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Original Article

Preliminary Phytochemical analysis and *In vitro* Antioxidant, FTIR Spectroscopy, Anti-diabetic activity of *Acacia catechu* ethanolic seed extract

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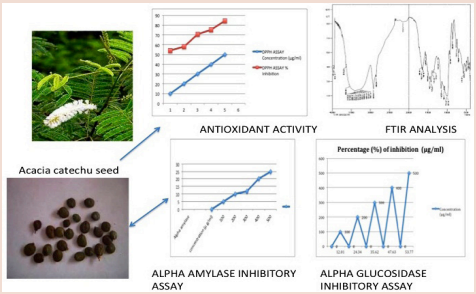
ABSTRACT

Objective: To evaluate the preliminary phytochemical analysis and *in vitro* antioxidant activity, anti-diabetic effect of ethanolic seed extract of *Acacia catechu* against the alpha amylase and alpha glucosidase digestive enzymes in the pancreas and small intestine. **Methods:** Preliminary phytochemical analysis was done by adopting the method of Evans. Antioxidant assay is performed by DPPH, ABTS and FRAP assay, Anti diabetic activity was determined by modified method of miller, the extract at different concentrations was tested for mammalian alpha amylase and alpha glucosidase enzyme inhibitory assay under the controlled experimental conditions and subjected to determination of absorbance. **Results:** The present study reveals the presence of few secondary metabolites and the extract exhibits potent Antioxidant activity and a concentration dependent inhibition of Alpha amylase and Alpha glucosidase. **Conclusion:** From the present study it can be concluded that ethanolic seed extract of *Acacia catechu* possessed marked *in vitro* antioxidant and anti-diabetic effect. The effect was plausibly due to the presence of phenolic contents of *Acacia catechu*.

Key words: *Acacia catechu* seed, Antidiabetic, Antioxidant, Alpha-amylase, Alpha glucosidase, FTIR Spectroscopy, Phytochemical.

SUMMARY

- *Acacia catechu* seed exhibited significant anti-oxidant and free radical scavenging activity.
- *Acacia catechu* seed extract inhibits alpha amylase and alpha glucosidase enzyme thus possessing anti-diabetic activity.
- *Acacia catechu* seed is used in management of diabetes mellitus.
- *Acacia catechu* seed exhibited significant anti-oxidant and free radical scavenging activity.
- FTIR analysis reveals the presence of the functional groups in *Acacia catechu* seed extract.



PICTORIAL ABSTRACT

Abbreviations used: DPPH: 2,2-diphenyl-1-picrylhydrazyl, TPC: Total phenolic content, TAC: Total Antioxidant content, TAE: Tannic acid equivalent, *A. catechu*: *Acacia catechu*, FRAP: Ferric ion reducing antioxidant power, ABTS: 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), FTIR: Fourier Transform Infrared Spectroscopy.

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INTRODUCTION

Antioxidants improve the quality of life by preventing the human body from various disorders like cancer, atherosclerosis, hypertension, cardiac failure, cognitive impairment, Alzheimer's disease which are caused by free radicals.[1] These free radicals which cause oxidative damage is inhibited by antioxidants in plants and animals. Antioxidants contain enzymatic and non enzymatic system which is used to scavenge the free radicals during oxidative stress.[2] Many plant extracts are rich in antioxidants like vitamin C, flavonoids, carotenoids, polyphenols which provides a nutritional value, and maintains proper physiological functions of the body.

Hyperglycemia in diabetes patients is of a serious concern today. Several factors may contribute to hyperglycemia in people with diabetes, including food and physical activity choices, illness, non-diabetic medications, or not taking enough glucose-lowering medication. It is essential to treat postprandial hyperglycemia, because if left untreated, hyperglycemia can become severe and lead to serious complications requiring emergency care, such as diabetic coma. In the long term, persistent hyperglycemia, even if not severe can lead to complications affecting vital organ functions of the body.

