ISOLATION, PURIFICATION, AND CHARACTERIZATION OF ANTIDIABETIC COMPOUNDS DERIVED FROM MORCHELLA ESCULENTA

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Clinical Biochemistry

By

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DECLARATION

I, hereby declared that the presented work in the thesis entitled, **"Isolation, Purification, and Characterization of Antidiabetic Compounds Derived from *Morchella esculenta*"** in fulfilment of degree of Doctor of Philosophy (Ph.D.) is outcome of research work carried out by me under the supervision of **Dr. Pranav Kumar Prabhakar,** working as **Professor** in the **School of Allied Medical Sciences** of Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This has not been submitted in part or full to any other University or Institute for award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **"Isolation, Purification, and Characterization of Antidiabetic Compounds Derived from *Morchella esculenta*"** submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy** (Ph.D.) in the School of Allied Medical Sciences, is a research work carried out by **Keshav Anand**, (Registration No.) **11919206**, is bonafide record of his original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

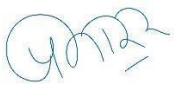

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TABLE OF CONTENTS

List of Figures

List of Tables

LIST OF ABBREVIATIONS

ADA	American Diabetes Association
CVD	Cardiovascular Disease
CDC	Centers for Disease Control and Prevention
DS	Dietary Supplements
DPP	Diabetes Prevention Program
DKA	Diabetic Ketoacidosis
DM	Diabetes mellitus
DNA	Deoxyribonucleic Acid
EC	Ethics Committee
FTIR	Fourier-Transform Infrared Spectroscopy
FBG	Fasting blood glucose
FBS	Fasting Blood Sugar
FPG	Fasting Plasma Glucose
HPLC	High Performance Liquid Chromatography
HDL	High-Density Lipoprotein
IDF	The International Diabetes Federation
ICMR	Indian Council of Medical Research
IFG	Impaired Fasting Glucose
IR	Insulin resistance
LDL	Low-Density Lipoprotein
LSD	Lysergic Acid Diethylamide
LC–MS	Liquid chromatography–mass spectrometry
mABs	Monoclonal Antibodies
M. esculenta	*Morchella esculenta*
MEP	*Morchella esculenta* Polysaccharides
NK	Natural Killer
NMR	Nuclear magnetic resonance
NCDs	Noncommunicable Diseases
NDIC	National Diabetes Information Clearing House
PBS	*Phosphate-buffered saline*
PSP	Polysaccharide Peptide
PCR	Polymerase Chain Reaction

PPBS	Postprandial Glucose Test
PEF	Pulsed Electric Field
SD	Standard Deviation
T1DM	Type-2 diabetes mellitus
T2DM	Type-2 diabetes mellitus
TC	Total Cholesterol
TLC	Thin Layer Chromatography
WHO	World Health Organisation

ABSTRACT

Insulin resistance, reduced pancreatic β-cell activity, and dysregulated glucose homeostasis are the hallmarks of type 2 diabetes mellitus (T2DM), a metabolic illness that encompasses multiple aspects and is characterized by diverse symptoms. Therapeutic treatments that are both new and complex are required because the prevalence of type 2 diabetes is increasing all over the world. *Morchella esculenta* (*M. esculenta),* a highly regarded culinary fungus, is the subject of this study, which aims to conduct an exhaustive analysis of the medicinal potential of the fungus, with a particular emphasis on the profound implications it has for the management of type 2 diabetes. During the first stage of our investigation, we are examining the antioxidant properties of *M. esculenta* extracts. This is done in recognition of the central role that oxidative stress plays in the development of type 2 diabetes and the difficulties that are connected with it. Using conventional taxonomic keys, samples were obtained from Jammu and Kashmir, India, in April 2021. The samples were morphologically identified as belonging to the genus *M. esculenta* that was collected. This article highlights the importance of *M. esculenta* from both a pharmacological and nutritional standpoint. Studies have been conducted to investigate the nutritional components of a variety of edible mushrooms; however, the pharmacological aspects of *M. esculenta*, particularly its hypoglycemic actions, have not been investigated to a significant extent. According to the findings of the study, there is a significant amount of room for further research into the therapeutic characteristics of *M. esculenta.* The presence of saponins, tannins, terpenoids and steroids, glycosides, flavonoids, alkaloids, quinones, phenols, curcumins, and carbohydrates was determined through qualitative analysis using chloroform, methanol, and ethanol. Additionally, an analysis was performed to determine the presence of carbohydrates. In addition, the samples exhibited free radical scavenging activity in experiments including DPPH, ABTS, Lipid peroxidation, Hydrogen peroxide scavenging activity, and the Nitric Oxide Scavenging Assay using three distinct solvents, namely ethanol, methanol, and chloroform. At a concentration of 1 mg/ml, ascorbic acid—the reference chemical for ABTS—displayed free radical scavenging activity of 64.71%. Ethanol was shown to be the most effective solvent for extracting antioxidant chemicals from *M. esculenta*, with consistently higher percentage inhibition values than chloroform and methanol at all doses. Ethanol showed the greatest percentage inhibition at 50.28% at the maximum concentration (1.0 mg), indicating strong antioxidant activity. The inclusion of bioactive components with antioxidant capabilities, such as phenolic compounds or flavonoids, may explain this efficiency. The reference chemical for DPPH, ascorbic acid, demonstrated a free

radical scavenging activity of 80.71% at a concentration of 1 mg/ml. The findings point to the antioxidant capabilities of *M. esculenta* extracts, as the percentage suppression of the DPPH radical increases with concentration for all three solvents. At most doses, chloroform showed a greater % inhibition value compared to ethanol and methanol, according to the extract comparison. The ethanol extract showed the greatest percentage inhibition among the solvents at the maximum concentration (1.0 mg), reaching 18.06%. At a concentration of 1 mg/ml, the anti-free radical scavenging activity was 70.1% for ascorbic acid, the gold standard chemical for hydrogen peroxide scavenging activity. Results showed that ethanol, chloroform, and methanol were in that order of H_2O_2 scavenging capacity. As the quantities of all solvents increased, the results showed that the extracts could scavenge more and more hydrogen peroxide. In comparison to chloroform and methanol, ethanol's consistently higher percentage inhibition results for hydrogen peroxide scavenging were indicative of its superiority. The existence of powerful bioactive components with substantial hydrogen peroxide scavenging properties was indicated by the highest percentage inhibition at 46.15% at the highest concentration (1.0 mg) of the ethanol extract. At 1 mg/ml, the reference chemical ascorbic acid exhibited an inhibitory activity of 75.71% in the lipid peroxidation assay when tested with *M. esculenta* extracts at different doses. Ethanol, chloroform, and methanol were determined to reduce lipid peroxidation in that order. Lipid peroxidation is a process that can damage cell membranes; the results show that the antioxidant qualities of the extracts can prevent this process, as the percentage inhibition increases with increasing concentrations of all solvents. When comparing chloroform, methanol, and ethanol at different concentrations, ethanol always showed a higher percentage inhibition value, suggesting that it inhibits lipid peroxidation more effectively. The presence of strong bioactive components that protect cell membranes from oxidative damage was suggested by the fact that the ethanol extract showed the highest percentage inhibition at 43.18% at the maximum quantity tested (1.0 mg). At 1 mg/ml, the standard chemical ascorbic acid exhibited 66.1% inhibitory activity in the nitric oxide scavenging assay, which was conducted on extracts of *M. esculenta* at different doses. Ethanol, chloroform, and methanol were shown to have the highest nitric oxide scavenging abilities. The results demonstrate that the percentage of inhibition increases as the concentrations of all extracts rise, suggesting that they have the ability to scavenge nitric oxide and reduce inflammation and oxidative stress caused by excess nitric oxide. At most concentrations, ethanol showed a greater % inhibition value than chloroform and methanol, indicating that it had a superior ability to scavenge nitric oxide. The ethanol extract showed a remarkable scavenging activity at the highest dosage tested (1.0 mg), with a percentage

suppression of nitric oxide of 25.34%. The α-glucosidase inhibition assay showed that different doses of *M. esculenta* extract inhibited the enzyme to varying degrees. At 1 mg/ml, the standard compound acarbose showed the strongest inhibitory effect, at 76.71%. The capacity of ethanol, methanol, and chloroform to inhibit α-glucosidase was seen in that order. The ability of the extracts to block the α-glucosidase enzyme, which is involved in carbohydrate digestion and glucose absorption, is suggested by the fact that the percentage inhibition increases as the concentrations of all solvents rise. The better inhibitory activity of ethanol against α-glucosidase was indicated by the consistently higher percentage inhibition values it displayed at most doses when compared to chloroform and methanol. It appears that the α-glucosidase enzyme can be inhibited by the bioactive chemicals found in the ethanol extract of *M. esculenta*. The ethanol extract showed the greatest percentage inhibition of α-glucosidase activity at 49.06% at the highest dosage tested (1.0 mg), indicating its potential to modulate carbohydrate digestion and glucose absorption. Using acarbose as the standard, the α-amylase inhibitory experiment showed that *M. esculenta* extracts at different doses inhibited the enzyme to the greatest extent (68.71%). In a manner that was dependent on concentration, the chloroform extract had the second-highest inhibition rate at 39.57%±1.44, IC50=1.33 mg/ml, while the ethanolic extract showed the greatest inhibition rate at 50.4%±1.14, IC50 = 1.0384 mg/ml. The results show that the extracts can hinder the action of the α-amylase enzyme, which breaks starch into simpler sugars. At most concentrations, ethanol showed a larger % inhibition value than chloroform and methanol, indicating that it had a stronger inhibitory effect on α-amylase. This suggests that bioactive substances capable of suppressing α-amylase activity are present in the ethanol extract of *M. esculenta*. Significant inhibitory potential was indicated by the ethanol extract, which showed the highest percentage inhibition of α-amylase activity at 50.4% at the highest dosage tested (1.0 mg). The outcomes of a glucose absorption assay carried out on L6 myotubes cell lines with varying doses of *M. esculenta* extracts. The basal glucose uptake of the untreated control group was around 33.70%, which was consistent across all three solvent extracts. An increase in glucose uptake was observed in the experimental group when treated with *Morchella* extracts at concentrations of 25, 50, and 100 µg/ml, as compared to the control group. This suggests that *Morchella* extracts may have the ability to improve glucose uptake in L6 myotubes cell lines. Glycemic absorption percentages were consistently higher in the ethanol and methanol extracts than in the chloroform extract across all concentrations when comparing the three solvent extracts. It appears that the bioactive chemicals found in the ethanol and methanol extracts have the ability to enhance glucose absorption in L6 myotubes cell lines. The glucose absorption activity was most pronounced in

the methanolic extract. From the methanolic extract, we then isolated a pure chemical. We used column chromatography to accomplish this, and the compound was separated into 12 fractions, each of which contained a single constituent. Next, the conformation of these fractions—labelled A, B, C, D, E, F, G, H, I, J, K, and L—was examined on thin-layer chromatography (TLC) plates. To ensure the presence of polysaccharides, qualitative tests were carried out following the extraction process. Fractionation is commonly accomplished using column chromatography. Using this method, chemicals can be separated from one another by classifying them according to how strongly they bind to the stationary phase of the column. We moved on to the next eleven fractions after the polysaccharide quantitative test (Molish test) indicated that the tenth fraction was positive. Afterwards, the compound identification process was carried out quantitatively on the 10^{th} (J) component utilizing Mass spectrometry, FTIR, and NMR. FTIR, NMR, and Mass Spectroscopy revealed diacetyl trehalose in the isolated chemical. FTIR study showed OH groups, ester groups, and ether linkages, which are characteristic of diacetyl trehalose. NMR and Mass Spectroscopy added to the evidence. These methods revealed diacetyl trehalose's functional groups and structural properties. The 33% glucose absorption of diacetyl trehalose showed its biological action. This shows that diacetyl trehalose may improve glucose absorption, which could benefit physiological processes including blood glucose homeostasis. The MTT assay for diacetyl trehalose shows cell viability at various doses. The cells had a high vitality of 99.31% at 6.25 µg/ml, showing negligible impact on cell health. Positive trend: 98.41% vitality at 12.5 µg/ml, highlighting the compound's safe impact on cell viability. At a dose of 25 µg/ml, cells still had a high viability of 97.47%, indicating a slight drop from lower concentrations. Increasing the concentration to 50 µg/ml slightly reduces cell viability to 96.28%. Despite a dose-dependent response with a minor effect on cell viability, cells are still viable at this concentration. Diacetyl trehalose is well-tolerated by cells, with a comparatively high viability of 95.98% at the maximum studied dose, 100 µg/ml. Since the minimal viability was 95.98%, no IC50 value could be computed. These data indicate that diacetyl trehalose is biocompatible in the measured concentrations and does not harm cells. On L6 myotubes, the glucose absorption experiment with the final product diacetyl trehalose showed good results. The untreated control group had a baseline glucose uptake of 30.0%, like the sample and insulin-treated groups. When given diacetyl trehalose at doses of 25, 50, and 100 µg/ml, glucose absorption increased compared to the control group. In L6 myotube cell lines, the chemical may increase glucose absorption. The percentage of glucose uptake did not change concentration-dependently, showing that diacetyl trehalose stimulates glucose uptake regardless of dose. When compared to the positive control group

treated with insulin, diacetyl trehalose did not exceed insulin's glucose uptake stimulation. This means that diacetyl trehalose enhances glucose absorption but is less effective than insulin. Diacetyl trehalose, a new chemical in *M. esculenta*, may treat diabetes, according to the study. *M. esculenta*, rich in nutrients, possesses antioxidant and anti-hyperlipidaemic characteristics that preserve mice's livers. The current study's antioxidant assays support *M. esculenta's* antioxidant characteristics. Mushrooms contain proteins, carbohydrates, vitamins, and fibre. *M. esculenta* polysaccharides may decrease colon cancer proliferation and reduce tumors and stimulate the immune system. *M. esculenta* polysaccharides have been studied for their effects on gut microbiota and potential treatment of diabetes and metabolic disorders. Assays on *M. esculenta,* including α-glucosidase inhibition, α-amylase inhibition, and glucose uptake, suggest its potential as a natural source of antidiabetic chemicals by regulating carbohydrate digestion and glucose absorption. Polysaccharides can treat diabetes by hypoglycaemia, hypolipidemia, β-cell apoptosis suppression, and β-cell growth. Research is needed to identify polysaccharides and understand their methods of action, according to the study. Polysaccharides from many plants have been studied for diabetic treatment. The naturally occurring disaccharide trehalose lowers glycaemic response and may reduce insulin resistance. Diacetyl trehalose, a molecule in *M. esculenta* that has antidiabetic properties, needs further study to determine its mechanisms of action and synergy with other substances. *M. esculenta,* particularly diacetyl trehalose, may be an antidiabetic drug, although further research is needed to understand the processes and interactions with other mushroom components.

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Dedicated to my Grandmother Smt. Sunita Anand

CHAPTER-1

INTRODUCTION

Summary: This chapter is about the introduction to the Research work. The chapter has been divided into seven parts.

Chapter-1

Introduction

1. INTRODUCTION

1.1. Origin of Mushrooms

The words "fungus" and "mykes" are where the English word "mushroom" originates from. Mushrooms are deemed edible provided they do not cause any health problems when consumed in sufficient quantities. It is possible to classify it into one of three categories according to its edibility: “edible (*Lepiotaprocera*), inedible or toxic (*Lepiotamargani*), and non-poisonous” [1]. Since thousands of years mushrooms are being used as both food and medicine, and many nations in Asia and S.Asia employ “traditional wild edible mushrooms as appealing and healthy foods”. These include countries like include Japan, Taiwan, China and India. As a direct consequence of this, there was a significant demand for mushrooms on the international market in 2013, with output reaching thirty-four billion kilograms and 4.7 kg/person (average consumption) [2]. Few of the most important consumable mushrooms that are typically available include “Chanterelles (*Cantharellaceae*), Puffballs (*Lycoperdon spp*. and *Calvatia spp*.), Shaggy mane (*Coprinus comatus*), two oyster mushrooms (*Pleurotus ostreatus* and *Pleurotus cystidiosus*), Boletes (*Boletaceae*), Sulfer shelf (*Laetiporus sulphurous*)” [3,4].

1.2. Nutritional aspect

Not only are wild edible mushrooms excellent in terms of their chemical and nutritional properties, but they are also superior in terms of the amount of protein and vitamins they contain, which include the vitamins B, D, K, A & C (occasionally) [5-7]. In addition, “mushrooms have a low fat content and a high content of dietary fibre, nutraceuticals, and polysaccharides” all of which gives beneficial effects in terms of health by helping in a variety of ailments with their properties involving immunomodulation and anti-inflammation [8,9]. Mushrooms are rich in polysaccharides, which are complex carbohydrates that have a variety of functions in the body. The “homoglucans (ß-1,3 glucan), heteroglycans, heterogalactans, and heteromannans” found in mushrooms are the primary structural polysaccharides found in these

organisms. These terpenoids and polysaccharides are 2° metabolites, but they have a crucial role to play in "glucose homeostasis by inhibiting -glucosidase, assisting the actions of glucose transporter 4, and reducing inflammatory factors to improve insulin resistance and lipid metabolism" [10,11]. Such polysaccharides and terpenoids are essential for maintaining normal glucose levels.

1.3. Diabetes

The phrase diabetes mellitus/DM comes from the Greek word ***diabetes***, which means ***syphon, to pass through***, and the Latin word mellitus, which means ***sweet***. Together, these two words form the term ***diabetes mellitus***. Diabetes mellitus is a collection of non-communicable metabolic illnesses that are defined by chronic hyperglycemia that results from abnormalities in insulin secretion, insulin action, or both [12]. This ailment tempers with the body's process to keep in check the sugar levels. According to the American Diabetes Association, 1997 (ADA), diabetes mellitus can be *+broken down into three categories:

Table 1.1. Types of diabetes mellitus

Type	Description
Type 1 diabetes mellitus (T1DM)	Insulin-dependent or juvenile-onset; accounting for 3–10% of cases.
Type 2 diabetes mellitus (T2DM)	Non-insulin-dependent or adult-onset; accounting for 85–90% of cases.
Gestational diabetes mellitus	Hyperglycaemia occurs during the second or third trimester

In 2021, the IDF suggested that the number of adult people suffering with diabetes is more than 537 million. 541 million people are estimated to have impaired glucose tolerance in 2021 with 80% of those people coming from families with middle- or low-incomes. If this trend continues, the count will reach 643 million by 2030 [13] [14]. In most cases, type 1 diabetes is associated with an AI problem, that is responsible for the loss of β cells in the pancreas, as well as abnormalities in lipid metabolism, increased hyperglycemia - mediated oxidative stress, malfunction of endothelial cells, and apoptosis [15,16]. In contrast, type 2 diabetes is associated with lipotoxicity,

glucotoxicity, apoptosis and endoplasmic reticulum-induced stress, together, which causes progressive β cell loss [17]. Particular symptoms include polydipsia/ phagia/ uria & nocturia.

Diabetic Complications:

Table 1.2. Complications in diabetes mellitus

Type of complication	
Microvascular	Retinopathy, nephropathy, and neuropathy
Macrovascular	Ischemic heart disease, peripheral vascular disease, and cerebrovascular disease

Polydipsia, polyphagia, polyuria, and nocturia are specific symptoms. Insulin resistance, also known as IR, has been shown by a number of studies to be the primary cause of worry when it comes to problems caused by diabetes [18,19]. Other prevalent risk factors for resistance to insulin include "oxidative stress, hydrolytic enzymatic inhibition, inflammation, hereditary behavioural variables, environmental factors, dyslipidemia, obesity, and epigenetic modulations" [20,21]. IR is also a risk factor for insulin resistance. Therefore, numerous pathogenic variables can lead to insulin resistance, even if the precise mechanism behind this condition is not well understood at this time.

1.4. Popularity of Mushrooms

Because of their one-of-a-kind taste, mushrooms have long been regarded as a necessary component of gourmet cooking all over the world. Thanks to their incomparable appeal, mushrooms have been revered by people everywhere as a veritable culinary miracle. There are more than 2,000 varieties of mushrooms found in nature, but only around 25 are utilised in culinary applications, and only a small percentage are farmed for sale. They are of substantial interest due to the organoleptic quality, medical characteristics, and economic relevance of mushrooms [22,23]. Mushrooms have a high value both nutritionally and functionally, and they are also recognised as foods that include nutraceutical capabilities. The line between mushrooms that are edible and those that are used for medicinal purposes is not as clear cut as one might think. This is due to

the fact that many of the species that are commonly consumed for their edible qualities also have medicinal use [24]. Species like *Agaricus bisporus* of mushroom are cultivated around the world, seconded by *L. edodes*, several species of *Pleurotus*, and *F. velutipes*. China is already the biggest mushroom producer around the globe [22,25,26], and mushroom output is only expected to continue rising. On the other hand, wild mushrooms are gaining in significance due to the nutritional, sensorial, and particularly pharmacological properties that they possess [23].

1.5. Pharmacological aspect

However, mushrooms may also include various 1° metabolites which include peptides, oxalic acid and proteins. Most of the antimicrobial substances found in mushrooms are 2° metabolites, which include "terpenes, steroids, anthraquinones, benzoic acid derivatives, and quinolones". Numerous species, such as Lentinus edodes, appear to possess an antibacterial action that works efficiently in the fight with bacteria be it gram -ve or +ve [27]. Because of their immunomodulatory and anti-cancer characteristics, many different cultures have traditionally used a wide variety of mushrooms for the keeping themselves healthy and in prevention & therapy of diseases. Tremendous growth has been observed in the interest regarding mushrooms' pharma potential in this decade.

It's been hypothesized that a lot of types of mushrooms are like small pharma factories themselves which can spew chemicals and compounds with life-saving therapeutic aspects [7,28]. This line of thinking has been supported by a number of studies. In addition, the expansion of understanding on the molecular basis of carcinogenesis and metastasis has made it possible to find novel treatments that combat abnormal biochemical/molecular pathways that may be causative of tumours [29].

Mushrooms & fungi are responsible for the production of more than one hundred different therapeutic functions. Most vital of them include *antioxidant, anticancer, antidiabetic, antiallergic, immunomodulating, cardiovascular protector, anticholesterolemic, antiviral, antibacterial, antiparasitic, antifungal, detoxification, and hepatoprotective effects.* Mushrooms and fungi also offer protection from tumor formations & other processes that are inflammatory [30,31].

Various compounds that are synthesised by macro-fungi are said to be bioactive. The bioactive molecules that can be obtained in fruit bodies, cultured mycelium, and cultured broth include *polysaccharides, proteins, fats, minerals, glycosides, alkaloids, volatile oils, terpenoids, tocopherols, phenolics, flavonoids, carotenoids, folates, lectins, enzymes, ascorbic acid, and organic acids*. Generally, in modern medicine polysaccharides are considered the most important compounds& β-glucan the best researched and adaptive. These are considered to have a wide range of biological action spectrum [7,30,32,33].

