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IN VITRO (α)- AMYLASE INHIBITION AND ANTI-OXIDANT ACTIVITIES OF TINOSPORA SINENSIS LEAVES EXTRACT

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ABSTRACT

In the present study, the methanol, Ethylacetate, and hexane extracts of *Tinospora sinensis* leaves were studied for α- amylase inhibition using an in vitro model. The plant extracts were also examined for its antioxidant activities by using free radical 1,1-diphenyl-2-picryl hydrazyl (DPPH) scavenging method, radical scavenging ability, reducing power capacity, estimation of total phenolic content, flavonoid content. The study exposed that the different concentrations of the extract possessed a very good amount of total phenolics, flavonoid and exhibit potent radical scavenging activity using DPPH as substrate. The methanol and ethyl acetate extract exhibited significantly α-amylase inhibitory activities with an IC50 value 202.2 and 327.1 dry extract respectively and well compared with standard acarbose drugs. Thus, it could be concluded that due to the presence of antioxidant components the plant extract have well prospective for the management of hyperglycemia, diabetes and the related condition of oxidative stress.

Keywords: *Tinospora sincensis*, leaves extract, Antioxidant activities, DPPH radical activity and α-amylase inhibitory activity

1. INTRODUCTION

Medicinal plants have been used as a source of medicine with their own personal recipes, which have been passed from one generation to another. Plant-derived substances have recently become of great interest owing to their versatile application **[1]**. Natural products present in the medicinal plants have been used as a source of drugs in the traditional medicine and some of them have been scientifically explored **[2]**. In recent years, the use of natural antioxidants has been promoted because of the concerns on the safety against synthetic drugs **[3]**. Antioxidants play an important role in protecting against cell damaged by reactive oxygen species and decreasing the adverse effects of these free radicals on normal physiological functions in humans. Phenolic compounds are the class of antioxidant agents, which can act as free radical terminators **[4]** and possess scavenging ability due to their hydroxyl group **[5]**. The antioxidant 698 the activity of phenolic is mainly due to the redox properties that allow them to act as reducing agents, hydrogen donors, and singlet oxygen quenchers.

Diabetes mellitus is an important chronic metabolic disorder that affects the metabolism of carbohydrates, fat, and protein. It includes a group of metabolic diseases characterized by hyperglycemia, in which blood sugar levels are elevated either because the pancreas does not produce enough insulin or cells of the body do not respond properly to the insulin produced. The effects of diabetes mellitus include long-term complications include heart disease, stroke, dysfunction, and failure of various organs **[6]**. There are three forms of diabetes. The three main types of diabetes are type 1, type 2, and gestational diabetes.

Another effective method to control diabetes is to inhibit the activity of α-amylase enzyme which is responsible for the collapse of starch to more simple sugars (dextrin, maltotriose, maltose, and glucose) **[7]**. This is contributed by α-amylase inhibitors, which delay the glucose absorption rate thereby maintaining the serum blood glucose in hyperglycemic individuals **[8]**. Some inhibitors currently in clinical use are acarbose and miglitol which inhibit glycosidases such as α-glucosidase and α-amylase while others such as and voglibose inhibit α-glucosidase. However, many of these synthetic hypoglycemic agents have their limitations, are non-specific, produce serious side effects, and fail to elevate diabetic complications. The main side effects of these inhibitors are gastrointestinal viz.,

bloating, abdominal discomfort, diarrhea, and flatulence **[9]**.

Thus, in this study, the antioxidant and antidiabetic activities were carried out, the methanol, ethyl acetate and hexane extract of *Tinospora sincensis* leaves. To determine the potential of *Tinospora sincensis* stem extract as antidiabetic agents, we investigated the effect of extracts on the α-amylase inhibitory activities.

2. MATERIALS AND METHODS

2.1 Materials

Chemicals: The solvents, and reagents were of analytical reagent grade or the highest quality commercially available and were purchased from Sigma, Aldrich, Fluka, Merck, and Spectrochem used as received without further purification.

Plant material: The leaves of *Tinospora sincensis* was collected from Mettur, Salem district during November and December (38 ± 1°C) in the year 2019 and this image shown in **Figure 1a**.

2.2 Methods

2.2.1 Soxhlet Extraction

The powdered leaves were subjected to Soxhlet extraction at 35^{o}C using solvents like hexane, ethyl acetate, acetone, methanol in increasing polarity.

