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SCIENTIFIC VALIDATION OF A NOVEL PLANT (*CROTOLARIA GENISTOIDES*) TO EXPLORE THE WEALTHINESS OF SIDDHA PHARMACOLOGY FOR ITS HYPOGLYCEMIC EFFECT- AN IN VITRO ASSAY

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ABSTRACT

Introduction: Siddha is the first and foremost traditions of healthcare with many therapeutic benefits. There is lot of scope and opportunities for Siddha. Only there is lack of scientific validations at various levels. In the plant kingdom there is remedy for every diseases. WHO estimates that 80 percent of the people in the developing countries of the world rely on traditional medicines for healthcare. **Methodology:** Plant extracts were made from the different solvents to get the detailed information about the phytochemicals present in the plant. Activity guided fractionation is the method employed to identify biologically active compound using Column chromatography, GCMS and then glucose diffusion and alpha amylase experimentation is for a pharmacological action of the fractioned compound. **Result and Discussion:** From this technique, it is revealed that chemical compound are separated and the glucose inhibitory test shows *Crotolaria genistoides* to have maximum inhibitory effect to the diffusion of glucose across dialysis membrane but it showed the least inhibition of alpha amylase activity. **Conclusion:** Thus, this exploration of this chemical compounds possess hypoglycemic effect and pave the way to increase the scope of Siddha medicine.

KEYWORDS: Siddha, WHO, Crotolaria Genistoides, Hypoglycemic Effect.

1. INTRODUCTION

Siddha medicine is bitter, but it was better than other medicine. Siddha system is an ancient system of medicine which has got enormous herbal values to cure various diseases without any Side effects in today's world the usage of siddha medicine is inevitable.[1] There is lot of scope and opportunities for Siddha. Only there is lack of scientific validations at various levels. In the plant kingdom there is remedy for every diseases. Lifestyle diseases better known as non-communicable diseases (NCDs) and diseases of civilization, characterize those diseases whose occurrence is primarily based on the daily habits of people and also as a result of an inappropriate relationship of people with their environment. It is a medical condition or disease which is non-infectious and non-transmissible, but is likely to continue progressively unless intervened. According to WHO, Lifestyle diseases (LSDs) may be chronic diseases of long duration and slow progression or they may result in more rapid death.[2] The major reason of this study is to explore our Siddha Pharmacology to Global level (By applying Botanical and Biochemical Parameters into our pharmacology) and there by Exploration of certain compounds from the plants as a natural emerging compound globally for its biological effects.

2. MATERIALS AND METHODS

2.1 Collection of Plant Material

Initially the *Crotolaria genistoides*[3] leaves were collected from Salem district. First the dirty part of leaves was removed and dried at room temperature, then it was grinded into fine powder using mechanical grinder.

2.2 The Powdered *Crotolaria genistoides* leaves (5 g) was macerated in 100 ml of methanol and was kept in shaker device at 100 rounds per minute for 72 hrs at room temperature. The result was extracted and filtered by using filter paper (What Man No 1.5 What Man Lted., England). The extract was decanted, filtered and the filtrate was evaporated. The residue of the extract was used for further analysis.

2.3 Phytochemical Screening

Preliminary qualitative phytochemical analysis was performed on the methanolic extract using standard procedures to identify phyto-constituents as described by Harborne (1973); Trease and Evans (1989); Sofowara (1993) and Kokate *et al*., (1996).

2.3.1 Test for Alkaloids

Reagents: a. Wagner's reagent: 2g of Iodine and 6 g of potassium iodide (KI) were dissolved in 100 ml of distilled water.

b. Hydrochloric acid, 1.5% v/v: 3.73 ml of commercially available hydrochloric acid (11 M) was mixed with and made up to 100 ml with distilled water.

c. Mayer's reagent: 1.358 g of mercuric chloride ($HgCl_2$) was dissolved in 60 ml of distilled water and poured into a solution of 5 g potassium iodide (KI) in 10 ml of distilled water and made up to 100 ml with distilled water.