Because of their abundance in critical bio-macromolecules such as polynucleotides, polysaccharides, and proteins, in fields of culinary and medical, mushrooms are being widely sought and priced. The disciplines of biochemistry and pharmacology have paid a lot of attention these years to some ***"bioactive polysaccharides"*** that were extracted from therapeutic mushrooms.

Few mushroom extracts offer promise medicinal benefits for cancer, heart-related illnesses, DM [24] and cancer of colon (which can be fatal). *M. esculenta* is among the highly expensive mushrooms around the globe. It is among the wild species of mushrooms that are most valuable to the economy and ranks among the most important. It is also known by a variety of different names, including "***Guchhi, morel, yellow morel, sponge morel, true morel, common morel, real morel mushroom, and true morel"*** [34].

1.6. *M. esculenta*

This mushroom is exceedingly pricey; hence it's known by ***"growing gold of mountains".*** *M. esculenta* is frequently located in the deep coniferous that are known to have humus-rich loamy soil. It is only found growing in nature at high altitudes in chilly environments. It inhabits woodland environments at elevations ranging from around 2500 to 3500 metres [35]. It is a common occurrence in the form of a mycorrhizal or saprobic connection with coniferous and hardwood trees [36]. The months of March through July make up its growing season [37]. The Indian states of Jammu & Kashmir and Himachal Pradesh each have woods that are home to this type of mushroom.

Traditional hill societies make use of *M. esculenta* for both medical and healthcare purposes. The plant is commonly referred to as Guchhi in the local language [38,39]. This particular adjective originates from the Latin word "***esculenta***", which literally translates as ***"Edible"***. Because it contains a large variety of bioactive molecules, which include proteins, polysaccharides, trace elements, vitamins &dietary fibres, *M. esculenta* is consumed for both its nutritional and therapeutic properties [40].

Antioxidant activity can be shown in the fruiting body of the *M. esculenta* plant [41]. *M. esculenta* 's mycelia have been shown to contain β-carotene and linoleic acid, both of which have been shown to have antioxidant properties [42]. *M. esculenta* is known to exhibit anti-inflammatory and anticancer effects, both of which were linked to the presence of polysaccharides in this fungus [43,44]. Because it's a scarce wild resource and its growth and development through standard ways is extremely difficult, an alternate method known as submerged fermentation was developed and put into use. Some of the polysaccharides that are isolated from *M. esculenta* have shown the property of tumor growth inhibition [45]. "*Staphylococcus aureus, Salmonella typhimurium, Listeria monocytogenes, Escherichia coli, and Enterobacter cloacae*" are some of the bacteria that are inhibited by the antibacterial properties of this mushroom's extracts [46].

Fig. 1. Dried *Morchella esculenta*

1.7. Rationale

M. esculenta is a pharmacologically and nutritionally important fungus. Studies have been already done on the dietic and nutritional component of this and other edible mushrooms. In addition to those studies have been done on various pharmacological

aspects of other mushrooms and some aspects of *M. esculenta*. But it has not been studied for many pharmacological properties including hypoglycemic activities. Tremendous scope is available for the exploration of its unexplored medicinal properties. Very less work is reported on diabetes mellitus using *M. esculenta*. The motive and objective of this study was to do the same and fill this research gap.

CHAPTER-2

LITERATURE REVIEW

Summary: This chapter is about the literature review on the existing literature done on the topic.

Chapter-2

Reviews of Literatures

2. LITERATURE REVIEW

2.1. DIABETES

The most prevalent non-communicable disease in India, sometimes known as the ***"Diabetes Capital of the World,"*** is diabetes mellitus [47]. One-seventh of all diabetes patients worldwide are affected by it. In India, there are currently close to 80 million diabetics, and till 2045 the count is expected to reach 130 million. In India, prevalence has climbed dramatically among all age categories, and among the younger population, it has increased by more than 10%. According to research released in November 2017 by the ICMR, Institute for Health Metrics and Evaluation, and the Public Health Foundation of India , the prevalence of diabetes in India showed a steep rise of sixty-four percent during the past two to three decades.

According to research by the International Diabetes Federation, 90% of diabetic cases are type 2 [47]. Patients with diabetes are vulnerable to a variety of sophisticated medical conditions. One of the most significant lifestyle diseases, it has significant short- and long-term negative consequences on health. Cardiovascular illnesses are one of the primary causes of death, and a prominent role in this is played by diabetes [48]. Microvascular and macrovascular disorders which include stroke & CHD are some examples of the associated medical conditions that follow diabetes.

2.1.1. Definition

Diabetes mellitus/ diabetes is a bunch of disorders/ailments defined as elevated blood sugar levels brought on by flaws in the body's capacity to make and/or use insulin [49]. It is a state of hyperglycemia that causes micro and macrovascular damage including retinal, neuro - & nephro-associated pathies and IHD, PVD & stroke, respectively. It's been associated with a decreased life expectancy, increased morbidity, and decreased quality of life. The pathogenetic processes that cause diabetes development are varying. These include procedures that result in insulin resistance and the destruction of the pancreatic β cells, which leads to an insulin deficit. The anomalies

in the metabolism of carbohydrates, fats, and proteins are caused by the desensitization of targeted tissues with respect to insulin, which produces inefficient results and metabolism [50]. Typical signs and symptoms of DM include “polydipsia, polyphagia, thirst, polyuria, blurred eyesight, tiredness and weight loss”. Frequently, symptoms present mildly or are even non-existent.

2.1.2. History

Diabetes has been acknowledged as a fatal and debilitating condition for more than 2000 years. A Greek physician named Aretaeus characterized the devastating characteristics of the illness in the first century A.D. He gave it the name "diabetes" derived from “syphon”, a Greek word. Aretaeus and other ancient doctors identified the characteristics of diabetes but couldn’t accomplish any feat in terms of its cure. In 17th century, Dr. Thomas Willis of London took samples of urine from patients to ascertain whether they had sugar overload in their blood and in turn suffered from diabetes or not. He would identify them by checking for the sweet taste of honey in them. Up until the 20th century, this diagnostic method stood the test of time. Nothing much could be done for diabetes patients before insulin was discovered. They lived longer thanks to low-calorie diets, but they also became frail and nearly starved. But in 1921, physicians in Canada did the miracle of treating near death diabetic patients to health and bringing their very high sugar blood levels down. Since that time, medical advancements have kept extending and improving the lives of persons with diabetes. In 1950s, two forms of diabetes—"insulin sensitive" (type 1) and "insulin insensitive" (type 2)—were discovered. Since Aretaeus referred to diabetes as "the strange sickness," two thousand years have passed. The search for a remedy has been a protracted and gruesome task carried by a large number of doctors & scientists in subsequent generations. In a modest laboratory in Canada, insulin was discovered due to the commitment and shared knowledge and work of these people. Since then, medical advancements have kept making diabetes patients' lives simpler. The hunt for a cure for diabetes is still ongoing in the twenty-first century. Currently, it is uncertain in terms of what novelty we may achieve in this regard. Maybe there’s going to be another miraculous and hefty discovery like insulin very soon or maybe we need to be satisfied with a gradual progressive journey of betterment and discovery in this aspect [51].

2.1.3. Pathophysiology

Understanding the basics in terms of how insulin acts and carbs are metabolized is prominent to understand the diabetic pathophysiology. Following food consumption, the carbs get broken down into molecules of glucose which are then absorbed into the blood and cause the glucose level to rise in it. The pancreatic β cells release more insulin following this. Most cells require insulin to enable glucose entry. Insulin enables the glucose to enter the cells which then is used to take energy, by binding to certain cellular receptors on the cell. Rise in insulin production from the pancreas, causing glucose consumption in cells results in decreased glucose levels in blood. Lower glucose levels then lead to less insulin being secreted. Blood glucose dynamics will alter if an illness alters insulin synthesis and secretion. Blood glucose rises if the production of insulin is decreased since it will restrict glucose entrance into cells. Normal insulin production occurring but not being used properly by the target tissue results in the same problem. When an enhancement occurs in insulin production due to the entry of a large amount of glucose in tissues, the glucose levels the in-blood decrease exponentially and it is termed hypoglycemia. Glycemia is impacted by many hormones. The lone hormone which decreases glucose levels in blood is insulin. In addition to their other actions, “the counter-regulatory hormones glucagon, catecholamines, growth hormone, thyroid hormone, and glucocorticoids” all increase glucose level in blood [52].

2.1.4. Complications

Diabetes complications cause a large amount of “disability, lowered quality of life, and mortality” around the globe. Diabetes problems can affect different body parts and present differently in each person. Several major health issues are associated with diabetes. It causes ED, decreased levels of testosterone & issues even involving the emotional spectra, that can affect sexual desire in men, such as depression, anxiety, and stress. Diabetes can be particularly difficult for women. Pregnancy increases the risk of gestational diabetes, even for people without diabetes. According to data from the American diabetes association, in women suffering with diabetes, diseases of the heart top the reason for mortality. Additionally, diabetic women are more likely to experience eating disorders and depression, as well as have problems with their sexual health. Every aspect of the body, “including the feet, the eyes, and the skin”, can be impacted by

diabetes. In fact, these issues can be a person's first indication that they have diabetes. Foot issues can worsen and result in more significant issues like neuropathy, skin abnormalities, calluses, foot ulcers and poor circulation [53].

2.1.5. Diagnosis

When a patient shows the characteristic symptoms/signs of increased glucose levels in the blood and in random blood glucose test shows=/>200 mg/dL (11.1 mmol/L), which is later verified, the diagnosis of DM could be done with ease.

The basic diagnosis is made using the tests listed below:

A. When a person hasn't eaten in at least 8 hours, their blood glucose level is measured with the FPG i.e., fasting plasma glucose test. Diabetes & prediabetes can be identified with the test.
B. An OGTT i.e., oral glucose tolerance test checks blood sugar levels after the suspected patient fasts for 8 hours at least and hasn't had any beverage containing glucose in the last 2 hrs. Diabetes and prediabetes can be identified using this test. For detecting diabetes, the FPG test is frequently used due to its affordability and ease. OGTT is better than FPG, in the fact that it even catches some diabetic and pre-diabetic cases that might have gone undiagnosed with FPG. The FPG test is most sensitive if done early in the morning. The OGTT is less practical to administer but according to research is more sensitive than the FPG test for detecting prediabetes.
C. A casual random plasma glucose test evaluates blood sugar without taking the subject's most recent meal into account. The test is done to check for diabetes, not pre-diabetes and reviews the symptoms. Confirmation is only ascertained when a 2nd test is done on a different day and it comes positive [54].
D. FPG should not exceed 7.0 mmol/l (126 mg/dl), and 2-hour plasma glucose should not exceed 11.1 mmol/l (200 mg/dl) [50].

2.1.6. Diabetes mellitus- Types

The WHO released its first widely used categorization in 1980 [55]. Two main types of DM are proposed: Type 1- IDDM and Type 2-NIDDM. Along with

gestational diabetes, other forms were also covered. Internationally, the revised version from the year 1985 is taken into consideration [56]. Since patients have been segregated based on the therapeutic aspect and not the pathophysiology, it was advised that the words ***"insulin-dependent diabetes mellitus"*** and ***"non-insulin-dependent diabetes mellitus"*** no longer be used. Type 1 & Type 2, instead, were proposed to be utilized to make a separation between diabetic cases due to pancreatic islet b cells & insulin secretion abnormalities, respectively [57].

2.1.6.1. Type 1 Diabetes

Type 1 cases of diabetes only account for 5–10% of the total ones. Even with that factor, it's on rise around the world and has important short/long-term effects. Type 1 diabetes is defined by the pancreatic b cell loss and insulin is necessary to avoid morbidity and mortality in this condition and to prevent the onset of ketoacidosis, coma, and death [50]. The ideal way to manage Type 1 diabetes is in the context of a multidisciplinary health team and needs ongoing focus on a variety of issues, such as insulin administration, blood glucose monitoring, meal preparation, and screening for problems associated with diabetes. These consequences, which are primarily responsible for the disease and death associated with Type 1 diabetes, include microvascular and macrovascular disease [58].

2.1.6.2. Type 2 Diabetes

Type 2 is the most commonly occurring type of diabetes. Worldwide, tens of lakhs of people have been diagnosed with type 2 diabetes, and many more go untreated. If diabetes is untreated or poorly managed, individuals are more likely to experience cardiovascular conditions including a heart attack and stroke. Slower or delayed wound healing, especially of lower limbs, leading to cases of amputation of the affected area and kidney failure requiring transplantation or life-long dialysis treatment are additional dangers that are increased for them [59]. People virtually are always suffering from pre-diabetes before moving on to developing diabetes Type 2, which is characterized by blood glucose levels that are elevated but not yet high enough to be classified as diabetes. Recent studies have revealed that long-term bodily damage, especially to the circulatory system including heart might be already in process during prediabetes [60]. In type 2

diabetes, the cells either do not respond to insulin or the body does not create enough of it. Insulin is needed to change the glucose into energy unit of the body. After a meal, starches and sugars are converted into glucose by our body, the primary energy source for cells. Insulin helps the cells absorb sugar from the blood. Diabetes problems can result from glucose not entering the cells as it should, which causes an accumulation in the blood.

2.1.6.3. Delay of/ Prevention of Type 2 Diabetes

Prediabetes, or blood glucose levels that are higher than usual but not yet high enough to be diagnosed as diabetes, almost invariably exists before Type 2 diabetes does. It is possible to address the significant medical condition of prediabetes. People with prediabetes can reduce the chances of moving to diabetes by diet changes and increase in exercise and physical activity, according to a published study conducted in US. The need for lifestyle modifications cannot be overstated. A healthy weight can be maintained, along with a longer lifespan and a lower risk of developing diabetes, by eating a balanced diet and engaging in more physical exercise. The findings of ***"Diabetes Prevention Program (DPP)"*** demonstrated that exercise and dietary changes resulting in weight loss can prevent and delay diabetes Type 2 [61]. This program was an important multi-national clinical research trial designed to determine if metformin (Glucophage), an oral diabetes medication, changes in diet, steeped up exercise and activity can delay/prevent the initiation of diabetes Type 2 in the recruitees.

2.1.6.4. Gestational Diabetes- Diabetes in Pregnancy

When a woman is pregnant, gestational diabetes is discovered for the first time. Gestational diabetes is more likely to affect overweight women, women who have already had gestational diabetes, and women with a strong family history of diabetes. The infant could experience issues if gestational diabetes is left untreated. Lifelong, higher risk of acquiring Type 2 diabetes is seen for both the mom and child in these cases [62].

2.1.7. Risks

Uncontrolled fat build-up and a lifestyle not complimented with exercise and physical activity are 2 diabetic risk factors that are under our control. However, other unavoidable risk variables, such genetics and race, can have a significant impact. A history of this chronic, lifelong illness in the family is the main risk factor for type 1 diabetes. A significant risk factor is having diabetes in the family. A person with 1° relative suffering with diabetes type 1, should get tested for the disease, according to the American Diabetes Association's standards of medical care in diabetes from 2007 [63]. Diabetes Type 1 can be diagnosed with a quick blood test.

Additionally, type 1 diabetes can result from pancreatic disease or injury that prevents the organ from producing insulin. Numerous relatively uncommon ailments and infections can damage the pancreas which can follow up into diabetes Type 1 [64]. Obesity, diet and physical inactivity, growing older, insulin resistance, a family history of diabetes, genetic variables, and race and ethnicity are risk factors for type 2 diabetes. According to research on genetic influences, having diabetes is more likely when specific gene mutations are present. These genes may contribute to decreased insulin synthesis, impaired insulin sensitivity in body tissues, and an increased risk of obesity. However, some ethnic groups, mostly including the ones found in global South, show increased diabetic rates which can be attributed to ethnic and racial factors. These groups are more susceptible to diabetic progression and heart diseases. This is mainly because these groups have greater incidences of diabetes, high blood pressure, and obesity. In contrast to other racial groups, African Americans have a higher risk of developing type 2 diabetes [65]. Even while genecity and racial profile are risk factors, they aren't the sole determiners of illness occurrence. Rapid technological advancement and urbanization-related dietary changes and declines in physical activity have caused a substantial rise in the number of persons contracting diabetes. History of substance use, has been seen as a prominent diabetes type 2 risk factor in terms of its early onset (age wise), according to research [66]. Cannabis and cocaine are the most often used illegal drugs , although diabetic primary care patients also frequently use illicit substances such as amphetamines, heroin, hallucinogens, and non-medical inhalants (ibid).

2.2. APPROACHES TO TREATMENT: OLD AND NEW

2.2.1. Pharmacological approach

Dietary changes and oral hypoglycemic drugs, which have previously been used to treat this chronic, progressive condition, have not been found to be effective, and insulin therapy only provides a temporary fix. Patients continue to experience macro and microvascular problems even with the most recent pharmaceutical treatments. Diabetes is linked to an increase in morbidity and mortality due to *"heart disease and stroke, kidney failure, blindness, and 60% of lower-limb amputations performed without trauma"* [67]. Alternative therapies aimed at various illness models need to be carefully and responsibly analyzed. As demonstrated below, after gastrointestinal bypass procedures, diabetes can be controlled without the use of insulin.

2.2.1.1. Insulin therapy

Diabetes places a significant financial strain on health care systems because it's a prominent reason for cardiac diseases and death in Western nations [68]. Therefore, effective glucose control (achieving normal HbA1C, prandial, and postprandial glucose levels) is important to stay away from fatal side effects of this condition. The hormone insulin is used to treat it by blood sugar/glucose level regulation. It is either derived from pork (porcine), beef (no longer accessible in the U.S.), or is genetically engineered to be exactly like human insulin when administered as a pharmaceutical [69].

When insulin isn't produced by the body, type 1 patients need to rely on insulin from external sources, which is typically administered subcutaneously. Type 2 patients/ diabetes mellitus are either insulin resistant, produce very little insulin, or both; if other drugs are ineffective at controlling blood glucose levels, some amount of the hormone might be required. To treat diabetes, a variety of insulin kinds are available. They are classed on the basis of rapidity of onset of function, the time required for peak activity, and residual effect in the body.

Types of Insulin: [70]

Table 2.1. Types of Insulin

Types of Insulin	
Insulin with a rapid onset of action	Acts quickly and lasts for a few hours
Short-acting or regular insulin	Starts working after around 30 minutes and lasts for 3 to 6 hours
Intermediate-acting insulin	Effects can last up to 18 hours and require 2 to 4 hours to take action.
Long-acting insulin	Enters the bloodstream between 6 and 10 hours after injection but continues to function all-day

Both subcutaneous and vein injections of insulin are options for treating diabetes (intravenously). Most patients with Type 2 diabetes and total patients of type 1 continue to receive subcutaneous insulin injection as their primary form of therapy. "A needle and syringe, a cartridge system, or prefilled pen systems" can all be used to administer insulin. There are other insulin pumps available. The patient's weight determines the initial dose and also takes into account their unique insulin sensitivity. When administered subcutaneously, 2/3rd of daily dose of insulin is given in the morning and rest in the evening [71].

2.2.1.2. Complications of the insulin therapy

Diabetes mellitus is a class of metabolic illnesses characterised by hyperglycemia that, if left untreated, can result in micro- and macrovascular problems as well as long-term repercussions. In various diabetes populations, it has been proposed that strict glycemic control combined with intensive insulin therapy can lower the incidence of such problems; nevertheless, this strategy is not without dangers and difficulties. Low sugar levels, hypertrophy at the site where injections with insulin are injected and rashes over the injection site/whole body are the main side effects of insulin used to treat diabetes (rare). Increased appetite, fatigue, chills, tremor in upper limbs, raised anxiety, and confusion are a few of the symptoms of the most common problem, low blood sugar. They could be symptoms of an insulin overdose, which many diabetic individuals experience and is very harmful [72]. Fortunately, most insulin-related incidents can be avoided if patients follow a few straightforward guidelines. Another

insulin-related issue is DKA i.e., diabetic ketoacidosis, resulting due to insulin intake insufficiency. In that situation, elevated sugar levels promote excessive urine, which severely dehydrates the body. At the same time, cells of the body imitate starvation, since there isn't enough insulin to for sugar absorption. Patients with type 1 diabetes experience severe increase in sugar levels of blood in the absence of insulin. Urine glucose is increased because of abnormal fluid and electrolyte loss. In addition to the breakdown of existing fat and protein stores, a lack of insulin also prevents fat and protein from being able to be stored. Release of ketones into the bloodstream &the ketosis process are the results of this imbalance. Diabetic ketoacidosis is a condition when ketones cause the blood to become acidic (DKA).

Constipation, vomiting, and nausea are diabetic ketoacidosis symptoms. Patients with DKA can quickly enter shock, unconsciousness, and even pass away without early medical attention (ibid). Infections, stress, or trauma can all result in diabetic ketoacidosis (DKA), which may need using more insulin. Missing insulin doses is another clear important risk for DKA development. Intravenous fluid, electrolyte, and insulin administration is used as an urgent treatment for diabetic ketoacidosis, in ICUs. When a person comes with DKA, severe dehydration maybe presented and a replacement of 5-7 L of fluids isn’t unusual. Infections are treated with antibiotics. Patients can recover astonishingly well with treatment, which can rapidly rectify abnormal sugar levels in blood, release of ketones, dehydration and acidosis.

Hyperosmolar hyperglycemic nonketotic syndrome (HHNS), which can be fatal, causes extreme dehydration similar to DKA. It is a very dangerous complication that in type 2 diabetes can result in diabetic coma and possibly death. Compared to DKA, hyperosmolar hyperglycemia is substantially less common and more frequently affects elderly, obese type 2 diabetic individuals [71].

Once they develop, these insulin problems need to be treated at a hospital. Correction of fluid deficits, electrolyte imbalances, and hyperglycemia serve as the cornerstones of treatment for both HHNS and DKA. Additionally, identifying and treating the underlying trigger condition is crucial in HHNS. The precipitating condition and comorbidities frequently conceal the hyperosmolar hyperglycemic nonketotic syndrome; therefore, it must be actively searched out and the precipitating illness should

be recognised and addressed. Additionally, the mortality rate for HHNS is significant. When compared to diabetic ketoacidosis, the fluid deficit is twice as large. Until the patient's mental state becomes better, the hyperosmolality goes away, and the goal blood sugar level is met, the insulin therapy should be continued.

