

Evaluation of inhibitory activity of *Vitex nirgundo* and *Terminalia chebula* by alpha amylase inhibition assay in management of diabetes

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ABSTRACT

*The present study was aimed to determine anti-diabetic activity of *Vitex nirgundo* and *Terminalia chebula* by alpha amylase inhibition assay. Powder of the herbal plants was subjected to cold maceration successively by petroleum ether, chloroform, ethyl acetate, n-butanol and water for 72 hrs and the extracts were filtered. Extracts were concentrated and weighed to get respective extractive values. Phytochemical screening was done to determine constituents present in every extracts. TLC fingerprinting done to determine the maximum constituents present in extracts of different solvents. The enzyme inhibition was determined by alpha-amylase inhibition assay by UV spectrophotometer at 540 nm using standard curve.*

Key words: Diabetes, *Vitex nirgundo*, *Terminalia chebula*, alpha-amylase assay

INTRODUCTION

Diabetes is a chronic disorder in metabolism of carbohydrates, proteins, and fat due to absolute or relative deficiency of insulin secretion with/without varying degree of insulin resistance^[1, 2].

Diabetes is classified into Type I diabetes (Insulin-dependent diabetes), Type 2 diabetes (Non-insulin dependent diabetes) and Gestational diabetes. Regardless of the type of diabetes, patients are required to control their blood glucose with medications and/or by adhering to an exercise program and a dietary plan. Due to modernization of lifestyle, non-insulin dependent diabetes mellitus is becoming a major health problem in developing countries. Oral antidiabetic agents exert their effects by various mechanisms: (1) stimulation of beta cells in the pancreas to produce more insulin (sulfonylureas and meglitinides), (2) increasing the sensitivity of muscles and other tissues to insulin (thiazolidinediones), (3) decreasing gluconeogenesis by the liver (biguanides), and (4) delaying the absorption of carbohydrates from the gastrointestinal tract (alpha-glucosidase inhibitors). These treatments have their own drawbacks, ranging from the developing of resistance and adverse effects to lack of responsiveness in large segment of patients population.

Alternative to these synthetic agents, plants provide a potential source of hypoglycemic drugs and are widely used in several traditional systems of medicine to prevent diabetes. Several medicinal plants have been investigated for their beneficial use in different types of diabetes. The effects of these plants may delay the development of diabetic complications and correct the metabolic abnormalities using variety of mechanisms. A considerable number of plants were subjected to clinical trials and were found effective. Moreover, during the past few years many phytoconstituents responsible for antidiabetic effects have been isolated from hypoglycaemic plants.

α -Amylase is an enzyme that hydrolyses alpha-bonds of large alpha-linked polysaccharides such as starch and glycogen, yielding glucose and maltose. It is the major form of amylase found in humans and other mammals. α -Amylase and α -glucosidase are key enzymes involved in starch breakdown and intestinal glucose absorption, respectively. The inhibition of these enzymes can slow down the passage of carbohydrates into the bloodstream

significantly decreasing the postprandial increase of blood glucose level after a mixed carbohydrate diet and therefore can be an important strategy in the management of type 2 diabetes¹³

Terminalia chebula :has been extensively used in ayurveda, unani & homoeopathic medicine and has become cynosure of modern medicine. The Sanskrit name 'Haritaki' is rich with meaning, referring to the yellowish dye (harita) that contains the god Siva (Hari, i.e. the Himalayas) and that it cures (harayet) all the diseases. Its other commonly used Sanskrit name, Abhaya, refers to the 'fearlessness' it provides in the face of the disease. According to Indian mythology, this plant originated from the drops of ambrosia (Amrita) which fell on the earth when Indra was drinking it. *T. Chebula* possesses a wide variety of activities like antimicrobial, antioxidant, antiviral, anticarcinogenic, hypocholesterolemic, radioprotective, antispasmodic & antipurgative^[4,5, 6, 7]

***Vitex negundo* L.** (Verbenaceae) is a hardy plant, flourishing mainly in the Indian subcontinent. All parts of the plant, from root to fruit, possess a multitude of phytochemical secondary metabolites which impart an unprecedented variety of medicinal uses to the plant. It is interesting to note that a single plant species finds use for treatment of a wide spectrum of health disorders in traditional and folk medicine; some of which have been experimentally validated. The plant is a component of a number of commercially available herbal formulations and has also shown potential as an effective bio-control agent. Employment of techniques such as cell and tissue culture would provide means of rapid propagation and conservation of the plant species and, from the point of view of phytochemistry, give scope for enhancement of the quality and quantity of the bioactive secondary metabolites occurring in the plant^[8, 9]