About 25 g of dry powdered leaves of *Tinospora sincensis* was taken in the porous thimble made of tough filter paper and then placed in the inner tube of the soxhlet apparatus. The apparatus was then fitted to RB flask of appropriate size containing the solvent (500 ml) and boiling chips, to a reflux condenser. The solvent was boiled gently; the vapour passed off through the tube was condensed by the condenser and the condensed solvent filled the body of the soxhlet. A plug of wool was placed within the bottom of the thimble. The solute to solvent ratio maintained was 1:10. When the solvent reached the top of the tube, it shiponed over into the RB flask and thus removed the portion of the substance which it had extracted from the porous thimble.

For the continuous extraction of a solid by a hot solvent it is better to use a soxhlet extraction apparatus as shown in the figure.

The process was repeated continuously until complete extraction. The total duration consumed by this process was 16 hours and the inference was from green color slowly turned to pale green and finally to colorless. For the accuracy of the extraction, it has been carried out using a sequence of solvents with increasing order of polarity. The solvent sequence is shown below.

Hexane < Ethyl acetate < Methanol

The petroleum ether (500 ml) was initially used to remove lipids and some other impurities if present. The further solvent was used step by step to separate the desired antioxidant contents.

2.2.2 Phytochemical Analysis

2.2.2.1 Identification of Constituents by Phytochemical Test

The extracts were subjected to qualitative tests for detection of phytoconstituents present in it viz. alkaloids, terpenoids, carbohydrates, phenolic compounds, tannin, proteins, free amino acids and saponins .

1.Test for alkaloids

Small quantities of crude extracts were treated with few drops of diluted hydrochloric acid and filtered.

Dragendroff's reagent test: Filtrates were treated with dragendroff's reagent. Formation of red precipitate indicates the presence of alkaloids.

Hager's reagent test: To a few ml of filtrate 1 or 2 ml of Hager's reagent (saturated aqueous solution of picric acid) were added. A prominent yellow precipitate indicated the test as positive.

Wagners test: Filtrates were treated with Wagner s reagent. Formation of brown/ reddish brown precipitate indicates the presence of alkaloids.

2. Terpenoids

The Ethanolic extract is treated with 2ml of chloroform and 1ml of conc. H_2SO_4. The reddish brown color formation is observed.

3. Phenolic compounds and Tannins

Ferric chloride test: The extract is treated with 2ml of water and 10% aqueous ferric chloride solution. The Blue or green coloration is observed.

Test for Lead acetate test: The Ethanolic extract is treated with few drops of 1% lead acetate solution. The Yellow or red precipitate formation is observed.

Gelatin test: To the extract, 1% gelatin solution containing sodium chloride was added. Appearance of white precipitate indicates the presences of tannin

4. Test for carbohydrates

Molisch's test: The filtrate was treated with 2-3 drops of 1% alcoholic alpha naphthol and 2ml of concentrated sulphuric acid was added along the sides of the test tube.

5. Test for Flavonoids

Ammonia test: 5 mL of dilute ammonia solution were added to a portion of the crude extract followed by addition of concentrated H_2SO_4. Formation of a yellow colouration in the extract indicates the presence of flavonoids. The yellow colouration disappears after some time.

6. Proteins and amino acid

Ninhydrin : The extracts were heated with 5% Ninhydrin solution in boiling water bath for 10 min and development of purple or bluish color indicated the presence of amino acids.

$CuSO_4$: 2 ml of extract was treated with 2 ml 5% NaOH and 2 ml 1% $CuSO_4$ solutions. Violet or purple coloration indicated presence of proteins and free amino acids.

7. Glycosides

Keller – Killani Test: 5ml of each extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. This was under layered with 1ml of concentrated **H_2SO_4**. A brown ring of the interface indicated a deoxysugar characteristic of cardenolides.

8. Saponins

Frothing Test: A small quantity of different extract was diluted with 4 ml of distilled water. The mixture was shaken vigorously and then observed on standing for stable brake.

