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Original Article

Analytical Characterization and *in vitro* Study of Antidiabetic and Antioxidant Activity of Hydroalcoholic Extract of Neem (*Azadirachta indica*)

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ABSTRACT

Objectives: The antidiabetic and antioxidant activity of the hydroalcoholic extract of the *Azadirachta indica* (Neem), a member of the family *Meliaceae* is studied by the *In vitro* evaluation that is by phytochemical screening, α-amylase inhibition assay, DPPH and H_2O_2 assays. The neem leaves extract is also analysed by chromatographic methods like HPLC, TLC and FTIR. **Materials and Methods:** Neem leaves were dried and powdered for 300 g and macerated to obtain hygroscopic greenish leaves extract. The extract underwent phytochemical screening by TLC which confirmed the presence of quercetin. Antidiabetic activity of neem leaf conformed by α-amylase inhibition assay, and antioxidant activity evaluation by DPPH and H_2O_2. HPLC was performed to identify the percentage purity of the neem extract and FTIR performed to confirm the presence of specific compounds in the neem and aids for the antidiabetic activity. **Results:** Neem leaves hygroscopic greenish leaves extract confirmed the presence of phytochemical components. TLC confirmed the quercetin content with an RF value 0.46. In α-amylase inhibition, maximum enzyme inhibition was 77.77% at 500 µg/mL. DDPH maximum inhibition of 47.05% at 250 µg/mL, FTIR and HPLC conformed function groups and purity. **Conclusion:** Neem leaf has antidiabetic and antioxidant activity which is confirmed by performing TLC, HPLC, FTIR, H_2O_2 and α-amylase inhibition assay.

Keywords: *Azadirachta indica* (*A. indica*), FTIR, H_2O_2, HPLC, DPPH, TLC, α-Amylase inhibition.

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INTRODUCTION

Diabetes is the most common health issue worldwide since the many long back years, typically from ages. Over the past ten years, its prevalence has risen up to 50%, highlighting its rapidly expanding burden. According to ancient Egyptian, Persian, Indian, and Greek civilizations, diabetes is one of the oldest recorded diseases. Around 250-300 BC Apollonius of Memphis first introduced the term "diabetes". The sweet nature of urine accompanied with the disease led to the term "diabetes mellitus".[1]

Globally, diabetes is considered one of the major and most common diseases, which does not have any permanent solution and need to be forever on medication once the individual is diagnosed with diabetes. Diabetes is considered a global problem because it has no permanent cure and major body functional issues are related to it and diabetes is diagnosed in most of the young adults which in severe cases it even leads to death. Diabetes formally also called Diabetes mellitus.[1]

Diabetes mellitus

The commonly used term for diabetes mellitus is high blood sugar, hyperglycaemia or sometimes it is just called 'sugar'. Diabetes mellitus is of different types, that is type I diabetes mellitus, type II diabetes mellitus, gestational diabetes and prediabetes.[1] In type I diabetes mellitus, the immune system destroys insulin producing cells in the human body. This requires daily intake of insulin. In type II diabetes mellitus, it is a very common diabetes where the body does not produce enough insulin cells. Gestational diabetes is usually very common during the time of pregnancy. Prediabetes is a condition in which blood glucose levels are typically high. Diabetes has become very common such that it is diagnosed in many individuals all over the world globally irrespective of age and gender.[2,3] As per the data of World Health Organization (WHO), nearly 347 million people globally were living with diabetes in 2008, accounting for nearly 9.5% of the adult population. The prevalence is projected to almost double by 2030.[1] Diabetes is growing rapidly across the world because various conditions that may be genetic, ill food habits, stress, lack of sleep and bad life stay, etc. Nearly 85% of all cases of diabetes are diagnosed with autoimmune condition diabetes

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(type II Diabetes). In the early stages, the body compensates with elevated insulin levels and maintains glucose homeostasis. Over time, however, insulin secretion gradually declines insulin secretion decreases resulting in sustained hyperglycaemia.[4] It is essential to differentiate diabetes mellitus from diabetes insipidus, which is a distinct disorder involving defective water regulation and the passage of large amounts of dilute urine.[4] Individuals with diabetes insipidus face risks such as dehydration, elevated sodium levels, and circulatory instability, though the condition does not affect glucose metabolism.[5] The drugs that are developed to regulate blood sugar levels are termed as antidiabetic agents. Currently hypoglycaemic medications are available and other medical treatments for the control of diabetes mellitus. Despite therapeutic advances, long-term disease control remains difficult, especially in regions with limited resources. Consequently, there is growing interest in affordable and plant-derived treatment options.[6]

