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In-vitro and *In-vivo* Inhibition of Postprandial Hyperglycemia (Type II Diabetes) by Use of *Adhatoda vasica* in Wistar Rats

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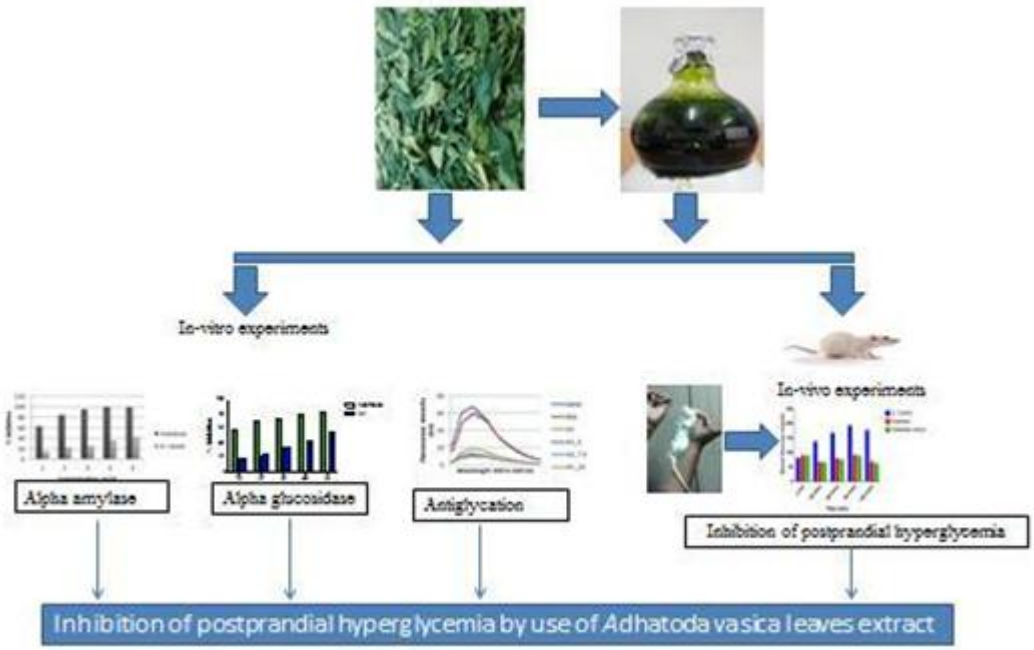
Keywords: *Adhatoda vasica*, α-amylase, α-glucosidase, DPPH, anti-glycation, postprandial hyperglycemia, type II diabetes

ABSTRACT

Adhatoda vasica (L.), commonly known as malabar nut, and locally known as adulsa. In the traditional system it has been known for its medicinal properties. It is commonly used in the treatment of cold, cough, fever, stomachache, asthma and tuberculosis. The present study was conducted to investigate the possible effect of *Adhato*da *vasica* leaves extract for inhibition of postprandial hyperglycemia in wistar rats. Phytochemical analysis, free-radical scavenging activity (DPPH), α- amylase inhibition, α- glucosidase inhibition, anti-glycation assay were selected for this study. Experimental study was conducted on wistar rats. Phytochemical screening revealed the presence of phytoconstituents like phenols, flavonoids, saponins, terpenoids and tannins. Total phenolic content present is 260µg/ml. The extract exhibit good free radical scavenging activity (73% to 78%). The extract exhibited α-amylase and α-glucosidase inhibitory activity with (15.25-43.22%) and (20-58%), when compared with Acarbose (99%). The extract showed potent antiglycation activity with inhibition of glycated BSA (upto 92%). Experiment in the wistar rats revealed that *Adhatoda vasica L.* extract given to wistar rats via oral route at 50 mg/kg b.w. produced significant inhibition of postprandial hyperglycemia. Together, *in-vitro* and *in-vivo* results suggest that *Adhatoda vasica L.* extract inhibited the postprandial hyperglycemia (type II diabetes) and is related to its ability to inhibit α-amylase, α- glucosidase, and AGEs production.