2.2.2.2 Determination of Total Phenol Content

Contents of total phenolics in the leaf extract of *Tinospora sincensis* were estimated by a UV-visible spectrophotometer array based on procedure described by Singleton and Rossi **[10]** with some modification. Basically 1ml of extract sample was mixed with 1ml of Folin and Ciocalteu's phenol reagent. After 3minutes 1ml of saturated sodium carbonate solution was added to the mixture and it was made up to 10ml with distilled water. The reaction was kept in dark for 90 minutes after which the absorbance was read at 725nm. Gallic acid was used for constructing the standard curve and the results were expressed as milligram of gallic acid equivalent per gram of extract.

2.2.2.3 Determination of Total Flavonoid Content

Flavonoids content in the extract was determined by the UV-visible spectrophotometer array described by Jia *et al*. **[11]** with some modifications. The *Tinospora sincensis* leaf extract (250µl) was mixed with 1.25ml of distilled water and 75µl of 5% sodium nitrate solution. After 5 minutes, 150 µl of 10% $AlCl_3.H_2O$ solution was added. After 6 minutes, 500 µl of 1M sodium hydroxide and 275 µl of distilled water was added to prepare the mixture. The solution was mixed well and the absorbance was read at 510 nm. (±) Catechin was used to calculate the standard curve and the results were expressed as mg of % RSA (±) Catechin equivalents per gram of extract.

2.2.2.4 DPPH Radical Scavenging Activity

Various concentrations of leaf extracts of *Tinospora sincensis* (0.3 ml) were mixed with 2.7ml of methanol solution containing DPPH radicals (6×10^{-5} mol/lit). The mixture was shaken vigorously and allowed to stand for 60 minutes in the dark. The mixture was shaken vigorously. The reduction of the DDPH radical was determined by reading the absorbance at 517nm **[12]**. The radical scavenging activity was calculated as a percentage of DPPH discolorations using the formula,

$$\% \text{ RSA} = \frac{ABS_{DPPH} - ABS_S}{ABS_{DPPH}} \times 100$$

Where ABS_S is the absorbance of the extract sample and ABS_{DPPH} is the absorbance of the DPPH solution. Ascorbic acid was used as standards.

2.2.2.5 Reducing Power Activity

Various concentrations of leaf extracts of *Tinospora sincensis* (2.5 ml) were mixed with 2.5 ml of 200mM sodium phosphate buffer (P^H =6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50˚C for 20 minutes. After 2.5 ml of 10% trichloroacetic acid (W/V) was added, the mixture was centrifugated at 1000 rpm for 8 minutes. (HERMLEZ 300K centrifuge). The upper layer (5ml) was mixed with 5ml of deionized water and 1ml of 0.1% ferric chloride and the absorbance was measured spectrophotometrically at 700nm. Ascorbic acid was used as a standard **[13]**.

2.2.3 In *vitro* α-amylase inhibition activity

The α-amylase inhibitory activities were investigated using the procedure reported by Nickavar and Yousefian **[14]**, which was originally proposed by Bernfeld (1955). The solution of compounds was prepared in DMSO with the various concentrations (100, 200, 300, 400, and 500 µg/mL). 500 µg/mL α-amysase solutions prepared in 0.02 M sodium phosphate buffer (pH 6.9) was added to different concentrations of the compounds and incubated for 15 min at 25˚C. After 10 min, 500 µg/mL of 1% starch in 0.02 M of sodium phosphate buffer was added to each tube. The mixture was further incubated at 25˚C for 10 min. Then the reaction was terminated by adding 1 mL of DNS reagent (12.0 g of sodium potassium tartrate tetrahydrate in 8 mL of 2 M NaOH and 96 mM 3,5-dinitrosalicylic acid solution) and the contents were heated in a boiling water bath for 5 min. The resulting reaction mixture was then diluted with 10 mL distilled water, and absorbance was measured at 540 nm. Acarbose was used as a positive control/standard. The antidiabetic activity of the compounds was determined by the inhibition of α-amylase that was expressed as

a percentage of inhibition and calculated by the following equation.

% inhibition = $[(A_C-A_S) / A_C] \times 100$

Where A_C and A_S are the absorbances of control and sample respectively

3. RESULTS AND DISCUSSION

3.1. Phytochemcial Screening

Phytochemical screening evaluation of the various extracts from the leaves of *Tinospora sincensis* listed in **Table 1.** Phytochemical screening revealed the presence of phenolic and flavonoids in a high level, which could be mainly responsible for the remarkable antioxidant effect of *Tinospora sincensis* leaves extracts. The other phytochemical constituents found to be present where flavonoids, glycosides, amino acid, carbohydrates, saponins, terpenoids, Oil and fat, or the presence of the phytochemicals. Terpene and saponin were detected only in hexane and methanol extracts.