Management of hyperglycemia is achieved by the inhibition of carbohydrate hydrolyzing enzymes like alpha amylase and alpha glucosidase.[3] These are termed as digestive enzymes that are mainly involved in digestion of carbohydrates and aid in intestinal absorption. Inhibiting alpha amylase and alpha glucosidase is a major goal in controlling the post prandial increase in blood sugar level.[4]

Herbal products have been used widely for management of diabetes mellitus. Various literatures revealed the use of herbal extracts in treatment of insulin dependent diabetes mellitus.[5] Plant extracts rich in polyphenols possess good antioxidant activity and shows activity similar effects like insulin in the management of type 2 diabetes mellitus by inhibiting Pancreatic alpha amylase and Intestinal alpha glucosidase enzyme.[6] Alpha glucosidase inhibitors can be used as monotherapy in conjunction with an appropriate diabetic diet and exercise, or they may be used in conjunction with other anti-diabetic drugs.[7]

Acacia catechu belongs to the family *fabaceae* also known as black cutch and karungali in Tamil. In Kerala people drink it in boiling water to pre-

vent digestive problems. Various part of the plant leaves, bark, heartwood possess diverse pharmacological actions for management of various disorders.[8,9] The pharmacological activities in various parts of the plant has been extensively studied. Phytochemical constituents like Catechin, Epicatechin, Cyanidol, Quercetin, Epigallocatechin gallate, Rutin, Isorhamnetin, Taxifolin is found to be present in *A.catechu*.[10] The various part of the extract reports the antipyretic, anti-inflammatory, antidiarrheal, hypoglycemic, hepatoprotective, antioxidant and antimicrobial activities including anti caries and anti plaque activity.[11,12] Hence, in this study the antioxidant and anti diabetic activities of *Acacia catechu* ethanolic seed extract were measured systematically using different assays using a standard protocol and first time being reported so far.

MATERIALS AND METHODS

Plant material collection and extraction

Acacia catechu seeds were collected from Hosur, Tamil Nadu and were authenticated by Green Chem lab, Bangalore.

Chemicals and reagents

DPPH, BHA, Folin-Ciocalteu, Alpha glucosidase, Alpha amylase, Trypsin, Tris-Hcl buffer, P-nitro phenyl- glucopyranoside, dinitrosalicylic acid, starch were procured from Sigma Chemicals (USA). Other reagents like dimethyl sulfoxide (DMSO), sodium carbonate per chloric acid, sulphuric acid, sodium phosphate and ammonium molybdate were purchased from Merck (India).

Preparation of ethanolic extract

Seeds were shade dried for a week. Dried seeds were milled to fine powder. Powder was passed through 100 mesh sieve and stored in a sealed polythene bag 2.5 kg of powdered *Acacia catechu* seeds were extracted with 10 liters of Ethanol, at 65°C temperature, for 1 hour, in a 20 liter round bottom flask with Graham condenser attached. Condenser was cooled circulating with chilled water. After 1 hour of extraction, round bottom flask was cooled to room temp and the extract were filtered and collected. The Marc was extracted repeatedly with 10 liters of Ethanol, twice. The extracts were filtered and collected. The combined extracts was evaporated to dryness under reduced pressure in a Buchi Rotary Evaporator (Switzerland) at 65°C, to obtain 150 g of powder extract. The w/w yield of the prepared extract was 6%. The extractwere stored at 4°C until used.

Preliminary phytochemical analysis

The ethanolic extract so obtained from the dried seed powder of *Acacia catechu*, were tested for the presence and absence of the phytochemicals–Tannins, Phlobatannins, Saponins, Flavonoids, Terpenoids, Phenols, Cardiac glycosides and Steroids according to method described by Evans.[13] The results were depicted in Table 1.

Table 1: Preliminary phytochemical analysis of *Acacia catechu* ethanolic seed extract

Tannins	+
Saponins	+
Flavonoids	+
Phlobatannins	-
Phenols	+
Terpenoids	-
Cardiac glycosides	-
Steroids	-

+=Presence; -=Absence.