Neem, the tree which is known for having many therapeutic benefits which has been used for centuries from our ancestors for the cure of many diseases. Every part of the neem tree has its own medicinal benefits that is from its leaves, fruits, bark and roots. Neem tree scientifically termed as *Azadirachta indica*, it belongs to the family *Meliaceae*. The various parts of neem have been utilized in the management of numerous conditions such as infections, inflammation, hypertension, cancer, and diabetes. Studies have reported that neem has more than 135 bioactive compounds. Neem contains various major phytoconstituents which include Azadirachtin, Nimbin, Quercetin, Nimbidin, Nimbolide, Nimbandiol, Nimbanene, and several limonoids. Among all the phytoconstituents Azadirachtin is considered one of the most biologically active constituents. There are various experimental studies which have depicted that neem extract possesses antimicrobial, anti-inflammatory, antioxidant, hypolipidemic, immunomodulatory, and potential antidiabetic properties.[7,8]

Antioxidant and antidiabetic potential

There is much scientific evidence that depicts neem leaves have the potential ability to cure many diseases in human beings. Likewise, neem leaves, hydroalcoholic extract has the benefits of curing diabetes in humans. With diabetes representing a global health challenge and current therapies having limitations, neem has emerged as a candidate for alternative therapy. Experimental works in animal models of diabetes induced by high fat diets and Streptozotocin (STZ) have reported beneficial outcomes. In a high-fat diet-induced rats with diabetes, daily intake of neem leaf extract of 400 mg/kg for the period of 30 days improved fasting glucose levels, glucose tolerance, lipid metabolism, glycogen storage, and insulin signalling. Other investigations have observed recovery of pancreatic islet function, increased insulin secretion, greater glycogen storage in liver and muscle, and improved antioxidant capacity.[9,10]

MATERIALS AND METHODS

Procurement and identification of raw materials

Azadirachta indica (Neem) leaves were procured from Yucca Enterprises, A-246, Antop Hill Warehousing Co., Barkat Ali Naka VIT Marg, Mumbai-400037, India. With purified water the procured leaves were washed to remove the dust particle and impurities. For one month it was shade dried in room temperature. After drying, the leaves were coarsely powdered and kept in in an airtight container until further study.

Preparation of hydroalcoholic extract

The powdered neem leaves were weighed 300 g and subjected to cold maceration with 1.5 L of 70% hydroalcoholic solvent (ethanol: water) for period of 15 days with periodic stirring. The macerated mixture was first filtered through cotton and then through Whatman filter paper. The filtrate was evaporated on a water bath at 40°C until a solid residue was obtained. The dried greenish extract was collected and stored for further analysis.[11]

Preliminary components detection

The determination of specific components in the neem leaves extract of *Azadirachta indica*, for the quantitative determination of the secondary metabolites.

Detection of Alkaloids

Mayer's test

It is performed by taking a small quantity of the sample solution that is hydroalcoholic extract of the neem and 2-3 drops of Mayer's reagent is added to the sample solution. This leads to the formation of off-white precipitate, which alkaloids in the sample solution.[11,12]

Iodo-potassium iodide

The sample quantity of the sample solution is taken and to this 2-3 drops of iodo-potassium iodide is added, this leads to the formation of brick red which identified the alkaloids in the sample solution.[11,12]

Potassium Bismuth Iodide Test

Nearly, 2-3 drops of Potassium Bismuth iodide are added to the small quantity of extracted sample solutions, which gives orange red precipitate. This confirmed the presence of alkaloids.[11,12]

Iron trichloride test

About 5% of Iron trichloride solution is added to the sample solution, showed presence of tannins by formation of blue-green or blue-black colouration.[11,12]

Flavonoids screening

The leaves extract was treated with dilute HCl and boiled, formation of characteristic coloration confirmed flavonoids presence. [11,12]

Saponins screening

The leaf extract was added with four fractions of water and agitated. The formation of the froth layer at approximately 2 cm height for 5 min indicated the presence of saponin.[11,12]

Screening of Steroids

Around, 2 mL of trichloromethane and concentrated sulfuric acid was added to the neem extract, this leads to the formation of red colour at the bottom layer of trichloromethane, which confirms steroids in neem extract.