3.2. Phytochemical Analysis

The successive extracts of *Tinospora sincensis* leaves have revealed the presence of industrially useful phytochemicals. Thus this preliminary screening tests may be useful in the detection of the bioactive principles and subsequently may lead to drug discovery and development of these classes of compounds (such as flavonoids, tannins, carbohydrates, saponins, terpenoids, fats, etc.) are known to have curative activity against several pathogens and therefore suggest their use traditionally for the treatment of various illness **[15]**. The curative properties of medicinal plants are perhaps due to the presence of various secondary metabolites as cited above.

The presence of these phytochemical compounds detected from leaf extracts of *Tinospora sincensis* leaves is known to have beneficial importance in industrial and medicinal sciences. Phenol compounds possess biological properties such as antioxidants, antiaging, antiapoptosis, anticarcinogen, anti - inflammation, cardiovascular protection as well as inhibition of angiogenesis and self proliferation activity. Most of this biological action has been attributed to their intrinsic reducing capabilities **[16]**. Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of the hydrolytic and oxidative enzyme, anti-inflammatory action **[17]**. They also show antiallergic, antimicrobial, antiviral, antibacterial, anticancer activity, etc.

3.3 Total Phenol and Flavonoid Content

The phenolic and flavonoid contents of the various solvent extracts are given in **Table 2**.

The comparative study of phenolic and flavonoid content is graphically represented in **Figure 1b**. The highest antioxidant activity was observed in methanol extract (0.607 and 0.891) proving efficient extraction. Whereas the flavonoid and phenolic content such as (0.455, 0.659) and (0.200, 0.463) of ethyl acetate and hexane the extract have been respectively. The determination of their content seems to be much important to explore the phytochemical properties/activities of this species.

Polyphenolic compounds have been labeled as "high level" natural antioxidants based on their abilities to scavenge free radicals and active oxygen species **[18]**. They contain conjugated ring structures and hydroxyl group that have the potential to function as antioxidants *in vitro* or cell-free systems by scavenging superoxide anion, singlet oxygen, lipid peroxy radicals and stabilizing free radicals involved in oxidative processes through hydrogenation or complexing with oxidizing species.

3.4 DPPH Radical Scavenging Activity

Various concentrations of *Tinospora sincensis* leaves extracts were found to increase with an increase in concentration evidenced by the quick disappearance of the violet colour of DPPH free radical. IC_{50} values calculated from the **Figure 1c** revealed the potency of acetone extract which are listed in **Table 3**. All the values of percentage RSA were compared against the standard, Ascorbic acid whose IC_{50} value.

DPPH is a very stable organic free radical with a deep violet colour which gives absorption maxima within the 515-528 nm. Upon receiving proton from any hydrogen donor, mainly from phenolics, it loses its chromophore and became yellow. As the concentration of phenol compounds or degree of hydroxylation of the phenol compounds increases DPPH radical scavenging activity increases and with its antioxidant activity. Because this radical is very sensitive to active ingredients at low concentration and can accommodate a large number of samples in a very short time. DPPH stable free radical method is an easy, rapid, and sensitive way to survey the antioxidant activity of a specific compound or plant extracts. However, due to the specificity of radical applied, which are not oxygen-related radical species, antioxidant activity assessed can be related to them only **[19]**.

Flavonoids widely distributed in plants have the ability to scavenge free

radicals, superoxide and hydroxyl radical by single-electron transfer. In this study various concentrations (0.25, 0.5, 1, 1.5, 2) extract were exposed to the DPPH radical for measuring their decrease in absorption.

The leaf extract acted rapidly after mixing the sample with DPPH solution leading to the quick disappearance of more than 80% of free radical within 1 minute. An appreciable IC_{50} value was obtained not only for methanol extract but also for hexane and ethyl acetate extracts present in 1mg/ml of the crude leaf extract, compared to the standard (Ascorbic acid).

