A. indica* was created and confirmed with an easy-to-use HPTLC densitometric method. The TLC profile may be accounted as reference for the authentication of flower. *A. indica* has been shown in studies to have significant impacts on *in-vitro* hepatoprotective activity against CCL_4-induced toxicity as well as *in-vitro* antidiabetic

benefits using the inhibitory assay. Analyses conducted in vitro suggested that the *A. indica* extract may function by inhibiting α-glucosidase and α-amylase as one of its modes of action in order to prevent and control hyperglycemia. The findings indicated that the extracts' suppression of intestinal α amylase and free radical scavenging activities might be a factor in their antihyperglycemic effects. The outcomes provide empirical evidence in favour of the application of Siddha medicine in the treatment of diabetes and its related problems. The primary liver injury-related enzyme levels in toxic control were raised in ALP, ALT, and bilirubin, according to the *in-vitro* hepatoprotective evaluation. However, when compared to the hazardous control, the liver indicators of SGPT, ALP, and bilirubin levels in the standard group and ethanolic extract from *A. indica* exhibit noteworthy results. The findings could support the notion that *A. indica* action on α-amylase and α-glucosidase indicates a possible tool for the creation of novel diabetes treatment strategies. To assess the medication's effectiveness in animal models, more in-vivo research is needed.

Statement of Competing Interests

The authors declare that none of the work reported in this study could have been influenced by any known competing financial interests or personal relationships.

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