For further experimental analysis, concentrated sulphuric acid and concentrated acetic acid of 2 mL was also added sample, showed the presence of steroids by formation to a greenish colour.[11,12]

Screening of Glycosides (Keller-Killian Test)

For this analysis, two test tubes were taken in which one of the test tubes contains sample solution and 2 mL of concentrated sulphuric acid. The other test tube contains 2 mL pure ethanoic acid and 2% of ferric chloride, now this mixture is transferred into the sample containing the test tube, this leads to the formation of a ring at the mid layer of the test tube that confirms the presence of glycoside.[11,12]

Terpenoids screening

About 2 mL of trichloromethane and it is mixed with the neem extract and it is subjected to heat until it gets evaporated and dried. To this concentrated sulfuric acid was added, which formed the brick red colour at the mid region, confirmed terpenoids content in neem.[11,12]

Carbohydrates Screening

The sample solution of 2 mL was taken and to this Fehling's reagent A and B of 1mL of each are added. This solution is heated in a water bath for about 2 to 5 min. The colour formed was red brick, which indicated the presence of carbohydrates.[11,12]

Quantitative Assessment of Phytochemical compounds

Estimation of Total Phenolic Content (TPC)

About 1 mL of standard gallic acid solution (ranges from 10-100 µg/mL), both sample and blank were taken in different test tube. Folin-Ciocalteu reagent 10% (5 mL) and 20% sodium carbonate solution (4 mL) was mixed in each test tube and the mixture was maintained at controlled room temperature in dark for 30 min and the absorbance was recorded at 765 nm using a UV-visible spectroscopy. Expressed as mg Gallic Acid Equivalent per gram of extract (mg GAE/g).[13]

$$\text{Total Phenolic Content} = \frac{C \times V}{M}$$

C=Concentration from calibration curve.

V=Volume of extract.

M=Weight of extract.

Estimation of TFC (Total Flavonoid Content)

About 1 mL of standard quercetin (ranges from 50-400 µg), both sample and blank were taken in different test tube. Distilled water (4 mL) and 5% sodium nitrite (0.3 mL) was added. Nearly, 0.3 mL of 10% aluminium chloride was added. Followed by 2 mL of 1 M sodium hydroxide. The final volume was adjusted to 10 mL with distilled water. The absorbance was recorded at 510 nm using a UV-visible spectroscopy. Expressed as mg Quercetin Equivalent per gram of extract (mg QE/g).[14]

$$\text{Total Phenolic Content} = \frac{C \times V}{M}$$

C=Concentration from calibration curve.

V=Volume of extract.

M=Weight of extract.

In vitro Alpha-Amylase Inhibition Assay

Around 1mL of leaves extract (100-1000 µg/mL) was treated with 1 mL of α-amylase enzyme solution and it was incubated at 37°C for 30 min. Starch solution (1 mL of 1%) was added and further incubated for 5 min. The reaction was terminated by adding DNS reagent (dinitro salicylic acid) the mixture was heated in a boiling water bath for 5 min and it was cooled. The absorbance was recorded 540 nm. The reference standard used as Ascorbic acid (Vitamin C).[15]

Antioxidant Assay

Evaluation of DPPH Radical Scavenging Activity

Nearly 10 mg of the *Azadirachta indica* leaves extract was dissolved in 10mL ethanol for 1000 µg/mL stock solution. The dilution was done ranging from 50- 250 µg/mL. About 2 mL of plant extract solution (each concentration) was taken. To that mixture adding of 3 mL of DPPH solution and the mixture was maintained at controlled room temperature in dark for 30 min. The absorbance was determined by UV-visible spectroscopy. The reference standard used as Ascorbic acid (Vitamin C).[16]

$$\text{Percentage of inhibition} = \frac{Ac - As}{Ac} \times 100$$

Ac- Absorbance of the blank mixture.

As- Absorbance of the sample mixture.

Table 1: Phytochemical test for *Azadirachta Indica* crude extract with various solvent.

Sl. No.	Test	Hydro-Alcoholic Extraction Of Neem [*Azadirachta indica*] Leaves
1.	Alkaloid	
	A] Mayer's Test	++
	B] Iodo-potassium iodide	+
	C] Potassium Bismuth Iodide Test	+
2.	Tannins	++
3.	Flavonoids	+
4.	Saponins	++
5.	Steroids	++
6.	Glycosides	+
7.	Terpenoids	++
8.	Carbohydrates	-

Note: +++ highly present, ++moderately present, + mildly present, - absence.

Hydrogen Peroxide Scavenging Assay

At different concertation solvent extract (50-250 µg/mL) was prepared in distilled water. The extract (1000 µL) was added to the hydrogen peroxide solution (600 µL) and the mixture was maintained at controlled room temperature in dark for 30 min. The absorbance was determined by UV-visible spectroscopy. The reference standard used as Ascorbic acid (Vitamin C).[16]

$$\text{Percentage of inhibition} = \frac{Ab - As}{Ab} \times 100$$

Ab- Absorbance of the blank.

