ISOLATION AND STUDY OF A POTENT INSULINOTROPIC AGENT FROM FENUGREEK SEEDS

Submitted in partial fulfillment of the requirements for the award of

Bachelor of Science in CHEMISTRY

by

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DEPARTMENT OF CHEMISTRY

SCHOOL OF SCIENCE AND HUMANITIES

SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY

(DEEMED TO BE UNIVERSITY)

Accredited with Grade "A" by NAAC

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SATHYABAMA

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **GRACE JESLIN .J (40030011)** and **CHANDRU .T (40030005)** who carried out the project entitled**"ISOLATION AND STUDY OF A POTENT INSULINOTROPIC AGENT FROM FENUGREEK SEEDS"**under our supervision from November 2022 to April 2023 .

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DECLARATION

We **Chandru.T**, **Grace Jeslin.J** student at **SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** hereby declare that the Project Report entitled **"ISOLATION AND STUDY OF A POTENT INSULINOTROPIC AGENT FROM FENUGREEK SEEDS"** Done by me under the guidance of **Dr. S. SUPRIYA Ph.D.,** at **SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** is submitted in partial fulfillment of the requirements for the award of **Bachelor of Science degree in CHEMISTRY**.

DATE: 08/05/2023

PLACE: CHENNAI

SIGNATURE OF THE CANDIDATE

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ABSTRACT

Diabetic mellitus is a heterogeneous metabolic disorder which is characterized by hyperglycemia resulting from defective insulin secretion, resistance to insulin action or both. Fenugreek is a plant containing a potential insulinotropic compound called 4-hydroxyisoleucine in bounty and has been discovered to be exceptionally useful for the treatment of hyperglycemia. Metal complexes of 4-hydroxyisoleucine have been reported to show insulin mimetic activity in in vitro experiments. 4-hydroxyisoleucine is extracted with methanol and the active component was identified using GC-MS which underwent lactonization. The separated component was made to form complex with metals such as copper in order to study the efficiency of 4-hydroxyisoleucine in treating diabetic condition.

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CHAPTER 1

INTRODUCTION

1.1 DIABETES MELLITUS:

Diabetes mellitus is a chronic metabolic disorder characterized by high levels of glucose (sugar) in the blood [1]. Glucose is an important source of energy for the body's cells and is regulated by the hormone insulin. However, in diabetes mellitus, there is either a deficiency of insulin or an inability of the body to use insulin effectively, resulting in elevated levels of glucose in the blood. There are three types of diabetes mellitus: type 1, type 2 and gestational diabetes.

Type 1 diabetes mellitus is an autoimmunedisease that occurs when the immune system mistakenly attacks and destroys the insulin-producing cells in the pancreas. This results in a complete deficiency of insulin, and people with type 1 diabetes require daily insulin injections to survive. Type 1 diabetes usually develops in childhood or adolescence but can occur at any age [2]. Type 2 diabetes mellitus is the most common form of diabetes, accounting for about 90% of cases. It typically develops in adulthood and is characterized by insulin resistance, where the body's cells become less sensitive to insulin, and the pancreas is unable to produce enough insulin to compensate. Type 2 diabetes is strongly associated with obesity, physical inactivity, and poor diet, and can often be managed with lifestyle changes, such as weight loss, exercise, and a healthy diet [3]. In some cases, medication or insulin therapy may be required. There are also other types of diabetes, such as gestational diabetes, which occurs during pregnancy [4], and rare forms of diabetes caused by genetic mutations or diseases of the pancreas.

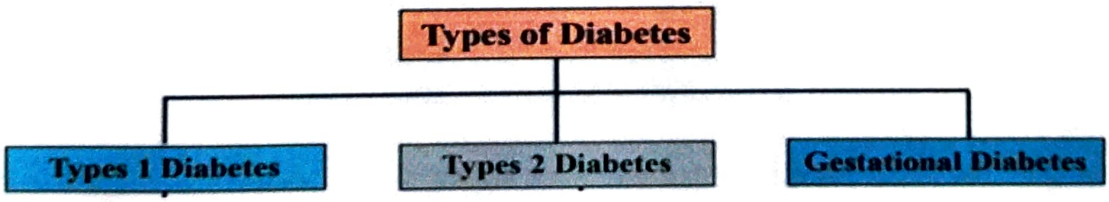

Fig1.1 Classification of Diabetes Mellitus

1.2 PLANT MEDICINE:

Plant medicine, also known as herbal medicine, is a traditional practice that involves the use of plants or plant extracts to prevent or treat illnesses. It is a form of alternative medicine that has been used for centuries in many cultures around the world. Plant medicine can be consumed in various forms such as teas, tinctures, capsules, or applied externally in the form of creams, poultices, or essential oils.