Procedure

Wagner's Test: 2 ml of methanolic extract was acidified by adding 1.5 ml of 1.5% v/v of HCl and a few drops of Wagner's reagent. Brown or yellow colour precipitate indicates the presence of alkaloids.

Mayer's Test

Extracts were dissolved individually in dilute Hydrochloric acid and filtered. Filtrates were treated with Mayer's reagent. Colour change indicates the presence of alkaloids.

2.3.2 Test for Flavonoids

Reagents

a.1% sodium hydroxide.

b. Dilute hydrochloric acid.

Procedure

Alkaline Reagent Test: To 1ml of extract few drops of 1% sodium hydroxide was added, an intense yellow colour was appeared which becomes colourless by adding dilute hydrochloric acid that indicates the presence of flavonoids.

2.3.3 Test for Glycosides

Reagents

a. Ferric chloride, 5%: Five g of ferric chloride was dissolved in 100 ml of distilled water.

b. Concentrated Sulphuric acid

c. Glacial acetic acid

Procedure

Keller-Killiani Test: To 2 ml of the extract, few drops of glacial acetic acid, one drop of 5% ferric chloride and concentrated sulphuric acid were added. Reddish brown colour appeared at the junction of two liquid layers and upper layer turned bluish green indicated the presence of glycosides.

2.3.4 Test for Tannins and Phenolic Compounds

Reagents

a. Ferric chloride, 5%: Five g of ferric chloride was dissolved in 100 ml of 90% alcohol.

b. Lead acetate, 10%: 10g of lead acetate was dissolved in 100 ml of distilled water.

c. Gelatin solution, 1%: 1g of gelatin was dissolved in 100 ml 10% sodium chloride (10 g sodium chloride dissolved in 100 ml of distilled water).

Procedure: To 1ml of extract, 2 drops of 5% ferric chloride solution was added. An intense green colour indicated the presence of phenolic compounds, whereas appearance of a violet colour indicated the presence of tannins. A buff coloured precipitate with lead acetate (10%) confirmed the presence of phenolic compounds. When gelatin solution (1% gelatin containing 10% sodium chloride) was added to the extract, formation of precipitate confirmed the presence of tannins.

2.3.5 Test for Saponin

Reagents

a. Sodium bicarbonate

Procedure

Froth Test: *1ml of extract was* taken in a test tube and shaken vigorously with small amount of sodium bicarbonate and distilled water. Formation of honeycomb like froth indicated the presence of Saponin.

2.3.6 Test for Steroids

Reagents

a. Concentrated sulphuric acid.

Salkowski Reaction

To 2 ml of the plant extract, 1.0 ml of concentrated sulphuric acid was added carefully along the sides of the tube. Formation of red colour indicates the presence of steroids.

2.3.7 Test for Terpenoids

Reagents

a. Acetic anhydride

b. Concentrated sulphuric acid

Procedure

Libermann – Burchard's Test

To 1ml of plant extract, 1ml of chloroform and 2 ml of acetic anhydride were added and mixed then, 2 drops of concentrated sulphuric acid was added. The dark green colouration of the solution indicated the presence of steroids and pink colouration of the solution indicated the presence of terpenoid.

2.4 Separation of Phytoconstituents Using Column Chromatography

In order to isolate the bioactive compound from the crude extracts they were further fractionated using column chromatography.

Stationary phase: Silica gel 60 (230 - 400)

Mobile Phase

Ethanol, methanol and distilled water was mixed in the proportion of 60:20:20.

A cylinder shaped glass column containing stationary phase (silica gel) was encountered slowly from the top with a liquid solvent (mobile phase) that flows down the column with the help gravity or external pressure applied. This technique was used for the purification of compounds from a mixture. Once the column is ready, the crude sample was loaded inside the top of the column. The mobile solvent was then allowed to flow down through the column. The compounds in mixture have different interactions ability with stationary phase (silica gel), and mobile phase, thereby will flow along the mobile phase at different time intervals or degrees. In this way, the separation of compounds from the mixture is achieved. The individual compounds are collected as fractions, totally 4 fractions were collected and analyzed further for structure elucidation.