As- Absorbance of the sample mixture.

Chromatographic Analysis

TLC (Thin Layer Chromatography)

The neem leaf extract was mixed in methanolic solution, Quercetin Dihydrate as reference standard and mobile phase of Toluene: Ethyl Acetate: Methanol (80:10:10). Spotting the standard and sample on a TLC plate.[17]

HPLC (High Performance Liquid Chromatography)

Neem purity was assessed by the HPLC system with detection at 254 nm. HPLC analysis of neem was carried out using RP-C18 column acetonitrile: methanol: triethylamine with pH 4 (70:30:1). The chromatogram revealed two peaks corresponding to neem and a minor impurity. Results were validated by comparison with published spectral data.[18]

Spectral Analysis

Vibrational Infrared spectroscopy

The Vibrational Infrared spectroscopy was performed to determine the major functional groups. This is performed by KBr pellet method in which the sample was mixed and grounded with dry KBr powder and it was grinded finely at 1:100 ratio. The mixer was compressed with a hydraulic press which formed a transparent plate. The transparent pellet is placed in the instrument for the detection at the wavelength of 400-4000 cm^{-1}. The final spectrum was taken in %T.[19]

RESULTS

Extraction Yield Results

The course neem leaf powder 300 g was processed by cold maceration with 1.5 L of 70% hydroalcoholic solvent (ethanol: water) for period of 15 days with periodic stirring. Macerate was filtered using a cotton plug and finally with Whatman filter paper. The remaining filtered solvent was evaporated on water bath at 40°C until a solid residue was obtained. Thus, final product was highly hygroscopic greenish leaves extract powder with a 6.78 percent yield.

$$\text{Percentage yield} = \frac{\text{Practical yield}}{\text{Theoretical yield}} \times 100 = \frac{20.36}{300} \times 100 = 6.78$$

Preliminary components detection

The preliminary qualitative screening of hydroalcoholic extract of *Azadirachta indica* leaves outlined in Table 1 identified tannins, alkaloids, flavonoids, saponins, and terpenoids.

Quantitative Assessment of Phytochemical compounds

Estimation of Total Phenolic Content (TPC)

Quantitative phytochemical analysis of *Azadirachta indica* for polyphenolic compound was carried out by standard spectrophotometric methods and phenolic compounds were detected. By Folin Ciocalteu method the TPC were determined and expressed as Gallic Acid Equivalent (GAE) in mg/gm. The TPC was calculated with the help of the observational table shown in Table 2 and calibration curve depicted in Figure 1. The standard curve equation was:

$$y=0.008x+ 0.452, \text{ where } R2=0.9834$$

The total phenolic contents (GAE, in mg/g) in the sample were calculated to be 47 mg/g.

Estimation of TFC (Total Flavonoid Content)

The neem leaves extract was screened phytochemically, and confirmed the flavonoid compounds. The total flavonoid contents were analysed (by aluminium chloride method), using the Quercetin Equivalent (QE) in mg/gm of the sample. The total

flavonoid content was determined using observational Table 3 and standard calibration curve is shown in Figure 2 graph, and the equation was:

$$y=0.0126x+0.006, \text{ where } R2=0.9878$$

The total flavonoid contents (quercetin equivalent, at mg/gm) in the sample were found to be 5.2 mg/g.

In vitro alpha-amylase inhibition assay

The assay was calculated using by observational Table 4 and graph of Figure 3 the neem leaf extract exhibited a maximum enzyme inhibition of 77.77% at 500 µg/mL, suggest the antidiabetic potential of neem leaf extract by evaluating its ability to inhibit carbohydrate-digesting enzymes.

Antioxidant Assay

Evaluation of DPPH Radical Scavenging Activity

The assay of the hydroalcoholic extract of *Azadirachta indica* leaves calculated by using Table 5 and graph of Figure 4 it shown maximum inhibition of 47.05% at 250 µg/mL, indicating moderate antioxidant activity. The flavonoid and phenolic constituents identified in the leaves extract significantly contributes to its antioxidant activity.

Table 2: Observation of total phenolic content for standard and sample.

Concentration	Absorbance (Standard)	Absorbance (Sample)
µg/mL	nm	nm
20	0.55	0.65
40	0.60	0.77
60	0.67	0.81
80	0.78	0.92
100	0.86	0.99

Hydrogen Peroxide Scavenging Assay

In hydrogen peroxide scavenging assay, the hydro-alcoholic extract of *Azadirachta indica* leaves calculated by using Table 6 and graph of Figure 5 showed a maximum inhibition of 14.7% at 250 µg/mL, indicating mild antioxidant activity against reactive oxygen species.