Plant medicine is often considered to be a holistic approach to healing that focuses on treating the whole person, rather than just the symptoms of an illness. Many plant medicines have been studied and found to have therapeutic properties that can be beneficial to our health. Some of the commonly used plant medicines include chamomile, ginger, echinacea, ginseng, and St. John's Wort, to name a few.

It is important to note that while plant medicine can be effective in treating certain conditions, it is not a substitute for professional medical advice and treatment. It is always best to consult with a qualified healthcare practitioner before using plant medicine, especially if you are taking any prescription medications or have underlying health conditions.

Fig1.2 Plant Medicine

1.3 FENUGREEK:

Fenugreek (Trigonellafoenum-graecum) is an herbaceous plant that belongs to the Fabaceae family, also known as the bean family. It is widely used in both culinary and medicinal purposes and is native to the Mediterranean region, Southern Europe, and Western Asia.

In traditional medicine, fenugreek has been used to treat a variety of ailments, including digestive problems, menstrual cramps, and skin inflammation. More recently, it has gained attention for its potential health benefits, which include improving blood sugar control, reducing inflammation, and boosting milk production in breastfeeding women [5].

Fig1.3 Fenugreek

1.4 USES OF FENUGREEK

1. Improving Digestion: Fenugreek is believed to help with digestion and relieve gastrointestinal issues such as indigestion, constipation, and stomach inflammation. It contains compounds like mucilage, which can help soothe the lining of the digestive tract.

2. Lowering Blood Sugar Levels: Fenugreek seeds have been shown to help lower blood sugar levels in people with diabetes, possibly by slowing down the absorption of carbohydrates in the intestine and increasing insulin sensitivity.

3. Reducing Inflammation: Fenugreek contains anti-inflammatory compounds that may help reduce inflammation throughout the body, potentially providing benefits for conditions like arthritis and other inflammatory conditions.

4. Promoting Healthy Skin: Fenugreek may help improve the health and appearance of the skin when applied topically. It contains antioxidants and other compounds that may help protect the skin from damage and promote healing. It is worth noting that while fenugreek has a long history of use in traditional medicine, more research is needed to fully understand its potential health benefits and to determine appropriate dosages and potential side effects.

1.5 4-HYDROXY ISOLEUCINE:

4-Hydroxyisoleucine (4-HI) is an amino acid derivative found in the seeds of the fenugreek plant (Trigonellafoenum-graecum), which has been used for medicinal purposes for centuries[6]. The compound has gained recent attention for its potential therapeutic effects on glucose homeostasis and insulin sensitivity, making it an attractive candidate for the development of novel antidiabetic drugs. 4-HI is a non-proteinogenic amino acid, meaning that it is not used in protein synthesis but has other important biological functions. Its chemical structure is similar to that of the amino acid isoleucine, but with an additional hydroxyl (-OH) group at the fourth carbon atom . This small modification has significant effects on the activity of the compound, as it alters the way it interacts with biological receptors and enzymes. Several studies have shown that 4-HI has insulinotropic properties[6], which means that it stimulates the secretion of insulin from pancreatic beta cells. This effect is mediated by the activation of specific receptors on the beta cells, which triggers a series of intracellular signaling pathways that ultimately lead to the release of insulin. The insulinotropic effect of 4-HI is dose-dependent and has been shown to be more potent than other insulin secretagogues such as sulfonylureas. In addition to its insulinotropic effects, 4-HI has been shown to improve insulin sensitivity in peripheral tissues such as skeletal muscle and adipose tissue. Insulin resistance is a hallmark of type 2 diabetes, and improving insulin sensitivity is a key strategy for its management. The mechanism by which 4-HI enhances insulin sensitivity is not fully understood, but it may involve the activation of specific signaling pathways involved in glucose uptake and metabolism. Several clinical studies have investigated the effects of

fenugreek extract, which contains 4-HI, on glucose homeostasis and insulin sensitivity in humans. One study showed that supplementation with fenugreek extract for 12 weeks improved glycemic control and insulin sensitivity in patients with type 2 diabetes. Another study showed that fenugreek extract improved glucose tolerance and insulin sensitivity in healthy subjects. However, more research is needed to establish the optimal dose and duration of treatment, as well as the long-term safety and efficacy of fenugreek extract therapeutic applications, such as anti-inflammatory and antioxidant effects. It has been shown to inhibit the production of pro-inflammatory cytokines and reactive oxygen species, which are implicated in the pathogenesis of several chronic diseases. However, more research is needed to confirm these effects and their clinical relevance. [5]

In conclusion, 4-Hydroxyisoleucine is a promising compound with potential therapeutic effects on glucose homeostasis and insulin sensitivity. Its insulinotropic and insulin-sensitizing properties make it an attractive candidate for the development of novel antidiabetic drugs[6]. However, more research is needed to fully understand its mechanisms of action and establish its long-term safety and efficacy .

