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RESEARCH ARTICLE

In-Vitro α-amylase Inhibitory and Anti-oxidant activities of Leaf Extract of *Vitex trifolia L.*

Monapati Suchitra[1], Binoy Varghese Cheriyan*[2]

[1]*Research Scholar, Department of Pharmaceutical Chemistry and Analysis, School of Pharmaceutical Sciences, VelsInstitute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai – 600 117, Tamil Nadu, India.*
[2]*Department of Pharmaceutical Chemistry and Analysis, School of Pharmaceutical Sciences, Vels Institute of Science, Technology and Advanced Studies (VISTAS), Pallavaram, Chennai – 600 117, Tamil Nadu, India*.

ABSTRACT

Lifestyle and stress play a major role in diabetic physiology. Natural antioxidants exist as therapeutic tools. *Vitex trifolia L.* is a flowering shrub indigenous to temperate regions. Aim and objective of the present work are to identify the potential of *in vitro* inhibitory activity of a α-amylase enzyme and antioxidant activity of leaf extract of *Vitex trifolia L.* Shade-dried finely powdered leaves of *Vitex trifoliaL.* were extracted with organic solvents of increasing polarity such as n-hexane, ethyl acetate, and ethanol 95%. The extract was subjected to phytochemical screening, quantitative estimation of total phenols, flavonoids and tannins. Antioxidant activity by DPPH, hydroxyl, nitric oxide, and hydrogen peroxide radicals were carried out for the extract. Different concentrations of the extracts were subjected to α-amylase inhibitory activity using soluble starch as substrate and the IC $_{50}$ value was calculated. Phytochemical screening revealed the presence of phytoconstituents like phenols, flavonoids, alkaloids, terpenoids, glycosides, saponins etc. The ethanolic fraction of the leaf extract exhibited the highest antioxidant activity for DPPH, hydroxy, nitric oxide and hydrogen peroxide radical methods with the IC_{50} values of 50.41, 50.14,49.64and 56.47µg/mL respectively compared to IC_{50} value of standard ascorbic acid. Almost all extracts have shown good α-amylase inhibitory activity but ethanolic extract exhibited significant activity with an IC $_{50}$ value of48.74µg/mL when compared with acarbose IC_{50} value of 48.25µg/mL. Furthermore, the result showed high levels of phenolic, flavonoid, and tannins content. Statistical analysis using Graph pad prism version 8.4.2(649) showed a significant correlation. This is the first time reporting antidiabetic activity of this species. The uniqueness of the work lies in proving the antioxidant and anti diabetic activities of the extract of *Vitex trifolia L.* which has got commercial and societal values.

Keywords: Anti diabetic activity; ethanolic fraction; IC_{50} value, porcine pancreatic amylase and *Vitex trifolia L*.

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Corresponding Author
Binoy Varghese Cheriyan
Department of Pharmaceutical Chemistry and Analysis,
School of Pharmaceutical Sciences,
Vels Institute of Science, Technology and Advanced Studies (VISTAS),
Pallavaram, Chennai – 600 117, Tamil Nadu, India.
MS-ID: IJPNM4326

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1. Introduction

Diabetes mellitus type II is characterized by hyperglycemia resulting from the deficiency of secretion of Insulin and lack of its action. The prevalence of diabetes is on the rise and the expected count of patients may reach 300 million by 2025with the majority of people suffer from diabetes type II(Mitra A *et al.,* 2007). Diabetes mellitus type II can be treated and controlled by oral hypoglycemic agents and lifestyle modification. A different class of oral hypoglycemic agents acts through different targets and brings down the elevated sugar levels such as stimulating the beta cells of the pancreas, peripheral utilization of glucose, and also inhibition of the α-amylase or glucosidase enzyme. The main adverse effect of large class of oral hypoglycemic agentsis hypoglycemia except for α-amylaseand glucosidase inhibitors. The action of the α-amylase enzyme is the conversion of starch into simple sugars (Ibrahim H.O *et al.,*2019). Acarbose, voglibose, and miglitol are some of the clinically used inhibitors ofα-amylase and glucosidase inhibitors. At the same time, they can produce adverse effects such as vomiting, bloating, and abdominal discomfort (Dineshkumar.B *et al.,*2010). Production of free radicals is closely related to oxidative stresswhich is the common complication in diabetes mellitus type II. Hence supplementation of antioxidants can be effective in reducing the severity of the diabetes (Fatmah A Matough *et al.,* 2012).