The roots, fruits, flowers, leaves and bark of nirgundi have great medicinal value and are used for medicinal purpose externally as well as internally. Nirgundi is extremely beneficial in cough, asthma, bronchitis and inflammatory conditions of pleura^[10, 11]

MATERIALS AND METHODS

Powder of *Vitex nirgundo* and *Terminalia chebula* plants was collected in March 2011 from Shree Akshar Aushadhi Bhandar, Malad(west), Mumbai.

Preparation of plant extracts

Plant powder of *Vitex Negundo* (leaves) and *Terminalia chebula* (fruits) were accurately weighed around 250 gm for extraction. Extraction from powder was done by cold maceration process for 72 hrs. successively by using five times quantity of petroleum ether, chloroform, ethyl acetate, N-butenol and water and filtered to get extracts of respective solvents. Collected extracts were evaporated to get soft extracts by using rota evaporator. Soft extracts collected after evaporation of water was weighed and extractive values from each extracts were calculated.

PHYTOCHEMICAL SCREENING

Preliminary phytochemical screening

The powdered of plants were extracted successively with petroleum ether, chloroform, ethyl acetate, n-butenol and water by cold maceration process. Different extracts were screened for the presence of various groups of phytoconstituents using different chemical tests.

TLC finger print profile

Thin layer chromatography of the petroleum ether, chloroform, ethyl acetate, n-butenol and water extracts was studied.

Enzymatic Assay of Alpha-Amylase

Reagent preparation

1) Starch solution:

0.5% (w/v) Starch Solution (Starch) was prepared in 20 mM Sodium Phosphate Buffer (pH-6.9) and solubilisation of starch was done by heating the starch solution in a glass beaker directly on a water bath using constant stirring for 15 minutes.

2) Colour Reagent Solution

Colour reagent was prepared by addition of Sodium Potassium Tartrate Solution to 96 mM 3,5-Dinitrosalicylic Acid Solution. With stirring, and was stored in amber colored bottle and protected from light.

3) 0.2% (w/v) Maltose Standard Solution was prepared by dissolving maltose monohydrate in to distil water.

4) Alpha-amylase Solution

Immediately before use, alpha amylase solution 2mg/ml in ice cold distilled water was prepared.

5) Standard Curve

A standard curve was plotted by pipetting (in milliliters) the following reagents into a volumetric flask. And kept on boiling water bath for exactly 15 minutes, and then cool on ice to room temperature and Water was added 9.00 ml in each flask and Mix by inversion and record the A540nm for the Standards and Standard Blank using a suitable spectrophotometer.

For test incubation, test and blank solutions were prepared.

For test solution preparation 1 ml starch solution was equilibrated at 25°C and 1 ml freshly prepared enzyme solution was added and solution was mixed and incubated at 25°C exactly for 0,1,2,3,6,12 and 24 minutes then 1 ml colour reagent was added and solution was kept on boiling water bath exactly for 15 minutes then cool the solution on ice to room temperature and 9 ml water was added.

For blank solution preparation 1 ml starch solution was equilibrated at 25°C and incubated at 25°C exactly for 3 minutes then 1 ml freshly prepared enzyme solution and 1 ml colour reagent were added and solution was kept on boiling water bath exactly for 15 minutes then cool the solution on ice to room temperature and 9 ml water was added.

Solutions were mixed and absorbance at 540 nm was recorded using uv spectrophotometer.

Table 1: Reagents and their quantity for preparation of standard curve

Standard	Std 1	Std 2	Std 3	Std 4	Std 5	Blank
0.2% w/v maltose solution	0.2	0.4	0.6	0.8	1.0	-
Distilled water	1.8	1.6	1.4	1.2	1.0	2.0
Colour reagent	1.0	1.0	1.0	1.0	1.0	1.0

Amylase Inhibitory Activity Assay

Crude α -amylase was dissolved in ice-cold distilled water to give a concentration of 4 unit/ml solution. Starch (0.5% w/v) in 20mM phosphate buffer (pH 6.9) containing 6.7mM sodium chloride, was used as a substrate solution. Experiments were performed with three replicate determinations for each experiment.