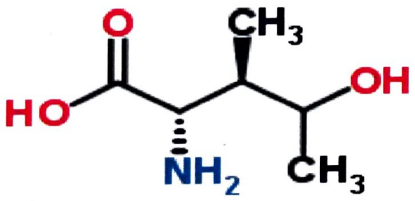

Fig1.4 4-Hydroxyisoleucine

CHAPTER 2

2.0 LITERATURE SURVEY :

Broca C, and his co-workers [6] reported that 4-Hydroxyisoleucine, a peculiar amino acid extracted from fenugreek seeds and never found in mammalian tissues, exhibits interesting insulinotropic activity and concluded that the insulinotropic properties of 4-hydroxyisoleucine, in the micro-molar range, are seen only in the presence of the linear major isoform; they also require carbon alpha in S-configuration, full methylation and carbon gamma-hydroxylation.

Jetté L, and her teammates [7] reported that the major isomer of 4-hydroxyisoleucine, an atypical branched-chain amino acid derived from fenugreek, is responsible for the effects of this plant on glucose and lipid metabolism. 4-Hydroxyisoleucine was demonstrated to stimulate glucose-dependent insulin secretion by a direct effect on pancreatic islets. 4-hydroxyisoleucine decreased elevated plasma triglyceride and total cholesterol levels in a hamster model of diabetes. Based on the beneficial metabolic properties that have been demonstrated, 4-hydroxyisoleucine, a simple, plant-derived amino acid, may represent an attractive new candidate for the treatment of type 2 diabetes, obesity and dyslipidemia, all key components of metabolic syndrome.

Pingali,and his fellow workers [8] studied that the new method was prepared for the extraction of 4-hydroxy isoleucine from fenugreek seeds. Similarly semi-synthetic derivatives were prepared with acceptable degree of purity and confirmed by spectral analysis. The anti-diabetic activity of semi synthetic derivatives was compared with the parent 4-hydroxy isoleucine and they showed increased activity than the parent compound.

GaddamA,and his teammates[9] reported This study provides evidence for the use of Fenugreek to delay the onset of diabetes in subjects with prediabetes. Fenugreek powder is useful to lower the blood glucose in prediabetes. From the results it can be concluded that Fenugreek showed hypocholesterolemic effects by reducing LDLc levels but without affecting serum TG, HDLc levels. Our results strongly suggest that the enhancement of serum insulin levels is due to insulinotropic effects. In conclusion, our results show that hypoglycemic effects are due to increasing levels of serum insulin and we suggest here that the mode of action of Fenugreek may be caused by their contents of alkaloids.

Solomon Habtemariam, [10] reported that the seeds of fenugreek (*Trigonellafoenum-graecum* L.) have a long history of usage as spices and medicine that attributes to the diverse chemical composition of fragrant small molecules, amino acid derivatives (4-hydroxyisoleucine)alkaloids (trigonelline), galactomannan rich polysaccharides, phenolic compounds of both flavonoids and aromatic acids, and complex saponins. The diversity of pharmacological activities induced by the seeds powder, extracts and active components in diabetes and hyperlipidaemic models are astonishing. This chapter provides the mechanistic basis and potential applications for diabetes and a range of associated disorders by assessing data from in vitro, extensive animal models and human trials. The contribution of the active components individually and as a group in the seeds preparations is discussed. Improvement of insulin sensitivity, insulin release, regulation of lipid metabolism and antioxidant and anti-inflammatory mechanisms of organoprotective effects under diabetes pathology are among the discussion topics.

Ramesh C. Gupta,and his co-workers [11]reported that based on published information, potential health benefits of fenugreek (Trigonellafoenum-graecum), one of the oldest medicinal plants. The scientific data summarizes for potential health benefits including management of blood glucose in type II diabetics, cholesterol-lowering effect, effect on growth hormone leading to modulation of metabolic syndrome, antiinflammatory and anticancer effects, enhancement of milk production in new mothers, increase in male libido, safety, and toxicity. These benefits are analyzed in terms of the weight of evidence for efficacy, effective human dose, extrapolation of animal data to humans, and the safety