Total phenolic content

Folin–Ciocalteu method was followed for the determination of the total phenolic content of the plant extract. Distilled water (500 µl) and Folin–Ciocalteu reagent (100 µl) were added to 100 µl of the plant extract and incubated for 6 min at room temperature. The final volume was made up to 3ml after addition of 1.25 ml of 7% sodium carbonate. The absorbance was measured at 760 nm using UV–visible spectrophotometer (Cyber-Lab, USA) after an incubation period of 90 min. The total phenolic content was expressed as milligrams of tannic acid equivalents per gram of dry weight (mg TAE/g DW) of the plant, using a standard plot of Tannic acid.[14]

Total Antioxidant content

The total antioxidant activity was estimated by phosphor molybdenum method. To the plant extract (0.5 ml), a reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) of 4.5 ml was added and the solution was maintained in a boiling water bath at 95^{0}C for 90 min. The solution was cooled to room temperature and the absorbance was measured at 695 nm using UV–visible spectrophotometer. The total antioxidants in the plant were expressed as mg TAE/g DW of the plant extract.

Free radical DPPH scavenging assay

The ethanolic seed extract of *A. catechu* was taken at various concentrations (10, 20, 30, 40 and 50 µg/ml), in small tubes and made up to 1 ml using methanol. 1 ml of 0.01 mM DPPH dissolved in methanol was added to all the test concentrations and maintained in the dark for 30 min, at room temperature. The absorbance of the solutions was read at 517 nm. The percentage inhibition and the IC_{50} values were calculated with DPPH as the control and butylated hydroxyanisole (BHA) as the reference. The concentration in µg of dry material per ml of solvent (µg/ml) that inhibits the formation of DPPH radicals by 50% is defined as IC_{50} value.[15]

$$\% \text{ Inhibition} = \frac{(\text{Absorbance of the control (Ac)} - \text{Absorbance of the sample (As)})}{\text{Absorbance of the control(Ac)}} \times 100$$

Ferric Reducing antioxidant power (FRAP) assay

1 ml of plant extract, 2.5 ml phosphate buffer (of 0.2 M, pH 7) and 1% potassium ferricyanide (2.5 ml) were mixed and incubated at 50°C for 30 min. To the solution, 2.5 ml of 10% trichloroacetic acid was added and centrifuged at 6500 rpm for 10 min. Distilled water (2.5 ml) and 0.5 ml of 0.1% $FeCl_3$ were added to 2.5 ml of the supernatant. The absorbance of the solution was measured at 700 nm using UV–visible spectrophotometer. The reducing ability of the plant was evaluated in terms of percentage by relating to the standard, $FeSO_4$·

$$\% \text{ Inhibition} = \frac{(\text{Absorbance of the control (Ac)} - \text{Absorbance of the sample (As)})}{\text{Absorbance of the control(Ac)}} \times 100$$

[2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] ABTS assay

A solution of 7 mM ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)] and 2.45 mM potassium persulphate was incubated in the dark for 12–16 h, after which the solution was diluted with ethanol till the absorbance reached 0.7 ± 0.02 at 734 nm. 1 ml of the diluted solution was mixed with 100 µl of plant extract and the absorbance was evaluated at 734 nm after 6 min. The percentage reduction against ABTS was calculated with reference to the standard, Tannic acid.

FTIR spectroscopy[16]

The infrared spectrum was recorded from KBr discs on a FTIR Perkin Elmer model 16 PC spectrophotometer (Perkin Elmer, Waltham, USA). Absorption maxima ($\check{H}_{max}$) were reported in wavenumbers (cm^{-1}). The spectra were used to determine organic groups indicative of the main classes of secondary metabolites. It reveals the presence of Hydroxyl, Alkanes, Amides, Alkyl halides, Aliphatic amines, Nitro groups.

Anti diabetic activity

α-Amylase inhibitory assay

The α-Amylase (0.5 mg/ml) was premixed with extract at various concentrations (100-500 µg/ml) and starch azure as a substrate was added as a 0.5% starch solution to start the reaction. The reaction was carried out at 37°C for 5 min and terminated by addition of 2 ml of DNS (3,5-dinitrosalicylic acid) reagent. The reaction mixture was heated for 15 min at 100°C and diluted with 10 ml of distilled water in an ice bath. α-amylase activity was determined by measuring at 540 nm using spectrophotometer (Perkin Elmer Lambda 25 UV-VIS). A control reaction was carried out without the test sample. The experiments were repeated thrice using the same protocol.

The % α-amylase inhibitory activity is calculated by the following formula;

$$(\%\ \text{Inhibition}) = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \times 100$$

The IC_{50} value was defined as the concentration of the sample extract to inhibit 50% of alpha amylase activity under assay condition.