Chromatographic Analysis

TLC (Thin Layer Chromatography)

During TLC analysis of *A. indica* leaf extract revealed a prominent spot with an R_f value of 0.46 corresponding to standard quercetin, confirming the presence of flavonoids as shown in Figures 6 and 7.

Mobile phase composition: Toluene, ethyl acetate, methanol (80:10:10)

Standard: Quercetin Dihydrate

$$Rf = \frac{Distance\ travelled\ by\ solute}{Distance\ travelled\ by\ solvent} = \frac{4.6}{10}$$

HPLC (High Performance Liquid Chromatography)

At 254 nm, HPLC evaluation revealed a major peak at a retention time of 2.897 min, corresponding to neem and accounting for 99.75% of the total peak area. A minor peak at 2.145 min (0.25%) likely represents trace impurity seen in Figure 8. The retention time and purity were consistent with literature standards, confirming that the sample meets the quality requirements for harmaline with a purity exceeding 99%.

Spectral Analysis

Vibrational Infrared spectroscopy

The results are illustrated in Figure 9. The FTIR spectroscopy showed absorption bands at 3400 cm^{-1} which corresponding to O-H stretch, 2925 cm^{-1} equivalent to C-H stretch, 1630 cm^{-1}

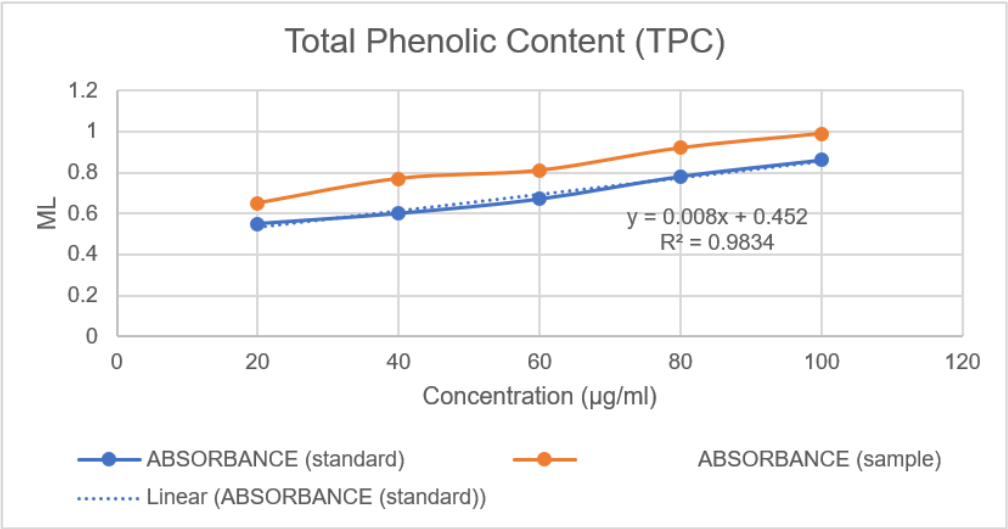


Figure 1: Graph of total phenolic content.

Table 3: Observation of Total Flavonoid Content for standard and sample.

Concentration (µg/mL)	Absorbance (Standard)	Absorbance (Sample)
µg/mL	nm	nm
50	0.02	0.03
100	0.03	0.04
150	0.04	0.07
200	0.06	0.08
250	0.07	0.09
300	0.08	0.12

Table 4: Observation of *In vitro* Alpha-Amylase Inhibition Assay for standard and sample.

Concentration (µg/mL)	% of inhibition (Standard)	% of inhibition (Sample)
µg/mL	(%)	(%)
100	11.11	22.22
200	33.33	33.33
300	44.44	44.44
400	55.55	66.66
500	77.77	77.77

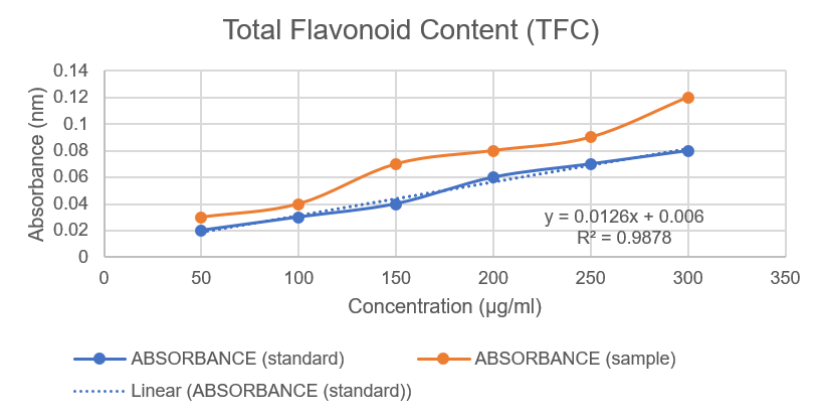


Figure 2: Graph of Total Flavonoid Content.