40 μ l of plant extract (20 mg/ml in DMSO), 160 μ l of distilled water and 400 μ l of starch were mixed in a screw top plastic tube. The reaction was started by the addition of 200 μ l of the enzyme solution. The tubes were incubated at 25 °C for a total of 3 min. Final concentrations in the incubation mixture were plant extract, 1 mg/ml, 0.25% (w/v) starch and 1 unit/ml enzyme. At intervals from addition of enzyme (1, 2 and 3 min), 200 μ l mixture was removed and added into a separate tube containing 100 μ l DNS colour reagent solution (96mM 3,5-dinitrosalicylic acid, 5.31M sodium potassium tartarate in 2M NaOH) and placed into a 85 °C water bath. After 15 min, this mixture was diluted with 900 μ l distilled water and removed from the water bath. α - Amylase activity was determined by measuring the absorbance of the mixture at 540 nm.

Control incubations, representing 100% enzyme activity were conducted in an identical fashion replacing plant extract with DMSO (40 μ l). For blank incubations (to allow for absorbance produced by the plant extract), the enzyme solution was replaced with distilled water and the same procedure was carried out as above. A separate set of incubations was prepared for the reaction of $t = 0$ min, adding samples to the DNS solution immediately after addition of enzyme.

Statistics:

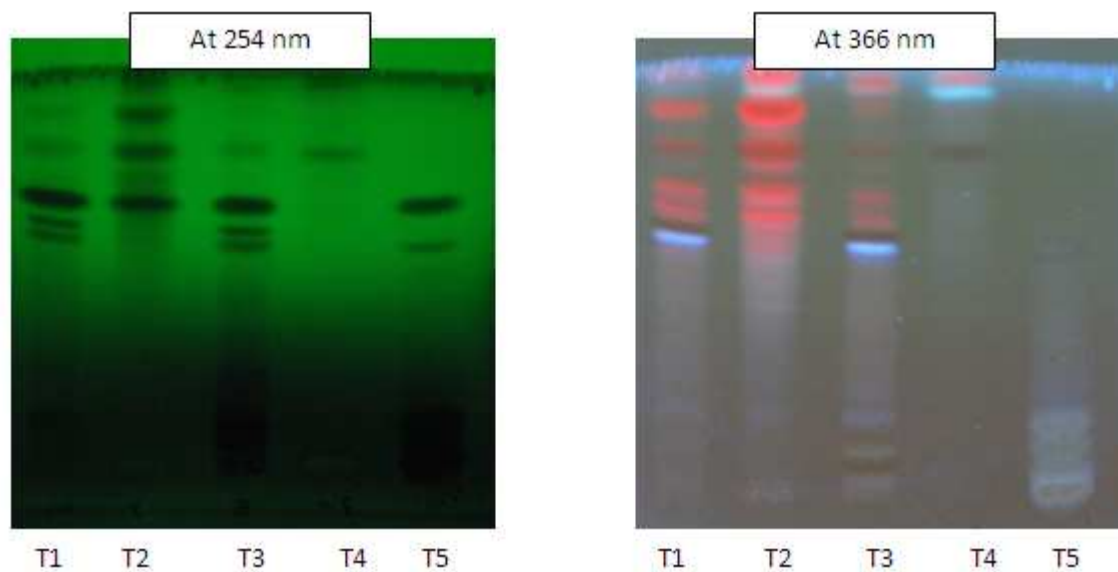
Data are expressed as mean \pm standard error of the mean. Statistical analysis was performed Graph Pad Prism 5.0 software for analysis of variance (ANOVA) followed by Dunnett's test. *p*-Values lower than 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

Preliminary physicochemical evaluation confirmed the authenticity and purity of the drug as the tests performed showed all the parameters such as ash value within the specifications.