Traditional medicines play a vital role in the treatment of hyperglycemia because of their effective therapeutic range, safety, and cost effectiveness compared to synthetic drugs (Wesam Kooti *et al.,* 2016). India is the land of Ayurveda and employs traditional medicinal plants for the treatment and cure of a wide range of diseases. So screening of medicinal plants scientifically may help to identify safe drugs and also can be used for further investigations. Medicinal plants such as *Cassia glauca, Euphorbia hirta,and Proteus vulgaris* showed potential inhibitory activity of α-amylase and the activity is attributed to phytoconstituents such asflavonoids, isoflavones, and anthocyanins. These secondary metabolites possess antioxidant properties (Anindita Banerjee *et al.,* 2017).

In India, traditional and indigenous medical practices are more empirical, local communities of Valaiyans of alagarkoil are known to use more than hundreds of plants as a source to treat diabetes (Palanichamy Ayyappan *et al.,* 2019).*Vitex trifolia L.* is one of the plants used and is available in alltropical and sub-tropical regions.It is the shrub that can grow up to 6 m heightbelongs to the family Lamiaceae and genus Vitex (McMillan X. A *et al.,* 1976).This species is effective and commonly used in folk medicine to treat a wide range of afflictions such as asthma, depression, diabetes, allergy, venereal diseases, wounds, skin diseases, snake bite, and gastroenteritis (Manjunatha BK *et al.,* 2007). Hence the study was directed to evaluate the *in-vitro* inhibitory activity α-amylase and antioxidant activity of *Vitex trifolia.* The leaves are simple, three foliate oppositely arranged acute at base approximately 4–7cm in length shown in Fig 1(Suchitra M*et al.,* 2018).

Fig1: Leaf of *Vitex trifolia*

2. Materials and Methods

All the reagents used were obtained from Sigma Aldrich and S.D. Fine-Chem Ltd.

Plant Material Collection and Processing

Leaves of *Vitex trifolia L.* were procured and authenticated in spring season from Davanagere locality in Karnataka.

Preparation of Plant Extracts

One week shade-dried under room temperature authenticated leaves of *Vitex trifolia L.* were coarsely powdered by a mechanical device (Morphy Richards Icon essential).Around 500 g per batch of coarsely powdered leavesof *Vitex trifolia L.* were successively extracted with organic solvents selected based on increasing polarityn hexane, ethyl acetate and ethanol (95%) by Soxhlet apparatus and the extracts wereconcentratedusing distillation apparatus. The same was used for screening of phytochemicals and for antioxidant and anti diabetic studies.

Qualitative Phytochemical Screening of the Extracts

The extracts of *Vitex trifolia L.* were screened for the phytochemical constituents as shown in the Table 1(Khandelwal KR *et al.,* 2006).

Table 1: Tests conducted for the presence of phytochemical constituents

Phytochemical constituent	Name of the test
Alkaloids	Mayer's test, Wagner's test, Hager's test, Dragendorff's test
Carbohydrates	Molisch's test, Fehling's test, Barfoed's test, Benedict's

	test
Amino acids and proteins	Biuret test, Ninhydrin test, Hopkins test
Phenols	Ferric chloride test, Potassium dichromate test
Flavonoids	Lead acetate test, Shinoda test, alkaline reagent Test
Tannins	Potassium dichromate,Gelatin, bromine water test
Steroids and triterpenoids	Libermann- Buchard, Salkowski test
Glycosides	Borntrager's test
Saponins	Froth and Foam test

Physical constants such as total ash, water soluble ash, acid insoluble ash, and alcohol soluble and moisture content extractive values were determined separately for air dried powdered leaves of the plant as per the official methods(Harborne, J.B *et al.,* 1998).

