

Section A-Research paper

PHYTOCHEMICAL EVALUATION, IN VITRO ANTIOXIDANT ACTIVITY AND IN-VIVO ANTIDIABETIC ACTIVITY OF ACACIA NILOTICA

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Abstract

In the current study, the total phenolic and flavonoid content, antioxidant activities, and antidiabetic activity of several leaf extracts from Acacia nilotica were assessed. Analysis of the extracts' phytochemical composition was also done. DPPH free radical scavenging experiment was used to calculate antioxidant potential. In comparison to pods and bark, the leaves were shown to have a higher total phenolic content, higher protein content, and higher antioxidant activity. The authors have tried to put all these classes of plants at a common platform so that the data and information of this review could be utilized in drawing strategies for use of medicinal plants in a way that can be extended for future scientific investigation in different aspects. The fact confirmed by reports from the World Health Organization (WHO) shows that India has the largest number of diabetic subjects in the world. Hyperglycemia can be handed initially with oral synthetic agent and insulin therapy. But these synthetic agents produce some serious side effects and are relatively expensive for developing countries. The clinical signs, severity, and treatment of oral antidiabetic drug toxicity vary greatly. Numerous plants have been touted as having therapeutic benefits for the treatment of diabetes mellitus in the natural medical system. Due to availability and affordability, a substantial rural population relies on medicinal herbs to cure their diabetes. Besides hyperglycemia, several other factors including dislipidemia or hyperlipidemia are involved in the development of micro and macrovascular complications of diabetes that are the major causes of morbidity and death. Leaves of Acacia nilotica used as anti-diabetic, for feeding sheep and goats in the Hissar district in India. In Kenya, the fleshy pods are readily eaten by goats, sheep and cattle, but some tribes believe they cause bloat. As a result, A. nilotica leaf extracts are a potential source of antioxidant and anti-diabetic chemicals.

Keywords: Acacia Nilotica, Hyperglycemia, Diabetes Mellitus, DPPH, Hyperlipidemia, Antidiabetic, Antioxidant.

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1. Introduction

In response to the expected improvement in modern herbal medicine and reflective of their growing demand for natural medicines, 73 % of the respondents to a consumer survey indicated they would depend more on herbal medicine in the future. Imports of herbs into Hong Kong in 2003 amounted to USD 166.4 million, a 6.8 % decrease over the 2002's imports. This reflects less imports of licorice roots of USD 0.2 (-23.8 %) and ginseng root of USD 123.2 (-8.8 %).

In India, around 15000 medicinal plants have recorded [6] however been traditional communities are using only 7,000 - 7,500 plants for curing different diseases. The therapeutic plant species for various ailments are recorded in numerous indigenous systems, including Siddha (600), Ayurveda (700), Amchi (600), Unani (700), and Allopathy (30). According to another estimate 17,000 species of medicinal plants have been recorded out of which, nearly 3,000 species are used in medicinal field. Many components, including saponins, tannins, alkaloids, alkenyl glycol-alkaloids, phenols, flavonoids. sesquiterpene lactones, terpenoids, and phorbol esters, are responsible for the beneficial medical qualities of various plants. Diabetes mellitus is a chronic metabolic disorder, mainly characterized by disruption in carbohydrates, protein, and fat metabolism caused by the complete or relative insufficiency of insulin action ^[17]. The hormone insulin is released from the pancreas when blood glucose levels rise, as they do, for instance, following a meal. As glucose is not digested and a large amount of glucose is circulating in the blood (hyperglycemia), insulin encourages muscle and fat cells to remove glucose from the blood and stimulates the liver to metabolize glucose. As a result, blood sugar levels decline to normal levels. The kidney eliminates surplus sugar from the blood and excretes it in the urine to maintain the blood's normal amount of glucose. Because glucose is not utilized by the body cells, the body is under constant impression of hunger, and that is why diabetics feel increased appetite (polyphagia) and eat more frequently. One of the most prevalent chronic diseases in the entire world is diabetes mellitus. It is a multifaceted, complex illness

that has an impact on a person's life's quantity, quality, and style. Diabetes mellitus affects people of all age groups and from all walks of life. There are estimated 150 million people worldwide sufferings from diabetes, which is almost 5 times more than the estimated number 10 years ago. Diabetes mellitus is a global health problem and the diabetic was constantly increasing. The number of diabetic people is expected to rise to 366 million in 2030 [28], and most of them were type 2 diabetes. As we all know that the hyperglycemia can damage many of the body's organs and systems, and then lead to renal failure, blindness, cerebrovascular disease and so on. One of the therapeutic approaches is to decrease the postprandial hyperglycemia.