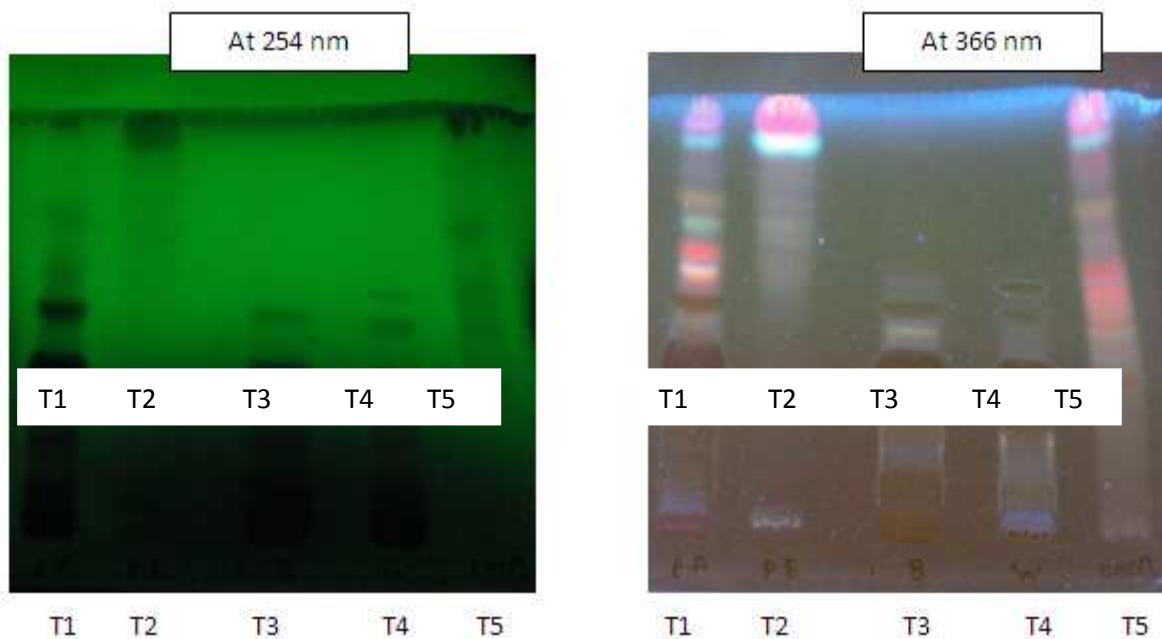
The preliminary phytochemical screening showed the presence of alkaloids, carbohydrates, fixed oils, sterols, Flavonoids, amino acids, proteins, and tannins.

The TLC fingerprinting showed the best separation in chloroform and ethyl acetate extracts in both the plant species using the mobile phase Toluene : Ethyl acetate : Formic acid : Methanol(3:3:0.8:0.2 v/v).



Where: T1=Ethyl acetate, T2=Chloroform, T3=N-butenol, T4=Petroleum ether, T5= Water

Figure 1: TLC of *Vitex Negundo* leaves



T1=Ethyl acetate, T2=Petroleum ether, T3=N-butenol, T4=Water, T5=Chloroform

Figure 2 : TLC of *Terminalia chebula*

**Enzymatic assay of alpha amylase:
Standard Curve of maltose.**

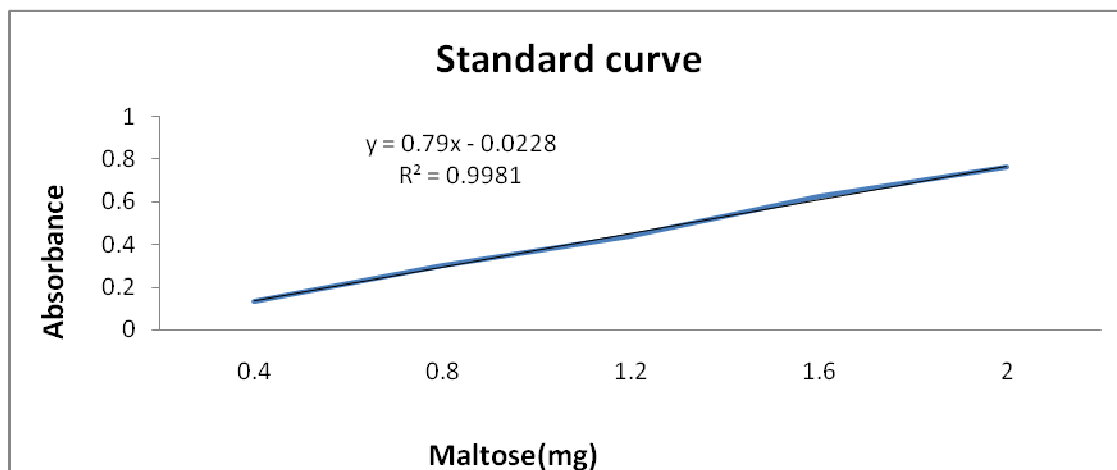


Figure 3 :Standard Curve of maltose.