Quantification of Secondary Metabolites

Estimation of total phenols

To the n hexane, ethyl acetate and ethanolic leave extracts of V*itex trifolia L.*(1mg/mL) added 10 µl of Folin – Ciocalteuphenol reagent (1:10) and 20 µl of aqueous 5% Na_2CO_3 **(**Singleton VL *et al.,* 1965**).** The absorbance was determined at 765 nm using UV-visible spectrophotometer (Schimadzu 1800, Kyoto, Japan) after keeping mixtures half an hour at room temperature. From the standard graph, the phenol content in the extract was determined using gallic acid as standard **(**Olayinka A Aiyegoro *et al.,* 2010**).**

Estimation of Total flavonoids

Total flavonoid content was determined using UV spectrophotometer. 1 ml of plant extracts (1mg/ml) was added to 0.2 ml of 10% $AlCl_3$ and 1 M potassium acetate and 5.6 ml of distilled water(Abdou Madjid O *et al.,* 2015).After keeping the mixture at room temp for 30 min, absorbance was measured at 420 nm using UV spectrophotometer(Schimadzu 1800, Kyoto, Japan). By taking the reference compound Quercetin plotted the standard curve to determine the content of flavonoid (Suman Chandra *et al.,* 2014).

Estimation of Total tannin content

Modified Folin - Ciocalteu method was used determination of tannins. About 1 ml of the each three extracts added 0.5 ml of Folin–Ciocalteuphenol reagent and 5 ml of 20% solution of sodium carbonate. Diluted with 10 ml of distilled water. The absorbance of the test and standard solutions were read at 725 nm after 5 min with an UV/Visible spectrophotometer (Schimadzu 1800, Kyoto, Japan). Gallic acid is taken as standard and tannin content is expressed in terms of mg of GAE /g of extract (**Vijay D Tambe *et al.*, 2014**).

Anti-oxidant activity of the leaf extracts

DPPH radical scavenging activity

About 3mlof different concentrations of test samples were added to 1ml DPPH in ethanol on triplicate way and kept at 37°Cfor 30 minutes (Harlalka VG *et al.,* 2007). The control contains freshly prepared DPPH without extract. (Arvind Kumar Goyal *et al.,* 2010).The absorbance was estimated at 517 nm by taking ascorbic acid as standard (Feng-Lin Hsu *et al.,* 2012). Freshly prepared DPPH solution was taken in test tubes and extracts were added followed by serial dilutions (100–1000µg) to every test tube such that the nal volume was 2 ml, and discoloration was measured Freshly prepared DPPH solution was taken in test tubes and extracts were added followed by serial dilutions (100–1000 µg) to every test tube such that the final volume was 2 ml, and discoloration was measured

Hydroxyl radical scavenging activity

To the 1 ml different concentrations of above said three extracts added 1 ml of de oxy ribose solution, $FeCl_3$ solution were added (Rajesh Kumar S *et al.,* 2011).To this added same quantity of EDTA solution, H_2O_2 in phosphate buffer (pH 7.4, 20 mM) solution, and 0.1 mM Ascorbic acid solution were added triplicate manner(Raja Sundararajan., *et al.,* 2018). Similarly standard also prepared and they were incubated for 30 min at 37°C followed by additionof ice-cold 15% w/v trichloro acetic acid(0.4 ml) and 1% w/v thiobarbituric acid in 0.25 N HCl(Kevin P *et al.,* 2013).The absorbance of both test and samples were measured at 532 nm and the percentage inhibition was calculated (Susantha kumar Mondal *et al.,* 2006).

Nitric oxide radical scavenging activity

To 4ml of standard and different concentrations of test solutions added 1ml of sodium nitro prusside solution in a triplicate manner and incubatedfor 2 h at 37°C, then added 1.2 ml Griess reagent (Josiah Bitrus Habu*et al.,* 2015).The nitric oxideradical chromophore absorbance was estimated at 570 nm andcalculated the percentage inhibition (Nabavi S.M *et al.,*2008).

Hydrogen peroxide scavenging activity

To the sample and standard solutions added 0.6 ml of 40mM H_2O_2 in triplicate manner and added phosphate buffer of pH 7.4 (Bibhabasu Hazra, *et al.,* 2008). After incubation for 10 min the absorbance was read in dark at 230nm and the percentage inhibition was calculated (Kumar.S, D Kumar, 2009). The Percentage of radical scavenging activity was determined by the following equation for all four methods(S.P.Choudhary et al., 2014).

% of radical scavenging activity= [(OD control- OD sample)/OD control] × 100

Where OD is the optical density

The effective concentration of sample required to scavenge free radical by 50% (IC_{50} value) was obtained by interpolation from linear regression analysis.