Types of diabetes mellitus

1. Type 1 diabetes

In Type 1 DM (previously called juvenile-onset or insulin-dependent), insulin production is absent because of autoimmune pancreatic β-cell destruction possibly triggered by an exposure genetically environmental in susceptible people. Type 1 DM generally develops in childhood or adolescence and until recently was the most common form diagnosed before age 30; however, it can also develop in adults (latent autoimmune diabetes of adulthood, which often initially appears to be type 2 DM).

2. Type 2 diabetes

In type 2 DM (previously called adult-onset or non-insulin-dependent), insulin secretion is inadequate because patients have developed resistance to insulin. Hepatic insulin resistance leads to an inability to suppress hepatic glucose production and peripheral insulin resistance impairs peripheral glucose uptake.

Plant Profile

Acacia nilotica Leaf Common Names (Hindi): Karuvela maram Classfication Kingdom: Plantae Order: Fabales Family: Fabaceae Genus: Vachellia Species: V. nilotica



Fig. 5 Acacia nilotica Leaf

A tree between 5 and 20 meters tall with a dense spherical crown, stems and branchlets that are often dark to black in color, fissured bark, and a grevish-pinkish slash that exudes reddish lowquality gum. Young trees have thin, straight, light-gray spines in axillary pairs that are typically 3 to 12 pairs long and 5 to 7.5 cm long. Mature trees often have no thorns. Leaves bipinnate, with 3-6 pairs of pinnulae and 10-30 pairs of leaflets each; leaflets 4-5 mm long and + / - tomentose, rachis with a gland at the bottom of the last pair of pinnulae. Flowers in globulous heads 1.2-1.5 cm in diameter of a bright goldenvellow color, set up either axillary or whorly on peduncles 2-3 cm long located at the end of the branches. Pods grey, thick, softly tomentose, straight or slightly curved, 5 to 15 cm long on a pedicel, 0.5 to 1.2 cm wide, with constrictions between the seeds giving a necklace appearance, fleshy when young, becoming black and hard at maturity (Andrews, 1952). Deeply constricted in a necklace fashion in subsp. tomentosa, lightly so in subsp. adstringens and glabrous but moniliform (in "pearl string") in subsp. nilotica. nilotica is characterized by glabrous, or nearly so, pods and twigs, while subsp. tomentosa has

strongly constricted white-grey hairy pods and subsp. *adstringens*, also known as var. *adansonii* (Guill. & Perrott.) Kuntze, exhibits hardlyconstricted or non-constricted, densely and persistently tomentose pods, and twigs.

Distribution and habitat

Caribbean, Africa, Northen Australia,

Parts used:- Flowers, Leave & Fruits

Uses

They are occasionally browsed by goats (Dougall and Bogdan, 1958). The wood is hard and heavy, difficult to work as it blunts tools for its high content in silica ; it is regarded, however, as excellent quality timber and service wood, poles, carpentry, boat and house construction, it is also considered a very good fire-wood and produces an excellent charcoal. Bark and pods are used in the tanning industry, leaves are readily browsed by stock, they have an average 12 % crude protein content. Young pods and seeds are eaten roasted by humans. Bark and leaves are used to treat haemorrhage, colds, diarrhoea, scurvy, dysentry and ophtalmia etc.

2. Material and Methods

Extraction

The correctly identified leaves of *Acacia nilotica* are dried and coarsely powdered. They should be extracted with methanol solvent in order to there increasing polarity to get correct and dependable retention factor to get significant results.