2.5 Alpha Amylase Inhibition Assay

Dinitrosalicylic Acid Method

Reagents

a. 0.02 M Sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride.

b. 2 N Sodium hydroxide

c. Dinitrosalicylic acid color reagent: Prepared by dissolving 1.0 gm of 3,5-dinitrosalicylic acid in 50 ml of reagent grade water. Add slowly 30.0 gms sodium potassium tartrate tetrahydrate. Add 20 ml of 2 N NaOH. Dilute to a final volume of 100 ml with reagent grade water. Protect from carbon dioxide and store no longer than 2 weeks.

d.1% Starch: Prepare by dissolving 1.0 gm soluble starch, in 100 ml 0.02 M sodium phosphate buffer, pH 6.9 with 0.006 M sodium chloride. Bring to a gentle boil to dissolve. Cool and bring volume to 100 ml, with water, if necessary. Incubate at 25°C for 4-5 minutes prior to assay.

e. Alpha amylase with concentration of 1 mg / ml.

Procedure

α-amylase inhibitory activity of extract and fractions was carried out using 3, 5 Dinitrosalicylic acid method.The extracts with different concentration such as 100 µl, 200 µl, 300 µl and 400 µl and fractions with concentration of 100 µl were added to 500 µl of 0.02M sodium phosphate buffer (pH 6.9 containing 0.006 M sodium chloride) containing 1 mg/ ml of α–amylase solution and were incubated at 37°C for 20 min. Then 500 µl of 1% starch was added as a substrate and incubated further at 37°C for 30 min; 100 µl of the DNS color reagent was then added and was kept in boiling water bath for 10 min. The absorbance of the resulting mixture was measured at 540 nm in UV - visible spectroscopy. Without test (extract and fractions) substance was set up in parallel as control. The results were expressed as percentage inhibition, which was calculated using the formula

$$\% \text{ Inhibition} = \frac{\text{Abs control} - \text{Abs test sample}}{\text{Abs control}} \times 100$$

2.6 Glucose Diffusion Inhibitory Studies: 1ml of the extract was then placed in a dialysis membrane (12000MW, HiMedia Laboratories, Mumbai, India) along with 1 ml of glucose solution (0.22mM in 0.15 M sodium chloride).It was then tied at both ends using thread and it was immersed in a beaker containing 40ml of 0.15 M sodium chloride and 10ml of distilled water. The control contained 1ml of 0.15M sodium chloride containing 22mM glucose and 1ml of distilled water. The beakers were then placed on orbital shaker and kept at room temperature. The movement of glucose into the external solution was monitored every half an hour. Simultaneously the glucose diffusion inhibition activity of fractions was carried out by replacing 1 ml of extract with the fraction.

2.7 Total Antioxidant Capacity

Phosphomolybdenum Assay

Reagent Preparation: Reagents was prepared by adding 1 ml each of 0.6M sulphuric acid, 28mM sodium phosphate and 4mM Ammonium molybdate. The final volume was made up to 50 ml with distilled water.

Procedure: The extracts with different concentration such as 100 µl, 200 µl, 300 µl and 400 µl and fractions with concentration of 100 µl was taken and 1ml of reagent solution was added to it and incubated in a boiling water bath at 95°C for 90 min. After 90 min, the absorbance of the solution was read at 695 nm. Ascorbic acid (10mg/ml DMSO) was used as standard.

The Anti-oxidant capacity was estimated using the following formula

$$\text{Total antioxidant capacity (\%)} = \left[\frac{(\text{Abs. of control} - \text{Abs. of sample})}{(\text{Abs. of control})}\right] \times 100$$

2.8 Gas Chromatography - Mass Spectroscopy (GC- MS) Analysis: The GC-MS analysis of the leaves extract was made in an Agilent 7890 A instrument under computer control at 70 eV. About 1 µl of the methanol extract was injected into the GC-MS using a micro syringe and the scanning was done for 45 min. As the compounds were separated, they eluted from the column and entered a detector which was capable of creating an electronic signal whenever a compound was detected (Imad et al., 2014a; Hameed et al., 2015d).