Graphical abstract

The components of *Adhatoda vasica* induced inhibition of postprandial hyperglycemia (type II diabetes) and it is possibly related to its ability to inhibit free radical (DPPH), α-amylase, α-glucosidase and antiglycation activity.



Keywords

Adhatoda vasica, α-amylase, α-glucosidase, DPPH, anti-glycation, postprandial hyperglycemia, type II diabetes.

INTRODUCTION

Modern lifestyle is a major cause of many diseases, especially diabetes. Diabetes is the worldwide **[1]**, multifactorial syndrome. Diabetes is a problem of every 2nd person in this world as compared to any other disease. India is a world capital of diabetes, especially type II diabetes. Indian people have more body fat at each BMI as compared to western people. Diabetes is a metabolic disorder in which person is suffering from abnormally high blood glucose level, it causes complications like neuropathy, retinopathy and cardiovascular diseases **[2]**. The hallmark of type II diabetes mellitus is insulin resistance as well as pancreatic β-cell dysfunction **[3]**. Postprandial hyperglycemia (PPHG) is associated with earlier detectable abnormalities in diabetes mellitus **[4].** Indian population eat diet which has higher content of carbohydrates, and this have been held responsible for the increased incidences of postprandial hyperglycemia in Indians around the world. Treating diabetes is economically a problem for the globe, and as a result it needs to be paid attention globally.

It is learned that postprandial hyperglycemia increases the oxidative stress and free-radicals which is a major cause of many chronic diseases **[5**]. Recently, it has been reported that oxidative stress and free-radicals is responsible for the patho-physiological link between cardiovascular disease (CVD) and diabetes and also related to the complications caused due to diabetes **[6].**

Therefore, inhibition of postprandial hyperglycemia and scavenging excessive free radicals leading to the consequent reduction in oxidative stress holds promise in reducing the risks responsible for the development of diabetes and diabetic complications **[7].**

With the acquaintance and the fact that „AGEs‟ (advanced glycation end-product) can lead to many chronic diseases, rise in the „AGEs‟ will lead to many harmful diseases which after many years will become chronic **[8].** There are many worldwide diseases, India is a hub of medicinal plants, and our conviction for positive health effects of medicinal plants has been documented by our ancestors, who have used several plants with medicinal properties for the treatment of various diseases. Allopathic drugs have been used immensely which also causes various side effects. Therefore, medicinal herbs and botanicals now appear to replace them at a fast pace like ever before. In the recent years, there has been tremendous research in the medicinal properties of plants for their use to cure chronic diseases. The history of medicinal plants is very impressive

with respect to the development of mankind. Driven by these studies, worldwide the herbal industries now appear to be a major source of wide variety of herbal medicines.

Adhatoda vasica

Adhatoda vasica is also known as Malabar nut or Adulsa in Indian Ayurveda. *Adhatoda vasica* (Acanthaceae) distributed throughout India up to an attitude of 1300 m.The leaves, flowers, fruit, and roots are extensively used for treating cold cough, whooping cough, chronic bronchitis and asthma as sedative, expectorant and antispasmodic **[9]**.

Over many years, every part of *Adhatoda vasica* has been used in Ayurveda. The leaves of are tend to show antibacterial, antifungal, antihypertensive, antiallergic, antitussive, antispasmodic properties. Greatly influenced by such research the use of herbal medicine is increasing day by day and is thought to increase in near future. In Ayurvedic medicine the *Adhatoda vasica* has been used for a long time and the long history of *Adhatoda vasica* shows that it is moderately safe to use it as a medicine for the treatment of type II diabetes.

In this present study, antioxidant scavenging, inhibition of two major carbohydrate hydrolyzing enzymes i.e. alpha amylase and alpha glucosidase as well as anti-glycation activity of the methanol extract of *Adhatoda vasica* leaves was analyzed. Also the effect of methanol extract on starch-induced postprandial hyperglycemia in Wistar rats was estimated.

2. MATERIALS AND METHODS

2.1 Chemicals

Chemicals used in this study were of high purity grade and purchased from Sigma-Aldrich chemicals (St Louis, MO USA), Merck Limited (Mumbai, India) and S.D. Fine Chemicals Ltd (Mumbai, India).