margin at the projected clinical dose. Effects of not only the whole seed or plant but also the main active components, such as diosgenin, trigonelline, 4-hydroxyisoleucine, and galactomannan, are identified and summarized. The advantages of using the aqueous or alcoholic extract(s) instead of whole-seed powder, thus mitigating any coumarin-associated potential dermal or anticoagulation-related adverse effects, are also discussed. There appears to be strong preclinical-based evidence for most of the benefits listed here, but the clinical data appear to be not robust and need stronger evidence by conducting well-controlled clinical trials, as performed for human pharmaceuticals. Overall, the effective dose for the whole-seed powder is too high to be practical but does not pose a safety concern with respect to dose-limiting toxicity, even after repeated dosing. The use of fenugreek should be avoided during pregnancy until safety of the developing fetus is established based on quality (GLP) embryo/fetal developmental toxicity studies in appropriate animal species. Also, fenugreek, as a member of the Leguminoseae family, could be allergenic to some subjects, particularly those known to be allergic to peanuts. Further research for reducing the clinically effective dose has been proposed by using the bioactive components alone or in combination.

Singh P,and his fellow mates reported [12] that Type 2 diabetes mellitus (T2DM) is a metabolic disorder characterized by chronic hyperglycemia and insulin resistance. 4-hydroxyisoleucine (4-HIL) is a non-proteinogenic amino acid isolated from the fenugreek seeds and has enormous pharmacological activities. The present study was undertaken to investigate the antihyperglycemic effect of 4-HIL in streptozotocin (STZ)-induced diabetic rats. Moreover, its toxicity was evaluated in vitro and in vivo employing human embryonic kidney cells (HEK-293) and healthy rats, respectively. In experiment 1, STZ-induced diabetic male rats were subjected to an oral treatment of 4-HIL (100 mg/kg), while experiment 2 deals with the effects of 4-HIL on healthy male and female rats following oral administration. The treatment (experiment 1) declined the elevated blood glucose level, feed intake, and increased body weight(s).

Additionally, blood glucose impairment was improved as observed by OGTT and IPGT tests. Pancreatic histopathology revealed mild changes in the 4-HIL group. Moreover, experiment 2 showed increased body weight, normal blood glucose levels (male-106.06 ± 7.49 mg/dl and female-100.06 ± 14.69 mg/dL), hematological parameters, and histopathological profiles in the treatment group. 4-HIL did not affect the viability of HEK-293 cells, and no signs of toxicity were observed in healthy rats. Therefore, the study concludes that 4-HIL has potential antihyperglycemic activity without any toxic effects.

CHAPTER 3

AIM & OBJECTIVE

AIM :

The Aim of our project is the extraction of 4-Hydroxy isoleucine from fenugreek by a simple method in it's pure form followed by metal complextion..

OBJECTIVE :

- To identify the potent Insulinotropic agent 4-Hydroxyisoleucine in fenugreek by GC-MS .
- To obtain 4-hydroxyisoleucine in its pure form by HPLC.
- 4- Hydroxyisoleucine is stabilized by complexing it with metal ions such as Cu.
- To characterize 4-hydroxyisoleucine and its complex by UV,FTIR, and GC-MS..

CHAPTER 4

MATERIALS & METHODS

4.1 MATERIALS

Fenugreek solution , Copper Sulphate salt , 4-Hydroxyisoleucine .

4.2 Method

Fenugreek seeds were kept for drying for 2 to 3 days in the sun and after drying ground them into a fine powder and soaked the powder in a mixture of water and methanol and kept for stirring for two days . After 2 days , clear methanol extract was filtered and subjected to GC-MS to identify the target molecule based on m/z values. After the identification of the region of appearance, the methanol extract was subjected to HPLC in order to obtain the particular liquid portion in its pure form.. After this, complexation was carried out using copper as a metal ion . Finally the complexed product was analyzed using FTIR and UV-visible spectroscopic methods.