In vitro inhibition of α-glucosidase

The enzyme α-glucosidase inhibitory activity was determined by premixing α-glucosidase (0.07 Units) with 100-500 µg/ml of extract. Then 3 mM p-nitro phenyl glucopyranoside was added as a substrate.[17] This reaction mixture was incubated at 37°C for 30 min and the reaction was terminated by addition of 2 ml of sodium carbonate. The α-glucosidase activity was determined by measuring the p-nitro phenyl release from p-nitro phenyl glucopyranoside at 400 nm. A control reaction was carried out without the test sample. The % α-glucosidase inhibitory activity is calculated by the following formula.

$$(\%\ \text{Inhibition}) = \frac{\text{Control OD-Sample OD}}{\text{Control OD}} \times 100$$

The IC_{50} value was defined as the concentration of the sample extract to inhibit 50% of alpha glucosidase activity under assay condition.

Statistical analysis

Experimental values are expressed as mean ± SD. Values are means of three independent analyses of the sample ± standard deviation (n=3). Independent Sample t-test was carried out for statistical comparison. Statistical significance was considered to be indicated by a *p value<0.05 in all cases.

RESULT AND DISCUSSION

Determination of total phenolic content (TPC), and total antioxidant content (TAC)

TPC and TAC of *Acacia catechu* seed extract were determined in terms of tannic acid equivalent (TAE). TPC with folin-ciocalteu method was reported as 47.32 ± 0.29 mg TAE/g DW, TAC with phospho molybdenum method was reported as 20.07 ± 0.23 mg TAE/ DW of the plant.[18] Table 2 represents the results of TPC and TAC recorded.[18]

Table 2: TPC and TAC of the ethanolic seed extract of *Acacia catechu*

***Acacia catechu* ethanolic seed**	
Total phenolic content (mg TAE/g DW)	47.32 ± 0.29 mg TAE/g DW
Total antioxidant content (mg TAE/g DW)	20.07 ± 0.23 mg TAE/ DW

Values are mean ± SD of triplicate samples.

DPPH free radical scavenging assay

The free radical scavenging ability of the ethanolic seed extract of *Acacia catechu* was carried out using DPPH. The IC_{50} value of the extract was recorded as 10-µg/ml. herbal extracts with IC_{50} values above 30 µg/ml are generally considered as non antioxidants.[19] Table 3 shows the results of DPPH free radical scavenging activity of *Acacia catechu* seed extract tested at different concentrations.

Table 3: 2,2-Diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging assay of *Acacia catechu* ethanolic seed extract

Concentration (µg/ml)	% Inhibition
10	54.11
20	57.89
30	71.00
40	75.56
50	84.44
IC_{50} (µg/ml)	
Sample	<10
BHA	25.78

The present study reveals that ethanolic seed extract of *Acacia catechu* of the Asian region showed better free radical scavenging activity (10 µg/ml). This might be due to the presence of strong biologically active components such as phenols and polyphenols, etc., which show remarkable activity against free radicals.[20] Lower the IC_{50} value higher will be the antioxidant free radical scavenging ability. When compared with BHA, the plant showed better IC_{50} value and this can be potentially used as a natural antioxidant.

Ferric reducing antioxidant power (FRAP) assay

In FRAP assay, the complex containing ferric ions is converted to ferrous ions due to the action of reducing agents antioxidants, thereby generating chromogenic complex.[21] The present study reveals the ability of *Acacia catechu* ethanolic seed extract to reduce the ferric ions (52.36%).

2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay

Stable radicals of ABTS are generated when ABTS is mixed with potassium persulphate and incubated under dark conditions. Spectrometric evaluation at 734 nm (the characteristic wavelength for the ABTS radicals), showed that the extract scavenged the radicals to a higher extent (97.93%).

The antioxidant assays performed represent the potential inhibition of the products of lipid oxidation as well as the ABTS radicals by *Acacia catechu* ethanolic seed extract. However, the plant exhibited low activity against the ferric ions in the FRAP assay. The differences in the different assays performed might be attributed to the different mechanisms involved.[22]

The results examined for DPPH free radical scavenging assay is presented in Figure 1. FRAP and ABTS assays is presented in Figure 2. TAE and TPC is presented in Figure 3.