***In Vitro*α-amylase inhibitory assay:**

To the plant extract added starch solution,(porcine pancreatic α-amylase)(500 µl) wasadded to initiatethereaction and incubated at 37 ºC. Added 300 µl of DNS Areagent (1g of DNSA, 30g of sodium potassiumtartarate and 20 ml of 2N sodium hydroxide was added and made up to a final volume of 100 ml with distilled water) and10 minutes kept in a boiling water bath for reaction**(**G.Keerthana *et al.,* 2013**)**. The reaction blend diluted with 2.5 ml of water and absorbance was estimated at 540 nm. The experiments were done in triplicate manner.

The inhibitory activity of α-amylase was calculated by usingIC_{50} linear regression analysis(Ali, Houghton PJ *et al.,* 2006**).**

% inhibition = (Ac-As)/AcX 100

Where Ac is the absorbance of the control and As is the absorbance of the sample

Statistics

Regression analysis was performed to calculate the dose-response relation between the extracts. The correlation coefficient can identify by linear regression analysis. The data were expressed as mean ± standard deviation by performing all assays in triplicate manner.Statistical analysis was done using Graph pad prism version 8.4.2(649). Column analysis followed by the RM One-way ANOVA test was performed. Significant values were consideredat $p < 0.05$. IC50 values were depicted by plottinga percent of inhibition versus concentration curve for positivecontrols in scavenging of DPPH, hydroxy, Nitric oxide,Hydrogen peroxide and α-amylase inhibitory assays.

3. Results and Discussion

Extraction yield values

The leaves of *Vitex trifolia L.* was extracted with solventsn-hexane, ethylacetate and ethanol and the yields were found to be 25.43g, 29.38g and 31.25g respectively depicted in Table 2.

Table 2: *Vitex trifolia L.*Extraction yield values

S.NO	Extract	Yield(for 500g)	Colour	Consistency
1	n-hexane	25.43g	Dark greenish brown	Oily viscous
2	Ethylacetate	29.38g	Pale brown	Powder
3	Ethanol	31.25g	Brownish yellow	Powder

Phytochemical screening: The screening of phytochemicals of different extracts of *Vitex trifolia L.* demonstrated the presence of carbohydrates, saponins, glycosides, flavonoids, tannins, amino acids and alkaloids Table 3

Table 3: Phytochemical screening and Quantification of Secondary Metabolite

	Phytoconstituents	Ethanol extract	n-hexane extract	Ethyl acetate extract
1	Alkaloids	+	-	+
2	Carbohydrates	+	+	+
3	Amino acids	+	+	+
4	Flavonoids	+	+	-
5	Glycosides	+	-	+
6	Phenols	+	+	-
7	Tannins	+	+	+
8	Steroids	-	-	-
9	Triterpenoids	+	+	+
10	Saponins	+	+	+

(+) means Present (-) means absent

Table 4: Evaluation of Physiochemical parameter

S.No	Physico chemical parameters	%w/w
1	Total ash	2.255
2	Water soluble ash	1.2
3	Acid insoluble ash	1.21
4	Alcohol soluble extractive	7.81
5	Water soluble extractive	3.89
6	Moisture content	3.9
7	Foreign organic matter	0.08

Estimation of Total phenol, flavonoid and Tannin content

The extracts were tested for its Total Phenol, flavonoid and tannin contents and the ethanol extract has shown highest levels of total phenol, flavonoid and Tannins contents. Results of three extracts of *Vitex trifolia* are depicted in Table 5.

Table 5: Total Phenol, Flavonoid and Tannin content

S. No	Extract	Total phenolic content (mg GAE/g dry weight of extract)*	Total flavonoid content (mg QE/g dry weight of extract) *	Total Tannins content (mg GAE/g dry weight of extract)*
1	Ethanol extract	100.2±0.2563	116.1±0.1447	158.6±1.107

2	Ethyl acetate extract	88.53±0.8401	102.4±0.7987	135.7±0.8454
3	n- hexane extract	73.30±0.6034	93.73±1.622	104.2±0.6652

* Mean value ± SD

Anti-oxidant study

The Free radical Scavenging of extracts were tested for its antioxidant nature against various free radical generating systems

Scavenging radical of DPPH:

In the assay of DPPH radical scavenging the standard drug ascorbic acid has shown dose related inhibition of free radical generated and the IC_{50} value was found to be 50.34µg/ mL. The extracts also showed similar dose dependent inhibition of free radical generation and the prominent inhibition of free radical was exhibited by ethanol extract followed by ethyl acetate and then n-hexane extract and the corresponding IC_{50} values and were found to be 50.41, 56.02and 62.08µg/mL respectively**.** The IC $_{50}$of tested compounds was done by linear graph method and represented in Figure 2. The significant correlation between all the extracts has been shown by statistical analysis, P value is 0.0065 ($P < 0.05$) is statistically significant.