The greater the concentration in the sample, the bigger the signal obtained which was then processed by a computer. The time from when the injection was made (Initial time) to when elution occurred is referred to as the retention time (RT).While the instrument was run, the computer generated a graph from the signal called chromatogram. Each of the peaks in the chromatogram represented the signal created when a compound eluted from the gas chromatography column into the detector. The x-axis showed the RT and the yaxis measured the intensity of the signal to quantify the component in the sample injected. As individual compounds eluted from the gas chromatographic column, they entered the electron ionization (mass spectroscopy) detector, where they were bombarded with a stream of electrons causing them to break apart into fragments. The fragments obtained were actually charged ions with a certain mass The mass/charge (M/Z) ratio obtained was calibrated from the graph obtained, which was called as the mass spectrum graph which is the fingerprint of a molecule (Imad et

al., 2014b). Before analyzing the extract using GC-MS, the temperature of the oven, the flow rate of the gas used and the electron gun were programmed initially. The temperature of the oven was maintained at 100°C. Helium gas was used as a carrier as well as an eluent. The flow rate of helium was set at 1 ml/min. The electron gun of mass detector liberated electrons having energy of about 70eV.The column employed here for the separation of components was Elite 1(100% dimethyl poly siloxane). The identity of the components in the extracts was assigned by the comparison of their retention indices and mass spectra fragmentation patterns with those stored on the computer library and also with published literatures. Compounds were identified by comparing their spectra to those of the Wiley and C:/Database/NIST11.L mass spectral libraries

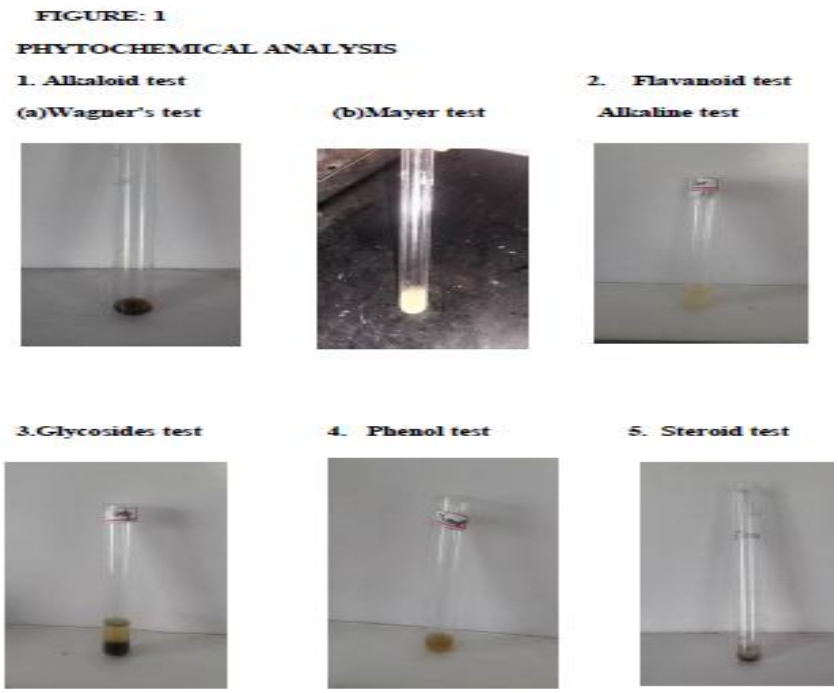
3. RESULTS

Plants serves as a vast source for varied phyto constituents exhibiting varied pharmacological property. Investigating such potential plants is of significance in medicine. Phytochemicals are bioactive chemicals of plant origin. They are regarded as secondary metabolites

3.1 Phytochemical screening of methanolic extract from the leaves of *Crotolaria genistoides:* Phytochemicals are bioactive chemicals of plant origin. They are regarded as secondary metabolites. In the present study, methanolic extract of *Crotolaria genistoides* was qualitatively examined for the presence of phytoconstituents .The results revealed the presence of Alkaloids, flavonoids, phenol, steroid and glycosides. Whereas Tannins, Saponin, terpenoids and quinine are absent (Table 1 and fig 1). any of these secondary metabolites, singly or in combination with others could be responsible for the anti-diabetic activity of the plant.