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Preparation of extract Hydroalcoholic extraction

Plant material was subjected to hot continuous extraction with Hydroalcoholic (80:20% v/v) (40-45°C) in a Soxhlet apparatus for 24 hours. The extraction procedure was ensured by pouring a few drops of extract from thimble left no residue on evaporation. After complete extraction the solvent was evaporated and concentrated to dry residue. % yield was calculated for extract after drying under vacuum.



Fig. No. Hydroalcoholic extract of Acacia nilotica

Determination of percentage yield

The percentage yield of each extract was calculated by using following formula: -

Weight of Extract Percentage yield = -----x 100 Weight of powder drug Taken

Phytochemical Screening: The chemical tests were performed for testing different chemical groups present in extracts.

A. Alkaloids

To the extract dilute hydrochloric acid was added. Then it was boiled and filtered.

i. Mayer's test

Take 2-3 ml of filtrate, add few drops of the Mayer's reagent was added. Formation of cream precipitate indicated the presence of alkaloids.

ii. Dragendorff's test

Take 2-3 ml of filtrate, add few drops of the Dragendorff's reagent was added. Formation of orange brown precipitate indicated the presence of alkaloids.

iii. Hager's test

To 2-3 ml of filtrate, few drops of Hager's reagent was added. Formation of yellow precipitate indicated the presence of alkaloids.

iv. Wagner's test

To 2-3 ml of filtrate, few drops of Wagner's reagent was added. Reddish brown precipitate obtained due to presence of alkaloids.

B. Carbohydrates

i. *Molisch's test* (General test)

In a test tube containing 2 ml of extract, 2 drops of freshly prepared 10 per cent alcoholic solution of α - naphthol was added. shake and add 2 ml Conc. sulphuric acid from sides of the test tube. So the violet ring was formed at the junction of two liquids, due to the presence of carbohydrates.

ii. *Fehling's test* (Reducing sugars)

To 2 ml of extract, equal volume of mixture of equal parts of Fehling's solution A and B were added and boiled for few minutes in boiling water bath. Red or brick red coloured precipitate was obtained due to the presence of reducing sugars.

iii. Benedict's test (Reducing sugars)

Equal volume of Benedict's reagent and test solution were added in a test tube and boiled for 5 min in a water bath. Green, yellow or red coloured precipitate was obtained depending on amount of reducing sugar present in test solution.

C. Flavonoids

i. Ferric-chloride test:

Test solution with few drops of ferric chloride solution shows intense green colour.

ii. Alkaline reagent test: To 2 ml of test solution add 2 ml alkali, gives yellow color, which disappears on addition of dil. HCl due to presence of flavonoids.

iii. Shinoda's test

In a test tube containing 0.5 ml of the extract, a small piece of magnesium was added. Then few drops of conc. hydrochloric acid was added. Formation of pink colour indicated the presence of flavonoids.

D. Proteins

i. Biuret's test (General test)

To 1 ml of test extract, 4% of sodium hydroxide solution and few drops of 1% copper sulphate

solution were added. formed violet red colour ppt. due to the presence of proteins.

E. Saponins

i. Foam test

The extract was shaken vigorously with water in a test tube. Formation of persistent foam indicated the presence of saponins.

ii. Haemolytic test

Few drop of extract solution was mixed with Blood, which indicates haemolysis, shows presence of saponin.

iii. Salkowaski test

Take 2 ml of test solution and add 2 ml conc. Sulphuric acid into it. The solution was shaken and allowed to stand. The colour of lower layer changed to yellow indicating presence of triterpenoids.

F. Steroids

i. Salkowski test

To 2 ml of extract and add 2 ml of chloroform and 2 ml of Conc. sulphuric acid and shaken, red color at lower layer indicated the presence of steroids.

ii. Liebermann-burchard reaction:

2 ml test sample was mixed with2 ml chloroform. Add 2 ml of acetic anhydride and 2 drops of conc. Sulphuric acid from the side of test tube were added. Change in colour first red, then blue and finally green indicated presence of steroids

G. Amino acid

i. Ninhydrin test (General test)

3 ml of test solution and 3 drops of 5% ninhydrin solution in a test tube were heated in boiling water bath for 10 minutes. Obtained Purple or bluish colour due to the presence of amino acid.

ii. **Millons test:** T.S (3 ml) and Million's reagent (5 ml) were mixed in a test tube.Due to the presence of proteins, appearance of white precipitate changing to brick red or dissolved and gave red color to solution on heating.

iii. **Xanthoprotic test:** To the test tube containing T.S (3 ml), 1 ml of conc. Sulphuric acid was added. Appearance of white precipitate which turns yellow on boiling and orange on addition of NH_4OH indicated presence of tyrosin and/or tryptophan containing proteins.