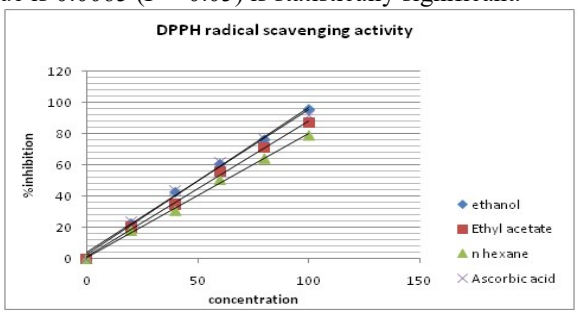

Figure 2: DPPH radical scavenging assay

Hydroxyl radical scavenging assay:

The standard drug ascorbic acid showed dose dependent inhibition of hydroxyl ion generated and the IC_{50} valuewas49.68µg/mL.The extracts also showed similar dose dependent inhibition of OH ion generation and the prominent inhibition of free radical was exhibited by ethanol extract followed by ethylacetate and thenn-hexaneand the corresponding IC_{50} value were found to be50.14,53.78, and 55.77µg/mLrespectively. The $IC_{50}$of tested compound on hydroxyl ion scavenging was done by linear regression method(Fig -3).The significant correlation between all the extracts was proved by P value is 0.0033 ($P < 0.05$) is statistically significant.

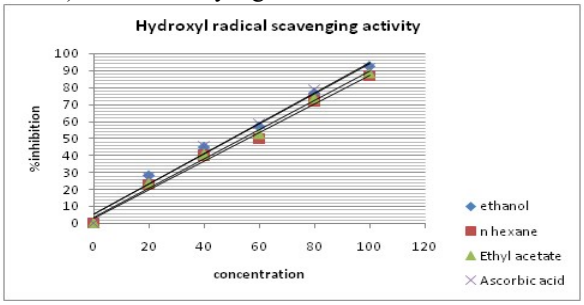

Figure 3: Hydroxyl radical scavenging activity

Nitricoxideradical scavenging assay:

The standard drug ascorbic acid showed dose dependent inhibition of NO generated and the IC_{50} value was found to be 47.71µg/mL.The extracts also showed a similar dose dependent inhibition of NO ion generation and the prominent inhibition of free radical was exhibited by ethanolic extract followed by ethylacetate and then n-hexane and the corresponding IC_{50} value were found to be-49.64,53.88, and 59.86µg/mLrespectively.The IC $_{50}$of tested compounds by NO scavenging was done by linear regression method (Fig-4). The statistical analysis showed significant correlation between all the extracts, P value is 0.0149 ($P < 0.05$) is statistically significant.

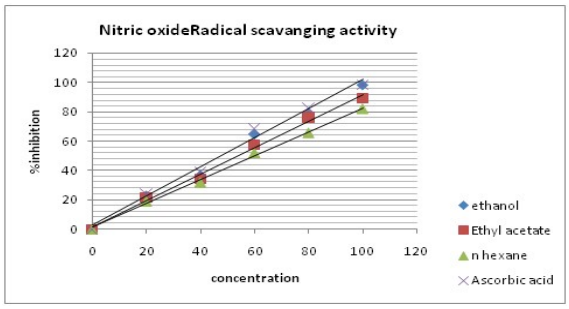

Figure 4: Nitric oxide radical scavenging assay

Hydrogen peroxide Scavenging assay

The standard drug ascorbic acid showed dose dependent inhibition of H_2O_2 generated and the IC_{50} value was found to be -50.24µg/mL.The extracts also showed a similar dose dependent inhibition of H_2O_2 ion generation and the prominent inhibition of free radical was exhibited by ethanol extract followed by ethyl acetate and then n-hexane and the corresponding IC_{50} values were found to be56.47, 62.53,and70.68µg/mL respectively.The IC $_{50}$ of tested compounds by H_2O_2 scavenging was done by graphical method Fig -5. The statistical analysis showed significant correlation between all the extracts, P valueis 0.0069 ($P < 0.05$) is statistically significant with good summary value.