2.2. Collection of plant material

The leaves of *Adhatoda vasica* were collected from the Bhima Shankar ghat region of Sahyadri hills in Maharashtra (India) in the month of January. Herbarium specimen of these plants was submitted to Botanical Survey of India (Pune) for authentication. BSI Pune has authenticated this

plant as *Adhatoda vasica.*

2.3. Preparation of plant leaves extract

The collected *Adhatoda vasica* plant leaves were shade-dried for about 7 days, finely crushed and ground into powered form. 300 gm of plant were extracted with 100% methanol at room temperature for 24 hrs. The extract was filtered with fresh cotton bed and 300 ml of solvent extract was collected in a separate container and concentrated in the reduced pressure below 50psi in a rotary evaporator vacuum. The filterate was concentrated with a rotary evaporator at low temperature (40º- 50ºC) and reduced pressure to provide *Adhatoda vasica* extract.

2.4. Preliminary Phytochemical Screening of *Adhatoda vasica* extract

The extract was tested for the presences of phytochemical such as Tannins, Flavonoids, Terpenoids, Alkaloids, Saponins, Phenols **[10] [11]**.

2.5. Determination of free radicals scavenging antioxidant potentials

2.5.1. DPPH radical scavenging activity:

The extract was evaluated for the scavenging of free radical, DPPH (2, 2-diphenyl-1-picrylhydrazyl) was used as a substrate with slight modification**[12]**.Briefly, the sample was prepared in methanol at various concentrations (25 - 1000µg/ml) in a 96-well microplate. The 100 µl DPPH solutions (0.5mM in methanol) were added to each well in dark on ice and it was incubated for 15 minutes. Reaction mixture was shaken well and absorbance was taken at 517 nm by 96-well plate reader. The control was prepared without plant extract. The percentage scavenging activity of DPPH was determined by using the following formula:

$$\frac{[\text{Absorbance control} - \text{Absorbance test}]}{\text{Absorbance control}} \times 100$$

2.6. Determination of total protein content

Protein content in the *Adhatoda vasica* methanol extract was determined using Bradford‟s dye **[13]**. Briefly, 10 mL of extract was mixed with 200 mL of 1 x Bradford reagent and absorbance was read at 595nm on a BioTek synergy4 multi-mode micro plate reader (BioTek Instruments,

Inc. Winooski, VT, USA). Protein concentration (mg/mL) was expressed using BSA standard curve ($y = 0.066x - 0.01$, $R^2 = 0.994$).

2.7. Determination of total phenolic content

Phenolic content in the *Adhatoda vasica* methanol extract was determined using Folin-Ciocalteu reagent with slight modification **[14]**. Briefly,1mg/ml of plant extract was prepared in methanol and diluted with 150 µl of milli-Q water and added to 96-well microplate, followed by 10µl of Folin-Ciocalteu reagent (1 M) and it was kept for 10 minutes incubation after incubation 30µl of Sodium carbonate (7.5%) was added. Reaction mixture was incubated in dark for 120 minutes and total Polyphenols content was measured at 765 nm by 96-well plate reader (BioTek synergy4 multi-mode microplate reader, BioTek Instruments, Inc Winooski, VT, USA). Results were expressed in terms of gallic acid equivalent per ml ($y = 0.0062x + 0.0389$, $R^2 = 0.999$).

2.8. α-Glucosidase inhibition assay

Inhibition of rat intestinal α-glucosidase enzyme was done by method as reported earlier **[12]**. Briefly, 50µl of plant extract (10 mg/ml in DMSO) with 100µl of α-glucosidase enzyme is incubated for 10 minutes in 96 well microplate, after the incubation 50 µl of substrate (5mM, p-nitrophenyl α-Dglucopyranoside in 100mM phosphate buffer pH, 6.9) was added. The reaction mixtures were incubated for 5 minutes at 25^{o}C .Release of p-nitrophenyl is measured at 405nm by 96 well micro-plate reader (BioTek synergy4 multi-mode microplate reader).The percentage inhibition is measured by using following formula mentioned above.