H. Glycosides

General test

Test A: 200 mg of extract were diluted with 5 ml of dilute sulphuric acid by warming on a water bath and filtered it. Then the acid extract was neutralized with 5% solution of sodium hydroxide. Add 0.1 ml of Fehling's solution A and B until it became alkaline and heated on a water bath for 2 minutes. Noted the quantity of red precipitate formed and compared with that of formed in test B.

Test B: 200 mg of extract was diluted with 5 ml of water instead of sulphuric acid. Then equal amount of water was added after boiling. Add 0.1 ml of Fehling's solution A and B until it became alkaline and heated on a water bath for 2 minutes. Noted the quantity of red precipitate formed. The quantity of precipitate formed in test B was compared with that formed in test A. If the precipitate in test A was greater than in test B then glycoside may be present. Since test B represents the amount of free reducing sugar already present in the crude drug, whereas test A represents free reducing sugar plus those related on acid hydrolysis of any glycoside in the crude drug.

i. **Baljet test:** 2 ml of the test solution was treated with 2 ml of sodium picrate solution. Obtained yellow to orange colour due to the presence of cardiac glycosides

ii. Legals test: To 2 ml of test solution, 1 ml of pyridine and 1 ml of sodium nitroprusside was added. Change in color to pink or red indicated presence of cardiac glycosides.

iii. Killer Killiani test: Glacial acetic acid (3-5 drops), one drop of 5% FeCl₃ and conc. Sulphuric acid were added to the test tube containing 2 ml of T.S. Appearance of reddishbrown color at the junction of two layers and

bluish green in the upper layer indicated presence of glycosides.

I. Tannins

i. Ferric chloride test

Extract solutions were treated with 5% ferric chloride solution. Formation of blue colours indicated the presence of hydrolysable tannins and formation of green colour indicated the presence of condensed tannins

ii. Lead acetate test

Extract solutions were treated with 5% lead acetate solution. Due to the presence of hydrolysable tannins white precipitate ws formed.

iii. Gelatin test

3 ml of test solution when treated with gelatin solution (3ml) gave white precipitate.

Total Phenolic content estimation:

Principal: The total phenolic content of the extract was determined by the modified Folin-Ciocalteu method.

Preparation of Standard: 50 mg Gallic acid was dissolved in 50 ml methanol, various aliquots of 25- 400 g/ml was prepared in methanol

Preparation of Extract:

1gm of dried powder of drug was extracted with 100 ml methanol, filter, and make up the volume up to 100 ml. One ml (1mg/ml) of this extract was for the estimation of flavonoids.

Procedure: 1 ml of extract or standard was mixed with 5 ml of Folin-Ciocalteu reagent (previously diluted with distilled water 1:10 v/v) and 4 ml (75g/l) of sodium carbonate. The mixture was vortexed for 15s and allowed to stand for 30min at 40°C for colour development. The absorbance was measured at 765 nm using a spectrophotometer.

Total Flavonoids Content Estimation Principal:

Determination of total flavonoids content was based on aluminium chloride method

Preparation of standard: 50 mg quercetin was dissolved in 50 ml methanol, and various aliquots of 25- 200 g/ml were prepared in methanol.

Preparation of extract

1gm of dried powder of drug was extracted with 100 ml methanol, filter, and make up the volume up to 100 ml. One ml (1mg/ml) of this extract was for the estimation of flavonoid.

Procedure: 1 ml of 2% AlCl₃ methanolic solution was added to 1 ml of extract or standard and allowed to stand for 60 min at room temperature; absorbance was measured at 420 nm.