Fig 4.1 Fenugreek Powder

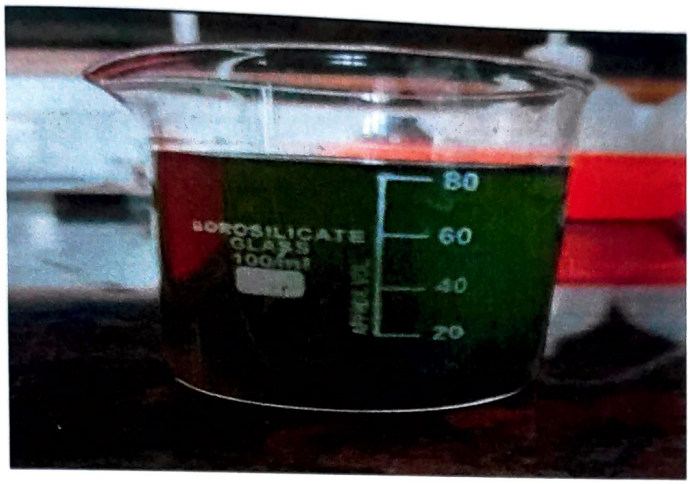

Fig 4. 2 Fenugreek Solution

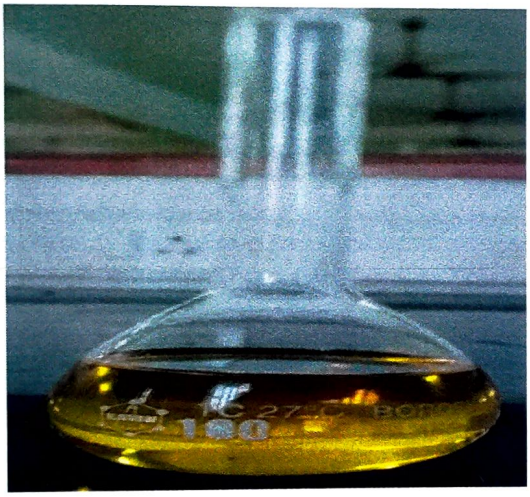

Fig 4.3 Methanol Extract

Fig 4.4 4-Hydroxyisoleucine complex

CHAPTER 5

RESULTS AND DISCUSSION

5.1 FTIR

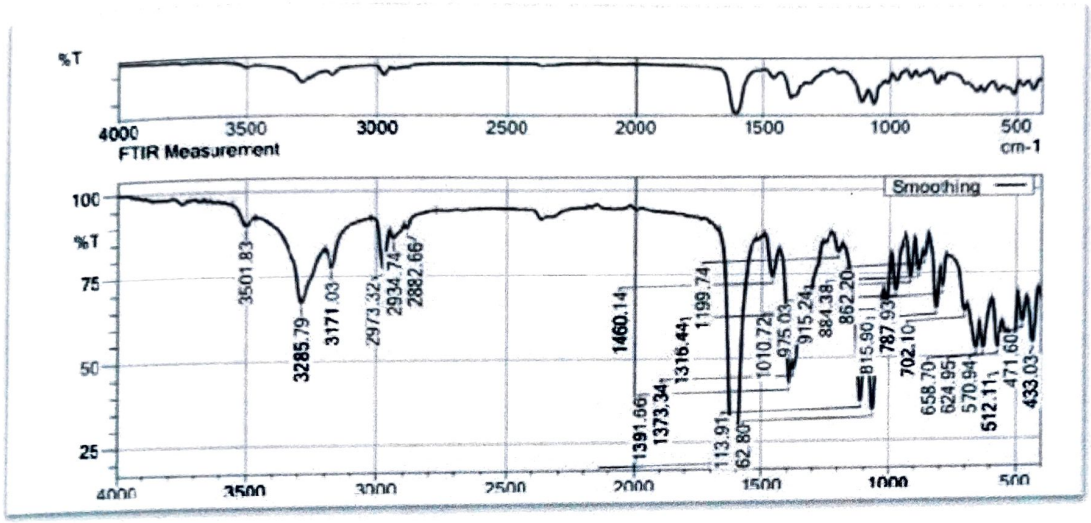

Fig 5.1 IR Spectrum Of 4-Hydroxyisoleucine

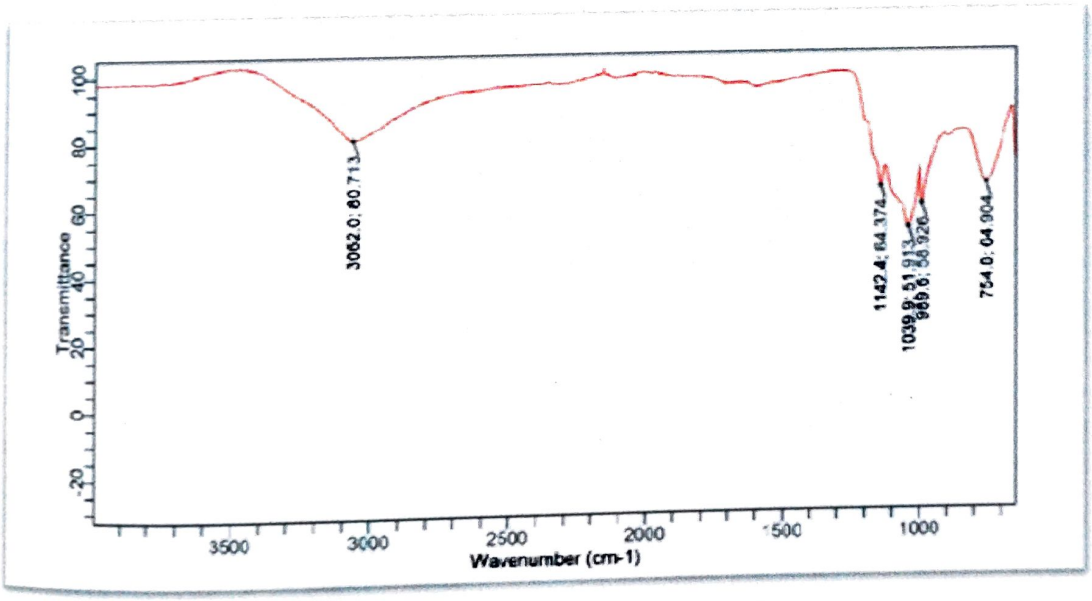

Fig 5.2 IR Spectrum Of Fenugreek

The bioactive compound 4-hydroxyisoleucine separated from the seeds of Trigonella Foenum Gracum was complexed with the metal ion Cu in order to understand the stability of the complex and to effectively use it for anti diabetic activity study. Similar complex was also synthesized by using 4-hydroyisoleucine prepared under laboratory conditions. Both the complexes were subjected to IR study to confirm the identity of the separated bioactive molecule from the fenugreek seeds. On comparison of the spectra, the two spectra were found to be matching but with the overlap of the peaks with respect to IR spectrum of the bio active molecule separated from the fenugreek seeds.

In the spectrum of the complex corresponding to that obtained from the laboratory synthesis of 4-Hydroxyisoleucine was found that the peak corresponding to OH at 3285cm^{-1}. The stretching frequency of CO group is at 1642 cm^{-1} . Peaks of low intensity at 3171 cm^{-1} correspond to NH stretching