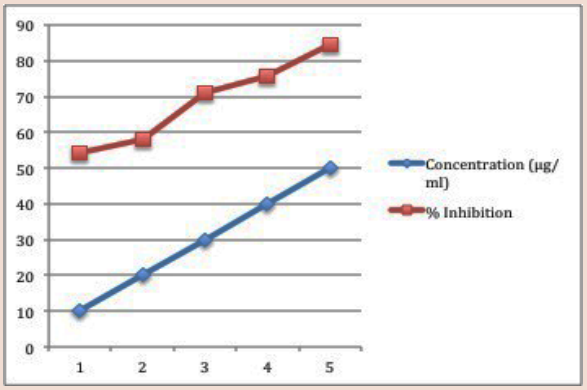

Figure 1: DPPH Assay of *Acacia catechu* seed

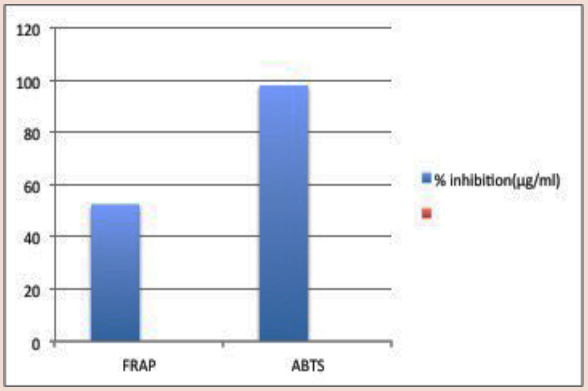

Figure 2: FRAP, ABTS Assay of *Acacia catechu* seed

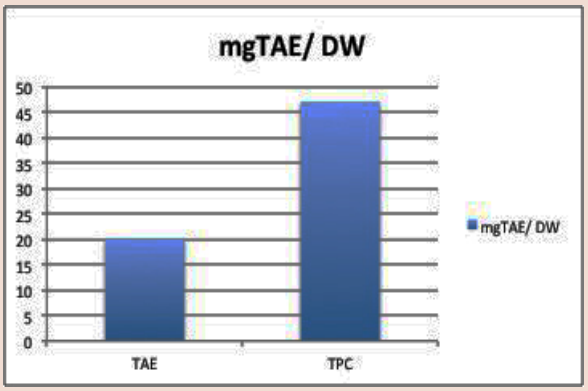

Figure 3: TAE, TPC of *Acacia catechu* seed

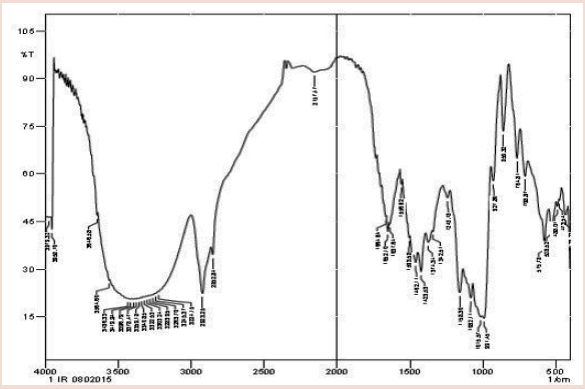

Figure 4: FTIR Spectroscopy of *Acacia catechu* seed

Table 4 :FTIR Spectroscopy

Peak	Intensity	Corr.Inte	Base (H)	Base (L)	Area	Corr. Area
400.25	30.214	1.603	413.75	399.28	6.078	0.143
428.22	46.399	0.882	430.14	419.54	3.387	0.016
473.54	51.666	0.981	476.44	460.04	4.502	0.099
488.01	49.81	1.819	495.73	477.4	5.414	0.162
528.52	43.342	6.47	545.88	496.69	16.304	1.566
575.78	38.538	8.443	593.14	546.84	16.626	1.611
708.87	89.247	8.52	742.63	689.58	10.094	1.059
764.81	64.946	14.775	821.71	743.59	8.802	2.748
859.32	73.411	18.873	877.65	822.68	4.503	2.738
927.8	57.81211.707	944.2	878.61	9.745	1.843	
991.45	14.544	11.34	1004.96	945.16	34.709	4.779
1015.57	15.013	1.846	1067.65	1005.92	46.399	2.759
1082.11	20.966	5.457	1135.16	1068.61	40.036	2.904
1158.3	22.625	13.69	1224.85	1136.12	40.274	5.71
1243.18	52.483	2.884	1274.04	1225.82	12.881	0.574
1342.51	41.882	1.225	1347.34	1275	21.772	0.142
1374.34	38.458	3.248	1391.7	1348.3	17.304	0.861
1423.53	29.187	8.55	1444.75	1392.66	24.024	2.292
1462.1	32.078	2.936	1481.39	1449.57	15.098	0.574
1503.58	39.917	1.715	1536.37	1499.72	12.912	0.451
1556.62	56.783	2.348	1567.23	1551.8	3.676	0.201