Table 2 : Optimization of time for alpha amylase enzymatic activity

Time(min)	Absorbance	Maltose liberated(mg)
0	0.1196	0.19
1	0.1639	0.24
2	0.2021	0.29
3	0.2607	0.35
6	0.2868	0.38
12	0.2931	0.39
24	0.3027	0.41

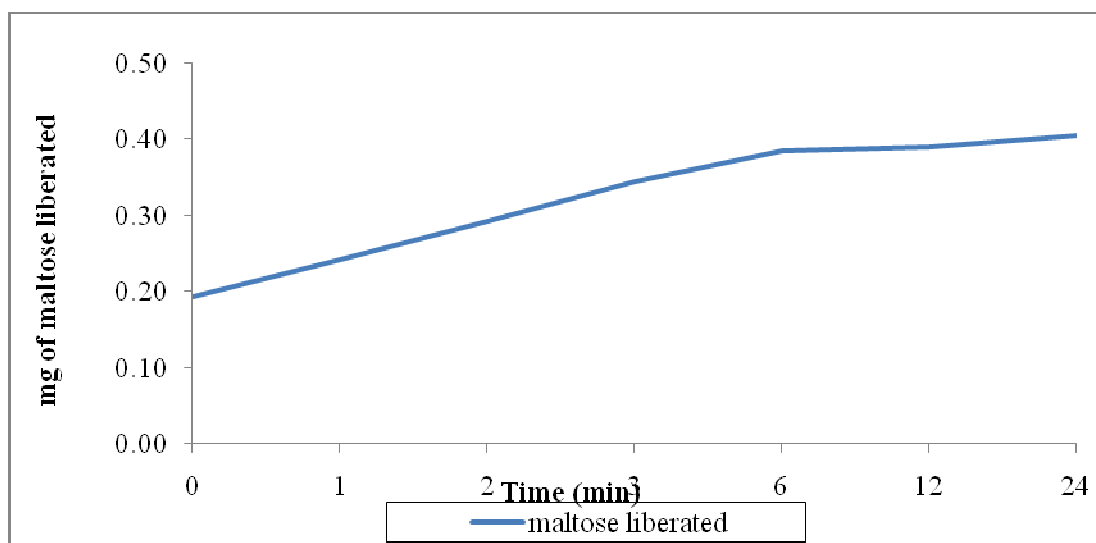


Figure 4 : optimization of time for enzymatic assay

Figure indicates that as time increases the amount of maltose liberated increases but after 6 min it remains almost constant till 24 minutes.so 6minutes is taken as cut off time for inhibition assay

Calculation:

Determination of the milligrams of Maltose liberated after three minutes using the standard Curve.

Absorbance of test solution at 3 min was found to be 0.2548 and the amount of maltose liberated was 0.7 mg

$$\text{Units/ml enzyme} = \frac{(\text{mg of Maltose released}) (df)}{\text{Volume of enzyme}}$$

$$= \frac{0.7 \times 1}{1}$$

$$= 0.7 \text{ Units/ml of enzyme}$$

So, stock solution of enzyme was made up of 2mg/ml

Therefore,

$$\text{Units/mg solid} = \frac{\text{Units/ml enzyme}}{\text{Mg solid/ml enzyme}}$$

$$= 0.7/2$$

$$= 0.35 \text{ Units/mg}$$

So the crude enzyme contains 0.35 Units of alpha amylase per 1 mg.