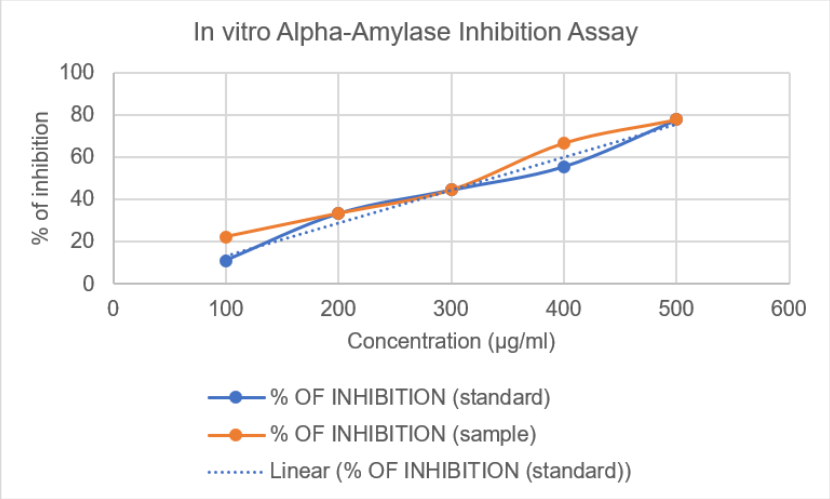


Figure 3: Graph of *in vitro* Alpha-Amylase Inhibition Assay.

representing C=O stretch and 1050 cm^{-1} corresponding to C-O stretch. These spectral peaks indicate the presence of phenolic, flavonoid compounds, and other secondary metabolites.

DISCUSSION

Azadirachta indica, commonly known as neem which, is a native tree for the Indian subcontinent that has many therapeutic benefits. It has several benefits from its leaves, fruits to root of the complete tree and have their own beneficial uses for curing many diseases in humans, from skin diseases and curing other internal circulatory or other organ related issues in the human body. There are many benefits from a neem tree such as, antimicrobial, antifungal, antiviral, antioxidant, antidiabetic and many more. The present study has focused on therapeutic benefits of neem leaves extract for antidiabetic and antioxidant activities. To support this study, few of the *in vitro* studies have been performed. Such as, TLC, DPPH assay, α-amylase inhibition assay, H_2O_2 assay, FTIR

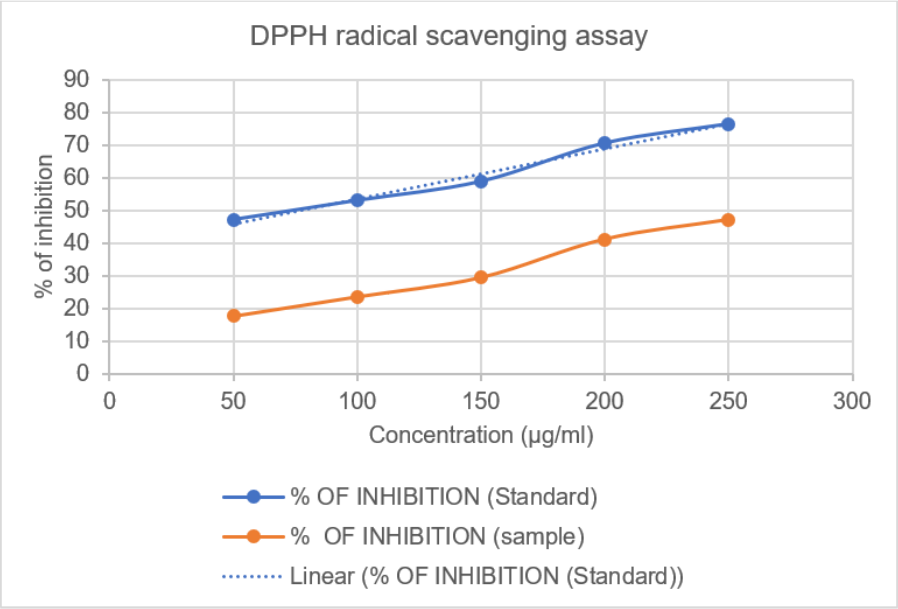


Figure 4: Graph of DPPH radical scavenging assay.