1637.64	44.309	0.283	1638.6	1585.55	15.572	0.132
1652.1	41.91	3.613	1661.75	1645.35	5.953	0.368
1664.64	46.523	0.912	1692.61	1622.71	9.022	0.349
2157.47	92.062	0.863	2222.09	2128.54	3.055	0.2
2852.84	34.601	3.55	2863.45	2359.04	82.066	0.838
2923.25	22.368	18.908	3002.33	2864.41	66.342	13.934
3224.15	22.948	0.438	3228.01	3003.3	110.894	3.084
3245.37	22.401	0.098	3248.27	3228.98	12.442	0.025
3263.7	21.967	0.212	3271.41	3249.23	14.521	0.041
3283.95	21.705	0.211	3290.7	3272.38	12.11	0.043
3303.24	21.48	0.914	3310.95	3291.67	12.832	0.036
3322.53	21.253	0.185	3328.31	3311.92	10.989	0.034
3340.85	20.991	0.209	3347.6	3329.28	12.368	0.039
3359.18	20.724	0.247	3366.89	3348.57	12.472	0.049
3378.47	20.616	0.189	3386.18	3367.86	12.529	0.04
3396.79	20.564	0.186	3407.4	3387.15	13.87	0.038
3419.94	20.564	0.192	3429.58	3408.36	14.532	0.043
3436.33	20.724	0.058	3450.8	3434.4	11.169	0.012
3564.6	26.539	1.188	3641.76	3560.75	37.873	1.515
3646.58	46.935	2.683	3667.8	3642.73	7.202	0.301
3958.1	42.491	9.95	3960.03	3944.6	3.073	0.561
3979.32	46.265	0.227	3988.96	3970.64	6.114	0.019

Table 5 : Alpha Amylase Inhibitory Assay

Concentration (µg)	Percentage activity (%)
100	4.71 ± 0.86
200	9.23 ± 0.33300
300	12.99 ± 0.46
400	19.02 ± 1.82
500	25.05 ± 1.18
IC_{50} *A.catechu*=341.20 ± 15.30	

FTIR spectroscopy

FTIR Spectroscopy emphasize the presence of the following functional groups present in *Acacia catechu* ethanolic seed extract at 3400 peak OH group, 2923 and 2852 C-H stretching vibrations that are mainly generated by lipids, 2157 peak generates Carboxylic acids, 1652 C=O (Amide), 1556 Asymmetric stretching for aliphatic and aromatic nitro compounds, 1462, 1374 peak generates Alkanes, 1423 N-H, 1243, 1082 peak generates alkyl halides, 1158 peak aliphatic amines are present, at 1015 and 991 C–O stretch (Esters) are seen.

The peaks generated in FTIR spectroscopy were depicted in Table 4 and Figure 4.

In vitro Anti diabetic activity

Treatment of diabetes in patients with insulin-dependent and non-insulin-dependent diabetes, diabetic retinopathy, diabetic peripheral neuropathy was made successful using lots of herbal products.[23] From the reports on their potential effectiveness against diabetes, it can be assumed that the herbal remedies have a remarkable role to play in the management of diabetes, which needs further exploration for necessary development of drugs and nutraceuticals from natural resources.