frequency. There are many peaks observed between 1010 and 433cm^{-1} corresponding to bending vibrations occurring with the CH_2 groups present in the molecule. Thus the spectrum of the complex obtained using 4-Hydroxyisoleucine synthesized under laboratory conditions is found to match with the proposed structure of the complex.

The IR spectrum of the complex formed with 4-HIL obtained from fenugreek seeds is found to have overlapping peaks. However, broad peaks at 3062cm^{-1} and 1142cm^{-1} denotes the presence of OH groups and bending vibrations of the CH2 groups present in the complex. Broad peaks indicates heavy polymerization existing between the synthesized molecules.

5.2 UV

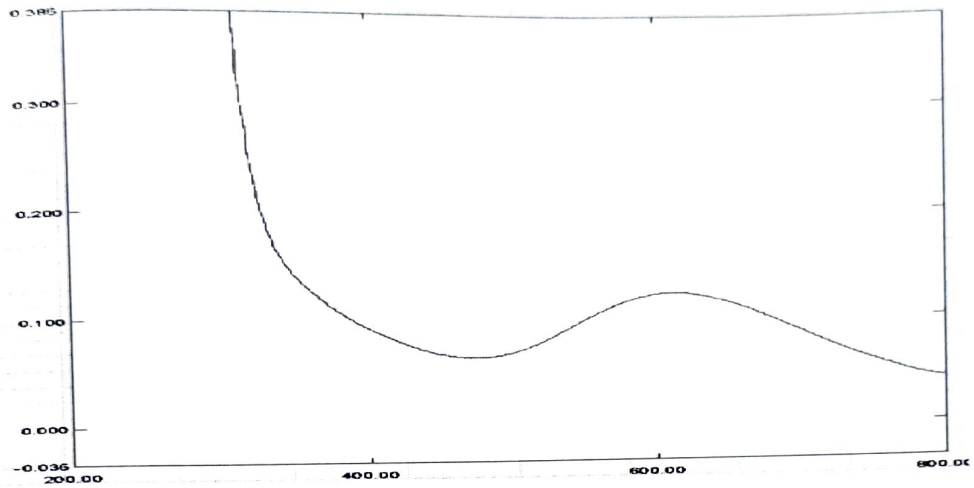

Fig 5.3 Complex Solubility

UV-visible spectrum of the prepared complex was taken using UV SHIMADZU model spectrophotometer as an aqueous solution. The UV spectrum of the product shows a well defined peak in the UV range corresponding to the organic part of the compound involved in complex formation. Presence of complex formation and incorporation of metal in the organic framework is confirmed from the d-d transition peak around 640nm. The Emax of the complex in the visible range is of low intensity compared to the peak in the UV region. Hence the formation of the targeted complex with copper as the metal ion is confirmed from by UV—visible spectroscopy.