2.2.1.3. Treatment of Non-Insulin Diabetes

Incretin mimetics, a family of relatively recent medications, imitates certain elements present in the stomach and intestines. These chemicals often trigger the production of insulin as response to food intake from the pancreas. Incretin mimetics function to elevate insulin release and aid in lowering blood sugar because this reaction is diminished in patients with type 2 diabetes. If other/earlier forms of medications are not being efficient, the doctor may advise incretinmimetics. Once or twice a day, injections are used to provide these drugs. DPP-4 inhibitors are drugs that can be used either alone or in conjunction with other diabetes treatments to treat type 2 diabetes. Incretin hormones are not broken down by DPP-4 inhibitors. The incretins can then assist the body in producing insulin to reduce the elevated sugar levels in the blood [72].

Oral Hypoglycemic Agents (OHA)

Any anti-diabetic drug can be referred to as an OHA. The following five categories are used to divide them: Thiazolidinediones, α glucosidase inhibitors, Biguanides (sensitizers), Sulphonylureas and analogs (secretagogues), and Incretine Analogues/Agonists [65]. Sulphonylureas and other secretagogues work by blocking the ATP-sensitive K+ channel, which in turn stimulates the release of insulin. When a patient is told they have Type 2 Diabetes, they are advised to be their first option. Hypoglycemia and the disulfiram reaction are side effects, and the competition for protein binding, metabolism, and secretion is an interaction. Tolbutamide (Orinase), acetohexamide (Dymelor), tolazamide (Tolinase), and chlorpropamide (Diabinese) are first-generation medications. Glipizide (Glucotrol), glyburide (Diaβ, Micronase, Glynase), and gliclazide (Diamicron) are second-generation medications. Glimepiride is a third-generation medication (Amaryl). The meglitinides are sulphonylurea-like short-acting secretagogues. They also open the Ca^{2+} channels and inhibit the ATP-

sensitive K+ channels, which promote the release of insulin. They can also cause hypoglycemia and weight gain as side effects.

- In terms of how sensitizers like the Biguanides work, they work on the liver in reducing glucose excretion and production while increasing the absorption of insulin by skeletal muscle. They are not hypoglycemic; rather, they are anti-hyperglycemic. They are recommended for polycystic ovarian syndrome and Type 2 Diabetes Mellitus. They may aid in weight loss and lower LDL cholesterol and triglyceride levels. They do not result in hypoglycemia, but cause other adverse effects including discomfort of gut, toxicity in kidney, diarrhea and acidosis (lactic).
- In terms of the glitazones' mode of action, like Thiazolidinediones, they function as selective agonists of PPAR receptors, activating the insulin-sensitive genes that control the metabolism of glucose and fat. They consequently improve the peripherial tissue's sensitivity to insulin. Hepatotoxic effects are one of their adverse effects (troglitazone).
- In terms of how α -glucosidase or α amylase inhibitors work, they reduce the absorption of dextrins, di-saccharides and starch on the intestine, which lowers postprandial plasma glucose. When combined with diet and insulin, they are suggested for both diabetes types (1 & 2). They can cause diarrhoea, gas, and malabsorption as adverse effects.
- Peptide analogues, like GLP-1 agonists, work by binding to a membrane-bound GLP-1 receptor. The enzyme dipeptidyl peptidase IV breaks them down (DPP-IV). They can cause nausea, hypoglycemia (when used with an insulin secretagogue), and exenatide-acute pancreatitis as side effects. Contrarily, the GPP-4 inhibitors' (gliptines') mode of action is to raise the blood concentration of the incretin GLP-1 (glucagon-like peptide-1) by preventing dipeptidylpeptidase-4 from degrading it (DPP-4). Although in comparison to OHA it has lesser side-effects, it should be kept in mind that they represent a new class of medications [72].

2.2.1.4. Type 2 diabetes- Metabolic surgery

There’s been a rapid development in the metabolic surgery field now, for developing surgical techniques specifically designed to treat diabetes. In the early 1980s, doctors discovered that many type 2 diabetes patients who underwent gastric bypass surgery to cure their morbid obesity achieved a full remission of their condition. This remission was long-lasting [73]. The best results for diabetes control and remission appear to come from surgeries like gastric bypass or biliopancreatic diversion that involve an intestinal bypass. Pories et al. (1995) observed that 83% of 608 patients who underwent gastric bypass had their Type 2 Diabetes resolved following a 14-year follow-up. Typically, remission is not noticed until several months after surgery, and then only after significant weight loss. Although the exact mechanism of diabetes remission following gastric bypass is still unknown, it does not appear to be only connected to weight loss. Following the surgery, days to weeks, remission is seen [74]. Furthermore, new research suggests that these results might be imitated in patients who aren’t obese too. Clinical studies further demonstrate that the impact of gastric bypass surgeries on diabetes is not just because of the loss in weight [60]. The operative management of diabetes may provide a solution to the coming generation's worldwide health challenge. The advantages of surgery must be evaluated against the possible hazards, though, just like with any other medical operations. Meaning, one must weigh the pros and cons, the complications arising out of surgery against the likelihood of a diabetes remission and a reduction in long-term morbidity and mortality from diabetes. Contrary to widely held misconceptions, the surgical profile of bariatric surgery is remarkably safe, and the associated mortality is low [75]. The advantages of surgically resolving diabetes are substantial. In a study, after Roux-en-Y gastric bypass, diabetes-related mortality was monitored for 7 years and dropped 92% in comparison to controls [76]. The betterment in case of metabolic syndrome, which lowers cardiovascular risk factors, is another advantage of the procedure. The metabolic syndrome, which includes type 2 diabetes, hypertension, elevated fasting glucose and triglycerides, decreased HDL, and abdominal obesity , has been reported to have significantly improved in all of its components, with holistic resolution reaching >95% after a year [77]. Surgery appears to be a further tool in the fight against diabetes. Despite the strong result data, each patient's risk factor should be considered when deciding whether to operate. Surgery carries hazards connected to both anesthesia and the procedure itself because it is by its very nature an

invasive therapeutic method. The advantages of metabolic surgery are actually very great. However, its application necessitates a reevaluation of diabetes treatment objectives and tactics. In the interim, research into the pathophysiology of diabetes is ongoing in an effort to identify the most effective therapy targets and therapies.

2.2.2. Non-pharmacological treatment

Lifestyle changes: As far as non-pharmacological treatment for diabetes mellitus is concerned, only this intervention has the ability to prevent the development of diabetes in individuals with impaired glucose tolerance, especially type 2 diabetes. In cases of early diabetes, it may potentially be the only treatment option. After receiving a diabetes diagnosis, one must change their habits and way of life. Due to its association with heightened risks such as kidney failure, visual impairment, foot ulcers, leg amputations, and heart attacks among individuals with diabetes, healthcare professionals should strongly recommend that all diabetic patients refrain from initiating tobacco use. Moreover, the primary focus for diabetic individuals who smoke should be on quitting this habit, as highlighted in a publication from Diabetes Care in 1993. The advantages of quitting smoking are substantial for those with diabetes, given that smokers exhibited a markedly higher occurrence of both microvascular and macrovascular complications compared to non-smokers, as indicated by a study conducted by Buysschaert in 2000 [69]. When it comes to alcohol, excessive consumption can lead to hypoglycemia, which can happen hours after drinking, especially if no food has been ingested prior.

2.2.2.1. Diabetes Mellitus and diet

Sedentary behavior and excessive calorie intake, which results in obesity, are the main environmental factors that contribute to type 2 diabetes [62]. Urbanized societies are more likely to have sedentary lifestyles.

Following a diabetes diagnosis, nutritional guidance is crucial. Typical guidance includes:

- Cutting back on fatty food consumption
- Consuming primarily veggies, cereal, fruits and rice (wholemeal products)

- Using refined sugar sparingly (jam, sweets etc.)
- Eat regularly spaced meals
- Keeping glucose pills, candy, or other supplies on hand in case of hypoglycemia
- Regular exercise reduces increase in blood sugar levels and by reducing obesity decreases the insulin resistance.

With healthy lifestyle modifications, the majority of instances can be avoided, and some can even be treated. Living in poverty is not the result of taking measures to prevent and manage diabetes. While maintaining a healthy diet is crucial, patients can still have a few cheats here and there. Carbohydrates are more important than fats and proteins, in impacting sugar levels in blood. Patients should generally avoid sugary drinks, candies, and snack meals, as well as highly refined carbs such white bread, pasta, and rice. Instead, concentrate on complex carbohydrates abundant in fibre, also referred to as slow-releasing carbohydrates. Because they are digested more slowly, slow-release carbohydrates assist maintains blood sugar levels by avoiding the body from overproducing insulin. Additionally, they give long-lasting energy and prolong satiety [78].

2.2.2.2. Diabetes Mellitus-Effect of exercise

Participating in regular physical activity can substantially decrease the risk of developing type 2 diabetes by approximately 30–50%. Even dedicating as little as 30 minutes to moderate exercise daily can lead to noticeable reductions in associated risks [72]. All forms of diabetes can benefit from improved glycemic control through regular exercise. The most effective strategy for addressing insulin resistance, a key contributor to type 2 diabetes, is consistent physical activity [57]. Various methods exist by which physical activity enhances insulin sensitivity. In cases of obesity, the primary factor behind insulin resistance is excess fat accumulation in the liver. Regular exercise can counter this fatty build-up, consequently reducing hepatic insulin resistance [61].

Tuomilehto et al. (2001) [70] recommend engaging in moderate exercise for 30 minutes daily or moderate physical activity, such as brisk walking, for at least 150 minutes weekly (Diabetes Prevention Programme research group in NEJM 2002). These

practices are linked to reduced body weight lowered insulin resistance leading to decreased symptoms associated with the metabolic syndrome like hypertension, dyslipidemia, and inflammation, as well as improved endothelial function [72]. The benefits of staying active extend beyond weight management and physical fitness. According to the American Diabetes Association, physical activity aids individuals with diabetes in effectively regulating blood sugar levels, reducing blood pressure, enhancing blood lipid profiles, and requiring less insulin and diabetic medication after weight loss. Furthermore, it diminishes the chances of developing additional health complications and assists in maintaining lost weight. Those who engage in regular physical activity often experience increased energy levels, improved sleep quality, and reduced levels of stress, anxiety, and depression. Physical activity also enhances flexibility and strengthens bones and muscles across all age groups [63].

2.2.2.3. Biological medications for the treatment of diabetes mellitus

Biological therapy aims to enhance or restore the immune system's ability to counteract diseases and infections within the body. Biotherapy and immunotherapy are additional names for biological therapy. Drugs with an active ingredient derived from a biological source are referred to as biological drugs. These medications were created using cutting-edge technology known as "genetic modification", which sets them apart from typical prescription medications. Today's biological medications are primarily monoclonal antibodies (mABs). These modifications are specifically tailored to target exact treatment locations and address particular illnesses with reduced side effects. However, they retain the capacity to combat diseases just like regular antibodies [61].

In Type 1 diabetes, the immune system targets and eliminates the pancreatic β-cells responsible for producing insulin. While synthetic insulin can be a lifesaver, it falls short of being a cure and preventing long-term complications. Many individuals with Type 1 Diabetes would go to great lengths to escape the daily routine of glucose testing and insulin injections. Despite promising clinical trials that have shown significant impact, discovering a definitive way to prevent and effectively treat the disease in human remains elusive. The interest in utilizing T cells with regulatory attributes, known as Treg cells, for biological therapy has gained momentum. The aim is to restore and maintain tolerance towards self-antigens (ibid), offering potential benefits. Type 1

Diabetes affects 5% of diabetes cases, where patients lose the ability to produce their own insulin due to the destruction of pancreatic -islet cells by autoreactive T-cells or the neutralizing effect of auto-antibodies against insulin. Currently, insulin replacement therapy is the sole available treatment. A notable example involves ActoBiotic, developed by ActoGeniX, which, when combined with low-dose anti-CD3, delivers pro-insulin and IL10 to the gut. This approach restores antigen-specific long-term tolerance and has even reversed diabetes in NOD mice. The mechanism involves enhancing the presence of local regulatory T cells, which multiply within the pancreatic islets and effectively suppress the autoimmune response in an antigen-specific manner (ibid). Monoclonal antibodies like Otelixizumab and Rituximab represent potential treatments for Type 1 diabetes.

Otelixizumab

Tolerx, Inc. developed the humanized anti-CD3 monoclonal antibody otelixizumab, also referred to as TRX4, as a therapeutic approach for autoimmune disorders like Type 1 Diabetes. Its primary objective is to target CD3, a T lymphocyte receptor involved in essential cell signaling processes. By bolstering regulatory T cells that counteract the harmful effects of effector T cells, otelixizumab aims to prevent the attack and destruction of insulin-producing β cells. This preservation of regulatory mechanisms helps sustain the β cells' ability to produce insulin. However, otelixizumab faced a setback in a pivotal Phase 3 trial back in 2011, prompting questions about the safety and efficacy of biological therapies. Despite its significant potential, otelixizumab still holds room for refinement. The results of further trials will play a pivotal role in defining its role in preventing Type 1 Diabetes [57].

Rituximab

Rituximab is a monoclonal antibody designed to selectively focus on the CD20 protein, primarily found on the cell surface. Some autoimmune diseases, like Type 1 Diabetes, are treated with it. It is thought that T lymphocytes' autoimmune destruction of -cells causes Type 1 diabetes. According to reports, a single Rituximab course can decrease in the very 1st year c-peptide loss [58]. It has been demonstrated that biological medications are effective in treating both diabetes and the complications that its sufferers

experience. They are utilised in particular to treat diabetic renal disease and foot ulcers. Patients can thus combat not only diabetes but also other annoyances. It has been established that prompt biological therapy can hasten the healing of diabetic foot ulcers.

2.3. Diabetes Complications: Management and Prevention

A category of chronic disorders known as diabetes are characterised by hyperglycemia. To prevent and manage hyperglycemia, modern medicine employs a wide range of dietary and pharmacological therapies. The objective of diabetes treatment is to reduce the risk of cellular damage caused by hyperglycemia and ensure the adequate provision of glucose to those tissues. It is crucial to safeguard the body from hyperglycemia because both types of diabetes have a high morbidity and mortality rate due to their effects on the vascular system of our body.

There are two main groups of diabetes complications:

a. Acute problems like hypoglycemia and comas brought on by DKA or HHNS (as mentioned above) as well as
b. Microvascular (pathies of retina, kidney and brain due to diabetes) or macrovascular chronic problems (CAD, PAD & stroke)

Microvascular

The ocular complications of microvascular diabetes are among the most severe. Diabetic individuals are strongly advised to undergo an annual eye examination. Diabetic retinopathy is the leading cause of blindness in the working population of Western countries. Almdal noted in 2006 that the duration and severity of hyperglycemia play a significant role in the development of diabetic retinopathy and other microvascular complications of diabetes [79]. Diabetic retinopathy encompasses two primary types: background and proliferative. A comprehensive understanding of the characteristics of each is essential for interpreting eye examination results and educating patients about the progression and prognosis of their condition. Background retinopathy, for instance, involves small hemorrhages in the intermediate layers of the retina, often

referred to as dot hemorrhages due to their appearance. Proliferative retinopathy, on the other hand, involves the growth of new blood vessels on the retina's surface, potentially leading to vitreous hemorrhage [80]. Diabetic nephropathy stands as the most common cause of renal failure in the United States. Approximately 20% to 30% of diabetic individuals exhibit signs of nephropathy. Prevention serves as the primary defense against diabetic nephropathy, as with all diabetes-related complications. Similar to other microvascular complications of diabetes, there are notable links between glucose management and the risk of developing diabetic nephropathy. To prevent or manage diabetic nephropathy, patients should aim for the lowest safe glucose levels achievable [78]. The treatment of diabetic nephropathy involves antihypertensive medications in conjunction with aggressive management of elevated blood glucose levels. Renin-angiotensin system blockade proves beneficial for diabetic nephropathy patients beyond its blood pressure-lowering effects.

Diabetic neuropathy is defined by the American Diabetes Association (ADA) as the presence of symptoms and/or signs of peripheral nerve damage in individuals with diabetes, after ruling out other potential causes [63]. The likelihood of developing diabetic neuropathy is inversely related to the severity and duration of hyperglycemia, similar to other microvascular complications, and some individuals may have a genetic predisposition to such issues. The precise mechanisms underlying the damage caused by hyperglycemia to peripheral nerves are not fully understood, but they likely involve processes such as polyol accumulation, damage from AGEs (advanced glycation end-products), and oxidative stress. Diabetic neuropathy can manifest in various forms, including sensory, focal/multifocal, and autonomic neuropathies. More than 80% of amputations are attributed to foot ulcers or wounds, often resulting from diabetic neuropathy [61]. Diabetic autonomic neuropathy also leads to significant morbidity and, in some cases, mortality among diabetic patients. Neurological dysfunction can affect multiple organ systems, resulting in symptoms such as anhidrosis, gastroparesis, constipation, diarrhea, erectile dysfunction, exercise intolerance, resting tachycardia, silent ischemia, and even sudden cardiac death [61]. Patients may experience symptoms like tingling, burning, numbness, or electric pain, while the impact is usually more pronounced in the feet rather than the hands.

Macrovascular

The primary pathological process behind macrovascular disease is the development of atherosclerosis, which results in a generalized narrowing of arterial walls throughout the body. Atherosclerosis is attributed to chronic inflammation and damage to the arterial wall, occurring in both peripheral and coronary vascular systems [81]. Diabetes significantly elevates the risk of cardiovascular disease (CVD). The precise mechanisms linking diabetes to the initiation of atherosclerotic plaque formation are not fully elucidated, but a substantial association exists between the two [82]. Cardiovascular disease is a severe outcome among diabetes patients, standing as the leading cause of premature mortality [40]. Diabetic individuals face a 2 to 6 times higher likelihood of experiencing complications like ischemic heart disease, cerebrovascular disease, and peripheral vascular disease compared to the general population [54].

Several studies, including the Framingham study, have demonstrated the connection between diabetes and coronary heart disease, a prominent macrovascular complication of diabetes [83]. While diabetes patients share the three main cardiovascular risk factors—smoking, high blood pressure, and hyperlipidemia—with the non-diabetic population, the condition amplifies these risks. Typically, individuals with diabetes live 7 to 10 years less than those without the condition. The metabolic syndrome, encompassing abdominal obesity, hypertension, hyperlipidemia, and increased coagulability, is a likely setting for the development of type 2 diabetes. These factors can also contribute to CVD. Even in the presence of other risk factors, type 2 diabetes independently serves as a risk factor for ischemic disease, stroke, and mortality (36). Similar to coronary artery disease, diabetes is also a significant independent predictor of stroke and cerebrovascular disease risk [79].

In response to the heightened risk of CVD, these conditions are being more aggressively managed for primary or secondary prevention of coronary heart disease. Intensive management of Type 1 diabetes has been linked to a lower resting heart rate, while individuals with higher levels of hyperglycemia tend to have a faster heart rate, which correlates with an elevated risk of cardiovascular disease (CVD) [84].

Compared to those with normoglycemia and macrovascular disease, diabetic patients experience a poorer survival prognosis once clinical macrovascular disease sets in. Female individuals with diabetes lose the protective qualities that women generally have against vascular disease development. Moreover, the coexistence of diabetes and hypertension poses a significant threat, escalating the risk of cardiovascular morbidity and mortality. Given that hypertension is more prevalent in diabetes patients and heightens the risk of complications, it should receive the same level of attention as glycemic control when formulating treatment strategies. Diabetes that is inadequately controlled can either cause hyperlipidemia or exacerbate its presence, thereby contributing to the risk of macrovascular disease. Approximately 25% of patients seen at diabetes clinics exhibit elevated lipid levels [85].

Infections

Diabetic patients face a heightened vulnerability to infections compared to healthy individuals. While conclusive data connecting diabetes to a general increased risk of infection are lacking, there is a propensity for diabetic individuals to be more susceptible to specific diseases, some of which are almost exclusive to them. Moreover, infections that do occur tend to be more severe and carry elevated risks of complications. The immunity of patients with diabetes is subject to several alterations. Evidence suggests that enhancing glycemic control can enhance immune function. Among the most commonly observed infections in diabetic individuals are fungal cystitis, rhino-cerebral mucormycosis, and community-acquired pneumonia [86].

Guidelines for managing diabetes mellitus complications include:

- Regular monitoring of blood pressure is imperative, with a target not exceeding 130/80 mmHg. Pharmacological treatment should be considered for patients with blood pressure below 140/90 mmHg alongside lifestyle and dietary modifications.
- For patients with blood pressure ranging from 130 to 139/80 and 89 mmHg, a three-month trial of behavioral and lifestyle interventions is recommended. If the target blood pressure isn't achieved, pharmaceutical therapy can then be considered.

- Lipid testing should be conducted annually for diabetic patients. Desired lipid levels for adults with diabetes include fasting triglycerides below 1500 mmol/l, HDL above 500 mmol/l, and LDL below 1000 mmol/l (or 700 mmol/l in individuals with overt CVD).
- All diabetic patients should restrict their intake of saturated fat, trans fat, and cholesterol.
- Comprehensive eye examination and dilation should be performed within three to five years of type 1 diabetes onset and at the time of type 2 diabetes diagnosis.
- Adequate glucose and blood pressure control should be pursued to minimize the risk of diabetic retinopathy or its progression.
- Annual screening for distal symmetric polyneuropathy is recommended for all diabetic patients.
- Patients with peripheral neuropathy should adopt proper foot care practices, including using specialized footwear to reduce the likelihood of ulcers.
- In addition to pharmaceutical recommendations, individuals with diabetes should be strongly encouraged to avoid starting or quit smoking to enhance their overall health and mitigate the risk of CVD.

2.4. Living With Diabetes Mellitus

People who are diagnosed with Diabetes must provide the majority of their own care , making it a unique sickness. There are many topics to discuss. They must combine insulin, medications, blood glucose monitoring, food, and exercise into their way of life in order to effectively manage their diabetes. Since the condition must be controlled on numerous different levels, there isn't simply one technique to deal with it. Effective diabetes management necessitates a wide range of therapies and tactics.

Upon receiving a diabetes diagnosis, individuals often experience a gamut of emotions. Among the common reactions to this unwelcome news are instances of denial, where they struggle to accept that something is amiss, feelings of self-blame for supposedly causing the diabetes themselves, and even episodes of anger. Although diabetes is a condition that affects a significant portion of the population, it's important to recognize that each patient requires tailored and individualized care [86]. The foundation of this care should be the medical professionals involved. Patients must

consistently bear in mind that they are the linchpin of their healthcare team, and it falls to them to candidly share their emotions and any challenges they've encountered.

In a broader sense, the goal of diabetes treatment is to emulate the natural balance of insulin and glucose within the body of a diabetic patient. Effective diabetes management necessitates the maintenance of blood glucose levels as close to the norm as feasible. For type 1 diabetes management, the key components encompass insulin, dosage adjustment of insulin, and dietary measures. In some instances of type 2 diabetes, blood glucose levels can be effectively regulated through diet and physical activity alone. Depending on factors like age, lifestyle, and overall health, a healthcare team can provide recommendations for a personalized lifestyle or exercise regimen [71].