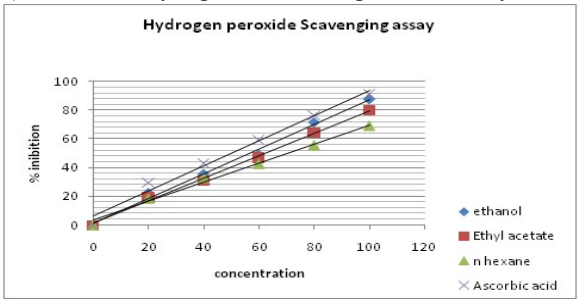

Figure 5: Hydrogen peroxide Scavenging assay

***In vitro* α-amylase inhibitory assay**

Different extracts of *Vitex trifolia L.* leaves exhibited α-amylase inhibitory activity at varying degrees. Standard

drug acarbose was used to compare the results and the IC_{50} value of enzyme inhibition was determined by linear regression analysis.Here also the prominent effect on α-amylase inhibition was observed with ethanol extract. The standard drug acarbose showed dose dependent inhibition of**α-**amylase enzymeand the IC_{50} value was found to be 48.25µg/mL.The extracts also showed a similar dose dependent inhibition ofα-amylase enzyme and the prominent inhibition was shown by ethanol followed by n-hexane extract and then ethyl acetate and the corresponding IC_{50} value were found 48.74µg/ml, 61.27µg/ml, 66.01µg/ mlrespectively depicted in Table 6.The IC $_{50}$ of tested compounds by α-amylase inhibitoryassay was done by graphical method Fig -6. The statistical analysis showed significant correlation between all the extracts, P value is 0.0063 ($P < 0.05$) is statistically significant with good summary value.

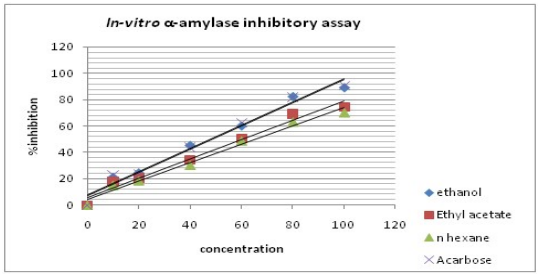

Figure 6: *In-vitro* α-amylase inhibitory assay

Table6: IC_{50} values of *Vitex trifolia L.* for *Invitro*α-amylase inhibitory assay(µg /mL)

Ethanol extract	Ethyl acetate extract	n hexane extract	Acarbose
48.74	66.01	61.27	48.25

Discussion

Diabetes Mellitus is a serious medical condition that occurs when the body has difficulty for regulating the glucose in the blood. Co morbid conditions such as myocardial infarction, neuropathy, atherosclerosis, nephropathy, retinopathy etc. may occur due to long term chronical elevated glucose levels of the disease. Current treatment of diabetes (type –II) is to take oral hypoglycemic agents and have life style modification which includes having more fibrous foods and regular exercise. Therefore a healthy living style with the use of herbals which possesses anti-diabetic activity and antioxidant activity in their daily diet would be ideal way of con-trolling the disease. In the current study all the extracts under study exhibited antioxidant activity and the same has direct correlation with the amount of polyphenolic, flavonoid and tannins present in the extracts (Padma R *et al.,* 2013). The extracts were also assessed for itsα-amylase inhibitory property and the results were similar to the anti oxidant activity. Antioxidants can reduce the oxidative stress in diabetes and Supplementation of antioxidant can reduce glycosylated hemoglobin (Madhu,C.G *et al.,* 2000).This study also scientifically validated the traditional claim of the plant that is widely prescribed for diabetic diseases. From the above findings by employing scientific methods the plant is tested for its phytoconstituents and the presence of phytoconstituents contributed to the antioxidant activity as well as α-amylase inhibition action of *Vitex trifolia L.*

4. Conclusion

The current study reveals that ethanolic extracts of leaves of *Vitex trifolia L.*exhibited potent α-amylase inhibitory activity and antioxidant properties and these properties are directly correlated to high phenolic, flavonoid and Tannin content in the extract. This potentially therapeutic extract could be used for the management of diabetes mellitus type II as it remarkably in. Further, this study directs future research in separating the bioactive compound responsible for the studied activity.

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Conflict of Interest

There is no conflict of interest.

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