Alpha amylase inhibitory activity assay

Table 3 : Comparison between +ve control and -ve control

Extracts	mg of maltose liberated(mean±SEM)				
	Control	Chloroform	EA	Pet ether	Water
Control	0.34±0.033	0.40±0.005	0.40±0.009	0.44±0.008	0.44±0.014
Chloroform	0.36±0.026	0.39±0.005	0.42±0.01	0.43±0.01	0.45±0.011
EA	0.31±0.016	0.37±0.008	0.42±0.011	0.44±0.005	0.47±0.014
Pet ether	0.27±0.008	0.32±0.009	0.42±0.004	0.45±0.007	0.47±0.008
Water	0.28±0.01	0.35±0.02	0.41±0.007	0.45±0.005	0.47±0.02

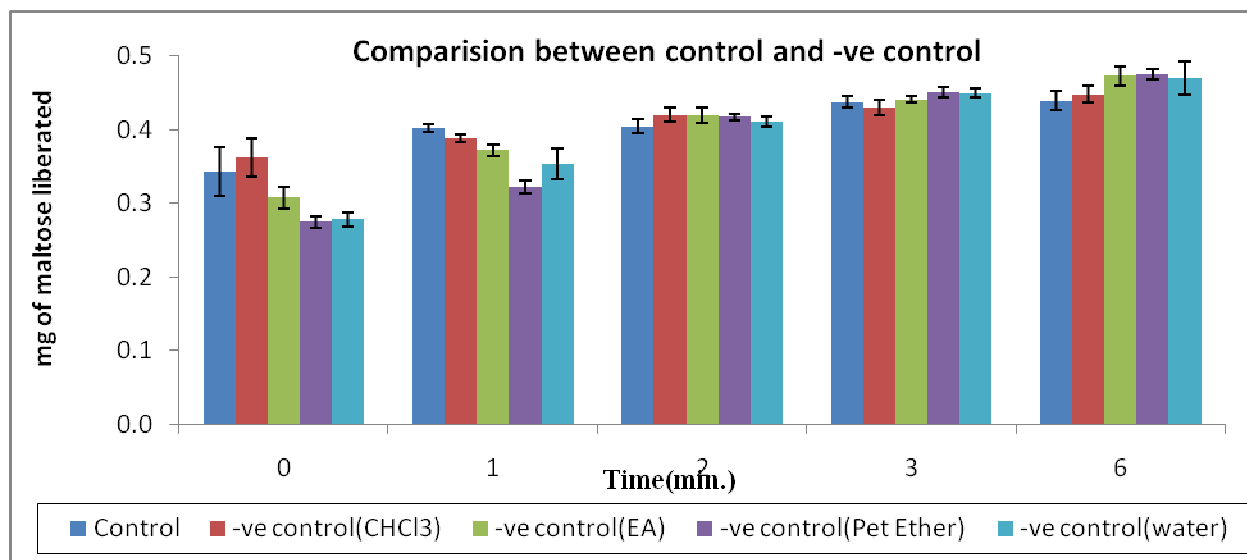
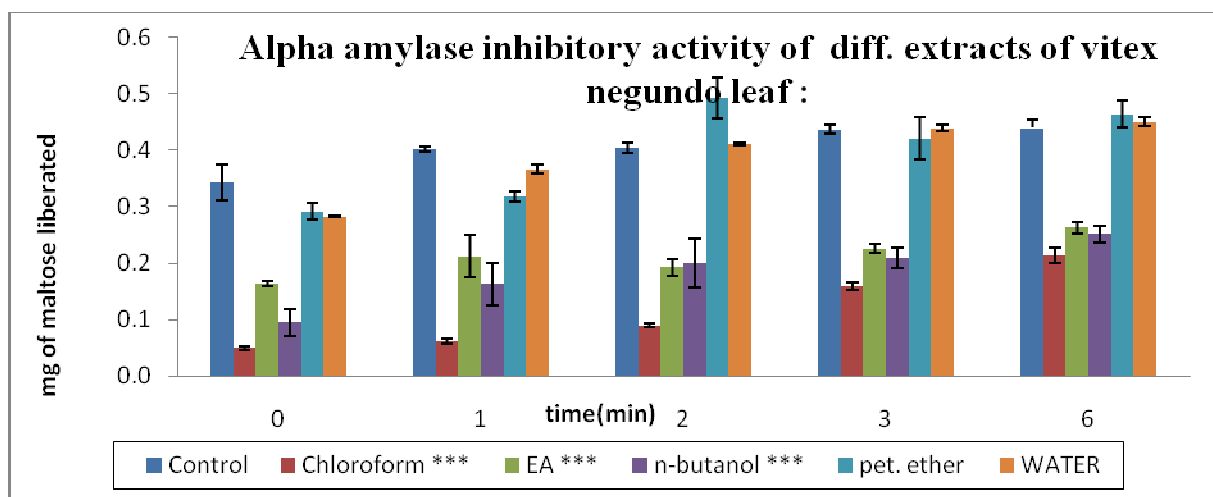


Figure 5: Comparison of +ve control and -ve control group

Comparison of positive control with negative control shows no significant difference which indicates the absorbance is produced due to plant extracts only.