2.4.1. Life-quality

Recognizing quality of life as the central objective of all medical interventions and a significant health outcome in its own right has gained prominence. More than 70 years ago, the World Health Organization emphasized that health encompasses not just the absence of disease and infirmity, but also physical, mental, and social well-being [87,88]. While healthcare practitioners might sometimes solely focus on medical outcomes when assessing the effectiveness of their interventions, those grappling with diabetes understand that these outcomes truly matter only to the extent that they influence physical, emotional, and social welfare – in essence, the quality of life. Dealing with diabetes is undeniably challenging. The sheer weight of the disease can be overwhelming, as individuals are often burdened by the ongoing demands of its management. Virtually all individuals with diabetes find that the condition exerts a substantial influence on their lives, imposing numerous demands that they must contend with. The acute distress brought about by hypoglycemia or hyperglycemia, coupled with the persistent physical discomfort due to diabetes-related complications, can exacerbate these emotional and social responsibilities, making them even more demanding.

Diabetes unquestionably shapes an individual's quality of life. However, what exactly does "quality of life" encompass? In its broadest sense, quality of life can be perceived as a multifaceted concept encompassing an individual's subjective perception of their physical, emotional, and social well-being. It also encompasses both cognitive

aspects like satisfaction and emotional elements like happiness [89]. Both individuals with diabetes and their healthcare providers assign considerable value to quality of life for various reasons. Diabetes can overwhelm and compromise self-care, subsequently worsening glycemic control, elevating the risk of complications, and intensifying the burdens of diabetes management in both the short and long term.

Just like in the wider population, specific demographic factors are linked to the quality of life in individuals dealing with diabetes:

- Men often report higher life quality than women do.
- In general, younger people report higher life quality than older persons.
- People with higher incomes and educational levels often report higher quality of life than people with lower levels of either.

Quality of life for men with diabetes can often be impacted by sexual problems. Both men and women can face sexual difficulties due to nerve and small blood vessel damage, but men are particularly susceptible to such issues. According to the National Diabetes Information Clearing House (NDIC), anywhere from 20% to 75% of men with diabetes may experience erectile dysfunction (www.diabetes.about.com). Additionally, challenges with the ejaculation process can also arise in men. Changes in sexual function linked to age affect everyone. However, men with diabetes might encounter these problems at an earlier age and with greater severity due to the slight blood vessel and nerve damage resulting from poorly managed diabetes. These issues can emerge 10-15 years earlier compared to men without diabetes. In fact, erectile dysfunction in men under 45 years old might signal existing diabetes or a high risk of developing it in the future. To mitigate the risk of sexual problems, men with diabetes should diligently manage their condition, adopt a healthy diet, engage in regular exercise, and maintain healthy abdominal fat levels. The initial step in addressing erectile dysfunction involves counseling and seeking emotional and psychological support; healthcare providers may also recommend specific treatment options [90].

2.4.2. Diabetes Mellitus and psychological aspects

Every potentially life-threatening illness brings about specific psychological effects, and diabetes is no exception to this. Family members of individuals with type 1

diabetes commonly traverse the familiar stages of grief, starting from anger and denial, progressing through bargaining and despair, and eventually reaching resolution or acceptance. A positive shift indicative of a deeper understanding of the pivotal role that behavioral and psychosocial factors play in the lives of diabetic patients is the growing emphasis on health-related quality of life overall, and specifically diabetes-related quality of life [71]. It is widely recognized that diabetes can profoundly impact a patient's overall quality of life, and their dedication to actively managing their diabetes is often influenced by their perception of their quality of life. As researchers delve into the intricate connections between quality of life and diabetes, its significance becomes increasingly apparent. Various psychosocial aspects, including personality traits, social support systems, coping strategies, and health-related beliefs, can significantly influence one's quality of life. These influences may be direct or indirect, either mitigating the adverse effects of diabetes or shaping how its demands are perceived. Intriguingly, these psychological factors often emerge as the most potent predictors of quality of life, often overshadowing the impacts of substantial disease-related factors like coexisting health conditions [91]. Recent studies have found correlations between enhanced Diabetes-Related Quality of Life (DRQoL) in adults and factors such as higher levels of social support, self-efficacy, engagement in physical activity, educational attainment, income, and the absence of health issues and co-occurring psychiatric disorders [53].

2.4.3. Depression with Type 2 Diabetes

Depression

Ensuring emotional well-being is crucial for individuals with diabetes, particularly when dealing with long-term conditions like diabetes. People managing diabetes may find themselves in need of emotional or psychological support due to the challenges posed by the disease or other factors. Accepting a diagnosis, coping with complications, managing medication side effects, and the ongoing demands of diabetes care can all have adverse effects on emotional health. This can sometimes lead to the development of phobias, eating disorders, anxiety, or feelings of sadness. Notably, individuals with diabetes are about twice as likely to experience depression compared to the general population. Managing diabetes, especially type 1 diabetes can prove to be both challenging and unappealing for patients and healthcare providers alike. Addressing

psychosocial barriers to diabetes self-management and overall quality of life becomes of paramount importance. Defining psychological barriers can be complex. As per the Webster's Encyclopedic Unabridged Dictionary of the English Language (1994), a barrier is an intangible factor that obstructs or separates. Researchers studying diabetes are particularly interested in understanding the social and psychological obstacles that hinder effective disease management and a satisfactory quality of life. In this context, depression emerges as a significant barrier due to its demonstrated negative impact on self-management efforts and overall well-being. Reports from www.diabetes.co.uk/diabetes-and-depression reveal that individuals diagnosed with chronic physical conditions like diabetes are three times more likely to be diagnosed with depression. Depression can detrimentally affect a person's health, their capacity to manage their illness, and their motivation to do so. The most prevalent psychiatric condition observed among the diabetic population is depression. While stress might trigger it, metabolic effects of diabetes on the brain could also contribute [92]. Some studies even suggest that diabetic women may experience depression at higher rates compared to their male counterparts [93]. Individuals with diabetes may perceive the management of their chronic condition and the potential for complications as a burdensome responsibility, particularly those who have recently been diagnosed. Many individuals with diabetes struggle to fulfill their responsibilities and often express feelings of being overwhelmed and disheartened. Failing to address diabetes with resilience and determination can lead to the prevalence of depression. Depression is linked to elevated blood sugar levels and an increased risk of diabetic complications. Conversely, effective treatment of depression is associated with better glycemic control. Additionally, depression has been associated with an increased risk of diabetic complications such as cardiovascular disease and retinopathy. Although the underlying mechanisms of these associations aren't fully understood, it's plausible that addressing depression can positively impact complications through improved glycemic control. In individuals with serious depression and diabetes, up to 50% may not respond fully to pharmacotherapy [94]. Managing the co-morbidities of diabetes and depression requires careful attention due to their negative effects on overall quality of life [90]. Depression can also influence a patient's ability to manage their diabetes effectively, including maintaining proper blood glucose levels. Research indicates that individuals with both

diabetes and depression often struggle with metabolic and glycemic control, exacerbating depressive symptoms [95]. Treatments such as psychotherapy, medication, or a combination thereof, can improve a patient's overall health and their ability to manage their diabetes [96].

Furthermore, it's been demonstrated that antidepressants can cause hypoglycemic side effects, posing significant challenges to diabetes management. Depressed individuals with diabetes are also more likely to deviate from their prescribed meal plans and medication schedules. This can lead to diminished quality of life and increased healthcare expenses. Treating depression has been shown to significantly improve glycemic control, mood, and overall quality of life [95]. In addition to depression, individuals diagnosed with chronic physical conditions like diabetes may also face other mental health issues, including bipolar disorder and anxiety disorders.

Taking both conditions into consideration

It can be challenging to successfully manage the two illnesses. Individuals are recommended to engage in diabetes behavior-focused programs as these have proven effective in aiding individuals with weight management, mitigating cardiovascular disease risks, enhancing metabolic regulation, and boosting overall fitness levels. Moreover, such programs can play a pivotal role in enhancing patients' emotional state and overall quality of life. Furthermore, the management of diabetes has seen advancements through psychotherapeutic interventions, with cognitive behavioral therapy, in particular, showing positive outcomes in addressing depression among individuals. The primary non-pharmacologic treatment for depression is psychotherapy. The focus of cognitive behavioral therapy is on the individual's thinking, as opposed to other factors, as the root of their moods and behaviors. To help their clients feel better, therapists concentrate on changing the way that they think [94].

2.5. Statistics

Statistics collected from across the globe demonstrate how common this illness is. Diabetes is on the rise throughout the world, and the epidemic might be linked to the sharp rises in overweight, obesity, and sedentary lifestyles. According to World Health Organization statistics, most diabetics in affluent nations are beyond retirement age,

while in poor nations, those most frequently affected are between the ages of 35 and 64 [96]. The prevalence of diabetes is increasing across the globe, currently affecting around 10 to 12 percent of populations in various countries. This surge is closely tied to the rising rates of obesity, unhealthy eating patterns, sedentary lifestyles, and economic challenges. It's essential to integrate diabetes prevention into broader population strategies aimed at curbing noncommunicable diseases (NCDs) collectively, as these same risk factors contribute to the development of other major NCDs like cancer, chronic respiratory ailments, and cardiovascular disorders, all of which have become urgent global health concerns [50]. The impact of this trend is particularly pronounced in developing nations. Noncommunicable diseases, encompassing conditions such as heart disease, stroke, cancer, chronic respiratory disorders, and diabetes, are now the primary causes of mortality worldwide. This concealed epidemic not only impedes the progress of numerous countries' economic development but also stands as a significant driver of poverty.

2.6. Traditional / Herbal Alternatives for Diabetic Therapy

Asian countries' traditional food habits and medicine are known for their distinctive traits. Several civilizations all over the world have a long history of using herbal medicine and plant derivatives, including fungus, as treatments for a wide range of illnesses and health issues. As complementary/alternate therapy to few medical ailments, such as cancer, HTN, hyperglycemia, and hyperlipidaemia, there has been "an increase in recent years in the use of herbal-based therapeutics that come from traditional medicines" [97,98]. Evaluating the MOAs and efficacy of these alternatives is an increasing field of interest in herbal medicine study. The National Health Service (NHS) has made a sizable number of supplementary medications available over the past 20 years, but the accessibility of those treatments lies on the regional interest & support [99]. Globally, about twelve hundred plants are utilised to treat T2DM . Just 350 of these, however, have been approved as anti-diabetic medications. Many eastern nations, like China, Egypt, and Iran, have successfully adopted such ancient medicinal therapies.

Since diabetes is one of the most incapacitating diseases and is becoming more common in the globe, additional research into herbal derivatives with anti-diabetic characteristics is becoming more and more appealing. Research on the efficiency of

herbal alternatives on DM may help us better understand the pathophysiology of diabetes as well as assist us find novel, effective anti-diabetic compounds.

2.6.1. Phytochemicals-Potential plant/algae/fungi derived compounds for anti-diabetic therapeutic uses

The complex and nonlinear physiological, pharmacological, and pathological processes that contribute to or promote diabetes, as well as the numerous signalling pathways that interact with one another, have been uncovered by advances in biomedical research. This justifies the use of many medications for various therapeutic targets or a single drug with potency for multiple targets in order to reverse all or the majority of the effects of diabetes. Concurrently, accessible conventional therapeutic measures for treatment are linked to serious side effects and resistance to a specific treatment during the course of prolonged medication administration. Also, the current traditional therapy strategy focuses on treating a specific disease or disorder's symptom or target rather than its underlying cause [100].

The hunt for a secure and efficient alternative source of treatment for this condition has been sparked by all these problems connected with conventional therapy. The best solution has been identified as traditional therapy with a comprehensive approach and thousands of years of experience. Lifestyle modification and medication using organic medicines, medicinal herbs, and minerals are all part of traditional therapy [101]. The main ingredient in the therapeutic formulations employed in the Indian, Chinese, Egyptian, Greek, and Roman systems of traditional medicine are medicinal plants. Several plant-derived chemicals and extracts have been employed in the treatment of diabetes, making plants an efficient and prospective source of hypoglycemic medications [102]. A modern scientific approach has been used to examine the therapeutic potential of medicinal plants in the form of extract or herbal formulation. Many active molecules have been isolated, identified, and evaluated for their function in the treatment of diabetes [103]. Many replies have been made about substances from all types of phytochemicals, including flavonoids. For their potential to treat diabetes, alkaloids, terpenes, polysaccharides, peptides, and proteins have been extracted and examined [104,105]. The next section explains the chemical make-up, origin, and precise molecular mechanism of action of various phytochemicals.

Alkaloids

Alkaloids are heterocyclic nitrogen compounds with an alkali nature and physiological activity that are derived from plants. They can be found in the plant in free form or can combine with an acid to generate salt. Typically, they are composed of one nitrogen atom as a primary, secondary, or tertiary amine. Alkaloids essentially come in three varieties. Real, proto, and pseudo alkaloids are all generated from amino acids, with the difference between them being the presence or lack of a heterocyclic ring. Pseudo alkaloids are derived from terpenoids and purines. Alkaloids have a wide range of pharmacological effects, including analgesics and narcotics (atropine, homatropine), stimulators of the neurological system (strychnine, brucince), mydriatics (atropine, homatropine), and myotics (physostigmine, pilocarpine), among others. *Leguminosae, Papaveraceae, Ranunculaceae, Rubiaceae, Solanaceae,* and *Berbridaceae* are only a few of the families that include biologically significant alkaloids. Alkaloids have a restricted distribution throughout the plant world. Alkaloid-rich fractions and pure alkaloids from several medicinal plants have been studied for their potential antidiabetic properties in in vivo and in vitro models because alkaloids are known for their diverse physiological activities.

Several studies have demonstrated the efficacy of alkaloid-rich fractions isolated from many medicinal plants, including Commelinacommumis, Percea americana, and Trigonella foenum graecum, in the management of diabetic diseases [106]. Purified alkaloids have also been investigated for their antidiabetic and hypolipidemic activities in addition to crude portions. Some common examples of these alkaloids that have demonstrated significant potential as hypoglycemic and hypolipidemic drugs include allicin, berberine, and boldine, which have been isolated from various plant species [107,108]. In particular, berberine-treated mice showed increased and additional antioxidant activities, AMPK activity, GLUT-4 translocation, glycolysis, and glucose metabolism [109]. Other alkaloids with the ability to affect several targets of lipid and carbohydrate metabolism, such as TNF-a, a-glucosidase, and G6pase, respectively, include caffeine (found in coffee), castanospermine derived from Castanospermusaustrale, and chlorogenic acids. Many alkaloids having insulinotropic properties, such as sparteine and P-carboline, have been identified from the active

portions of various plant extracts. Sparteine therapy causes a healthy guy to secrete more insulin and have lower blood sugar levels. While many b-carbolines, such as harmane, norharmane, and pinoline, cause isolated human islets of Langerhans to secrete insulin [110]. It has been demonstrated that an alkaloid termed urosolic acid, which was isolated from a wide range of plant species, inhibits the activity of PTP-113, a new target for the treatment of diabetes [111]. Several anti-diabetic alkaloids, such as thiospartine from Trigonella foeneum, tecomine from Tecoma stans, vindoline and vinolinine from catharanthus species, harmane and normane from Tribulisterrestris, and others, have also been isolated from various plants [112].

Carbohydrates

A class of chemical molecules known as carbohydrates typically contain the elements carbon, hydrogen, and oxygen in the ratio of 1:2:1. These are carbon, hydrogen, and oxygen-containing polyhydric alcohols that have aldehyde or ketone derivatives. The classification of carbohydrates into sugars and non-sugars is based on their complexity and how they behave during hydrolysis. The non-sugar carbohydrates known as polysaccharides have the general formula $C_nH2_nO_n$, where n is often a high number between 200 and 2500. It is possible for polysaccharides from various plants to change the glycemic index of the meal that is consumed, which greatly increases their potential to regulate post-prandial hyperglycemia. By limiting or delaying the absorption of carbs, dietary carbohydrates and fibres improve glycemic management. By lowering cholesterol absorption, they keep blood cholesterol levels lower. Also boost the excretion of bile acids. In high-fat diet and STZ-induced diabetic mice, polysaccharides from *Lyceum barbarum, Artemisia sphaerocephala,* Fucan from *Himanthalia elongate,* and a protein-bound polysaccharide from pumpkin lower blood glucose levels, improve insulin resistance, and enhance lipid profile. In addition to controlling post-meal glucose, Pleurotiscitrinopiliatus polysaccharides and Cordyceps sinensis polysaccharides CS-F30 and CS-F10 have a notable impact on the levels of the carbohydrate regulating enzymes G6Pase, hexokinase, and G6PDase [113].

An additional research investigation employing fractions abundant in insoluble fiber extracted from Cirrus sinensis' peel, including insoluble dietary fiber, alcohol-insoluble solids, and water-insoluble solids, unveiled their capacity to proficiently

regulate glucose, postpone the diffusion of glucose, and variably hinder α -amylase activity. These fractions could also be held accountable for diminishing the speed of glucose absorption and the levels of glucose concentration in the bloodstream after a meal [114].

Phenolic compounds

One or more aromatic benzene rings are found in phenolic compounds, which are joined by hydroxyl groups (C-OH). This vast class contains a wide range of structurally diverse plant chemicals. Eleven varieties of phenolic compounds exist, categorized according to the quantity of carbon atoms and their fundamental structure [115].

- Straight-chain phenols and benzoquinones
- Phenolic acids,
- Phenylacetic acid and acetophenone
- Polypropene, Coumarin, Isocaumarin, and Hydroxycinnamic Acid
- Naphtoquinone
- Xantone
- Anthrachinone with stilbene
- Isoflavinoids and flavonoids
- Neolignans and lignans
- Bisphenols
- Catecholmelanine and lignins (condensed tannins)

Flavonoids are the main class of substances in this group of different polyphenolic compounds. The flavone's C15 body is the source of flavonoids. Their core pyran ring's level of oxidation as well as their biological characteristics set them apart from other phenolic compounds. The three-ring systems hydroxylation and/or methylation patterns have a significant role in the flavonoid’s variability. They are extensively dispersed throughout plants and have unique pharmacological characteristics [116]. Flavonoids can be broadly categorised into various groups, including flavanols, flavones, catechins, flavanones, and anthocyanins, among others. Through changing the metabolism of glucose and oxidative substances, flavonoids exhibit hypoglycemic effects. In STZ-

induced diabetes mice, flavonoid-rich fractions from Loandra lacunose and Eugenia jambolana exhibit notable hypolipidemic and hypoglycemic effects [117]. An important flavonoid known for having a wide range of pharmacological actions is quercetin. Quercetin was given intraperitoneally to both healthy and diabetic rats induced with STZ. The diabetic animals' plasma glucose levels were significantly reduced, whereas those of the healthy rats showed no change. Furthermore, quercetin led to a notable reduction in plasma cholesterol and triglyceride levels, a significant increase in hepatic glucokinase activity, and the suppression of glucose levels in diabetic rats when subjected to glucose tolerance tests. These effects are likely due to quercetin promoting the insulin release from the diabetic rats' pancreatic islets [118,119]. When exposed to a glucose concentration of 20 mmol/I, quercetin, naringenin, and chrysin exhibited a substantial enhancement in insulin secretion from Langerhans pancreatic islets [120]. Within diabetic animals, citrus bioflavonoids like hesperidin and naringin demonstrate diverse impacts on hepatic glucose-regulating enzymes including fructose-1,6-bisphosphatase, PEPCK, hexokinase, as well as plasma insulin levels [121].

In addition to lowering glycated haemoglobin, narginin therapy in conjunction with vitamin-C significantly increased total haemoglobin and body weight. Furthermore, studies in “male and female obese Zucker rats, a model of type 2 diabetes”, have shown that the soy isoflavones genistein or daidzeiand also possess hypoglycemic activity. The “transcription of genes involved in lipid and glucose homeostasis”, as well as lipid metabolism inside the cell, is regulated by PPARs, promiscuous nuclear receptors. Because they function as a hypoglycemic PPARs agonist, isoflavones genistein and daidzein dramatically improved lipid and glucose metabolism. They also increased glucose tolerance and decreased glucagon levels. Proanthocyanidins, oligomeric-structured flavonoids, alleviate the harmful oxidative state of diabetes. Although grape seed procyanidins exhibit strong antihyperglycemic effects, possibly due to their insulin-mimetic properties. Moreover, it promotes glucose absorption in insulin-sensitive cells in a lab setting. In isolated rat soleus muscle, the flavonoid glycosides Kaempferol 3, neohesperidoside, and Kempferitrin (Kaempferol-3,7-O-(α)-l-di-rhamnoside) stimulate glucose uptake while inhibiting preadipocyte differentiation, indicating that the compound's ability to lower blood sugar is due to glucose transporter’s altered intrinsic activity [122]. Green tea flavonoid epigallocatechin gallate reduces hepatic glucose

synthesis, boosts insulin receptor tyrosine phosphorylation, and prevents insulin receptor substrate-I (IRS-1) Ser307 phosphorylation by activating AMPK. By the activation of 5'-AMP activated protein kinase (AMPK), it prevents hepatic gluconeogenesis and efficiently prevents fat buildup in the 3T3-L1 cell line. Furthermore, it enhances the functioning of PI3K, MAPK, and p70 (s6k), mimicking insulin effects, and reduces PEPCK gene expression through a mechanism dependent on PI3K. These findings strongly suggest that epigallocatechin gallate plays a pivotal role as a hypoglycemic agent [122]. Another flavonoid compound, (-)-epicatechin, has demonstrated insulin-like properties and the ability to safeguard albino experimental rats from the diabetogenic effects of alloxan [123]. Plants have water-soluble vacuolar pigments called anthocyanins. They come from a group of chemicals known as flavonoids. When compared to untreated animals, animals treated with anthocyanins from Cabernet sauvignen had lower concentrations of urine, blood glucose, and fatty acid metabolites [124]. Aside from suppressing the mRNA expression of enzymes involved in lipid metabolism (such as triglyceride synthesis and sterol regulatory element binding protein-1) within adipose tissue, the presence of cyanidin 3-O-β-D-glucoside in purple corn has been shown to counteract obesity and insulin resistance induced by a high-fat diet. Furthermore, it facilitated the buildup of triacylglycerols in white adipose tissue. In STZ diabetic rats, the blood glucose, serum lipid, super oxide dismutase (SOD), catalase, and fructosamine profiles are improved by pelargonidine, another anthocyanin (sugar-free component of anthocyanins). Prunus cerasus anthocyanins exhibit a variety of anti-diabetic benefits, including lowering fasting blood sugar and enhancing serum lipid and insulin profiles. Moreover, it treats fatty liver and raises levels of PPARa, which is a receptor that activates peroxisomes [125].