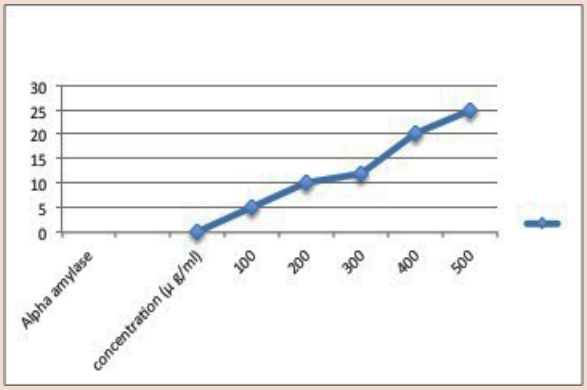

Figure 5: Alpha amylase inhibitory assay of *Acacia catechu* seed

Alpha amylase and Alpha glucosidase inhibitory assay

Acacia catechu commonly known as black cutch is used for treatment of diabetes and management of pain and inflammation by the traditional medicinal practitioners of Bangladesh.[24] *Acacia catechu* seed extract when tested *In vivo* in normal albino rats in combination of other extracts exhibited significant Anti-diabetic activity.[25] Synthetic α-glucosidase inhibitors are used widely but may cause severe gastro intestinal disturbances,[26] hence the use of natural products have raised to overcome the side effects caused by synthetic drugs.

The *in vitro* anti-diabetic study was carried to assess the α-glucosidase and α-amylase inhibitory activity of the extract to control postprandial hyperglycemia in Type-2 diabetes mellitus patients.[27]

The current study suggest that the presence of polyphenolic compounds of *Acacia catechu* ethanolic seed extract may have a prominent role in managing Type-2 diabetes. The α-glucosidase and α-amylase inhibitory activity of *Acacia catechu* ethanolic extract, 53.77 ± 0.86% and 25.05 ±

Table 6: Alpha Glucosidase inhibitory assay of *Acacia catechu* seed

Concentration (µg)	Percentage activity (%)
100	12.01 ± 3.83
200	24.34 ± 6.49
300	35.62 ± 3.03
400	47.63 ± 4.22
500	53.77 ± 0.86
IC_{50} A.catechu=187.80 ± 4.15	

Values are mean ± SD of triplicate samples.

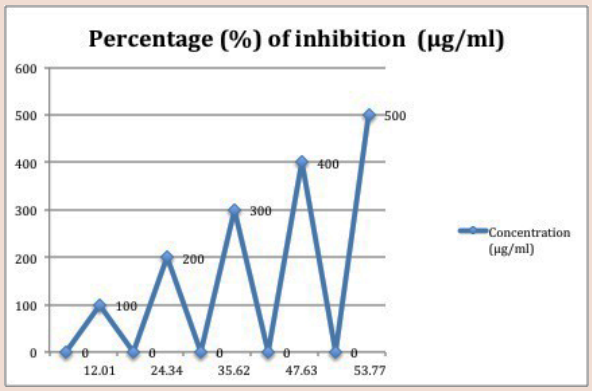

Figure 6: Alpha glucosidase inhibitory assay of *Acacia catechu* seed

1.18% was confirmed in this study at a concentration of 500 µg/ml. IC_{50} value for inhibition of α-glucosidase activity 187.80 ± 4.15 µ g/ml; IC_{50} value for inhibition of α-amylase activity 341.20 ± 15.30 µ g/ml. Lower the IC_{50} value better the inhibition activity of the extract tested. Values are expressed as mean ± SD. It was noted that the α-glucosidase activity is comparatively better than α-amylase activity.

The percentage inhibition of α-glucosidase activity by ethanolic extract of *Acacia catechu seed* is shown in Table 5 and Figure 5. The percentage inhibition of α-amylase activity by ethanolic extract of *Acacia catechu seed* is shown in Table 6 and Figure 6.

CONCLUSION

Inhibition of α-glucosidase and α-amylase enzyme activity leads to a reduction in disaccharide hydrolysis which has beneficial effects on glycemic index control in diabetic patients and can reduce the incidence of post prandial hyperglycemia. As the plant extract was reported for having anti-diabetic activity *in vivo*, its activity was evaluated *in vitro*. In conclusion, the present study indicates that *Acacia catechu* ethanolic seed extract shows prominent antioxidant and anti diabetic activity, The Antidiabetic activity reported is due to the presence of polyphenols in the tested plant extract, and thus the plant extract may provide the biochemical rationale for further animal and clinical trails.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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