Table. 1. Phytochemical screening of methanolic extract from the leaves of *Crotolaria genistoides*

S. No	Constituents	Methanolic extract from leaves of *Crotolaria genistoides*
1.	Alkaloids Wagner's test Mayer’s test	 + +
2.	Flavonoids Alkaline test	 +
3.	Phenols	+
4.	Tannins	_
5.	Glycosides	+
6.	Saponins	_
7.	Steroids	+
8.	Terpenoids	_
9.	Quinone	_



3.2 Alpha - Amylase Inhibition Assay

▶ In the present study, α-amylase was significantly inhibited by methanolic extracts of *Crotolaria genistoides*. Natural α-amylase inhibitors from food-grade herbal sources offer an attractive therapeutic approach to the treatment of postprandial hyperglycemia by decreasing glucose release from starch and may have potential for use in the treatment of diabetes mellitus and obesity [gray and Flatt. 1997].

▶ Table 2, indicates the concentration of methanolic extract , ranges from 100 µl , 200 µl , 300 µl , 400 µl and results a percentage inhibition of 72.3%, 73.1%, 77.6%, 80.7%.whereas fraction 1,2,3,4 results a percentage inhibition .As a result , fraction 3 showed a maximum inhibition of 85.2 % in 400 µl concentration.

▶ α-amylase is an enzyme that hydrolyzes alpha bonds of large, alpha-linked polysaccharides, such as starch and glycogen. It is the major form of amylase found in humans and other mammals. Since α-amylase plays an important role in digestion of starch and glycogen, it is considered a strategy for the treatment of disorders in carbohydrate uptake, such as diabetes and obesity to reduce postprandial glucose level. Hence α-Amylase inhibitors may be of value as novel therapeutic agents. The invitro α amylase inhibitory study demonstrated that *Crotolaria genistoides* has a good antidiabetic property.

Table. 2. Inhibition of Alpha Amylase as Compared to Control.

Concentration	Control %	Methanolic extract %	Fraction 1 %	Fraction 2 %	Fraction 3 %	Fraction 4 %
100 µl	0	72.3	59.2	62.3	73.1	70.4
200 µl	0	73.1	62.3	67.7	75.2	72.5
300 µl	0	77.6	65.4	69.2	79.2	75.3
400 µl	0	80.7	69.1	72.4	**85.2**	79.2

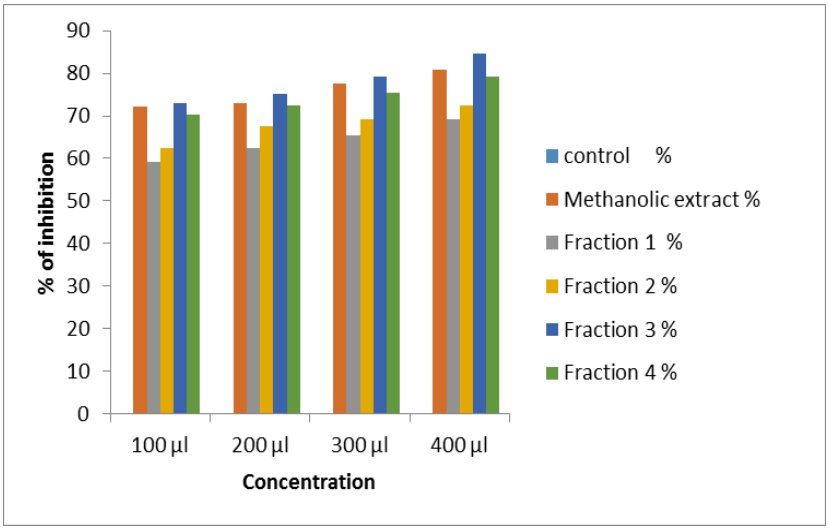


Figure. 2: Effect of Methanolic Extract & Fraction in Alpha – Amylase Assay.