2.9. α-amylase inhibition assay

The assay was performed with slightly modification. Briefly, the assay mixture containing 200 µl of 0.02M sodium phosphate buffer pH 7.0, 20µl of enzyme and the plant extract in concentration range 10-50µg/ml were incubated for 15 minutes at room temperature followed by addition of 200µl of starch in all eppendorf tube. The reaction was terminated with the addition of 500µl DNSA (3,5-dinitro salicyclic acid) reagent and placed in boiling water bath for 5 minutes, cooled and absorbance was measured at 540 nm. The control samples were prepared without plant extract. The percentage inhibition was calculated according to the formula **[15].**

$$\text{Inhibition (\%)} = \frac{[\text{Abs540 (control)} - \text{Abs540 (extract)}]}{\text{Abs540 (control)}} \times 100$$

Acarbose was used as the standard and the test was performed in triplicates.

2.10. *In-vitro* glycation of protein

Glycation of BSA protein was performed as described earlier in sterilized eppendorf tubes with slight modifications **[16]**. Briefly, BSA glycation reaction was carried out by incubating 1ml of 50mg/ml BSA in 0.1 M phosphate buffer (pH 7.4) and 0.5M dextrose monohydrate containing 5mM sodium azide as bacteriostat at 37°C for 7 days with extract and 15mM of above mentioned compounds. 30% DMSO and extract were used as positive control respectively. The BSA glycation was monitored for excitation at 330 nm and emission at 440 nm by using a spectrofluorometer (Thermo, Varioskan Flash Multimode Reader).Percentage inhibition of glycation was calculated by using the formulae;

$$\frac{(C-T) \times 100}{C}$$

Where C is the relative fluorescence intensity of glycated BSA in absence of an inhibitor and T is the relative fluorescence intensity of glycated BSA in presence of an inhibitor.

2.11. ANIMAL EXPERIMENT

For the postprandial glycemia test, animal experiments were performed with the male adult Wistar rats (180 to 250 gm body weight). Approval of experimental protocol was obtained from Institutional Animal Ethical Committee (SSBS/IAEC/2/4.3.2015).Experiments in live animals were performed in compliance with relevant laws and institutional guidelines. Experiments were carried out with the method reported earlier **[17]**.18 Wistar rats were randomly divided into three experimental groups after taking their body weight (Marked as Group I to III), where six rats was taken in each group. All animals were kept for overnight fasting. Diabetes was induced in one group (Group 1) of rats by oral administration of starch (2gm/kg body weight). The next day forenoon blood was collected from the retro orbital plexus in EDTA containing tubes. Blood glucose levels for the basal ("0" hour) value were measured by glucose- oxidase test method using auto- blood analyzer instrument (Bayer Express Plus, NY, USA). Rats were divided into

various groups (six rats in each group) as follows:

Group I (Diabetes Control): Diabetes rats received only distilled water followed with starch.

Group II (Positive Control): Diabetic rats body weight received reference antidiabetic drug Acarbose followed with starch (50 mg/kg).

Group III (Sample Treated): Diabetic rats treated with methanol leaves extract of *Adhatodavasica* at the rate of 50mg/kg body weight followed with starch.

Methanol extract of sample (*Adhatoda vasica*) were suspended in normal saline and administered to the respective group of animals at a random dose of 250 mg/kg body weight. Control group of animals were administered with normal saline. After 15mins of normal saline or extract administration, animals were fed with soluble- starch dissolved in normal saline at a dose of 2 gm/kg body weight.

Thereafter, blood samples were collected at intervals of 30 th, 60 th, 90 th and 120th mins post starch feeding. Blood glucose levels were estimated after 2 hrs at time intervals of 0, 30 th, 60 th, 90 th and 120 th minutes of drug and extract administration.

2.12. Statistical Analysis

Two analysis of variance followed by Turkey‟s multiple comparison tests was applied to compare the difference in animal study groups. Suitable regression analysis was applied to find correlations between the related parameters. The criterion for statistical significance was $P < 0.05$. Statistical analyses were performed by using GraphPad PRISM® Version 6.01 (GraphPad software, Inc., California, USA).