Stilbenes are organic substances with a functional group made of 1, 2-iphenylethylene. Stilbenoids are the hydroxylated derivatives of those compounds. Several novel compounds have recently been studied for their potential to treat diabetes. Resveratrol and pterostilbene are the two members of this family who stand out. According to reports, pterostilbene has an anti-diabetic impact via altering the activity of many enzymes that control carbohydrate metabolism. Pterostilbene has been demonstrated to considerably lower hyperglycemia and enhance liver glycogen content. Further chapters go into detail about its additional anti-diabetic characteristics and multi-target potency

for the treatment of diabetes. Pterosrilbene has demonstrated its efficacy for the management of diabetes after extensive research was done on various therapeutic targets of anti-diabetic treatment.

Terpenes/Terpenoids

With more than 20,000 known structures, terpenes are the largest class of naturally occurring plant metabolites. Terpenoids are compounds with oxygen-containing groups like hydroxyl or carbonyl groups that are biosynthesized from isoprene units (C5H8) to produce terpenes. Terpenes are divided into groups according to the number of isoprene units they contain, such as "monoterpenoids (n=2), sesquiterpenoids (n=3), diterpenoids (n=4)", etc. On the basis of the rings that are present in its structural makeup, this group is further classified into many sub-groups. Terpenoids' skeletal structures can differ from isoprene units added strictly by the deletion or shifting of a fragment, typically a methyl group.

Terpenoids are a collection of chemicals with a wide range of physiological effects that are widely distributed, chemically intriguing, and structurally diverse. Several terpenoids with anti-diabetic potentials have been identified from various plant groups. A diterpene stervial glycoside known as stevioside is obtained from the leaves of the Stevia rebudiana plant and has effects that are insulinotropic, glucagonostatic, and antihyperglycem. In both L6 myotubules and 3T3-Ll adipocytes, aviloside, a triterpenoid derived from bitter melon, exhibited anti-diabetic properties. It promotes fatty acid oxidation and GLUT-4 translocation. By activating the muscarinic (MC3) receptor in pancreatic cells, ginsenoside RH2 and intestinal metabolites of ginsenosides isolated from Panax ginseng root boost insulin secretion. They also have an antiobesity impact via the AMPK signalling pathway [126,127]. PTP- 1 U and gluconcogenesis are significantly impacted by the rosolic and urosolic acids found in banana leaves and Chinese medicinal plants, respectively. It has also been demonstrated that a steroid (tri terpenoid) derived from Azaractica indica may have hypoglycemic potential. Leptin and TNF-a were downregulated in white adipose tissue as a result of the therapy with the marine carotenoid fucoxanthone (tetraterpenoid). Moreover, it enhances insulin and blood sugar profiles. Lutein and zeaxanthia, two carotenoids found in plant chromoplasts, are effective free-radical scavengers that shield the retina from oxidative

damage. Furthermore, it has also been revealed that several terpenes, including b-sitosterol, lactucain A, and lactcaside, have strong antidiabetic activity in STZ-induced diabetic rats [128].

Protein and peptide

Another group of complex nitrogenous compounds are the molecules found in proteins and peptides. These are biopolymers made up of many different amino acids connected by peptide bonds. For research on their hypoglycemic effects, scientists have extracted and examined proteins and peptides from many medicinal plants and prokaryotes. These molecules, which resemble insulin, are known as glucokinin [129]. The existence or lack of glucoregulatory glucokinins in several plant species, however, is still up for debate.

Charantin is an insulin-like molecule that was discovered in a well-known bitter guard plant. There are numerous investigations on the separation and analysis of the activity of charantin from bitter guard [130]. In addition to bitter guard, hypoglycemic peptide molecules having the same sequence as insulin have been found in a variety of plant species, including bryophytes (mosses), psilophyta (whisk weed), Lycopodophytea (selaginela), gymnosperms (conifers, cycades), and angiosperms. Moreover, spinach, Lemna gibba G3, and other plants were used to isolate and describe insulin-like compounds. Moreover, Vigna unguiculata (cowpea) and Canovaliaensiformis (jack bean), two leguminous plants, have produced proteins with amino acid sequences that are similar to those of bovine insulin [131,132].

Conclusion

Medicinal plants, algae, and fungi have emerged as new resources for the treatment of various ailments because of their tremendous therapeutic potential. A number of phytochemicals have recently been identified, thoroughly investigated for their physiological effects, and have shown to be strong candidates for usage in commerce. These phytochemicals are safe compared to their synthetic competitors, but they also work well in situations where traditional therapeutic measures are unavailable or ineffective. The absence of standardisation and authenticity is the only disadvantage of using traditional medicines. Many solutions, along with modern analytical techniques

like GCMS, LCMS, HPLC, etc., have been developed to better overcome this disadvantage. Through the use of these techniques, a number of active phytochemicals from various medicinal plants and fungi with therapeutic potential have been isolated, and in some cases, herbal formulations made up of different plant extracts have been standardised based on qualitative and quantitative analysis of their active components. All these methods, in addition to aiding in the phytochemicals' isolation, also contributed to the proper validation of these substances, ensuring their continued safety for human use. The pharmaceutical industries now have a new focus on managing diabetes mellitus and other disorders as a result of recent scientific advancements and growing interest in the field of phytomedicines.

2.7. Fungi-In the World of Modern Medicine

The characteristics of fungi, such as their shape, ecology, metabolic processes, and phylogeny, are exceedingly diverse. Because they produce such a wide variety of bioactive substances, fungi are perfect for use in natural products. It is believed that medicinal mushrooms and fungi are capable of producing over 130 different therapeutic actions, one of which is anticancer activity [133,134]. Fungi have exhibited a diverse range of bioactive effects including immunomodulatory actions antioxidant properties and contributions to cardiovascular health, anti-parasitic, antiviral, antifungal, antibacterial activities, as well as radical scavenging, hepatoprotective, detoxification, and anti-diabetic effects [135]. Various fungal organisms produce an array of bioactive compounds such as polysaccharides, alkaloids, proteins, lipids, minerals, carotenoids, glycosides, terpenoids, folates, tocopherols, flavonoids, phenolics, volatile oils, ascorbic acid, lectins, enzymes, and organic acids.

It is critical to maintain a diet that is both nutritious and well-balanced for the purpose of disease prevention, particularly with regard to oxidative stress. Since ancient times, oriental medicine has made use of mushrooms as a preventative measure as well as for therapeutic purposesfor various diseases. Extracts of mushrooms are increasingly being made available as dietary supplements particularly for the purpose of boosting immune function and engaging in anticancer activities. The mushrooms, also known as macrofungi, are creatures that are aerobic, eukaryotic, and non-photosynthetic. They develop fruiting bodies that are easily recognisable. Based on their taxonomic

classification, the mushrooms can be broken down into two distinct categories: the Basidiomycetes and the Ascomycetes. It is presently believed that around 14,000 species of mushrooms, which have been classified into 30 different genera, could provide humans with possible therapeutic or preventative benefits. Of these 14,000 mushroom species, only 2,000 are edible. 270 different kinds of mushrooms that can be eaten [136]

The practice of mycophagy, which refers to the consumption of mushrooms, has historical roots. Within the vicinity of ancient archaeological sites in Chile dating back 13,000 years, researchers have uncovered edible mushroom varieties [137]. These edible fungi are sought after not only for their gastronomic and nutritional value but also for their ability to enhance the overall enjoyment of food. Various species of fungi produce edible mushrooms, which can either be gathered from the wild or cultivated intentionally. Edible mushrooms encompass the fleshy reproductive structures of specific macrofungi species, where these fungi possess fruiting structures large enough to be visible to the naked eye. These mushrooms can either grow above ground (epigeous) or below ground (hypogeous), and in either case, they can be manually extracted and utilized. While individual collectors may gather wild mushrooms on a small scale, markets often offer varieties that are easily cultivable or commonly found. Conversely, more elusive types are typically stocked by markets. The consumption of wild mushrooms is a widespread practice globally. Noteworthy examples of edible mushrooms include species like *Agaricus arvensis*, *Amanita caesarea*, *Handkeautriformis*, *Cortinariusvariicolor*, *Agaricus silvaticus*, *Ustilago maydis*, *Marasmiusoreades*, *Leccinumversipelle*, and *Suillus luteus*, among others. In China, both the medical and culinary applications of mushrooms carry significant importance, with the country holding them in high esteem. Mushrooms have found purpose in preventing, treating, or healing various ailments, contributing to a balanced diet. Medicinal mushrooms, in collaboration with fungi, exhibit around 130 therapeutic actions [138,139]. With a protein content ranging from 20 to 30 percent of dry matter, mushrooms provide all essential amino acids. Devoid of cholesterol, they exhibit low total fat content and a considerable portion of unsaturated fatty acids. Beyond serving as delightful culinary components and flavor enhancers, mushrooms have also been harnessed for medicinal formulations. In-depth research has unveiled the medicinal advantages of mushroom consumption [140]. The utilization of medicinal mushrooms

can yield diverse positive health outcomes. Contemporary scientific investigations continue to substantiate these longstanding health-promoting claims across civilizations worldwide. Numerous mushroom varieties have been included in the repository of traditional medicines [141]. Among them, highly beneficial species encompass *Agaricus brasiliensis, Ganoderma lucidum, Trametes versicolor, Lentinus edodes, Flammulinavelutipes, Agaricus bisporus, Tricholoma matsutake, Auricularia auricula, Pleurotusostreatus, Grifolafrondosa, Cordyceps sinensis, Coprinus comatus* [142]. Additional medicinal compounds derived from mushrooms include secondary metabolites, particularly low molecular weight substances such as lactones, alkaloids, terpenoids, a variety of antibiotics, and metal chelating agents [143]. These beneficial mushrooms have gained popularity in Western countries in recent years for valid reasons: their accessibility has increased, offering a multitude of advantages well-suited for modern fast-paced lifestyles. Mushrooms contain adaptogens, compounds that aid the body in adapting to internal and external stimuli, restoring balance and influencing various biological processes. Mushroom cultivation has a long history, yielding over twenty cultivated varieties. More than sixty countries participate in mushroom farming. However, due to the limited consumption of fungal species by humans, only a fraction of fungi are cultivated and commercially available. Despite this, cultivating certain species can be complex, while suitable cultivation methods are yet to be discovered for others.

2.7.1. Use of fungi/mushrooms as medicine traditionally

Mushrooms were a common ingredient in the cuisine of the ancient Romans and Greeks, particularly among those of higher social standing. Even the Emperors of Rome had food tasters work for them to make sure that the mushrooms they ate were safe to consume. Mushroom farming has a lengthy history, and there are approximately twenty species that are cultivated for commercial purposes at this time. Almost sixty countries around the world are responsible for their cultivation. Only a small percentage of the wide variety of fungi that are of value to humans are currently cultivated and sold in the marketplace. In days gone by, Europeans were not the only people who consumed mushrooms as a food source. Since the Neolithic period, people have looked to mushrooms for both food and medical treatment. Piptoporusbetulinus, known for its

antibacterial properties and natural parasitic-killing abilities, was uncovered within the medicinal inventory of the world's oldest human mummy, dated back 4,000 years [143]. In the realm of Egyptian hieroglyphics, mushrooms were depicted as the ***immortality plant***, often recognized as the ***sons of the gods***. Legend suggests that this plant was brought to Earth by lightning bolts and reserved exclusively for consumption by the privileged elite and pharaohs. The Aztecs, during their religious rituals, would partake in sacred mushrooms, occasionally referred to as ***the supper of the gods***.

In ancient China, some types of mushrooms, most notably the Reishi fungi, were highly valued for their properties as restorative herbs, and access to these mushrooms was strictly controlled. Knowledge about the medicinal benefits of fungi, which Buddhist monks and Taoist priests used in rituals, was spread from one monastery to another by monks who travelled from one Buddhist monastery to another [144]. It is supposed that the Buddha passed away as a result of ingesting a poisonous fungus. This psychedelic is now offered in a form more suitable for modern times. It is believed that the Vikings took LSD (lysergic acid diethylamide), a drug that contains psychedelic mushrooms, before to the conflict. This would have put them in the ferocious fighting mindset for which they are known. There is evidence to suggest that people in Europe employed enchanted mushrooms in religious ceremonies as far back as 6,000 years ago. This is exemplified by an ancient cave artwork in Spain, which could potentially depict hallucinogenic fungi from that era. Notable ancient Greek and Roman authors such as Pliny, Seneca, and Dioscorides, the author of De Materia Medica, engaged in debates about the medicinal use of mushrooms [145]. During the Eleusis festival, a celebration of birth, rejuvenation, and the arrival of spring, figures like Socrates and Plato, prominent in ancient Greek society, consumed the hallucinogenic elixir Kykeon. This elixir, a blend of ergot-infused barley and mint, was also referred to as ***the drink of the gods*** [146].

2.7.2. Some mushrooms with documented therapeutic use

A robust immune system is our strongest line of defence against illness. It is also the essential component of a preventative healthcare strategy that is aimed at achieving general and long-term wellness. These mushrooms are often recognised as being among the most potent superfoods for boosting the immune system [147]. Substances like β-

glucans, micronutrients, glycoproteins serving as antioxidants, triterpenoids, flavonoids, and ergosterols contribute to the cultivation of innate defenses against toxins and illnesses. They were utilised extensively as nutritional supplements, and this included mushrooms in a variety of different developmental stages. The functional characteristics of mushrooms are governed by the bioactive substances that they contain. It is possible to extract and purify the bioactive chemicals that are present in mushrooms for use as nutraceuticals or in the preparation of functional foods in order to battle chronic diseases that are associated to oxidative stress [148]. There are many different kinds of edible mushrooms, some of which have medicinal properties. Because of its flavour resembling almonds, the Agaricus subrufescens fungus is commonly referred to as the "***almond mushroom***." This has been mislabeled as Agaricus blazei and is grown commercially in the United States [149]. It finds application as a therapeutic dietary option in addressing cancer, diabetes, hyperlipidemia, hepatitis, arteriosclerosis, and prevention, while also serving as a source for multiple diverse bioactive compounds with potential for various treatments. Its capabilities encompass tumor growth inhibition, antibacterial and antiviral properties, along with immunostimulatory and antiallergic effects [150]. These are just some of its medicinal properties. The majority of the bioactive substances that may be recovered from this fungus are polysaccharides. These polysaccharides include riboglucans, α-glucans, and glucomannans. The lipid component ergosterol was found to have anticancer properties once their presence was uncovered [147,151].

"***Cinder Conk***" and the "***Chaga Fungus***" are both names for the same type of fungus that grows on birch trees in freezing climates all over the world. However, Russian herbalists are the ones who use these fungi most frequently as a cure to restore energy and maintain health [152]. In addition to polyphenols, sterols, and polysaccharides, the mushroom contains a number of different antioxidants. Some of these antioxidants include “superoxide dismutase, melanin, and triterpenes such as lupeol, inotodiol, and botulin” [143]. Birch bark contains a significant amount of betulinic acid, making this acid one of the key components that make up birch bark. Inonotus obliquus has a crusty layer that resembles charcoal and is melanin’s natural source [153].

The natural recurrent fungus known as maitake, sometimes known as "***signorina***" or "***hen of the woods***," grows in the same area year after year, typically in the vicinity of oak trees. Recently, it's being grown for the purpose of being marketed either fresh or dried, or as a supplement that has been manufactured. One of its key polysaccharides is called DFraction, and it is a special β-glucan that is attached to proteins [154]. This vitamin is credited as being one of the major components that contribute to the powerful immune-modulating capabilities of maitake [155]. Maitake is frequently prepared as a fresh form of mushroom and is considered to be a "gourmet mushroom" by chefs all over the world. It contributes a rich taste to a wide variety of dishes, including soups, sauces, and entrees [156]. Cordyceps sinensis is helpful for maintaining healthy adrenal function. In some circles, it is also referred as food for the kidneys and lungs. It stimulates sexual desire in both men and women, making it an effective aphrodisiac for both sexes [157]. In addition to -glucans and CO-1, cordyceps contain a molecule known as cordycepin, which is unique to this species of fungus. Cordyceps also contain this chemical. It is renowned as a plant that builds "Chi," which is a term that refers to the ability to "activate life forces," and the medical effects of this plant are frequently compared to those of ginseng. It is usual practise to make use of C. sinensis as an athletic support tonic for the purposes of "regaining physical stamina and improving performance" [158]. The Trametes versicolor (Coriolus) mushroom is a species that can be discovered in a variety of temperate regions all over the world. It is easy to distinguish because it "develops large shelf-like clusters on decaying trees and logs". This makes it easy to find. It is referred to as a "turkey tail" due to its shape, lines, and colours, all of which are reminiscent of the feathers seen on the tail of a wild turkey. One of the therapeutic mushrooms with the greatest research has been done on this one. The primary β-glucan-rich elements of Coriolus, sometimes known as turkey tail, are two polysaccharides known as kresin (PSK) and polysaccharide peptide (PSP). It is also sometimes referred to as "the cloud mushroom." The use of a prepared liquid or powdered extract of this fungus is the method that is considered to be the safest and most effective for therapeutic purposes [159].

Another name for the Brazilian mushroom species *Agaricus blazei* is "cogumelo do sol," which literally translates to "Sun mushroom." It is also referred to as "*Agaricus brasiliensis*". It's a distinct variety of mushroom "that may be consumed fresh and has

a flavour" that is comparable to that of sweet almonds [160]. It needs to be "treated with boiling water or an alcoholic solution" in order to bring out the beneficial medicinal components it contains [161].

Agaricus contains a notable proportion of β-glucans, recognized for their involvement in modulating immune function. A relationship exists between Agaricus and the white button mushroom *Agaricus bisporus*, alternatively referred to as cremini or portobella [162]. Acc. to the findings of the research, the white mushrooms that are now on the market do not have very many qualities that have a therapeutic effect. The market for edible mushrooms is dominated by the species *Agaricus bisporus*, which comes in a wide variety of subspecies in both Europe and North America. Meadows in Europe and North America are ideal environments for the growth of the Basidiomycetes fungus. This mushroom begins its life as a little, white smooth mushroom and eventually transforms into a massive light brown mushroom as it ages [163]. Documentation of Ganoderma lingzhi, also known as Reishi, may be found in some of the earliest pharmacopeia documents from Asian culture, which date back thousands of years. It is considered to be an effective Shen tonic due to the sedative impact that it has on the nerves as well as the kidney system. In Traditional Chinese Medicine, it is supposed to open the spirit, &Taoists have employed it as a means to attain Enlightenment or spiritual immortality [159]. *Ganoderma lucidum* is another type of mushroom that promotes neuron renewal and relieves insomnia. Because of these properties, it is often regarded as an effective dietary supplement for improving sleep quality. Mycelia from reishi mushrooms have been found to contain peptides, nucleotides, sterols, polysaccharides, triterpenoids, steroids& trace elements [164]. Shiitake mushrooms (*Lentinula edodes*) are highly popular edible gourmet mushrooms acclaimed for their robust flavor and substantial texture. Originating from the Lentinula genus, these mushrooms possess not only culinary appeal but also significant medicinal attributes, often overlooked [26]. The composition of Shiitake mushrooms includes β-glucans and other polysaccharides like lentinan, emitanin, and KS-2, which have garnered extensive research attention for their potential pharmacological properties [165]. These mushrooms are commonly employed both as a nutritional supplement and in hot water extracts, typically derived from the entire mushroom, mycelium biomass, or isolated bioactive components such as lentinan extracts, enhancing their therapeutic potency across various

applications [141]. The terminology lion is frequently used to symbolize both the nervous system and the brain. Due to its dual role as a nootropic and neurotrophic superfood, Shiitake mushrooms have gained prominence as a highly utilized medicinal option. Since 1990, research has demonstrated their effectiveness in stimulating the growth of nerve growth factors and the secretion of proteins vital for neuron preservation, regeneration, and survival. These properties have been substantiated [166,167]. Notably, Shiitake mushrooms contain unique compounds called hericenones and erinacines, making them suitable as nootropic dietary supplements to enhance cognitive functions. These substances hold promise for conditions such as dementia, Parkinson's disease, and Alzheimer's disease as indicated by several studies [167].

2.7.3. Therapeutic compounds from medicinal mushrooms

There are many species of mushrooms that fall under various taxonomies. Although some of these mushrooms are grown for a range of cuisines as well, they are increasingly used in nutraceuticals due to their immunomodulatory properties. Medicinal mushrooms contain essential immunomodulatory compounds such as terpenes, flavonoids, terpenoids, lectins, and polysaccharides, particularly D-glucans, alongside fungal immunomodulatory proteins. These components play a significant role in influencing the immune system [168].

Polysaccharides

In recent years, considerable attention has been directed towards polysaccharides for their potential to modulate the immune system. These polysaccharides encompass polysaccharopeptides and polysaccharide proteins, which may possess side chains or not [169]. Among the most notable polysaccharides derived from Schizophyllum commune are lentinan, sourced from shiitake (Lentinus edodes), and schizophyllan, both recognized for their immunomodulatory and anti-cancer effects [159]. Both lentinan and schizophyllan feature 1,3-D-glucans with 1,6 branches. Schizophyllan has demonstrated anticancer properties against head and neck cancer, while lentinan has shown immunomodulation characteristics against stomach cancer.

These medications received approval for chemotherapeutic use in Japan in 1986 [170] [171].

Other polysaccharide compounds with diverse branching patterns and similar core structures also exhibit immunomodulatory effects. Heteroglucans, which are complex polysaccharides, demonstrate immunomodulatory effects as well [172]. Immunomodulatory attributes have been attributed to Agaricus brazei and Macrocybe gigantea as well. In mice with tumors, a polysaccharide-protein complex (PSPC) derived from *T. giganteum* was observed to enhance and restore macrophage phagocytic activity [171]. Furthermore, AbM from A. brazei contains polysaccharide-protein chains with diverse chemical bonding, including a 6-glucan, 1,6- and 1,4-glucans, glucomannan, and 1,3-glucans. AbM has been discovered to possess immunomodulatory and anticancer effects [173].

Mushroom proteins and complex protein-protein interactions

Mushroom-derived therapeutic proteins and their conjugate-protein complexes are recognized reservoirs of immunomodulatory agents. Similar to polysaccharide-based compounds, these protein-based molecules with immunomodulatory properties within medicinal mushrooms can be classified. These compounds fall into two categories: lectins and fungal immunomodulatory proteins (FIPs) Unlike lectins, which involve specific carbohydrates bound to polypeptides, FIPs lack such conjugates [174].