3.5 Reducing Power

Reducing concentrations of the respective solvents of the investigated leaf extract was found to be active correspondingly with an increase in absorption. EC_{50} value calculated from the **Figure 1d** was found to be 0.154mg/ml for acetone, 0.162mg/ml for methanol, 0.235mg/ml for ethyl acetate compared against the standard, whose EC_{50} was 0.101mg/ml present in 1mg/ml of the crude extracts.

All the values of the respective crude solvent extracts are listed in **Table 4**. In this method of reducing ferric irons to ferrous iron accompanying with the colour change, the selected plant extract showed a gradual increase in absorption with an increase in concentration for almost all the extracts.

Reports prove that the reducing properties are generally associated with the presence of reductones, which have been shown to excerpt antioxidant action by breaking the free radical chain by donating a hydrogen atom. From **Figure 1d** it can be inferred that there may be a high amount of reductones in the extract **[20]**. The obtained absorption values (0.176, 0.259, 0.387, 0.490, 0.593) increases with increase in concentration (0.2, 0.4, 0.6, 0.8, 1.0) showing the extent of active reducing power of the leaf extract. Also the EC_{50} value of acetone extract (0.101mg/ml) was calculated from the graph, present in 1mg/ml of the crude extract against the standard, Ascorbic acid.

EC_{50} values of acetone and methanol were 0.154 & 0.162 present in 1 mg/ml. The principle behind the reducing power assay is based on its electron donating activity which is an important mechanism of phenolic antioxidant action. Reducing power activity is noteworthy that it has a gradual increase of absorption with an increase in various concentrations of the *Tinospora sincensis* leaf extracts. The increase of the plot in **Figure 1d** makes clear about the presence of reductones on flavonoids which readily

reduce the free radicals by donating its electron.

3.6 FT-IR studies for hexane, ethyl acetate and methanol extraction

FT-IR spectra were recorded with a Perkin Elmer-Spectrum. The spectrometer was continuously purged with dry nitrogen to eliminate atmospheric water vapour and carbon dioxide. Background spectra, which were collected under identical conditions, were subtracted from the sample spectra automatically. The frequencies for all sharp bands were accurate to 0.001 cm^{-1}. Each sample was scanned under the same condition for five times.

The IR spectrum of different extracts reveals structural information about major and minor constituents were shown in **Figure 2-4**. The peaks at 3418 and 3404 cm^{-1} assigned to the O-H or NH stretching vibration. In addition, the peak at 1732-1608 cm^{-1} assigned to the C=O stretching vibration of carbonyl compounds existed in the leaf extracts. Specifically, –C-O stretching vibrations show the IR spectral region like 1067-1074 cm^{-1}. The –CO and –OH vibrational frequencies exactly indicate, the extracts have phenolic –OH group. Further, C-C and C-H stretching vibration of corresponding values indicates, the bonding associated with aromatic skeletons. So, depending on the fingerprint characters of the peaks positions, shapes and intensities, the fundamental components may be identified.

3.7 *In vitro* α-amylase inhibition activity

The results of an antidiabetic activity using the pancreatic α-amylase inhibitory assay of the ethyl acetate and methanol extracts of *Tinospora sincensis* leaves are shown in **Table 5**.

The extract revealed a significant inhibitory action of α-amylase. The percentage inhibition at 100- 500 µg/ mg concentrations of *Tinospora sincensis* leaves extract showed a dose-dependent increase in percentage inhibition. The percentage inhibition varied from 24.38% - 87.37% for increasing concentration. Thus the inhibition of the activity of α-amylase by *Tinospora sincensis* leaves extract would delay the degradation of carbohydrates, which would in turn cause a decrease in the absorption of glucose, as a result, the reduction of postprandial blood glucose level elevation. A comparison of α-amylase inhibitory activity between the methanol and ethyl acetate extract of *Tinospora sincensis* leaves have been depicted in **Figure 5**.

In this study, acarbose was also used as a standard drug for α-amylase inhibitors. Acarbose at a concentration of (100- 500 µg/ mg) showed α-amylase inhibitory activity

from 48.55 to 98.1 % with an IC50 value of 94.8. This indicates that the ethyl acetate extract of *Tinospora sincensis* leaves is a very potent αamylase inhibitor in compared with methanol. This could be justified that the nature of some extract constituents (phenols, flavonoids saponins, steroids, alkaloids, terpenoids) present in the extract could be responsible for being effective inhibitors of α-amylase.