Antioxidant Activity Evaluation DPPH reducing power assay:

DPPH quenching ability of Acacia nilotica extract was measured according. A methanolic solution DPPH solution (0.01mM) was mixed with serial dilutions (20–200 \square g/ml) of the extract and after 20 min, the absorbance was read at 517 nm. The antiradical activity was expressed as IC50 (\square g/ml), (the antiradical dose required to cause a 50% inhibition). Vitamin C was used as standard. The ability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = (A_0-A_t/A_0) X 100

Where A_0 is the absorbance of the control at 30 min, and A_1 is the absorbance of the sample at 30 min. All samples were analyzed in triplicate.

In Vivo Anti Diabetic Activity Materials and methods:-Animals:-

Wistar rats (150–200 g) were group housed (n= 6) under a standard 12 h light/dark cycle and controlled conditions of temperature and humidity (25 ± 2 °C). Rats received standard rodent chow and water *ad libitum*. Rats were given regular rodent food and unlimited amounts of water. Prior to the experiments, rats spent 7 days becoming used to the lab environment. Between 8:00 and 15:00 hours, all studies were conducted in a room with no background noise. Each set of studies used a different group of rats (n=6). The Institutional Animal Ethics Committee (IAEC), established by the Ministry of Environment and Forests, Government of India, New Delhi, India, to oversee and supervise the use of experimental animals, gave its approval to the animal experiments.

Chemicals:-

The company's authorized distributor was where we obtained Alloxan (Central Drug House Pvt.Ltd., India) and Glibenclamide pills (Daonil; Aventis Pharma. Ltd., India). In this investigation, all additional compounds were used.

Acute toxicity

Toxicity studies were carried out in accordance with OECD guidelines, acute oral toxicity study of leaves hydroalcoholic extract of *Acacia Nilotica*. The *Acacia Nilotica* (50, 100, 150, 200, 300 mg/kg/day) was administered orally for 4 days of six groups of rats (n=6) and the animals were kept under examination for mortality as well as any behavioral changes.

Induction of Experimental Diabetes in Rats

After a period of fasting, diabetes was brought on by giving a single intraperitoneal injection of 'Alloxan monohydrate' in distilled water at a dose of 120 mg/kg body weight. To treat the drug-induced hypoglycemia, the animals were given access to 5% glucose solution to drink over the course of the night. These animals were tested for diabetes after 15 days and animals with blood glucose (fasting) were selected for experimentation ^{[64, 65].}

Experimental Protocol

Animals were divided into five groups of 6 rats each ^{[65].}

Group I: Rats served as normal-control and received the vehicle (0.5 ml distilled water/day/rat)

Group II: Rats served as diabetic-control and received the vehicle (0.5 ml distilled water/day/rat)

Group III: Rats (diabetic) were administered *Acacia Nilotica* (100 mg/kg p.o.) for 15 days.

Group IV: Rats (diabetic) were administered *Acacia Nilotica* (200 mg/kg p.o.) for 15 days.

Group V: Rats (diabetic) were administered Glibenclamide ($600\mu g/kg$ p.o.) for 15 days.

Statistical Analysis

The data were expressed as mean \pm SEM. The data of hypoglycemic activity, oral glucose tolerance test and antidiabetic activity were analyzed by one way analysis of variance

(ANOVA) followed by "Dunnett's test." p value less than 0.05 was considered as statistically significant.

3. Result And Discussion

Extraction of plant material

Table: 7.1 % yield value of plant Hydro-alcoholic extraction			
S. No. Solvent		% Yield (W/W)	
1.	Hydro alcoholic (80:20)	12.25%	

PHYTOCHEMICAL SCREENING OF EXTRACTS

Chemical Tests	Results
Alkaloids	
Mayer's reagent	+ ve
Hager's reagent	+ ve
Wagner's reagent	+ ve
Dragendorff's reagent	+ ve
Glycosides (+Ve)	
Baljet test	- ve
Legal's test	- ve
Keller-Kiliani	- ve
Phenols/Tannins	
Ferric chloride	+ ve
Gelatin Solution	+ ve
Lead acetate test	+ ve
Flavonoids	
FeCl3 test	+ ve
Alkaline reagent test	+ ve
Shinoda test	+ ve
Saponins	
Foam test	- ve
Hemolytic test	- ve
Lead acetate	- ve
Fixed oil/Fats	
Spot	+ ve
Saponification	+ ve
Gums & Mucilage	
Water	- ve
Carbohydrates	
Molish test	+ ve