Terpenes, Terpenoids

In mushrooms, terpenoids and terpenes are prevalent. They are a diverse group of organic molecules having the fundamental building block isoprene, which has five carbon atom units in its centre. The most well-known compounds in this class are likely the triterpenoids from *G. lingzhi* and *G. lucidum* [175]. These triterpenoids can help stop drug-induced inflammation and nephrotoxicity. There are numerous terpene derivatives found in *G. lingzhi* and *G. lucidum*, such as lucidone, ganodermanondiol and ganodermanontriol. These medicines are all anti-infective immunomodulatory and anti-cancer [171].

2.7.4. The Neuroprotective and Cardioprotective Effects of Mushroom derived compounds

Medicinal mushrooms, belonging to an elevated fungal class, offer an array of nutraceutical attributes. These properties encompass a low-fat content, a notable Fiber content, and a trans isomer of unsaturated fatty acids. Additionally, these mushrooms house biologically active compounds such as polysaccharides, alkaloids, steroids, polyphenols, polysaccharide-glucans, and terpenoids [176]. Extracts and fresh edible mushrooms exhibit a diverse range of therapeutic applications for human health benefits, including anti-diabetic qualities, cardioprotective properties, and an anti-obesity effect. These benefits have been validated through in vitro tests, animal models, and human study trials [177]. Certain mushrooms, particularly *L. edodes, G. frondosa, and P. ostreatus,* are particularly well-suited for low-fat calorie diets, aiding in the prevention of cardiovascular disease and promotion of a healthy lifestyle. This is attributed to the fiber, microelement, and protein content in mushrooms. A multitude of bioactive compounds extracted from *Boletus aestivalis, G. frondosa, L. edodes, G. lucidum, H. marmoreus,* and *Pleurotus* species contribute to maintaining healthy levels of low total cholesterol, high-density lipoproteins, and homocysteine, effectively averting arterial oxidative stress, hypertension, and cardiovascular disease [24].

Cardiovascular diseases (CVDs), including heart attacks and strokes, can inflict damage upon the circulatory system. Heart disease stands as the leading global cause of death. Elevated blood glucose levels, cholesterol levels, and arterial pressure are the primary risk factors for cardiovascular disease (CVD) [176]. Many medicinal mushrooms possess hypocholesterolemic and hypoglycemic properties, rendering them as natural, nutritious aids in disease prevention and cardiovascular health enhancement. Notably, medicinal mushrooms like Ganoderma lucidum and Cordyceps sinensis contain adenosine, an amino acid recognized for its cardioprotective effects. Adenosine interacts with a range of effector systems and specific receptors linked to G-proteins. Both endogenous and exogenous adenosines have demonstrated roles in safeguarding against myocardial ischemia, shielding the heart from the detrimental impacts of insufficient blood flow and oxygen supply [178]. Mushrooms from diverse taxonomic and ecological classifications are active sources of proteolytic enzymes, including fibrinolytic,

thrombolytic, and caseinolytic enzymes [179]. *F. velutipes* notably exhibits the discovery of proteases with fibrinolytic activity. Subsequent exploration examined the thrombolytic and fibrinolytic potential of select *basidiomycetous* mushrooms. Various studies have identified fibrinolytic proteases in the mycelia and fruiting bodies of medicinal mushrooms like *Armillariellamellea, Auricularia polytricha,* and *coprini* species. High blood pressure or hypertension poses significant strain and risks to heart function. Mushrooms, with their high potassium and low sodium content, serve as excellent dietary supplements for hypertension reduction. The potassium concentration ranges from 182 to 395 mg per 100 grams of mushroom. Studies on mushrooms like *Lentinula edodes, Pleurotusnarbonensis, Ganoderma lucidum, and Grifolafrondosa* have demonstrated their potential to lower blood pressure [159]. Consumption of antioxidants is a potent strategy for preventing various cardiovascular issues. Polysaccharides and phenolic substances found in mushrooms possess potent antioxidative properties. They elevate the activity of oxidative enzymes like catalase, Glutathione peroxidase, and superoxide dismutase while maintaining stable levels of malondialdehyde and glutathione. This effectively curtails the radicals' capacity to inflict harm. Examples of mushrooms with antioxidant properties include *Ganoderma lucidum, Termitomycesheimii, Coriolus versicolor, G. Lentinula edodes tsugae*, and *Termitomycesmummiformis* [180].

Atherosclerosis stands as a significant cardiovascular disease risk factor, characterized by the release of lipids and fibrous components in major blood arteries. An array of edible mushrooms, alongside their mechanisms of antiatherosclerotic activity, have been intensively researched [159]. *Grifolafrondosa, Pleurotusforida,* and *Hypsizygusmarmoreus* are promising contenders for potential anti-inflammatory agents.

2.7.5. Recent studies and clinical uses

Mushrooms, the fruiting bodies of macrofungi, encompass a range of bioactive constituents including unsaturated fatty acids, phenolic compounds, triterpenes, glycoproteins, and peptides. The prevalent -glucan polysaccharide in fungal cell walls is the -1,3-glucan polysaccharide, constituting 65–95% of the total -glucan content. B-glucans exhibit diverse structural attributes that potentially influence therapeutic

immune activation and protection against fungal infections. They also interact with CR3, a critical -glucan receptor on neutrophils and natural killer cells (NK), regulating phagocytosis and fungal killing in a complement-dependent manner, as noted by Khara Lucius et al., [181]. Furthermore, α-glucans can be metabolized by gut bacteria, potentially benefiting the host's microbiome [179,181]. Numerous medicinal mushrooms demonstrate robust immune-stimulating properties, affecting various immunological targets [182]. These immune-enhancing characteristics suggest significant therapeutic potential for individuals dealing with acute or persistent viral infections [183]. In adults with HIV who were not undergoing antiretroviral therapy, a proprietary supplement containing a blend of healing mushrooms, including *T. Versicolor, G. lucidum, Cordyceps militaris, Agaricus blazei, Cordyceps Sinensis, Grifolafrondosa, and Lentinula edodes*, led to a significant increase in CD4+ T lymphocyte counts [183]. The potential benefits of healing mushrooms for cancer patients have also garnered considerable attention. Varied oncology-related advantages might stem from altered cellular and humoral immunity, alongside ongoing anticancer effects [181]. Clinical trials involving individuals with different types of cancers, such as breast, prostate, colorectal, hepatic, and lung cancers, have explored the effects of mushrooms [181]. The method of extracting medicinal mushrooms, influencing the solubility of inhibitory mediators and stimulatory compounds, offers an avenue to evaluate the immunomodulatory effectiveness on immune cells [184].

2.7.6. Clinical Trials with mushrooms

Given its potential therapeutic significance, several fungal metabolites or extracts have been subjected to in-vivo evaluation in clinical trials involving human patients. However, only a limited number of these have demonstrated efficacy in in-vitro tests or preclinical investigations.

2.7.6.1. Clinical Trials on Medicinal Mushrooms for Cancer Treatment

Agaricus blazei Kyowa* Extract Trial [185]*:

In this randomized clinical trial (RCT), an extract of Agaricus blazei Kyowa was administered to 100 patients with various gynecological cancers (ovarian, cervical, and endometrial) undergoing chemotherapy. The treated group showed a significant increase

in natural killer (NK) cell activity, although no change was observed in monocyte activity. The study also found lymphokine-activated killer cells and reported benefits such as reduced chemotherapy-related side effects (e.g., emotional instability, hunger, baldness, weakness). Additionally, mood-related indicators such as anxiety, mental stability, and depression improved.

***A. blazei Murrill* Phase I [186]:**

In this phase I clinical trial, 78 cancer patients were treated with *A. blazei Murrill* extract known as Senseiro over six months. The study aimed to evaluate safety. Most patients experienced no adverse effects, and only nine cases of minor adverse effects, including digestive issues like nausea and diarrhea, were reported. One patient with liver dysfunction developed an allergic reaction, although these effects were not dose-dependent. The trial did not reveal any significant immunological response.

C. versicolor Mycelium Phase I Trial [187]:

A phase I two-center dose-escalation clinical study was conducted using powdered C. versicolor mycelium. The study assessed the tolerable dosage over six weeks for women who had breast cancer and had completed standard chemotherapy and radiotherapy. Three groups received varying dosages of medicinal mushroom powder. The study found that the dosages were well-tolerated, and there were only minor adverse events reported. Positive effects on the immune system, such as increased lymphocyte numbers, NK cell functional activity, and certain CD19+ B and CD8+ T cells, were observed at specific dosage levels. However, the study did not explore higher doses and had limitations in terms of sampling extent. These studies collectively contribute to the understanding of the potential benefits and safety of using medicinal mushrooms for cancer patients, particularly in terms of immune system activation and managing side effects of conventional treatments.

2.7.6.2. Medicinal Mushrooms for Neurological Conditions

***Ganoderma lucidum (Reishi)* for Neurasthenia [188]:**

A randomized, double-blind, placebo-controlled clinical trial was conducted by Tang et al. with Ganopoly, a preparation derived from Ganoderma lucidum (Reishi).

The study focused on neurasthenic patients, a condition characterized by fatigue and emotional disturbances. A total of 132 patients were given either a placebo or 1800 milligrams of Ganopoly three times a day for eight weeks. Patients who received G. lucidum showed improved well-being, as measured by a Visual Analogue Scale (VAS), and a consistent decrease in the severity of their symptoms as assessed using the Clinical Global Impression (CGI) scale.

***Hericiumerinaceus (Lion's Mane)* for Neurological Conditions [189]:**

Nagano et al. conducted a randomized, double-blind, placebo-controlled study to evaluate the effects of *Hericium erinaceus* (Lion's Mane) on various conditions related to the nervous system and brain function. The study involved assessments using the Kupperman Menopausal Index (KMI), the Center for Epidemiologic Studies Depression Scale (CES-D), the Pittsburgh Sleep Quality Index (PSQI), and the Indefinite Complaints Index (ICI). Thirty women participated, and they were given either placebo cookies or Hericiumerinaceus biscuits for a month. The treatment group showed significantly lower scores on the ICI and CES-D scales after consuming *Hericium erinaceus*. Certain symptoms related to emotional well-being, such as anxiety, palpitation, concentration, irritation, and anxiousness, were improved in the treatment group. The authors concluded that the mushroom's mechanism of action is distinct from nerve growth factor (NGF) enhancement, suggesting potential benefits for anxiety, panic attacks, and sadness.

These clinical trials provide insights into the potential neurological benefits of mushrooms such as Ganoderma lucidum and Hericiumerinaceus. The positive outcomes observed in these trials indicate that these mushrooms could have a role in improving mood-related indicators and addressing neurological conditions, although further research is needed to fully understand their mechanisms of action and potential therapeutic applications.

2.7.6.3. Toxicological Characteristics of Medicinal Mushrooms

Toxicological Study of *Ganoderma Lingzhi:*

Researchers have conducted extensive toxicological studies on Ganoderma Lingzhi (Reishi) mushrooms. In these studies, the polysaccharide component of the mushrooms was investigated. The results indicated that in 90-day studies, doses of 1200 mg/kg and 2000 mg/kg of the polysaccharide component did not result in any observed adverse levels (NOALs). In other words, no adverse effects were observed at these doses. The literature research also supported the conclusion that the substance from Ganoderma Lingzhi was non-toxic.

Toxicity Studies on *A. subrufescens and A. blazei:*

Regarding other species of mushrooms such as Agaricus subrufescens and Agaricus blazei, toxicity studies were conducted at a dose of 2000 mg/kg. These studies did not identify any toxicity in either of these mushroom species at this dose. However, it's mentioned that some reduction in locomotor activity was observed in rats given a dose of 2000 mg/kg of A. subrufescens.

These findings suggest that the studied mushrooms, including Ganoderma Lingzhi, Agaricus subrufescens, and Agaricus blazei, show promising results in terms of safety based on the toxicological assessments. However, it's important to note that these conclusions are based on specific doses and conditions of the studies, and further research is required to comprehensively understand the safety profiles of these mushrooms across various doses and populations.

2.7.6.4. Medicinal Mushrooms for Diabetes, Hyperglycemia, and cardiovascular diseases

***A. blazei Murrill* for Diabetes Management:**

In a randomized, double-blinded, placebo-controlled study, the effectiveness of *Agaricus blazei Murrill* (ABM) in managing diabetes was evaluated. The study included 72 individuals diagnosed with type 2 diabetes who were already taking medications like gliclazide and metformin. The participants were given Agaricus blazei mushroom extract at a dosage of 1500 mg/day for a period of 12 weeks. The homeostasis model assessment for insulin resistance (HOMA-IR) was used to analyze the results. The study found that therapy with ABM extract led to a significant reduction in insulin resistance.

This reduction in insulin resistance was likely attributed to an increase in plasma adiponectin concentration, which was higher in the ABM group compared to the placebo group.

***Pleurotusostreatus* for Metabolic Improvement:**

Another study investigated the efficacy of Pleurotusostreatus (a type of mushroom) in improving metabolic parameters in diabetic individuals. The study involved 89 participants who consumed 50 grams of cooked and boiled mushrooms three times a day for 24 days. This was done in a pattern of 7 days of consuming mushrooms followed by 7 days of not consuming them, and measurements were taken at the beginning and end of each week. The results showed significant improvements in systolic and diastolic blood pressure, total cholesterol (TC), triglycerides (TGs), and plasma glucose levels. Although weight and high-density lipoprotein (HDL) levels didn't show significant changes, systolic blood pressure, HDL, and total body weight remained unchanged when mushrooms were not consumed. Restarting the consumption of mushrooms led to the reappearance of the positive changes. This suggests that regular consumption of Pleurotusostreatus can benefit individuals with diabetes by improving their metabolic profiles. These findings indicate the potential benefits of certain medicinal mushrooms, such as Agaricus blazei Murrill and Pleurotusostreatus, in managing diabetes-related factors and improving metabolic parameters. Further research is needed to understand the underlying mechanisms and to establish the efficacy of these mushrooms for larger populations.

2.7.7. Research Gaps and Unresolved Issues

Development of Mushroom Products: The development of mushroom-based products, including dietary supplements (DS) and functional foods, involves considerations of safety, standardization, efficacy, and modes of action. Regulations, safety levels, and standardization methods need to be established for the creation of these products.

Standardization and Quality Control: Standardization of therapeutic mushrooms and fungi is a global challenge. There are no universally accepted standards and techniques for research, development, and testing of fungal-based products. The lack of consistent

quality can result in significant variation in composition and efficacy of medicinal fungus products.

Understanding Bioactive Components: The pharmacological effects of medicinal mushrooms are often attributed to complex mixtures of bioactive compounds. It's not clear whether these effects arise from single agents or the additive/synergistic effects of multiple components. The roles of low-molecular-weight compounds in fungal extracts and their contribution to pharmacological effects remain unclear.

Cultured Mycelium Research: While the teleomorph stage (fruiting bodies) is essential for species identification, fungal cultures in pure mycelial form are often overlooked. More research on vegetative mycelia of fungi grown in pure culture is needed for maintaining consistency and reliability in scientific studies.

Molecular Weight of Polysaccharides: Many therapeutic mushrooms contain polysaccharides with high molecular weights. These compounds, such as lentinan and schizophyllan, are often extracted from fruit bodies or cultured mycelium and are not easily synthesized, leading to high market prices.

Structural-Activity Relationship of Carbohydrate Polymers: Research into the structural-activity relationship of mushroom carbohydrate polymers (such as β-glucans) is necessary to understand their mechanisms of action. Factors like molecular weight, side chain length, linkage ratios, and ionization affect the solubility and pharmacological activity of these compounds.

Differentiating Glucans: Different types of glucans exist, including those from plants, yeast, and medicinal mushrooms. Plant glucans have different structural characteristics compared to those from medicinal mushrooms, and their biological activities require more research.

Oral Absorption of Glucans: The mechanism of orally administered glucan intestinal absorption is not fully understood. There are different theories, including absorption through various pathways like gap junctions, M cells, and Toll-like receptor interactions.

Bioactivity of Plant Glucans: The biological activity of glucans from plants is an area that needs more exploration, as most research has focused on yeast and medicinal mushroom glucans.

Solubility and Biological Activity: The solubility of glucans, including their water solubility and insolubility, affects their immunostimulatory properties. Different glucans have different solubility characteristics.

Different Types of Glucans: Distinct types of glucans, such as those from plants, yeast, and medicinal mushrooms, have differences in structure, solubility, and biological activity. Understanding these differences is essential for accurate research and application. Overall, these gaps in research and unresolved issues highlight the complexity of studying medicinal mushrooms and their bioactive compounds. Further research is needed to address these challenges and to better understand the potential benefits and mechanisms of action of these natural products.

2.8. Studies related to Bio prospectus of Mushrooms, *M. esculenta* and their role in therapeutic & preventive aspect of diabetes

Sunil et al. (2022) in their study titled "Mycochemical profile and health- promoting effects of morel mushroom *M. esculenta* (L.) – A review" mention that *M. esculenta* stands as a notable wild mushroom species that holds considerable medicinal significance and is economically advantageous. The authors additionally highlight that within *M. esculenta's* fruiting body, a diverse array of active elements can be found, including "vitamins, proteins, minerals, steroids, polysaccharides, and polynucleotides". *M. esculenta*, along with its active components, showcases notable attributes such as cardiovascular protection, anti-tumor effects, immunomodulation, anti-parasitic action, hepatoprotection, antibacterial and antiviral activities, as well as properties related to diabetes management. Their publication undertakes a comprehensive examination of *M. esculenta's* mycochemical makeup, nutritional value, and the scope of its biological activities [190].

Badshah et al. (2021) did a study titled "Molecules/ Isolation, Characterization, and Medicinal Potential of Polysaccharides of *M. esculenta".* The initial findings of this study indicated that the crude polysaccharides extracted from the chosen mushroom

variety, M. esculenta, exhibited significant antioxidant capabilities, particularly evident in the deproteinized crude polysaccharides. Consequently, this genre of mushrooms presents itself as a viable reservoir of both antioxidants and compounds with potential anticancer properties [191].

Wan et al. (2019) did a study titled "Anti-inflammatory effects of *M. esculenta* polysaccharide and its derivatives in fine particulate matter-treated NR8383 cells". Within this investigation, the polysaccharide denoted as FMP-1, originating from M. esculenta, demonstrated its ability to shield NR8383 cells against inflammation induced by PM2.5 exposure. The augmentation of protective effects was effectively achieved through processes involving sulfation and carboxymethylation. Among these, SFMP-1 displayed the most favorable outcomes, and its potential advantages appear to stem from its capability to impede the activation of the NF-κB pathway [192].

Wagay et al. (2019) did a study titled "Phenolic profiling and antioxidant capacity of *M. esculenta* L. by chemical and electrochemical methods at multiwall carbon nanotube paste electrode". This research undertook an exploration of the mushroom's antioxidative properties through a combination of chemical and electrochemical assessments. Through HPLC-UV analysis, the existence of eight distinct phenolic compounds was confirmed. Notably, these compounds encompassed "p-coumaric acid, tocopherol, catechol, rutin, hyperoside, quercetin, ellagic acid, and cinnamic acid, with quercetin" found to be the most abundant, accounting for the highest proportion (169.76%) [193].

Raman et al. (2018) did a study titled "*M. esculenta*: a herbal boon to pharmacology". This document provides an encompassing summary encompassing the introduction, functionalities, and medicinal attributes of *M. esculenta* , while also offering a concise depiction of its closely related species. It encompasses an extensive array of pharmacological traits, comprising antioxidant, antitumor, antimicrobial, and anti-inflammatory characteristics. Moreover, its role as an immune stimulant arises from its diverse set of active components. Its potential applications span a purgative and laxative role, acting as a body tonic and emollient. Additionally, it finds utility in addressing stomach-related concerns, facilitating wound healing, and addressing instances of general weakness [194].

Tietel et al. (2017) did a study titled "True morels (Morchella)—nutritional and phytochemical composition, health benefits and flavor: a review." The study unveiled the antioxidative and anti-inflammatory bio efficacies of the subject, along with its capacity for immunostimulation and anti-tumoral effects. Despite the burgeoning demand and escalating economic significance of morels, their cultivation remains constrained, necessitating reliance on wild harvesting or culture-based fermentation. This cultivation approach serves to harness their potential as functional food and flavor enhancers. The health advantages linked to morels predominantly arise from their active polysaccharides and diverse phytochemical composition, prominently featuring phenolic compounds, tocopherols, ascorbic acid, and vitamin D [195].

Kuo et al. (2012) did a study titled "Taxonomic revision of true morels (Morchella) in Canada and the United States". The article highlights those contemporary molecular phylogenetic investigations have unveiled the presence of no less than 50 distinct Morchella species on a global scale. These studies have further illuminated a pronounced prevalence of continental specificity within the Morchella genus. In this context, the paper provides an account of 19 distinct phylogenetic Morchella species originating from North America [4].

Liu et al. (2016) did a study titled "Characteristics and Antitumor Activity of *M. esculenta* Polysaccharide Extracted by Pulsed Electric Field". Within this research, an exploration was conducted into the chemical composition, as well as the anti-proliferative and antitumor attributes, of a polysaccharide named *M. esculenta* polysaccharide (MEP). This MEP was acquired through the utilization of pulsed electric field (PEF) during submerged fermentation. The preliminary assessment in this recent investigation focused on the impact of M2 on the in vitro proliferation of HT-29 cells. However, forthcoming inquiries are expected to delve into the mechanism of action and optimal dosages through studies involving tumor-bearing mice [196].

Pakistan Journal of Food sciences published an article titled "*M. esculenta* : An edible and health beneficial mushroom" (2015). This research underscores the diverse spectrum of pharmacological attributes inherent in *M. esculenta*, encompassing antioxidant, antitumor, antimicrobial, and anti-inflammatory characteristics. Furthermore, owing to the presence of a multitude of active components, it is recognized

for its immunostimulant capabilities. From an ethnobotanical perspective, it finds application as a laxative, purgative, emollient, and body tonic. Additionally, it holds a historical utilization in addressing stomach-related issues, aiding wound healing, and mitigating general weakness [197].

Sandrina et al. (2015) did a study titled "Nutritional value, bioactive compounds, antimicrobial activity and bio accessibility studies with wild edible mushrooms". Within this investigation, wild edible mushroom species were identified as abundant reservoirs of essential nutrients, minerals, and bioactive compounds, notably including phenolic acids. Subsequent in vitro digestion led to a reduction in antibacterial efficacy; however, no significant alteration was observed in antifungal and demelanizing attributes. Notably, in the majority of instances, the tested samples exhibited superior antibacterial and antifungal potential when compared to established standards. The presence of bioactive compounds, particularly phenolic acids, was more pronounced within the phenolic extracts prior to in vitro digestion. This aligns with the augmented antibacterial potency demonstrated by these extracts [198].