Table 1: Phytochemical screening on leaves extracts of *Tinospora sincensis*

Test	Hexane	Ethyl acetate	Methanol
1) Alkaloid test			
A) Drangen droff	+	+	+
B) Hagers test	+	++	+
C) Wagner's test	+	+	+
2) Test for terpenoids			
A) H_2SO_4 test	++	-	+
3) Test for Phenols and Tannins			
A) Lead acetate test	+	-	++
B) $FeCl_3$	-	+	-
C) Gelantin	+	–	+
4) Protein & amino acid			
A) $CuSO_4$	–	–	–
B) Ninhydrin	–	–	–
5) Carbohydrate			
A) Molish test	+	-	+
B) Benedict test	++	++	++
C) Fehling (A+B)	-	-	++
6) Glycosides			
A) Keller-killani test	++	+	+
7) Saponin	++	-	+
	++	-	-

Table 2: Total antioxidant Activity of different extract of *Tinospora sincensis* leaves

Total antioxidant activity	Hexane	Ethyl acetate	Methanol
Total Flavonoid	**0.200**	**0.455**	**0.607**
Total Phenol	**0.463**	**0.659**	**0.891**

Table 3: DPPH Radical Scavenging Activity of different extract of *Tinospora sincensis* leaves

Concentration (mg)	Hexane	Ethyl acetate	Methanol	Ascorbic acid
0.25	**1.193**	**1.335**	**0.194**	**0.026**
0.5	**1.134**	**0.919**	**0.094**	**0.022**
1	**1.075**	**0.552**	**0.094**	**0.016**
1.5	**1.015**	**0.296**	**0.093**	**0.012**
2	**0.866**	**0.193**	**0.097**	**0.009**