Alpha amylase inhibitory activity assay:**Alpha amylase inhibitory activity assay of *Vitex negundo* leaf****Table 4 : Alpha Amylase Inhibitory Activity Assay of *Vitex negundo***

Extracts	mg of maltose liberated (mean±SEM)				
Control	0.34±0.033	0.40±0.005	0.40±0.009	0.44±0.008	0.44±0.014
Chloroform	0.05±0.004	0.06±0.004	0.09±0.003	0.16±0.007	0.21±0.014
Ethyl acetate	0.16±0.004	0.21±0.038	0.19±0.015	0.23±0.009	0.26±0.010
N-butanol	0.09±0.025	0.16±0.037	0.20±0.043	0.21±0.018	0.25±0.015
Pet. Ether	0.29±0.015	0.32±0.008	0.49±0.036	0.42±0.038	0.46±0.024
Water	0.28±0.002	0.37±0.0080	0.41±0.003	0.43±0.006	0.45±0.008

**Figure 6 : Alpha-Amylase Inhibitory Activity of Different Extracts of *Vitex Negundo***

Comparison of different extracts of leaf with control *** $p < 0.001$ compare to control.

Discussion : Extracts of *Vitex Negundo* leaf were investigated for α -amylase inhibition assay. chloroform, ethyl acetate and n-butanol extracts of leaf showed significant α amylase inhibition as compare to control's results. Again out of these three solvent extracts, chloroform extracts showed good inhibitory action compare to other extracts. While other extracts (pet ether and aqueous) of leaves didn't show any significant inhibition. Which indicate leaves are having α amylase inhibitory potential.

Alpha amylase inhibitory activity assay of *Terminalia chebula***Table 5: Alpha Amylase Inhibitory Activity Assay of *Terminalia chebula***

Extracts	mg of maltose liberated (mean±SEM)				
Control	0.34±0.033	0.40±0.005	0.40±0.009	0.44±0.008	0.44±0.014
Chloroform	0.15±0.053	0.20±0.013	0.23±0.016	0.20±0.006	0.26±0.011
Ethyl acetate	0.38±0.032	0.41±0.004	0.47±0.029	0.40±0.021	0.40±0.008
N-butanol	0.29±0.007	0.38±0.007	0.47±0.025	0.45±0.14	0.48±0.011
Pet. Ether	0.32±0.043	0.29±0.007	0.43±0.007	0.44±0.016	0.42±0.013
Water	0.29±0.004	0.37±0.008	0.41±0.004	0.44±0.007	0.44±0.008