Sandrina et al. (2013) did another study titled "A comparative study of chemical composition, antioxidant and antimicrobial properties of *M. esculenta* (L.) Pers. from Portugal and Serbia". Sandrina A. Heleno's study aimed to establish a comparison between two distinct M. esculenta (morel) samples originating from different countries—Portugal and Serbia. The analysis encompassed a thorough evaluation of both samples. It was evident that both samples exhibited significant contents of carbohydrates, encompassing free sugars, and proteins. Furthermore, the presence of numerous bioactive constituents was identified, including organic acids, phenolic compounds, and tocopherols. Among these, polyunsaturated fatty acids ranked as the predominant constituents, followed by mono- or saturated fatty acids [46]. Comparing the samples, the Portugal-originating sample (SP) displayed heightened activity in terms of radical scavenging and reducing power. Conversely, the Serbia-originating sample (SS) exhibited a more pronounced capacity for inhibiting lipid peroxidation. Impressively, both samples demonstrated antibacterial efficacy against five different bacterial strains.

De Silva et al. (2012) did a study titled "Medicinal mushrooms in prevention and control of diabetes mellitus". Within this comprehensive analysis, the focus is directed toward the intrinsic biological characteristics of diabetes. Specifically, the examination delves into several mushrooms that hold promise due to their experimental anti-diabetic attributes, which could potentially hinder or diminish the progression of diabetes mellitus. The exploration encompasses the significance of medicinal mushrooms not just as components of medical nutrition therapy but also as potential candidates with supportive roles in diabetes prevention and management, drawing attention to the utility of their metabolites [199].

Kim et al. (2011) did a study titled "Antioxidant and NF-κB inhibitory constituents isolated from *M. esculenta* ". This research undertook a process of activity-guided fractionation and isolation, specifically focusing on the antioxidant compounds found in *M. esculenta*. This endeavor involved evaluating intracellular reactive oxygen species (ROS) levels within HT-29 colon cancer cells. Among the fractions, the methylene chloride extract from *M. esculenta* exhibited the most substantial antioxidant activity [200]. Because the medicinal qualities of only a small number of mushrooms have been looked into in the recent times, there's a great deal more to find out. Moreover, good-quality, randomized, placebo-controlled human trials that are multiple-blind, in performed in longer time and with larger pool of samples are required, in addition to modern statistical and bioinformatics techniques. These studies must be conducted for an extended period of time. More research is required to identify the specific mushroom extracts that are most effective in treating a variety of diseases and conditions.

Chapter-3

RESEARCH GAP

Chapter-3

Research Gap

M. esculenta is a pharmacologically and nutritionally important fungus. It has not been studied for many pharmacological properties including hypoglycemic activities. There is tremendous scope available for the exploration of its unexplored medicinal properties. Insulin resistance and poor glucose management are the hallmarks of type 2 diabetes, a metabolic condition that persists over time. Natural chemical research, especially from plants like *M. esculenta*, provides a potential new direction for the development of safer and more effective anti-diabetic medications, while there are also a number of pharmaceutical therapies available. There has been a lot of study on this mushroom, however finding certain polysaccharide components with low cytotoxicity to manage type 2 diabetes effectively is still missing. Several investigations on the effects of *M. esculenta* on type 2 diabetes have yielded encouraging results. Nevertheless, there is a significant lack of study into the isolation of certain polysaccharide compounds derived from *M. esculenta* that may offer new therapeutic approaches to the treatment of type 2 diabetes. While several research have looked into the bioactive components of this fungus, few have concentrated on isolating and characterizing the polysaccharides that may have anti-diabetic effects. We set out to fill this need by discovering and developing new polysaccharide compounds with potent anti-type 2 diabetes effects in *M. esculenta*. Our main objective goes beyond just identifying these chemicals; we also want to highlight their potential for therapeutic uses. Evaluating and ensuring that these new polysaccharides display minimal cytotoxicity is an important part of our work, along with their anti-diabetic capabilities. Developing therapeutic solutions for people with diabetes mellitus that are safe and have minimal adverse effects requires this factor to be considered. The goal of our methodical extraction, isolation, and characterization approaches is to discover polysaccharide compounds from *M. esculenta* that have a good safety profile and show strong anti-diabetic benefits. Our work intends to fill this knowledge gap in the hopes of advancing the area of natural product-based medicine by providing new, safe therapeutic agents for type 2 diabetes management. This could lead to exciting new possibilities for successful and well-tolerated treatments.

Chapter-4

OBJECTIVES OF THE RESEARCH

Chapter-4

Objectives of the Research

- To identify and evaluate the anti-diabetic potentials of *M. esculenta* crude extract.
- To obtain the novel compound from extract through extraction techniques
- Evaluation of efficacy of a novel compound derived from *M. esculenta* against type 2 diabetes mellitus in the L6 myotubes cell line.

Chapter-5

MATERIALS AND METHODS

Chapter-5

Materials and Methods

5.1 Sample Collection:

Samples were collected from a natural habitat in Jammu and Kashmir, India during the month of April 2021. The collection was carried out in accordance with the regulations set by the Ministry of Forestry.

5.2 Morphological Identification and certification:

The collected specimen was morphologically identified as *M. esculenta* using standard taxonomic keys and reference materials specific to the genus *Morchella*. Morphological features such as cap shape, color, and texture, as well as the characteristics of the stipe and hymenium, were carefully examined and recorded. After morphological identification, the specimen was sent to the office of the forest range officer for certification. The certification process involved validation by qualified taxonomists who confirmed the identification of the specimen as *M. esculenta* based on its morphological characteristics.

5.2.1 DNA Extraction, ITS amplification and Sequencing:

Genomic DNA was extracted with the established CTAB method [201]. The extraction was carried out on a small piece of the mushroom tissue, carefully removed from the specimen. Briefly, cell walls of the sample were broken down by grinding with mortar and pestle in the presence of liquid nitrogen. The CTAB extraction buffer was then added, and after incubation at 65°C for 1 hour, the protein was precipitated with equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1). The mixture was then centrifuged at 12,000 rpm for 5 minutes and the aqueous layer transferred to a fresh tube. Two volumes of chilled isopropanol were added and the tubes were kept at -20°C for 1 h for DNA precipitation. After incubation the tubes were centrifuged at 12,000 rpm for 5 minutes and the resultant DNA pellet was washed with 70% ethanol. Air dried and the DNA pellet was dissolved in 50µl of pure water.

CTAB Buffer recipe

- 1 M Tris-HCl pH 8.0
- 5 M NaCl
- 0.5 M EDTA
- 2% CTAB
- 2% PVP
- 0.2%β-mercapto ethanol add right before use.

DNA Quantification

The DNA obtained was quantified and checked for purity using a µDrop plate reader (ThermoScientific). Quality of the obtained DNA was analysed by subjecting it to agarose gel electrophoresis. The Internal Transcribed Spacer (ITS) region of the extracted DNA was amplified using PCR (Polymerase Chain Reaction). Primers specific to the ITS region were used for the amplification. The PCR reaction was carried out in 20µl reaction mixture containing 10µl Takara mix, 1µl of 10µM forward and reverses primers respectively, 100-200ng of template DNA. The final volume was made up with nuclease free water. Amplication of the ITS region was carried out in a thermal cycler (Applied Biosystems) with an annealing temperature of 52ºC and a cycle number of 35. The obtained PCR amplicon was visualized using agarose gel electrophoresis. The PCR amplicons were purified using a DNA purification kit to remove excess primers and nucleotides. The purified amplicons were then sent to a sequencing facility for Sanger sequencing. Sequencing was performed using the appropriate sequencing primers for the ITS region. The obtained sequence data were analyzed using bioinformatics tools. Around 750bp of good quality sequences obtained as a forward read and reverse read were used to construct a consensus sequence. The 1543 bp consensus sequence obtained was subjected to NCBI- nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi#) against ITS database.

5.3 Preparation of mushroom extract

The collected mushroom samples were used for the extract preparation. Briefly, the 250g of the dried and powdered mycelia was extracted with three different solvents i.e., methanol, ethanol, chloroform for 16-18h at the boiling points of the respective solvents. The extract was concentrated at low temperature and the solvent was completely evaporated under vacuum with help of rotary evaporator. The obtained extract was stored at 4^0C.

Fig 5.1. Preparation of extract using a Soxhlet apparatus

5.4 Determination of the extract yield

The yield of soluble mass was determined with respect to the original mass of powdered *M. esculenta.*

$$Yield\ \%se = \frac{Mass\ of\ dried\ extract}{Mass\ of\ Morchella\ esculenta\ powdered}\ X\ 100$$

5.5 Qualitative Phytochemical analysis

The extract solution was prepared by dissolving 200 mg of the extract in 3 ml of solvents and made up to 20 ml with distilled water. Concentration: 10mg/ml. We had followed same procedure for three solvents i.e., methanol, ethanol, chloroform.

5.5.1 Detection of Saponins

The saponins were detected by foam test. To 2 ml of the extract solution, 2 ml of distilled water was added and shaken vigorously; formation of foam was considered to be a positive indication of the presence of saponins [202].

5.5.2 Detection of tannins

1 ml of the extract solution was mixed with 2 ml of $FeCl_3$. Formation of a greenish black colour was considered to be a positive indication of the presence of tannins [203].

5.5.3 Detection of Terpenoids and steroids

Terpenoids and steroids were detected by Salkowski's test 1 ml of the extract solution was mixed with chloroform and concentrated H_2SO_4. An appearance of reddish-brown colorant the interface was considered to be a positive indication of the presence of terpenoids. Appearance of red colour at the lower chloroform layer was considered to be a positive indication of the presence of steroids [204].

5.5.4 Detection of Glycosides

Borntrager's test detection for Glycosides as follows; 1 gm of extract solution add 5–10 ml of dilute HCl boil on water bath for 10 min and filter. Filtrate was extracted with CCl4/ benzene and add equal amount of ammonia solution to filtrate and shake. A reddish brown colour formation indicated the presence of glycosides [204].

5.5.5 Detection of Flavonoids

Flavonoids were detected by alkaline reagent test. 2ml of extract solution was subjected to the addition of sodium hydroxide and dilute hydrochloric acid. Formation and disappearance of yellow colour indicated the presence of flavonoids [204].

5.5.6 Detection of Alkaloids

2ml of the extract solution was subjected to the addition of concentrated hydrochloric acid. Appearance of a green colour or a white precipitate following the addition of a few drops of Mayer's reagent indicated the presence of alkaloids [204].

5.5.7 Detection of Quinones

1 ml of the extract solution was subjected to the addition of concentrated sulphuric acid. Formation of a red color indicated the presence of quinines [204].

5.5.8 Detection of Phenols

Phenols were detected by ferric chloride test. 1ml of the extract solution was diluted using 2 ml of distilled water and subjected to addition of 3-4 drops or 2ml of 10% ferric chloride. Formation of a blue green or a bluish black color indicated the presence of phenols [204].

5.5.9 Detection of Coumarins

1 ml of sodium hydroxide was added to the extract solution. Formation of a yellow color indicated the presence of Coumarins [204].

5.5.10 Detection of Carbohydrates

1 ml of Molisch reagent was added to the extract solution followed by the addition of 1ml of concentrated H_2SO_4. The mixture was then allowed to stand for 2-3 minutes. Formation of a purple red or dull violet colour at the interphase of the two layers indicated the presence of carbohydrates [204].

5.6 Quantitative phenol estimation

One ml of sample from (10mg extract/ml stock) was taken and subjected to the addition of 3.0 ml of distilled water. Folin-Ciocalteau reagent (0.5ml) and 2mL 20% Na_2CO_3 were added and the tubes were placed in a boiling water bath for exactly one minute. The tubes were cooled and the absorbance was read at 750 nm in a spectrophotometer against a reagent blank. Standard gallic acid solutions (2.5-100µg/ml) were also treated as above [205].

5.7 Determination of antioxidants

5.7.1 ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay

The free radical scavenging capacity of the samples were tested using ABTS radical cation decolorization assay as per reported protocol of RE et al.,1999 [206]. ABTS dissolved in water to get 7mM concentration. ABTS radical cation ($ABTS^{*+}$) was produced by reacting ABTS stock solution with 2.45mM potassium persulphate (final

concentration) and allowing the mixture to stand at room temperature in the dark for 12-16 hrs before use. The free radical was stable for more than two days, when stored in the dark room temperature. For the study of the test samples, the ABTS*$^{+}$ solution was diluted with absolute ethanol to an absorbance of 0.700 (±0.02) at 734 nm and equilibrated at 30°C. Reagent blank reading was taken (A_0). After addition of 2.0 mL of diluted ABTS*$^{+}$ solution $A734\ nm$ = 0.700 (±0.02) to 50 µL of test sample (20 mg/mL), the absorbance reading was taken at 30°C exactly 6 min after initial mixing (A_t). Appropriate solvent blanks were run in each assay. All determinations were carried out at least three times. The percentage inhibition of absorbance at 734 nm was calculated using the above formula and decrease of the absorbance between A_0 and A_t.

$$\%se\ inhibition\ of\ ABTS\ radical = \left[\frac{(A_{C(0)} - A_{A(t)}}{A_{C(0)}}\right] X100$$

where $A_{C(0)}$ is the absorbance of the control at t = 0 min; and $A_{A(t)}$ is the absorbance of the antioxidant at t = 6 min. Ascorbic acid was used as standard

5.7.2 DPPH (1,1-diphenyl-2-picrilhydrazyl) free radical Scavenging assay

The effect of given samples on DPPH radical was estimated according to the procedure described by Von Gadowet al. (1997) [207]. Two mL of 6 ×10^{-5} M methonolic solution of DPPH were added to 50 µl of a methonolic solution (20 mg ml^{-1}) of the sample. Absorbance measurements commenced immediately. The decrease of absorbance at 515 nm was continuously recorded in a spectrophotometer for 16min. at room temperature. Methanolic/ Ethanolic/ Chloroform extract of *M. esculenta* were tested at 1 mg/ml concentration. The scavenging effect (decrease of absorbance at 515 nm) was plotted against the time and the percentage of DPPH radical scavenging ability of the sample was calculated from the absorbance value at the end of 16min. duration as follows:

All determinations were performed in triplicate. The percentage inhibition of the DPPH radical by the samples was calculated according to the formula of Yen and Duh (1994).

$$DPPH\ scavenging\ assay = \left[\frac{(A_{C(0)} - A_{A(t)}}{A_{C(0)}}\right] X100$$

Where $A_{C(0)}$ is the absorbance of the control at t = 0 min; and $A_{A(t)}$ is the absorbance of the antioxidants at t = 16 min, Ascorbic acid was used as standard.

5.7.3 Hydrogen peroxide scavenging activity

Scavenging activity of hydrogen peroxide by the extract was estimated using the method of Ruch et al., 1989. The extract (4 ml) prepared in distilled water at various concentration was mixed with 0.6 ml of 4 mM H_2O_2 solution prepared in phosphate buffer (0.1 M pH 7.4) and incubated for 10 min. The absorbance of the solution was taken at 230 nm against blank solution containing the extract without H_2O_2. The reaction mix containing H_2O_2 without extract served as control [208]. The amount of hydrogen peroxide radical inhibited by the extract was calculated using the following equation.

$$H_2O_2\ scavenging\ assay = \left[\frac{(A_C - A_T}{A_C}\right] X100$$

Where; Abs control is the absorbance of H_2O_2 radical; Abs sample is the absorbance of H_2O_2 radical + sample extract. Ascorbic acid was used as standard.

5.7.4 Lipid peroxidation Assay

Lipid peroxidation was estimated as thiobarbituric acid reacting substances (TBARS) by the method of Ohkawa et al. (1979). The reaction mixture contained 0.1ml of the extract in Tris-HCl buffer (20mM, pH 7.0; KCl (30mM); $FeSO_4$ (NH_4) $SO_4.7H_2O$ (0.06 mM). 0.4 ml of the mixture was treated with 0.2 ml sodium dodecyl sulphate (8.1%), 1.5 ml thiobarbituric acid (TBA) (0.8%) and 1.5ml of trichloroacetic acid (20%). The total volume was made up to 4.0ml with distilled water and then kept in a water bath at 95°C for 1 hour. After cooling, 1.0 ml of distilled water and 5.0 ml of n-butanol and pyridine mixture (15:1) were added to the reaction mixture, shaken vigorously and centrifuged at 4000g for 10 min. The butanol pyridine layer was removed and its absorbance was measured at 532 nm [209]. Inhibition of lipid peroxidation was

determined by comparing the optical density (OD) of the test sample with that of the control using the following formula. Ascorbic acid was used as standard.

$$Percentage\ Lipid\ Peroxidation\ assay = \left[\frac{(A_{C\ at\ 532\ nm} - A_{T\ at\ 532\ nm}}{A_{C\ at\ 532\ nm}}\right] X100$$

5.7.5 Nitric Oxide Scavenging Assay

Nitric oxide generated from sodium nitroprusside in an aqueous solution at physiological pH was measured by the Griess reaction Marcocci et al., 1994. The reaction mixture (3ml) containing sodium nitroprusside (10mm) in phosphate buffer saline and the test extract (10, 25, 50 and 100µg/ml) was incubated at 25ºC for 150min, after incubation 1.5ml of the reaction mixture was removed and 1.5ml of the Griess reagent (1% sulphanilamide, 2% orthophosphoric acid and 0.1% Napthylethyline diamine hydrochloride) was added. The absorbance of the chromophore formed was read at 546 nm. Percent inhibition of nitric oxide scavenging was calculated using the formula. Ascorbic acid was used as standard [210].

$$Nitric\ oxide\ scavenging\ assay\ (\%se) = \left[\frac{(A_C - A_A}{A_C}\right] X100$$

5.8 α-glucosidase inhibition assay

α-glucosidase inhibition assay was performed based on the method of Watanabe et al., 1997. Each extract was dissolved in MeOH (24µM, final concentration). Yeast IX-glucosidase (0.1 µg/ml) was dissolved in 10 mM phosphate buffer (pH 7.0) containing 0.2% BSA and used as an enzyme solution. p-Nitrophenyl-~-D-glucopyranoside (NPG) (5 mM) in the same buffer (pH 7.0) was used as a substrate solution. Phosphate buffer (10 mM, pH 7.0. 2 ml), substrate (200µl) and inhibitor (100µl) were mixed in a test tube and incubated for 5 min at 37°C. Enzyme solution (200µl) was then added and the reaction was done for 5 min at 37°C [211]. The reaction was stopped by adding 0.25 M Na_2CO_3 (1.5 ml). The increase in absorbance at 405 nm was measured. Acarbose was used as standard.

$$Percentage\ inhibition\ of\ \alpha - glucosidase\ \left[\frac{(A_{C\ at\ 405\ nm} - A_{T\ at\ 405\ nm}}{A_{C\ at\ 405\ nm}}\right] X100$$

5.9 α -amylase inhibitory assay

The method developed by McCue and Shetty, 2004 was adapted and used in the execution of this experiment. Briefly, α-amylase (0.5 mg/mL) dissolved in 0.02 M sodium phosphate buffer (pH 6.9), followed by addition of 250µL sample solution (0.2mg/ml, 0.4 mg/ml, 0.6 mg/ml, 0.8mg/ml, 1mg/ml). Before being incubated at 25°C for 10 minutes, this solution was preincubated at 25°C for 10 minutes [212]. Following this, 250µl of 1% starch solution in 0.02 M sodium phosphate buffer (pH 6.9) was added at scheduled intervals .500 µl of dinitrosalicylic acid (DNS) reagent was added to stop the process. After 5 minutes in boiling water, the experimental samples were removed and allowed to cool to room temperature. The absorbance at 540 nm was measured after diluting the reaction mixture with distilled water. A place of distilled water was made in the same way as the extract. Acarbose was used as standard. Percentage inhibition of α-amylase was used to determine the inhibitory activity:

$$\alpha - \text{Amylase inhibitory activity}(\%) = \left[\frac{(A_0 - A_1}{A_0}\right] X100$$

Where A_0 is the absorbance of negative control (α-Amylase) and A_1 is the absorbance of test sample.

5.10 Glucose uptake assay

The L6 myotubes cell line is the best characterized cellular model origin to study glucose uptake and GLUT4 translocation. There are reports that medicinal plants can enhance the glucose uptake by GLUT4 translocation and were proven by *in vitro* glucose uptake models. *In vitro* models comprising of skeletal muscle cells and adipocytes are widely used to study glucose uptake activity of drugs. Hence in this study we have used L-6 muscle cells to determine the enhancement in the glucose uptake activity of the test compounds *in vitro*.

Glucose uptake activity in L6 cells was estimated by the methods described by Guptha et al. (2010) with slight modifications [213]. Cells were cultured on 48 well

plates and incubated for 48 hours at 37°C in a CO_2 incubator. When semi-confluent monolayer was formed, the culture was renewed with serum free DMEM containing 0.2% BSA and incubated for 18 hours at 37°C in the CO_2 incubator. After 18 hours, the medium was discarded and cells were washed with PBS (pH 7.4) buffer once and treated with 1000 µg/ml glucose along with test compound (25, 50, 100 µg/ml) for 1 hour. Glucose uptake was calculated as the difference between the initial and final glucose content in the incubated medium. The final glucose concentration was estimated by anthrone method with the aid of a glucose standard graph. The glucose uptake in L6 cells treated with test compounds were compared with that of control cells (untreated). If the treated cells showed improved glucose uptake compared to that of control cells we can assume that the compound have medicinal value. The glucose content was analysed using anthrone method. 4mL of anthrone reagent was added to the incubated medium followed by heating for 8 min. in a boiling water bath. The test sample mixture was cooled rapidly and the absorbance of the obtained green colour was read at 630nm. The standards were prepared similarly but taking different concentrations of glucose (200-1000µg/ml) in each test tube instead of the incubated medium. Similarly, after glucose uptake assay the remaining glucose present in the control and treated wells were assayed by anthrone method as mentioned above with the aid of standard curve.

5.11 Isolation of pure compound using column chromatography

5.11.1 Separation of Markers

Compounds were isolated from the methanol fraction by column chromatography and fractions were monitored on TLC. Methanol: Ethyl Acetate was used as the developing solvent used for the TLC in various proportion. The spots were visualized by spraying the chromatogram with 0.5% Anisaldehyde sulphuric acid [214].

5.11.2 Slurry Formation

Dried methanol fraction was taken and dissolved in the minimum quantity of DCM and then adsorbed on weighed quantity of silica gel, to get free flowing material.

Weight of silica gel used	=	2g
“Weight of methanolic extract	=	2 g

5.11.3 Packing of column

A neat and dried column was taken and a plug was put at the base the column. Solvent was poured into the column and packed with slurry of silica gel prepared by suspending it into the solvent. The adsorbed extract was then charged into the column.

Table 5.2. Prepration of Column

Weight of methanolic extract	2.00 gm
Weight of slurry	2.00 gm
Slurry prepared by added silica gel (60-120 mesh)	
Silica packed in coloumn	220g
Column length	60 cm
Column diameter	3 cm

5.11.4 Collection of fractions by elution

The column was first eluted with hexane.