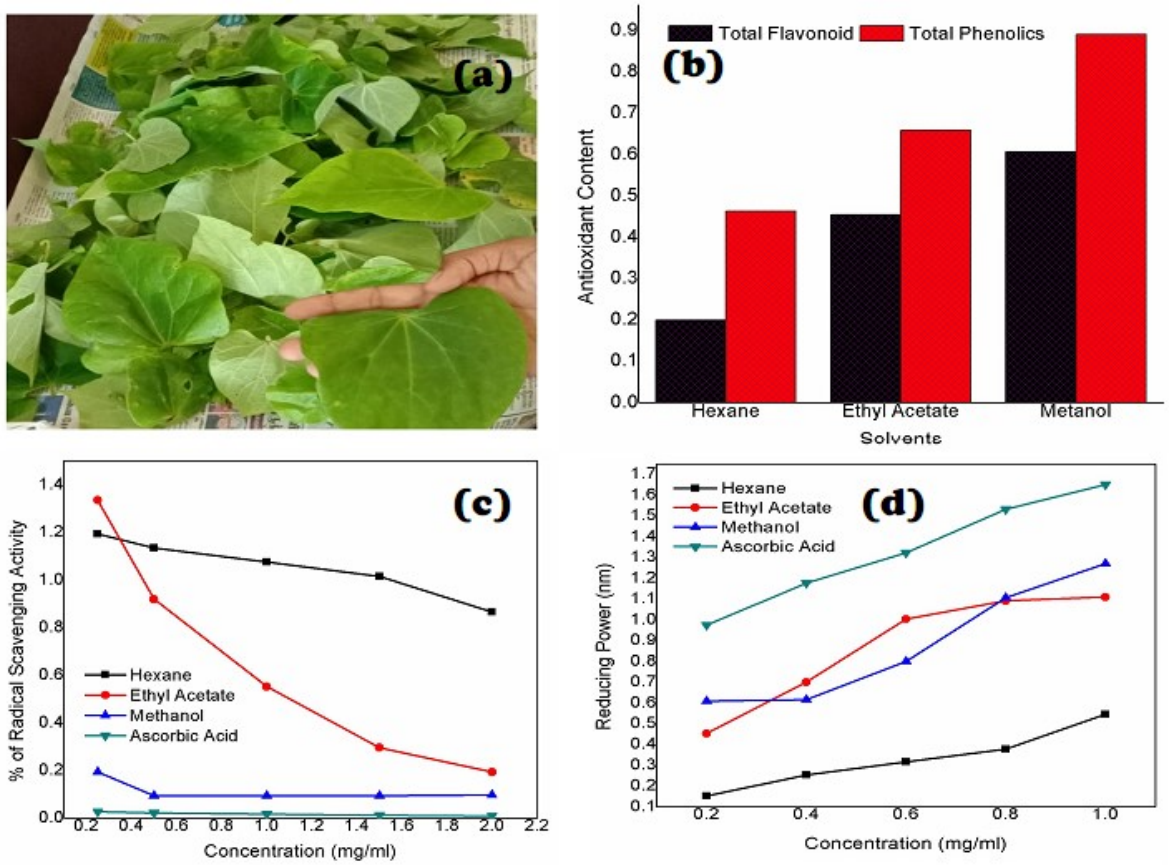

Figure 1 (a) Green leaves of Tinospora sinensis (b) Total antioxidant Activity (c) DPPH Radical Scavenging Activity (d) Reducing Power

Table 4: Reducing Power of different extract of *Tinospora sincensis* leaves

Concentration	Hexane	Ethyl acetate	Methanol	Ascorbic acid
0.2	0.153	0.453	0.609	0.975
0.4	0.253	0.7	0.616	1.177
0.6	0.317	1.003	0.799	1.323
0.8	0.378	1.091	1.107	1.531
1	0.546	1.109	1.271	1.651

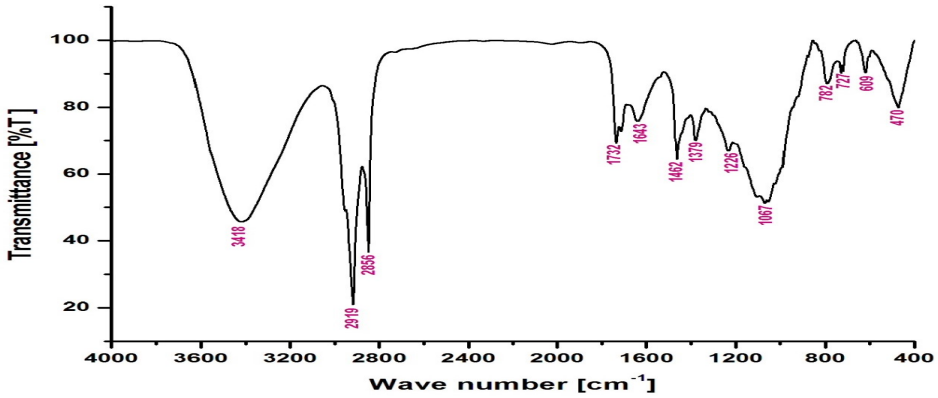

Figure 2: FT-IR spectrum for hexane extract of *Tinospora sincensis* leaves

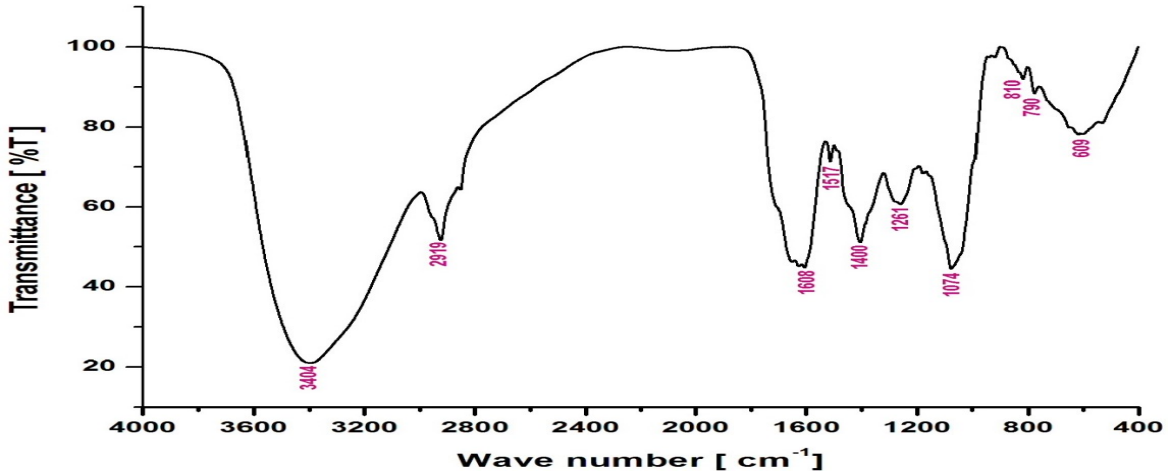

Figure 3: FT-IR spectrum for Ethylacetate extract of *Tinospora sincensis* leaves