5.3 GC-MS

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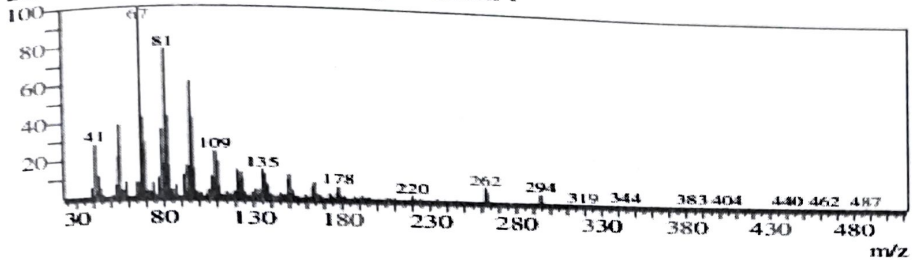

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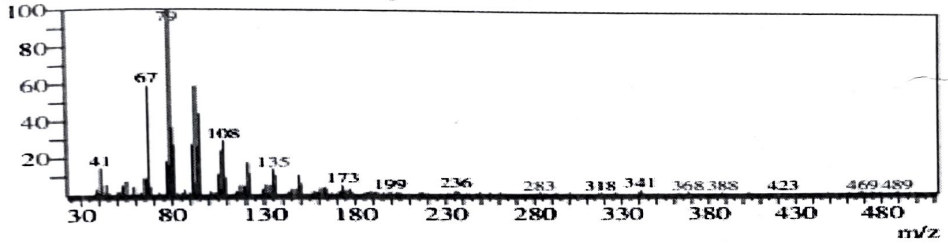

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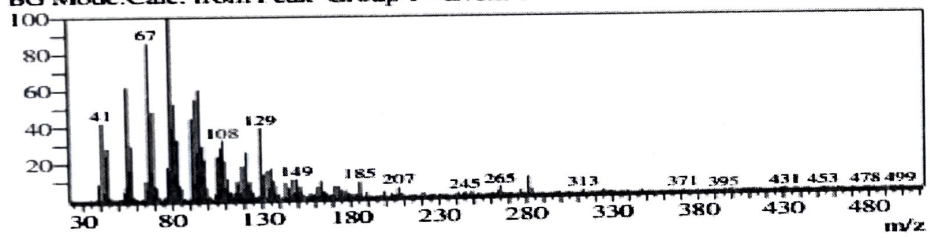

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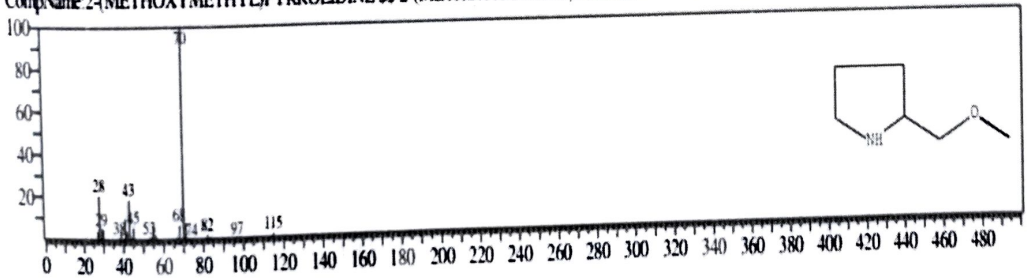

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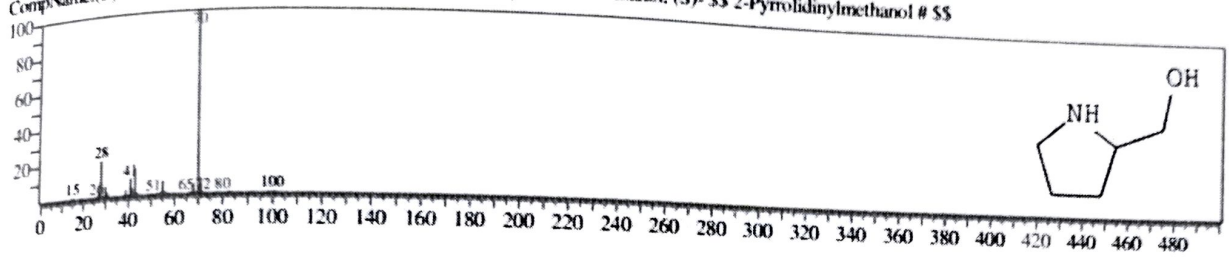