3. RESULTS

Table 1. Primary phytochemical screening of methanolic extract of *Adhatoda vasica*

s.no	Phytochemical	Methanol Extract
1	Phenols	+
2	Tannins	+
3	Alkaloids	+
4	Terpenoids	+
5	Saponins	+
6	Flavonoids	+

+ **-present; - absent**

3.2. Yield of methanolic leaves extract, total protein content and total phenolic content

The yield obtained from methanolic extract was 63%. Total protein content was obtained to be 0.27 µg/µl and total phenolic content was obtained to be 260 µg/µl.

3.3. DPPH free radical scavenging

Methanol extract of *A. vasica* has showed potent DPPH radical scavenging activity It has shown 73 to 78% of inhibition at different concentrations (25 µl to 1000 µl . Ascorbic acid was used as a standard (fig1).

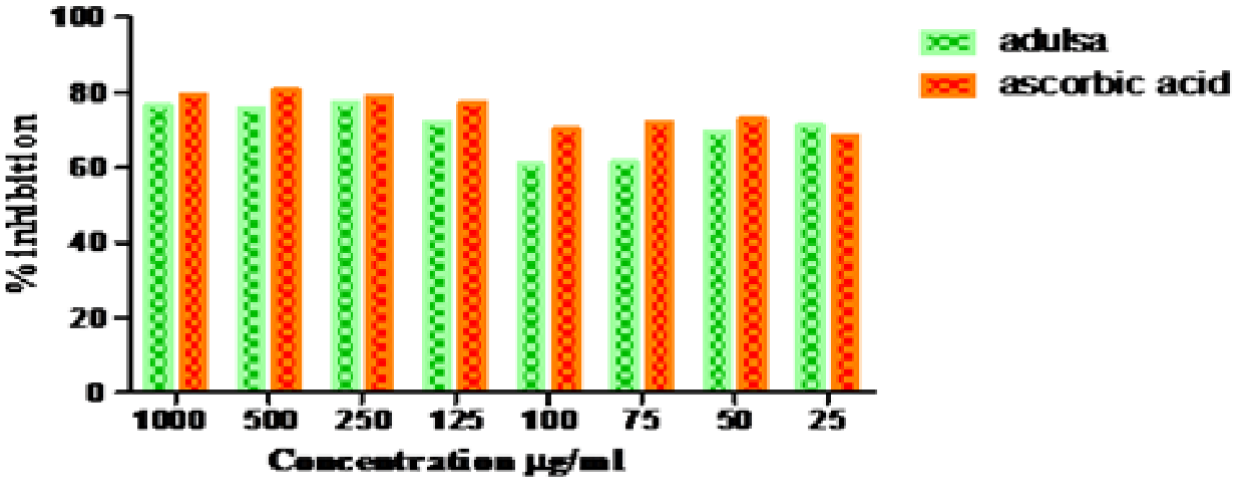


Figure 1: DPPH scavenging activity of the methanolic extract of *A.vasica.*

3.4. Alpha–amylase inhibition assay

Figure2 reveals that acarbose is a standard drug for alpha amylase inhibitor. Acarbose at a concentration of (10-50 µg/ml) showed alpha amylase inhibitory activity from 63.21 to 99.75 %. Methanol extract (10-50 µg/ml) of *Adhatoda vasica* exhibited alpha amylase inhibitory activity but the inhibition is not above 50%. As the concentrations get higher the percentage inhibition with plant methanolic extract increases from 15.25 to 43.22 %.