3.3 Glucose Diffusion Inhibitory Assay

▶ Glucose diffusion studies of methanolic extract and fraction 3 reveal significant inhibitory effects on glucose movement into external solution across the dialysis membrane. Fraction-3 has shown most effective movement when compared to extract (Table 3 and Figure 3,4).

▶ Dialysis creates and maintains a concentration differential across the membrane. On liquid-to-liquid interface all the molecules will try to diffuse in either direction. Because of its close related function as that of cells, it is extensively used in in vitro assays as replicate to in vivo. Separation by dialysis membranes is based on diffusion, convection and adsorption. The adsorption is one of the important criteria options in treating diabetes. The mechanism of inhibition of glucose diffusion of plants is due to slow absorption of carbohydrates and inhibition of glucose transport. Present study attributes potential evaluation of phytochemical efficiency retarding the diffusion and movement of glucose diffusion.

Table. 3: Effect of Methanolic Extract and Fraction on Diffusion of Glucose Out of Dialysis Membrane Over 180 Minutes.

Time (mins)	Control Mean ± SEM	Methanolic extract Mean ± SEM	Relative movement %	Fraction 3 Mean ± SEM	Relative movement %
30	0.007± 0.0003	0.004 ± 0.0015	61.43	0.003 ± 0.0017	57.14
60	0.03 ± 0.0015	0.024 ± 0.0017	81	0.0126 ± 0.0022	54.33
90	0.117 ± 0.0058	0.093± 0.0085	79.48	0.0063 ± 0.0017	44.45
120	0.128 ± 0.0064	0.1 ± 0.002	78.36	0.057 ± 0.006	48.39
150	0.149 ± 0.0074	0.118 ± 0.0042	79.40	0.115 ± 0.0042	52.32
180	0.152 ± 0.0076	0.12 ± 0.0046	78.94	0.101 ± 0.0046	55.73

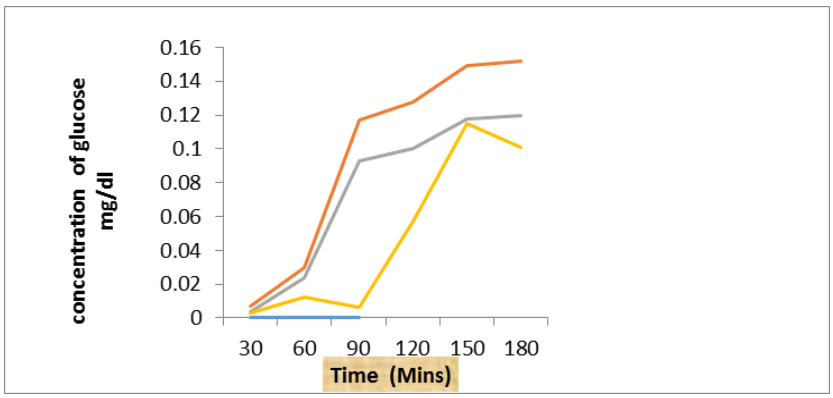


Figure. 3: Effect of Extract & Fraction on the Diffusion of Glucose Out of Dialysis Membrane.