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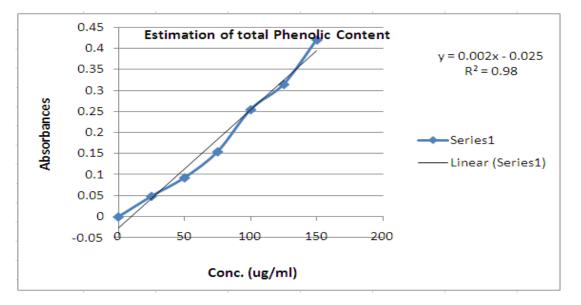
Fehling's solution test	+ ve
Benedict's test	+ ve
Amino acids	
Ninhydrin Test	+ ve
Millons Test	+ ve
Xantoprotein Test	+ ve
Lieberman Burchard Test	+ ve
Salkowski test	+ ve
Steroids	
Lieberman Test	- ve
Protein	
Biuret test	- ve

Total Phenolic content estimation (TFC)

The content of total Phenolic compounds (TPC) and to tal tannin content was expressed as mg/100mg of gallic acid equivalent of dry

extract sample using the equation obtained from the calibration curve: Y = 0.002X + 0.025, $R^2 = 0.980$, where \times is the absorbance and y is the tannic acid equivalent (GAE).

S.No.	Conc.	Absorbance
0	0	0
1	25	0.049
2	50	0.093
3	75	0.155
4	100	0.255
5	125	0.315
6	150	0.421



Total flavonoids content estimation (TFC) Using the equation based on the calibration

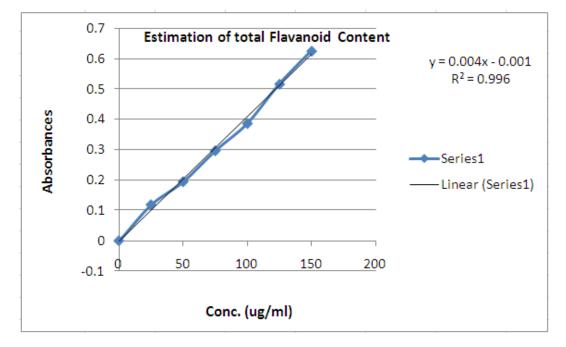
Using the equation based on the calibration curve: Y=0.004 X+0.001, R2=0.996, where X is

the absorbance and Y is the quercetin equivalent (QE), the total flavonoid content was determined as quercetin equivalent (mg/g).

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S.No.	Conc.	Absorbance
0	0	0
1	25	0.118
2	50	0.196
3	75	0.295
4	100	0.388
5	125	0.516
6	150	0.627



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Table: 7.2 Estimation of Total	phenolics and Total flavonoids content

S. No	Extracts	Total phenolic content	Total flavonoids content
		(%)	(%)
1	Hydroalcoholic Extract	0.81	1.12

Results Of Invivo Antidiabetic Activity

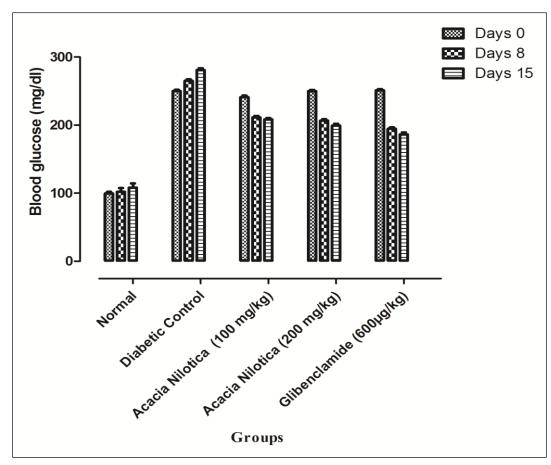
Group	Treatment	Blood glucose (mg/dl)		
		Days 0 15	Days 8	Days
Ι	Normal	99.6 ± 2.36 6.32	102.0 ± 5.61	108.1±
II	Diabetic Control	250.1 ± 2.0 2.80 [#]	265.1 ± 2.11 [#]	280.8 ±
III	Diabetic + Acacia Nilotica (100 mg/kg)	241.0 ± 2.5 2.0***	211.3 ± 2.16***	208.3 ±