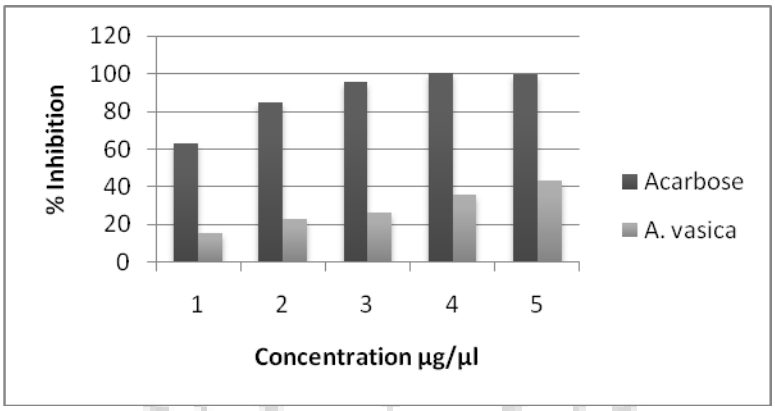


Figure 2: % inhibition of porcine pancreatic alpha amylase enzyme by methanolic leaves extracts of *Adhatoda vasica,* and standard alpha amylase inhibitor i.e. acrabose

3.5. *α* –glucosidase inhibition assay

Figure 3 shows the inhibition of rat intestinal *α* -glucosidase by extract. It was observed that as the concentration increases the inhibition also increases.

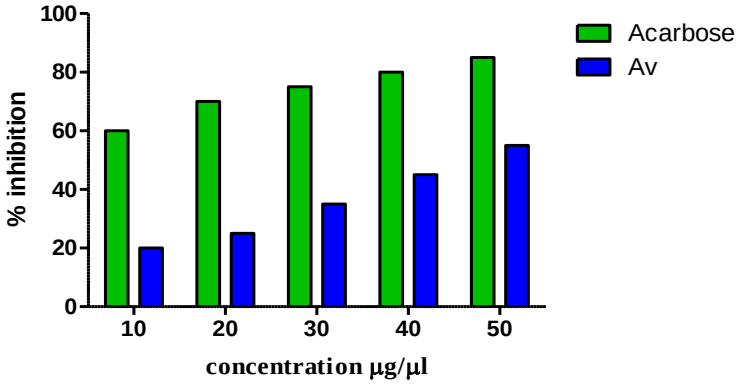


Figure 3: % inhibition by *A.vasica* leave extract and acarbose for *α*-glucosidase from rat intestine.

The inhibition effect of *A.vasica* on the ***α***-glucosidase is 20%, to 58.00%, at the concentration of 10-50 µg/mL.

3.6. Anti-glycation Fluorescence inhibition

Using AGE fluorescence assay *Adhatoda vasica* leaves methanolic extract was found to be a Potent AGE inhibitor as detected by decreased AGE fluorescence (Fig. 4).

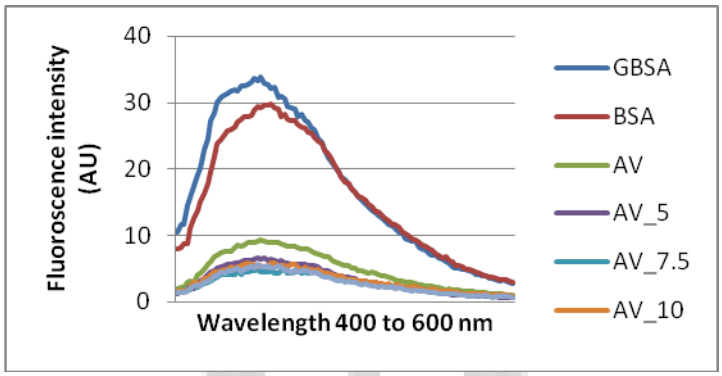


Figure 4 (a): AGE fluorescence (Ex/Em 370/440 nm) spectra of BSA, glycated BSA or glycated BSA treated with different conc. of *A.vasica*