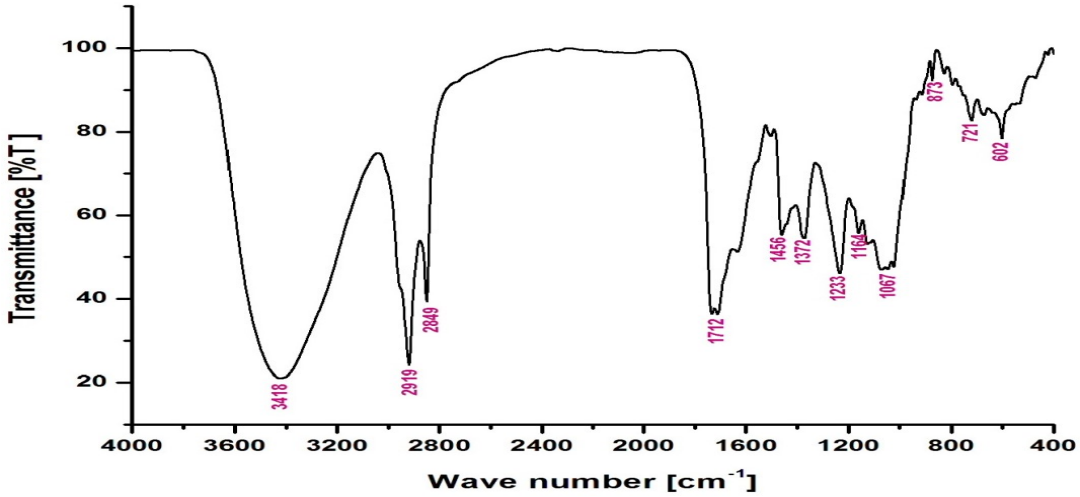

Figure 4: FT-IR spectrum for Methanol extract of *Tinospora sincensis* leaves

Table 5: *In vitro* α-amylase inhibition activity of *Tinospora sincensis* leaves extracts

Solvent crude	% of inhibition					
	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml	500 µg/ml	IC_{50} µg/ml
Ethyl acetate	38.34	49.39	62.08	71.84	86.65	202.2
Methanol	24.38	36.25	49.53	60.19	66.27	327.1
Acarbose	48.55	64.38	76.91	89.28	98.1	94.8

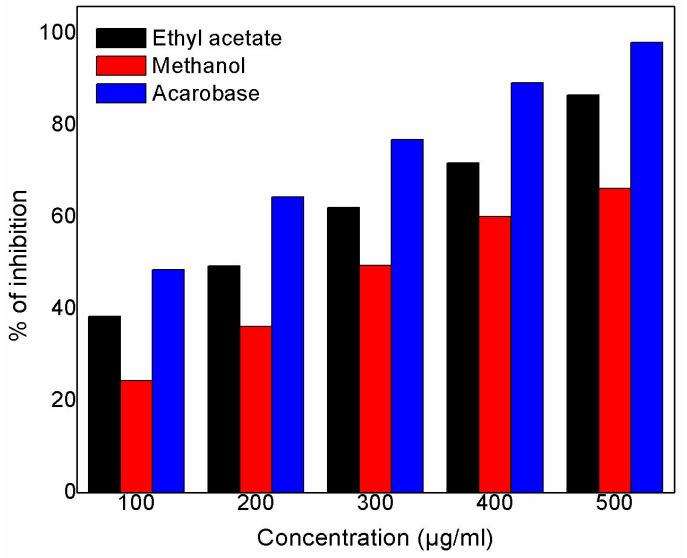

Figure 5: Alpha amylase inhibitory activity ratio of crude sample and standard

4. CONCLUSION

The study revealed that the different solvent of the extract possessed a very a good amount of total phenolics, flavonoid and exhibit potent radical scavenging activity using DPPH. Particularly, methanolextract posses good antioxidant activity than that of ethyl acetate and hexane extracts. The FT-IR spectrum of crude fractions showed that the presence of phenolic OH, aromatic C=C and C-O groups. The ethyl acetate extract exhibited significant α-amylase inhibition activity.

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