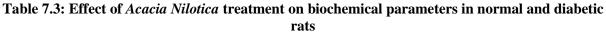
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IV	Diabetic + Acacia Nilotica (200 mg/kg)	249.7 ± 2.0 2.7 ***	$206.4 \pm 2.17^{***}$	199.3 ±
V	Diabetic + Glibenclamide (600µg/kg)	251.1 ± 1.9 3.1***	194.5 ± 2.15***	186.2 ±

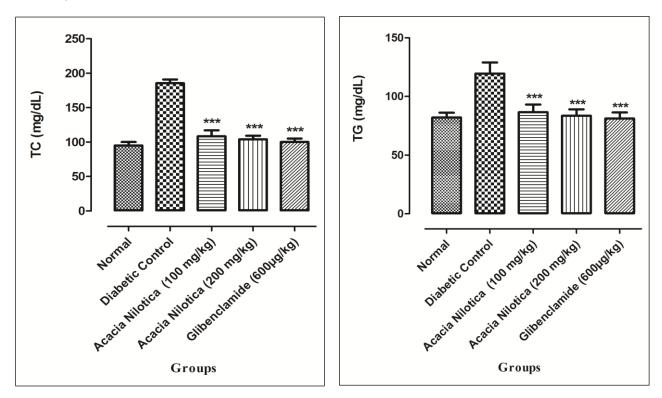
Values are expressed as mean \pm S.E.M (n = 6).Values are statistically significant at [#]p<0.001 vs. normal group; *P < 0.001, **P < 0.01vs. diabetic control group (One-way ANOVA followed by Tukey's post hoc test).

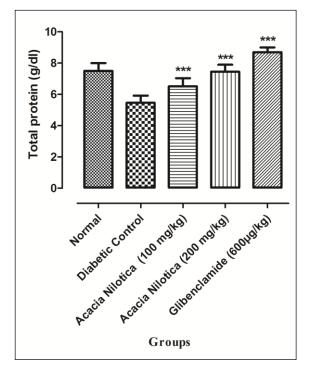




Group	Treatment	TC (mg/dL)	TG (mg/dL)	Total protein(g/dl)
Ι	Normal	90.1 ± 1.12	86.16 ± 5.5	8.00 ± 1.0
II	Diabetic Control	191.0 ± 1.00	129.01 ± 10.5	5.92 ± 1.1
III	Acacia Nilotica (100 mg/kg)	117.1 ± 1.12***	$93.12 \pm 6.19^{***}$	$7.03 \pm 1.1^{***}$
IV	Acacia Nilotica (200 mg/kg)	$109.2 \pm 1.10^{***}$	89.13 ± 8.15***	$7.90 \pm 1.0^{***}$
V	Glibenclamide (600µg/kg)	$105.2 \pm 1.23^{***}$	$86.44 \pm 6.10^{***}$	$8.40 \pm 1.0^{***}$

Values are expressed as mean \pm S.E.M (n = 6).Values are statistically significant at p < 0.001 vs. normal group; P < 0.001, P < 0.001, P < 0.01 vs. diabetic control group (One-way ANOVA followed by Tukey's post hoc test).





4. conclusion

The current research concludes that the extracts of leaves of Acacia nilotica, based on acute toxicity studies are safe at the decided dose level of 100 & 200 mg/kg of body weight. Extract showed the significant hypoglycemic activity which may lower the blood glucose level in hyperglycemia condition and may be helpful in antidiabetic study. Our study provides a way to study the antidiabetic study of the extract for the development of antdiabetic formulation. In our study the biochemical parameters are significantly reduced which may be helpful in diabetic complication. We can say that intake of this plant product may help not only in glycaemic control but also in minimizing the complications associated with diabetes. In future the activity of product and in house combination could be checked

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