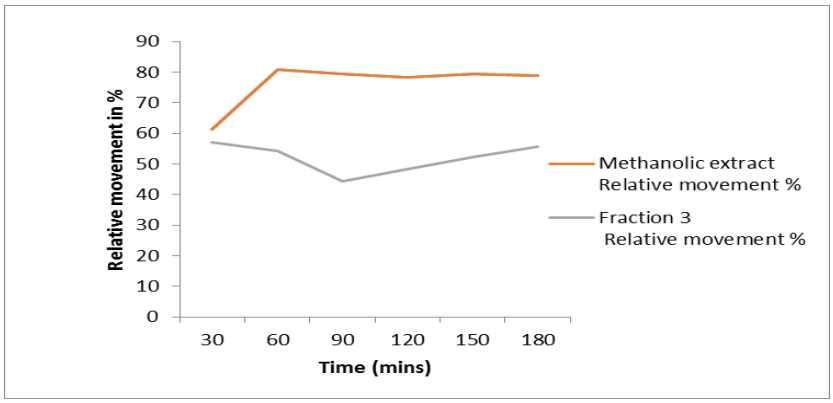


Figure. 4: Relative Movement of Glucose Across Dialysis Membrane.

3.4 Total Antioxidant Activity: The phospho molybdenum method is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compound and the formation of a green phosphate/Mo(V) complex with a maximal absorption at 695 nm. Increase in absorbance indicated the increase of the total antioxidant capacity. The antioxidant capacities of methanolic extract, were measured spectrophotometrically. Potent antioxidant capacity has been found in all samples (Table 4 and Figure 5).

Table. 4: Total Antioxidant Capacity.

	TAC %		
Concentration (µg / ml)	**Ascorbic acid**	**Methanolic extract**	**Fraction 3**
100	59	45	49
200	62	52	55
300	72	61	65
400	81	65	69

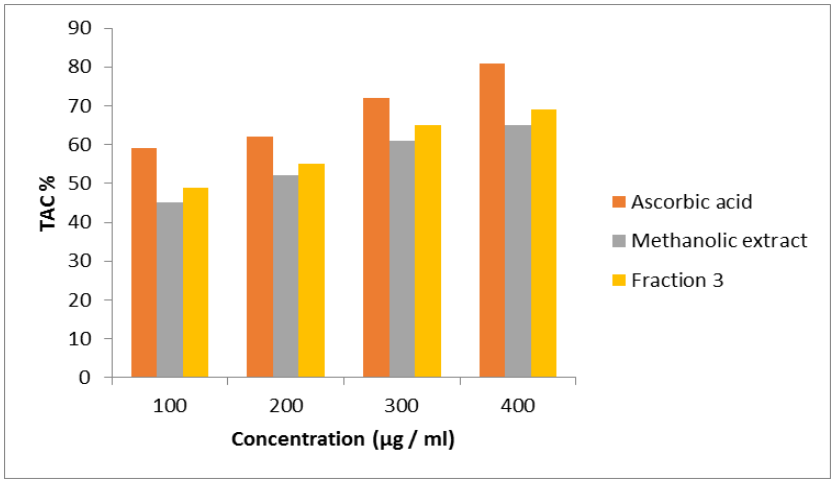


Figure. 5: Total Anti-Oxidant Capacity.

3.5 Gas Chromatography- Mass Spectroscopy (GCMS) Analysis: GC-MS is the best technique to identify the bioactive constituents of long chain hydrocarbons, alcohols, acids, esters, alkaloids, steroids, amino acid and nitrocompounds (Muthulakshmi et al., 2012). In the present study, Gas Chromatography Mass Spectroscopy analysis was carried out in fraction of *Crotolaria genistoides* leaves. The compounds were identified through mass spectrometry attached with GC with respect to their peak area and retention time.

The result of the GC-MS analysis identify the various compounds present in the fraction 3 (Figure 8 and Table 5). Figure showed the gas chromatogram of the fraction which shows distinct peaks identified by GC-MS while the compounds identified through comparing their spectra to those of the Wiley and C:/Database/NIST11.L mass spectral libraries. The major compounds present in the methanolic fraction *Crotolaria genistoides* leaves as identified by GC-MS was Thymoquinone, Trilinolein, 9, 12 octadecadienoic acid ethyl ester, linolenic acid ethyl ester .The compound thymoquinone shows greatest peak percentage of 12.54 may be responsible for the maximum inhibition of alpha amylase. Hence, the bioactive compound thymoquinone identified may be responsible for the antidiabetic activity of *Crotolaria genistoides* leaves.