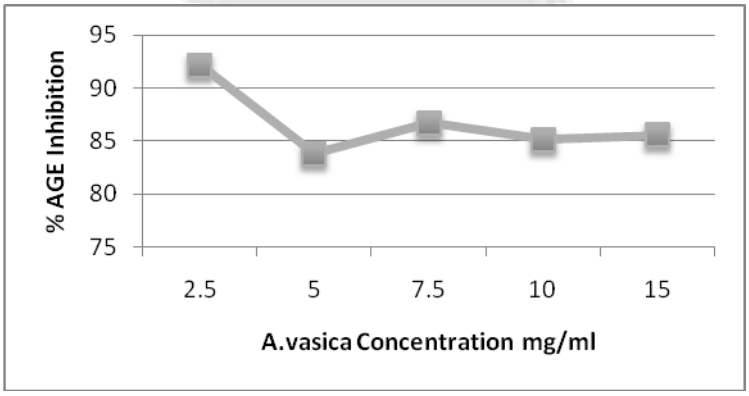


Figure (b): % inhibition of AGE by *Adhatoda vasica*

3.7. In-vivo inhibition of postprandial hyperglycemia in wistar rats

Figure 5 shows the effect of administration of methanol extract of *Adhatoda vasica* leaves in different groups of overnight fasted wistar rats. It was observed that the pretreatment of rats with methanolic leaves extract moderated starch induced the load of postprandial hyperglycemia. The

methanolic extract of *Adhatoda vasica* leaves, however, significantly (P< 0.05) inhibited postprandial glycemic load. These observations show that polyphenols and antioxidants maybe responsible for the antidiabetic effect of the methanol extract.

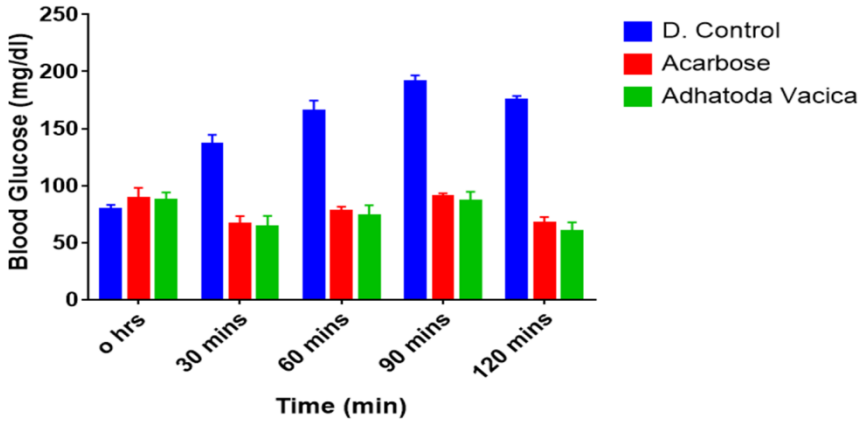


Fig 5. The blood glucose concentration graph following starch administration to overnight fasted rats with or without pre-treatment of study materials.

Table 2 :

	o hrs	30 mins	60 mins	90 mins	120 mins
D. Control vs. Acarbose	ns	****	****	****	****
D. Control vs. Adhatoda Vacica	ns	****	****	****	****
Acarbose vs. Adhatoda Vacica	ns	ns	ns	ns	ns

Table 2- Two way analysis of variance followed by Turkey's multiple comparison tests was applied to compare the difference in an animal study groups. The criterion for statistical significance was *$P < 0.05$. The postprandial glycemic load was found positively correlated with fasting blood glucose values [Fig 5]. The reduction in the blood glucose level was observed after 30 minutes; however there is a little increase in the blood glucose level at 90 minutes, the reduction in the blood glucose of rats pre-treated with the *A.vasica* following starch administration was found more than control after 60th minutes to 120th minutes.