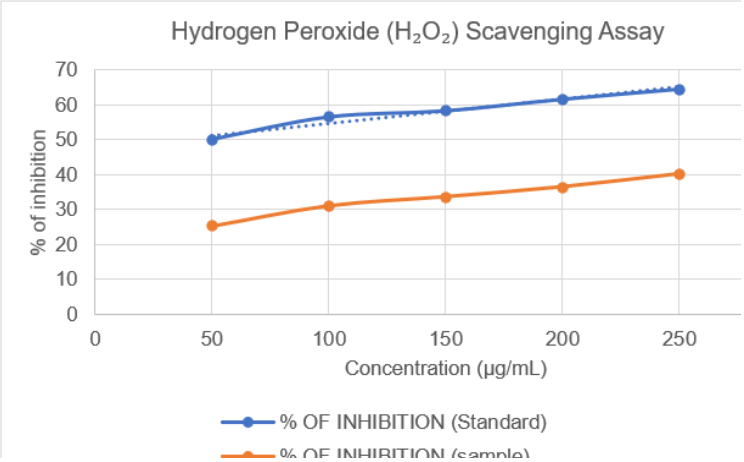


Figure 5: Graph Hydrogen Peroxide (H_2O_2) Scavenging.

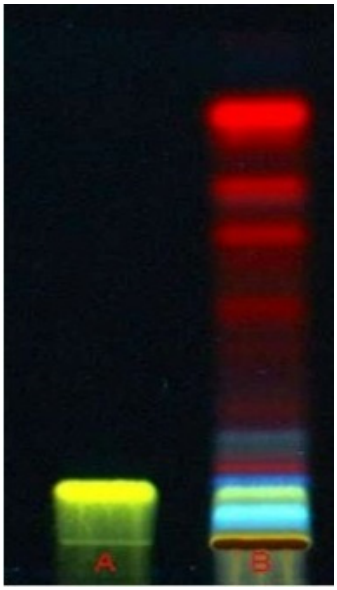


Figure 6: Natural Product Reagent. A- Reference compound Quercetin Dihydrate. B- Sample (Neem leaf extract).

Table 6: Observation of Hydrogen Peroxide (H_2O_2) Scavenging Assay for standard and sample.

Concentration (µg/mL)	% of inhibition (Standard)	% of inhibition (Sample)
µg/mL	(%)	(%)
50	50	25.26
100	56.49	31.05
150	58.26	33.65
200	61.61	36.48

Table 5: Observation of DPPH radical scavenging assay for standard and sample.

Concentration (µg/mL)	% of inhibition (Standard)	% of inhibition (Sample)
µg/mL	(%)	(%)
50	47.05	17.6
100	52.94	23.5
150	58.8	29.4
200	70.58	41.1
250	76.47	47.05

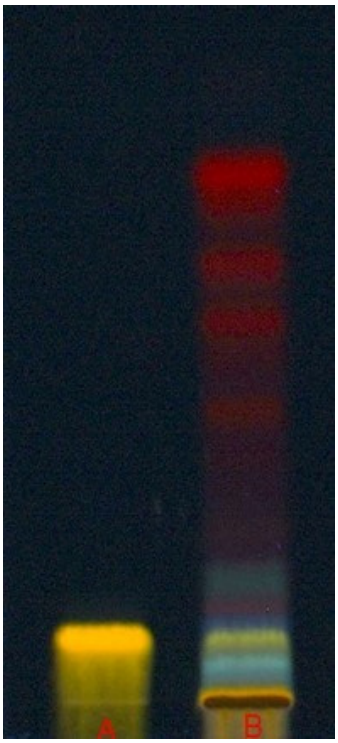


Figure 7: Natural product reagent +PEG. A- Quercetin Dihydrate Reference compound. B- Sample (Neem leaf extract).