Table. 5: Phytochemical Compound Identified in The Methanolic Fraction of *Crotolaria Genistoides.*

S. No	RT	Peak Area %	Name of the compound
1.	17.352	1.54	Hexadecanoic acid, methyl ester pentadecanoic acid, 14 methyl ester
2.	17.691	4.57	n Hexadecanoic acid Octadecanoic acid
3.	18.018	3.41	Ethyl 14 methyl - hexadecanoate undecanoic acid, 2,4,6 Trimethyl - methyl ester
4.	18.956	12.54	Thymoquinone
5.	19.017	9.77	9 Octadecenoic acid (Z)- methyl ester 9 Octadecenoic acid (E)
6.	19.309	10.35	Trilinolein
7.	19.362	4.96	9, 17 Octadecadienoi 7- pentadecyne
8.	19.564	10.53	Iinoleic acid ethyl ester 9, 12 Octadecadienoic acid , ethyl ester
9.	19.624	8.41	Oleic acid.

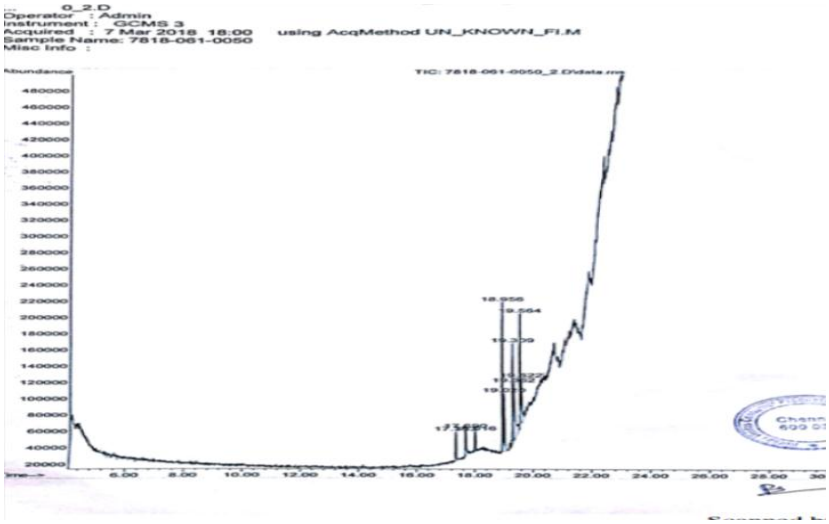


Figure. 6. *Crotolaria Genistoides* Showing their Retention Value And area Percentage.

DISCUSSION

► In the present study, the Photochemical Analysis of leaves of ***Crotolaria genistoides*** in Extract and fractions proved the presence of various phytochemically active constituents. The invitro ant diabetic activity of extracts and fraction of ***Crotolaria genistoides*** leaves on Alpha amylase inhibition and glucose diffusion inhibition were studied and it was observed that both the extract and fraction have an ant diabetic activity but when compared to Extract , fraction have high inhibitory activity on Alpha amylase and glucose diffusion. The GC-MS analysis of the prepared fraction showed the presence of 9 compounds. The major constituents are Thymoquinone, 9, 15 Octadecadienoic acid, methyl ester (z, z) 9, 12

Octadecadienoic acid, methyl ester (EE) (20.62%). Thymoquinone was identified as a predominant compound present in the methanol leaves extract of *Crotolaria genistoides* on GCMS Instrumentation. Hence it was concluded that thymoquinone compound from this plant may render hypoglycemic activity, and it may be used as drug through future studies.

▶ Finally, I conclude that Exploration of this chemical compounds with such suitable scientific validation in the Siddha Pharmacology will led to a Global acceptance of Siddha medicine worldwide and also I request you to explore such scientific validations and pave the way for discovery of new effective drug in Siddha Pharmacological industry.

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