4. DISCUSSION

In Indian traditional system people consumed medicinal plants for the treatment of many diseases as it has many bioactive components **[18]**. Plants have a various group of phytochemicals including alkaloids, flavonoids, tannins, proteins, terpenoids, saponins **[17]** and they are said to the major bioactive components which are responsible for the antioxidants and other reaction in the biological system **[19]**. First time we have reported that the methanolic extract of the leaves showed potent ***α***-glucosidase inhibitory activity indicating the presence of potential inhibitors in the form of alkaloids, phenols, flavonoids etc. We also demonstrated the anti-glycation ability of *Adhatoda vasica* by in vitro experiments. Salivary and pancreatic α‑amylase and intestinal α‑glucosidase are key enzymes responsible for digestion of carbohydrates into glucose. The inhibitors which cause the inhibition of ***α***-amylase and ***α***-glucosidase are also called as starch blockers since they delay the hydrolysis of starch in the body. This is done by blocking the hydrolysis of 1,4-glycosidic linkages of starch and other oligosaccharides into maltose and other simple sugars **[20]**. In our study, the methanolic extract showed ***α***- amylase(fig 2) and potent ***α***-glucosidase inhibitory activity (fig 3) which could be attributed to the presence of polyphenols (260µg/ml) along with other phytochemicals because polyphenols are not only capable of reducing oxidative stress but also of inhibiting carbohydrate hydrolyzing enzymes**[21]**. Drugs that inhibit starch hydrolyzing enzymes have been demonstrated to decrease postprandial hyperglycemia and improve impaired glucose metabolism without promoting the insulin secretion of NIDDM patients. The results of in-vitro and in-vivo studies reveal that *Adhatoda vasica* leaves methanolic extract inhibits ***α***-amylase and ***α***-glucosidase activity. Natural products were clearly indicated as a promising avenue for the prevention of chronic diseases like diabetes also. **[22]**.

In Biological system, multiple metabolic reactions take place which lead to the production of number of free-radicals and therefore antioxidants perform their mechanism to fight against these free-radicals.

AGEs are formed as a result of a series of non-enzymatic reactions between reducing sugars and proteins. Advanced glycation end products (AGEs) have been implicated as one of the major causal factors in pathogenesis of diabetes and its complications **[23][24]**. In diabetes there is

increase in the AGEs formation due to hyperglycemia whereasplasma proteins go and interact with the receptors (RAGE). The AGE-RAGE interaction leads to oxidative stress which causes the development of various degenerative diseases such as diabetes, atherosclerosis, chronic renal failure and Alzheimer's disease **[24][25][26]**. AGEs display a characteristic fluorescence (Ex 370/Em 440 nm) that can be used to evaluate the extent of AGE-modification **[27]**.Using AGE fluorescence assay *Adhatoda vasica* was found to be a potent AGE inhibitor as reflected by decreased AGE fluorescence(Fig.4A). Therefore, AGE fluorescence assay was used to determine the percentage inhibition of AGEs formation which was found to be inhibited (85-92%) (Fig.4B). This indicates that the methanolic extract of *Adhatoda vasica* is the potent inhibitor of AGEs.

Postprandial hyperglycemia (PPHG) is one of the earliest detectable defects in diabetes prone individuals **[28]**. Although PPHG is an independent risk factor for development of vascular complications in type-2 diabetes, the control of acute postprandial glycemic spikes and consequent triggering effect of acute glycemic excursions on oxidative stress needs integration into glycemic disorders **[29]**.In this experiment, pretreatment of rats with *Adhatoda vasica* extract resulted in above 50 % ($P< 0.05$), less increase in blood glucose level after 30^{th}, $60^{th}$and 90^{th}minutes and 120^{th}minutes, respectively of starch feeding in comparison to the control group of rats (Fig. 5). This may be due to the reason that *Adhatoda vasica* is a α-glucosidase inhibitor hence suitable for in-vivo starch tolerance test. The present study indicated that *Adhatoda vasica* could be useful in management of postprandial hyperglycemia. The plant extracts produced a slightly weak α-amylase enzyme inhibition when compared with α- glucosidase. It also produces the potent anti-glycation activity.

5. CONCLUSION

Analysis of this research finds that methanolic extract of *Adhatoda vasica* leaves are rich source of biological antioxidant and may help in counter balancing hyperglycemia induced imbalances through diverse mechanisms of antioxidant defense. The study reveals that *Adhatoda vasica* exhibit α-amylase and potent α-glucosidase inhibitory activity. *Adhatoda vasica* extract also shows inhibition of advance glycation end-product which is the main culprit of future complications due to PPHG. We are stating that *Adhatoda vasica* leaves extract may become

very good option to the available drugs in the market for the treatment of world‟s leading syndrome i.e, diabetes mellitus. The mechanism by which *Adhatoda vasica* leaves inhibited blood glucose which lowers the risk of PPHG respectively requires further investigation.

6. ACKNOWLEDGEMENT

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