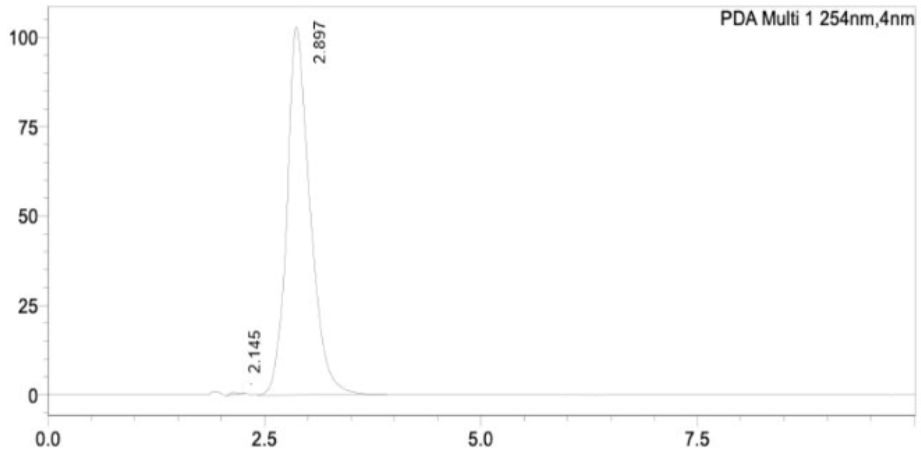


Figure 8: HPLC profile of *Azadirachta indica*.

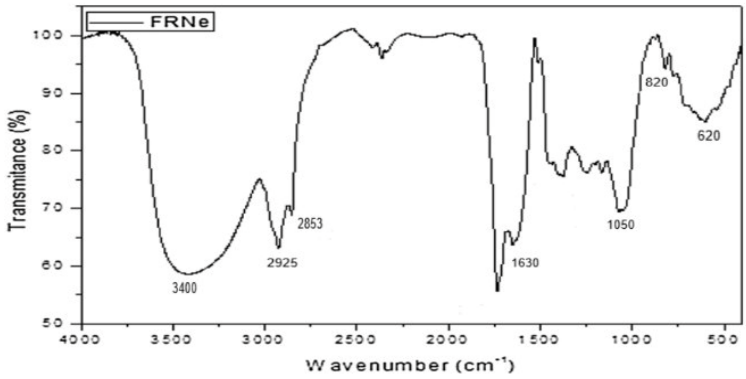


Figure 9: FT-IR profile of *Azadirachta indica*.

and HPLC, these *in vitro* studies confirmed that the neem leaves assess the antidiabetic and antioxidant activities. As the neem leaves contain major functional groups and enzymes that slow the absorption of glucose and are also effective in managing postprandial hyperglycaemia, etc. These studies and *in vitro* test results showed the natural benefits of the hydroalcoholic extract of neem leaves for the antidiabetic and antioxidant activity in human.

CONCLUSION

In conclusion, the hygroscopic greenish extract of neem leaves showed antidiabetic and antioxidant activities, this is confirmed by performing the *in vitro* analysis of the neem leaves extract. The phytochemical tests were performed to confirm the presence of specific components such as phenols, alkaloids, flavonoid, glycoside, saponins, steroid, terpenoids, and tannins, etc., which aids to the antidiabetic activity and α-amylase inhibition also confirmed the antidiabetic activity. TLC confirmed the quercetin content by comparing it with the quercetin standard. Chromatographic analysis of HPLC and FTIR was performed to confirm the presence of a major functional group and purity of neem. Therefore, *Azadirachta indica* leaves extract has therapeutic activity for diabetes management. The antioxidant activity is justified by DPPH and H_2O_2 assay that is neem leaves extract has the potential in reducing the oxidative stress in human body, which was confirmed by DPPH assay. It also showed the reduction in the hydrogen peroxide which leads to the protection against induced cell damage which was confirmed by H_2O_2 assay.

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ABBREVIATIONS

HPLC: High Performance Liquid Chromatography; **DPPH:** 2,2-Diphenyl-1-Picrylhydrazyl; **TLC:** Thin Layer Chromatography; **FTIR:** Fourier Transform Infrared Spectroscopy; **R_f:** Retention Factor; **WHO:** World Health Organization; **H_2O_2:** Hydrogen Peroxide; **DNS:** Dinitro salicylic acid; **TPC:** Total phenolic content; **TFC:** Total flavonoid content; **GAE:** Gallic acid equivalent; **QE:** Quercetin equivalent; **HCl:** Hydrochloric acid; **KBr:** Potassium bromide; **STZ:** Streptozotocin; **PDA:** Photodiode array; **PEG:** Polyethylene glycol.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

SUMMARY

The present study, that is analytical characterization and *in vivo* study of antidiabetic and antioxidant activity of hydroalcoholic extract of neem (*Azadirachta indica*) showed the potential benefits in the human body for its antioxidant and antidiabetic activities, to prove these certain analytical tests were performed, which showed the justifiable results that support the study on hydroalcoholic extract of neem. Such as TLC, HPLC, phytochemical tests, and assays. So, the present study confirmed the potential benefits that will reduce diabetes, oxidative stress and induced cell damage in the human body.

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