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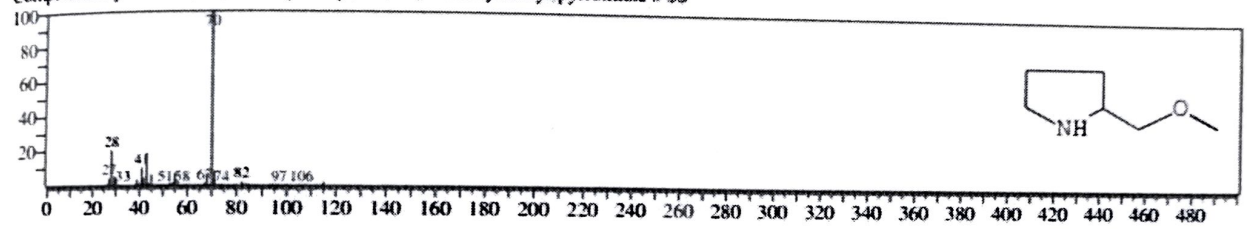

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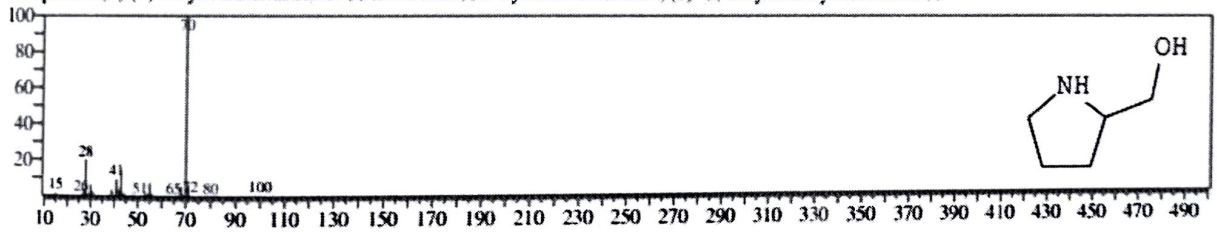

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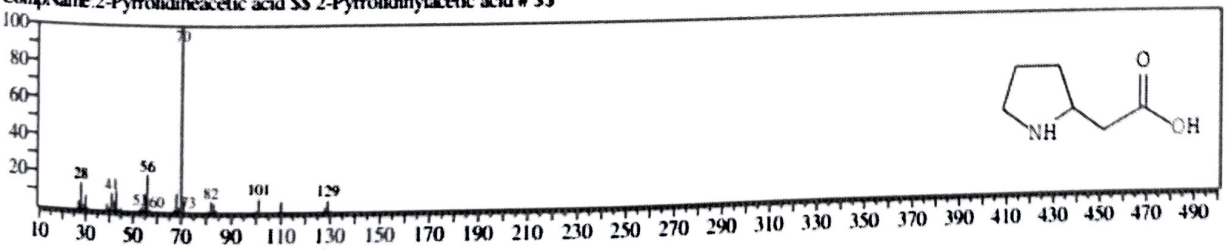

Fig 5.4 Mass Spectrum

The fenugreek powder was mixed with methanol and stirred for about 3 hours. The methanolic extract was subjected to GC-MS analysis. The spectrum revealed the presence of various fractions from the m/z values of the components. The targeted component was found to have the least retention time of 5.1 sec and labelled as line 1. The active component was found to be present as the second fraction with the retention time of 5.8 sec. From the spectral analysis based on the m/z values, the component 4-hydroxyisoleucine was found to be highly sensitive and unstable to light and heat which corroborates with the previous findings [13]. The molecule is found to undergo lactonization with different fragmentation patterns. Based on the molecular weight and the corresponding m/z values the lactonized structure of the targeted component 4-hydroxyisoleucine has been suggested as given in Fig [5.4].Because of unstability and high sensitivity of 4-hydroxyisoleucine, it is proposed to chelate the molecule with metal ions for improving its stability and thereby to increase its antidiabetic activity. Metal complexes of unstable ligand moities are converted to highly stable complexes in coordination with metal ions. Hence metal complexation is carried out to prevent lactonization of the active molecule so as to make it available in its original form for high insulinotropic activity.

CHAPTER 6
CONCLUSION

An attempt to isolate 4-hydroisoleucine from dried fenugreek powder was made. Analysis of the methanolic solution of fenugreek powder by GC-MS confirmed lactonization of the molecule which corroborates with the previous reports.The 4-hydroxyisoleucine fraction was complexed with copper metal ion in order to stabilize the bioactive insulinotropic molecule and the formed complex was characterized by UV-visible and IR spectroscopic methods.

CHAPTER 7

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