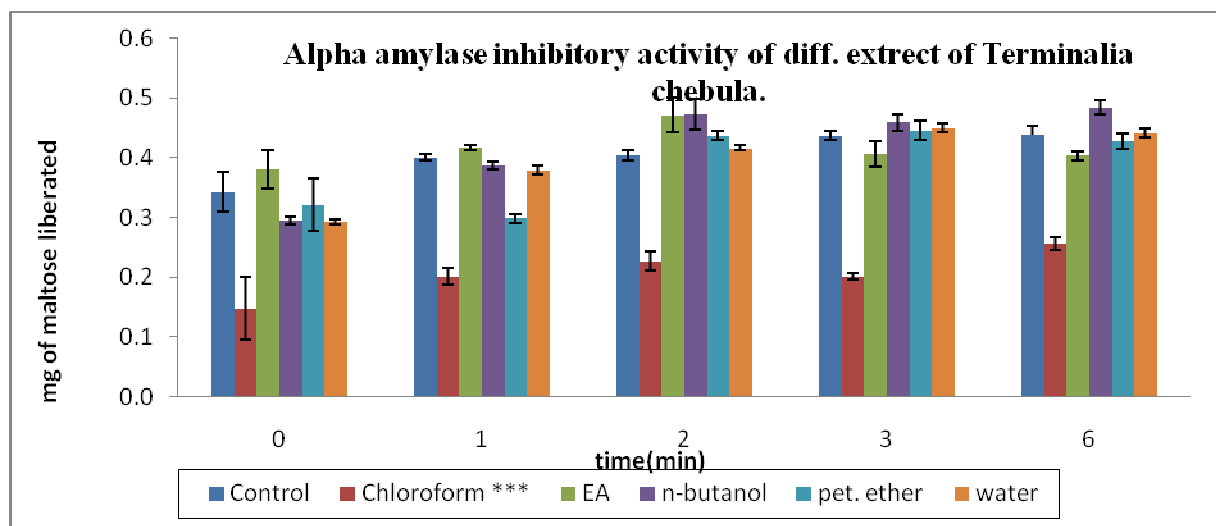


Figure 7 :Alpha-Amylase Inhibitory Activity of Diff. Extracts of *Terminalia chebula*

Comparison of different extracts of leaf with control *** $p < 0.001$ compare to control.

Discussion : Extracts of *Terminalia chebula* Fruits were investigated for α -amylase inhibition assay. Chloroform extracts of fruits showed significant α amylase inhibition compare to control's results. While other extracts (ethyl acetate , n-butanol, pet ether and aqueous) of fruits didn't show any significant inhibition

Summary

Diabetes mellitus (DM) is a chronic disease caused by inherited or acquired deficiency in insulin secretion and by decreased responsiveness of the organs to secreted insulin. Such a deficiency results in increased blood glucose level. Various synthetic agents have been developed for the treatment of diabetes but the complete cure for the disease has not been found yet. Herbal medicines have also been investigated for their anti-diabetic potential since many years and thus plants provide invariable source for the anti-diabetic drug development.

Many natural resources have been investigated with respect to suppression of glucose production from carbohydrates in the gut or glucose absorption from the intestine. α -Amylase catalyses the hydrolysis of α -1,4-glucosidic linkages of starch, glycogen and various oligosaccharides and α -glucosidase further breaks down the inhibition of their activity, in the digestive tract of humans, is considered to be effective to control diabetes by diminishing the absorption of glucose decomposed from starch by these enzymes.

In this study we have investigated hypoglycemic potential of the Indian herb *Vitex Negundo* and *Terminalia chebula* by α amylase inhibition assay.

Preliminary physicochemical evaluation confirmed the authenticity and purity of the drug as the tests performed showed all the parameters such as ash value within the specifications.

The preliminary phytochemical screening showed the presence of alkaloids, carbohydrates, fixed oils, sterols, Flavonoids, amino acids, proteins, and tannins.

The TLC fingerprinting showed the best separation in chloroform and ethyl acetate extracts in both the plant species using the mobile phase Toluene : Ethyl acetate : Formic acid : Methanol(3:3:0.8:0.2 v/v).

Vitex Negundo Leaf :

Extracts of *Vitex Negundo* leaf were investigated for α -amylase inhibition assay. chloroform, ethyl acetate and N-butanol extracts of leaf showed significant α amylase inhibition as compare to control's results .Again out of these three solvent extracts chloroform extracts showed good inhibitory action compare to other extracts as described in table no. 4. While other extracts (pet ether and aqueous) of leaves didn't show any significant inhibition. Which indicate leaves are having α amylase inhibitory potential.

***Terminalia chebula* Fruits:**

Extracts of *Terminalia chebula* Fruits were investigated for α -amylase inhibition assay. Chloroform extracts of fruits showed significant α amylase inhibition compare to control as result described in table no.5. While other extracts (ethyl acetate , n-butanol, petroleum ether and aqueous) of fruits didn't show any significant inhibition.

CONCLUSION

The results in the present study confirmed the alpha-amylase inhibitory action of both the plants species but it was observed that *Vitex Negundo* had a far better influence as far as alpha amylase inhibition was concerned . chloroform , n-butanol and ethyl acetate extract of the leaf showed potential hypoglycemic activity for *Vitex Negundo* while only chloroform extract of the fruits showed potential hypoglycemic activity for *Terminalia chebula* , While other solvent of Extracts didn't show any significant inhibition of α amylase.

So, *both plants* might be a potential source of research for the treatment of hyperglycemia by reduction of post-prandial glucose absorption

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