Table 5.3. Elute fractions with different Solvent systems

Sr. No.	Fraction no.	Column Solvent system	TLC Solvent system
1	A	Hexane	Hexane
2	B	50% in EtOAc in Hexane	55% in EtOAc in Hexane
3	C	100% EtOAc	5% Methanol 95% EtOAc
4	D	20% Methanol 80% EtOAc	25% Methanol 75% EtOAc
5	E	30% Methanol 70% EtOAc	35% Methanol 65% EtOAc
6	F	35% Methanol 65% EtOAc	40% Methanol 60% EtOAc
7	G	40% Methanol 60% EtOAc	45% Methanol 55% EtOAc
8	H	45% Methanol 55% EtOAc	50% Methanol 50% EtOAc
9	I	50% Methanol 50% EtOAc	55% Methanol 45% EtOAc
10	J	50% Methanol 50% EtOAc	55% Methanol 45% EtOAc

11	K	60% Methanol 40% EtOAc	65% Methanol 35% EtOAc
12	L	60% Methanol 40% EtOAc	65% Methanol 35% EtOAc

The column was eluted with the solvent by gradually increasing the percentage of EtOAc in hexane and then methanol in EtOAc. Each fraction of 50 ml was collected and concentrated on water bath. A total of 90 fractions were collected and TLC of all 90 fractions was performed using different developing solvents. The fractions were pooled on the basis of the TLC pattern shown by them.

A total of 12 set of fractions were obtained after elution and which were pooled based on the similarity of spots obtained on a TLC plate. Methanol: Ethyl Acetate was used as the developing solvent used for the TLC in various proportion. The spots were visualized by spraying the chromatogram with 0.5% Anisaldehyde sulphuric acid**.** These fractions were named as A, B, C, D, E, F, G, H, I, J, K, and L. Re-chromatography was performed on these fractions as they seemed to be a mixture of compounds. The obtained fraction was dried using Rota evaporator and subjected to measurement of weight.

To verify the presence of polysaccharides, qualitative tests were carried out following the isolation process. The purpose of these assays was to offer preliminary proof that the isolated material contained the necessary components, specifically polysaccharide since polysaccharides/ carbohydrates are responsible for antidiabetic activity. We continued with the 12 fractions, to detect the polysaccharides by quantitative test (Molish test)

5.12 Molish test (Detection of polysaccharides)

Molisch's test is done by using molisch reagent which is a colourimetric method for the analysis of the polysaccharide's presence in our fractions. A solution of naphthol in ethanol (95%) is known as Molisch reagent. It's also known as the purple ring test. Other than the Molisch reagent, concentrated sulfuric acid is also used in the test.

5.13 Spectral Analysis of the isolated compound

5.13.1 Fourier-transform infrared spectroscopy (FTIR) analysis

The FTIR (Fourier Transform Infrared) spectra of the sample was recorded in FTIR instrument (Perkin Elmer Spectrum IR Version 10.6.1), with PC based software-controlled instrument operation and data processing. The data of infrared transmittance was collected over a wave number ranged from (4000-400cm inverse) [215]. All the samples were analyzed in triplicates with plain KBr pellets as blank. The spectral data were compared with a reference to identify the functional groups existing in the sample.

5.13.2 Nuclear magnetic resonance (NMR) analysis

The H^1 and C^{13}NMR spectra were recorded on a Bruker-AC 400 MHz spectrometer in DMSO using tetramethylsilane (TMS) as the internal standard. Chemical shifts are reported in parts per million by taking tetramethylsilane as a standard. In H^1 NMR spectra, $\delta H = 2.50$ ppm for DMSO and in C^{13} NMR spectra, $\delta C = 39.52$ ppm. In H^1 NMR, data are reported as chemical shift values, multiplicity (s = singlet, d = doublet, t = triplet, br = broad, and m = multiplet), and coupling constant (Hz). [217]

5.13.3 Liquid chromatography–mass spectrometry (LC–MS) analysis

The fractions, exhibited antidiabetic activity, were further processed for identification of bioactive compounds by LC MS/MS (n=2-9) (Make: Thermo Model: LTQ-XL) analysis. The detection was performed through direct injection mode with Electron Spray Ionization (ESI) probe, at positive-mode. The MS parameters for each compound were optimized to ensure the most favorable ionization, ion transfer conditions and attained optimum signal of both the precursor and fragment ions by infusing the analytes and manually turning the parameters. The source parameters were identical for all of the analytes [216].

5.14. MTT assay

5.14.1. Principle

The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. This colorimetric assay is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells. The viable cells contain NAD(P)H-dependent oxidoreductase enzymes

which reduce the MTT to formazan [218]. The insoluble formazan crystals are dissolved using a solubilizing solution (100% DMSO) and the resulting purple colored solution is quantified by measuring absorbance at 570 nm using an ELISA plate reader [219].

5.14.2. Cell lines and maintenance

The cell line / cell lines were procured from the National Centre for Cell Sciences (NCCS), Pune, India.

5.14.3. Cell culture media and maintenance

The cells were cultured in Dulbecco's Modified Eagles Medium (DMEM-Himedia), supplemented with 10% heat inactivated Fetal Bovine Serum (FBS) and 1% antibiotic cocktail containing Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). The cell containing TC flasks ($25cm^2$) were incubated at 37°C at 5% CO_2 environment with humidity in a cell culture incubator.

5.14.4. Assay Procedure

The cells (2500 cells/well) were seeded on 96 well plates and allowed to acclimatize to the culture conditions such as 37°C and 5% CO_2 environment in the incubator for 24 hours. The test samples were prepared in DMEM media (10 mg/mL) and filter sterilized using 0.2 µm Millipore syringe filter. The isolated compound were further diluted in DMEM media and added to the wells containing cultured cells at final concentrations of 6.25, 12.5, 25, 50, 100 µg/mL respectively. Untreated wells were kept as control. All the experiments were done in triplicate and average values were taken in order to minimize errors. After treatment with the test samples the plates were further incubated for 24 hours. After incubation period, the media from the wells were aspirated and discarded. 100 µL of 0.5 mg/mL MTT solution in PBS was added to the wells. The plates were further incubated for 2hours for the development of formazan crystals. The supernatant was removed and 100 µL DMSO (100%) were added per well.

The absorbance at 570 nm was measured with micro plate reader. Three wells per plate without cells served as blank. All the experiments were done in triplicates. The cell viability was expressed using the following formula

$$Percentage \text{ of cell viability} = \frac{\text{Average absorbance of treated}}{\text{Average absorbance of control}} \times 100$$

5.14.5. IC_{50} value

The IC_{50} value is the half maximal inhibitory concentration of the sample. The IC_{50} values were calculated using the equation for slope (y = mx + C) obtained by plotting the average absorbance of the different concentrations of the test sample (6.25-100 µg/mL) in Microsoft Excel.

5.14.6. Cell Morphology

Representative photomicrographs of cells belonging to each experimental group were taken using an inverted phase contrast microscope (LABOMED, TCM 400, USA). Magnification- 10x. Total magnification- 100x (10x Objective lens × 10x Ocular lens).

5.15. Glucose uptake assay

5.15.1 Principle:

The L-6 myotubes cell line is the best characterized cellular model origin to study glucose uptake and GLUT4 translocation. Isolated compound can enhance the glucose uptake by GLUT4 translocation and were proven by *in vitro* glucose uptake models. *In vitro* models comprising of skeletal muscle cells and adipocytes are widely used to study glucose uptake activity of drugs. Hence in this study we have used L-6 muscle cells to determine the enhancement in the glucose uptake activity of the test compounds *in vitro* [213]. Glucose uptake activity in L6 cells was estimated by the methods described by Guptha et al. (2010) with slight modifications. Cells were cultured on 12 well plates and incubated for 48 hours at 37°C in a CO_2 incubator. When semi-confluent monolayer was formed, the culture was renewed with serum free DMEM containing 0.2% BSA and incubated for 18 hours (serum starved overnight) at 37°C in the CO_2 incubator. After 18 hours, the medium was discarded and cells were washed with PBS (pH 7.4) buffer once and treated with 1000 µg/ml glucose along with test compound (25, 50, 100 µg/ml) for 1 hour. Glucose uptake was calculated as the difference between the initial and final glucose content in the incubated medium. The final glucose concentration was estimated with the aid of a glucose standard graph. The glucose uptake in L6 cells treated with test compounds were compared with that of

control cells (untreated). If the treated cells showed improved glucose uptake compared to that of control cells we can assume that the compound have medicinal value.

Chapter-6

Results and Discussion

Chapter-6

Results and Discussion

6.1 Results

6.1.1 Identification of the sample

6.1.1.1. Morphological identification and certification

The collected mushroom sample was identified and certified as *M. esculenta* using standard taxonomic keys and reference materials specific to the genus *Morchella*. The certification process involved validation by qualified taxonomists who confirmed the identification of the specimen as *M. esculenta* based on its morphological characteristics. Figure 6.1 shows the certificate provided by the office of the forest range officer.

OFFICE OF THE FOREST RANGE OFFICER BANI

J&K Forest Department
UT (J&K) 184206
E-mail id. - rangeofficebani@gmail.com

Dispatch No: 82/RO/BNI /22-23 Dated: 03/06/2022

MINOR FOREST PRODUCE AUTHENTCATION CERTIFICATE

The Mushroom specimen which has been sent by you for authentication is identified as "*Morchella esculenta*". The identified specimen is returned here with for preservation in your college / Department / institution Herbarium.

Forest Range Officer
Bani, Forest Range
Bani

FOREST RANGE

To
Mr. Keshav Anand
Reg. No. 11919206
Ph.D. Research Scholar
Department of Medical Laboratory Sciences
Lovely Professional University
Phagwara, Punjab- 144411

Fig. 6.1. Certificate of identification provided by the office of the forest range officer

6.1.1.2. DNA Extraction, ITS amplification and Sequencing and taxonomic identification:

The DNA obtained from the sample using the CTAB extraction method was found to be within the standards of quantification and quality for proceeding to PCR. An absorbance ratio of 1.8 and 2.0 were obtained for $A_{260/280}$ and $A_{260/230}$ respectively corresponding to a concentration of 296µg/ml.

Sample code	Concentration (µg/ml)	Purity A260/280
sample	296	1.8

The quality and integrity of the obtained DNA was analysed using agarose gel electrophoresis. The PCR was performed using ITS1 and ITS4 primers and the amplified product was also analysed using agarose gel electrophoresis. The gel images of the DNA and ITS amplified product is provided in figure 6.2.

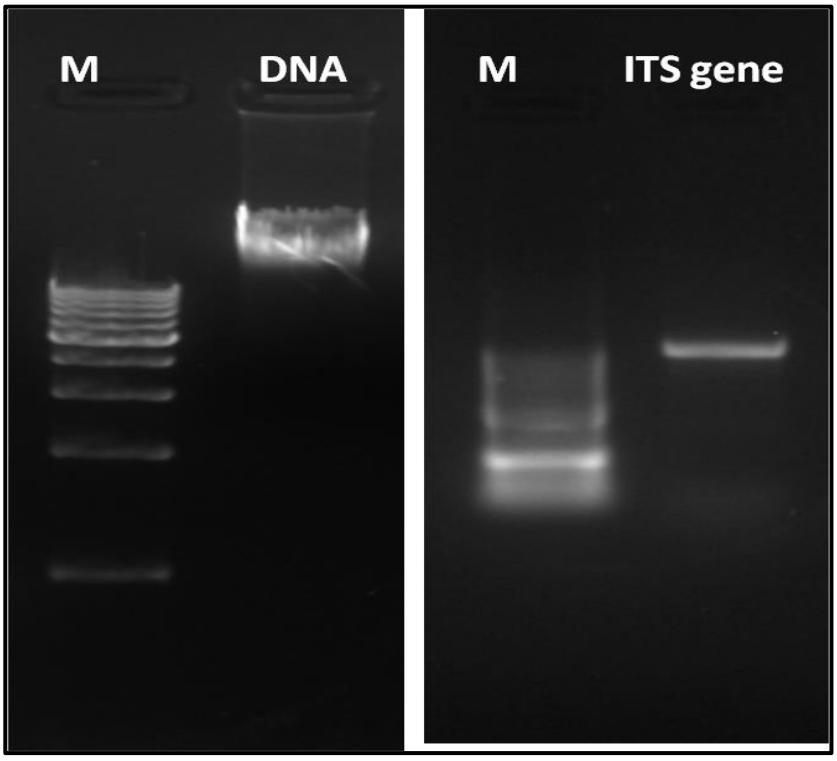


Fig. 6.2 Agarose gel images of left: total DNA from the samples (with 1Kb DNA ladder Takara) and right: ITS amplified products of the samples (with 50bp plus DNA ladder Takara)

Sequencing results of the ITS amplified product using the forward and reverse primers were obtained as follows:

>ITS1

AAAGGGATCGCGCTCGCGCTGGGCTCGATAGACGCGCTAGCACCCGTCAGGCCCGGAGGGCGA
ACCAGGCCCCTGGGGCCGGCAGGTTAAACCCAAGGTACCCATCCAAGGTGGACAGTCCTCCCA
GAGTTCGCCCTTCGGCGTCTGCGAGCCATCGGGGCATGGAACCTTGGGGGCTGTCGGTTTCCC
GCTGGGCATACGTCCGATGCCGCCTCGGGAGGCGAAACGTGGCTCGTGCAGCGTCGCAGCGTT
GGTCCCCCACTCACTCACCAAAACCCTCTGCGTACCTTGCCCACTTGCTTCCCCCGGCCCCCC
CGGCCGGGGGGCGACACCAAAAACCAAACTCTTTGCGATGAACCGACGTCTGAATGCCAAAAG
CAAAACAAAAACAGTTAAAACTTTCAACAACGGATCTCTTGGCTCCCACATCGATGAAGAACG
CAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA
CATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATAAAACCCCCTCCCCCT
CGGATTAAATGTTCCTTGGGGGGTATTGGCCAATGGGATTGCGAGATAGCAATTGTTACCCAG
GCGCCCAAATGCATCAGTACCCCGCCATTTACAGATTTACCAGCACCGAGGCCGTCAACCGTG
GAGTTATGGGAATTATAGGCTTGCAGTAATATGCTCACCTCTCTCCATACGCAGGCGGCACAC
CGGGTAGAGTCGCGGGCGTAAAGCGGAATCCGAAACGGCCCTCACGGGTAGAGGAGGATCCTT
GCGGGCTAGTCACCCCTCATCACAATTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTA
AGCCTATCATAAAGCGGAAGAAAACA

>ITS4

ATCCTCCTGATCCGAGGTCAAATTGTGATGAGGGGTGACTAGCCCGCAAGGATCCTCCTCTAC
CCGTGAGGGCCGTTTCGGATTCCGCTTTACGCCCGCGACTCTACCCGGTGTGCCGCCTGCGTA
TGGAGAGAGGTGAGCATATTACTGCAAGCCTATAATTCCCATAACTCCACGGTTGACGGCCTC
GGTGCTGGTAAATCTGTAAATGGCGGGGTACTGATGCATTTGGGCGCCTGGGTAACAATTGCT
ATCTCGCAATCCCATTGGCCAATACCCCCCAAGGAACATTTAATCCGAGGGGGAGGGGGTTTT
ATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGATTCG
ATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGT
GGGAGCCAAGAGATCCGTTGTTGAAAGTTTTAACTGTTTTTGTTTTGCTTTTGGCATTCAGAC
GTCGGTTCATCGCAAAGAGTTTGGTTTTTGGTGTCGCCCCCCGGCCGGGGGGGCCGGGGGAAG
CAAGTGGGCAAGGTACGCAGAGGGTTTTGGTGAGTGAGTGGGGGACCAACGCTGCGACGCTGC
ACGAGCCACGTTTCGCCTCCCGAGGCGGCATCGGACGTATGCCCAGCGGGAAACCGACAGCCC
CCAAGGTTCCATGCCCCGATGGCTCGCAGACGCCGAAGGGCGAACTCTGGGAGGACTGTCCAC
CTTGGATGGGTACCTTGGGTTTAACCTGCCGGCCCCAGGGGCCTGGTTCGCCCTCCGGGCCTG
ACGGGTGCTAGCGCGTCTATCGAGCCCAGCGCGAGCGCGATCCCTCGCTCATGTGTTTTGGTT
CTTGGTAATGATCCTTCGCAGGTCACCCCACGGAAAGGATCTTTACCAGAACCAAAACCCTTG
AGCGGGGATCGCGCTCGCTCGGGCCATAAAACCGCCCCCCTCCGGCGAAGGGGAAAAAGCCCT
CGGGCGCGGGTTAAACAAGGAACCTCCCGGGGGAAGGACCCCAACTACATGCGTTGCGACCTC
GGGCGGGAAACTAAGGGGGGGGGGGTTCCCCCGGCCAACCCCCAACCCCCCCCCGGCAAAAAG
GATGGGGGGGGCCCCCCCCCCCCCTCCCTCCCCCCCCTCCCTCCCCCCGCCCGCGCCGGGGCG
GGGGGCGCAGCGAGACACAGAGAACAGAGAACAGGAAAGCACA

A consensus sequence was constructed using the forward and reverse sequences as follows:

>consensus sequence

GGGATCGCGCTCGCGCTGGGCTCGATAGACGCGCTAGCACCCGTCAGGCCCGGAGGGCGAACC
AGGCCCCTGGGGCCGGCAGGTTAAACCCAAGGTACCCATCCAAGGTGGACAGTCCTCCCAGAG
TTCGCCCTTCGGCGTCTGCGAGCCATCGGGGCATGGAACCTTGGGGGCTGTCGGTTTCCCGCT
GGGCATACGTCCGATGCCGCCTCGGGAGGCGAAACGTGGCTCGTGCAGCGTCGCAGCGTTGGT
CCCCCACTCACTCACCAAAACCCTCTGCGTACCTTGCCCACTTGCTTCCCCCGGCCCCCCCGG
CCGGGGGGCGACACCAAAAACCAAACTCTTTGCGATGAACCGACGTCTGAATGCCAAAAGCAA
AACAAAAACAGTTAAAACTTTCAACAACGGATCTCTTGGCTCCCACATCGATGAAGAACGCAG
CGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT
TGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATAAAACCCCCTCCCCCTCGG
ATTAAATGTTCCTTGGGGGGTATTGGCCAATGGGATTGCGAGATAGCAATTGTTACCCAGGCG
CCCAAATGCATCAGTACCCCGCCATTTACAGATTTACCAGCACCGAGGCCGTCGGGATCGCGC
TCGCGCTGGGCTCGATAGACGCGCTAGCACCCGTCAGGCCCGGAGGGCGAACCAGGCCCCTGG
GGCCGGCAGGTTAAACCCAAGGTACCCATCCAAGGTGGACAGTCCTCCCAGAGTTCGCCCTTC
GGCGTCTGCGAGCCATCGGGGCATGGAACCTTGGGGGCTGTCGGTTTCCCGCTGGGCATACGT
CCGATGCCGCCTCGGGAGGCGAAACGTGGCTCGTGCAGCGTCGCAGCGTTGGTCCCCCACTCA
CTCACCAAAACCCTCTGCGTACCTTGCCCACTTGCTTCCCCCGGCCCCCCCCGGCCGGGGGGCG
ACACCAAAAACCAAACTCTTTGCGATGAACCGACGTCTGAATGCCAAAAGCAAAACAAAAACA
GTTAAAACTTTCAACAACGGATCTCTTGGCTCCCACATCGATGAAGAACGCAGCGAAATGCGA
TAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCCCT
GGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATAAAACCCCCTCCCCCTCGGATTAAATGTT
CCTTGGGGGGTATTGGCCAATGGGATTGCGAGATAGCAATTGTTACCCAGGCGCCCAAATGCA
TCAGTACCCCGCCATTTACAGATTTACCAGCACCGAGGCCGTCAACCGTGGAGTTATGGGAAT
TATAGGCTTGCAGTAATATGCTCACCTCTCTCCATACGCAGGCGGCACACCGGGTAGAGTCGC
GGGCGTAAAGCGGAATCCGAAACGGCCCTCACGGGTAGAGGAGGATCCTTGCGGGCTAGTCAC
CCCTCATCACAATTTGACCTCGGATCAGGTA

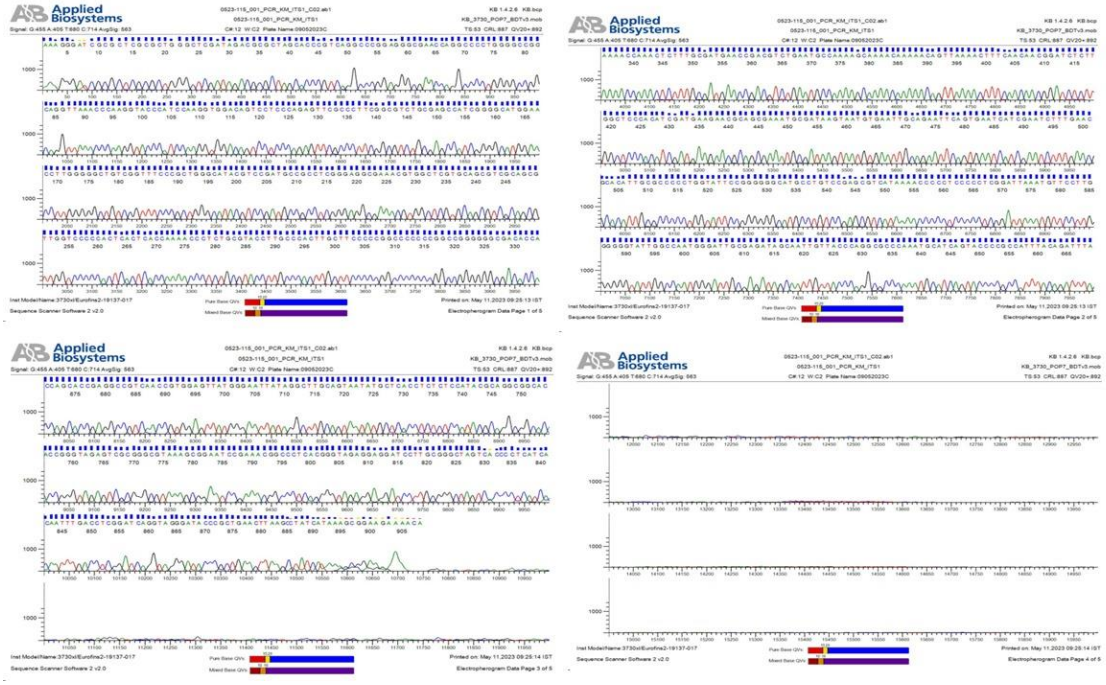


Fig. 6.3. Blast report ITS1

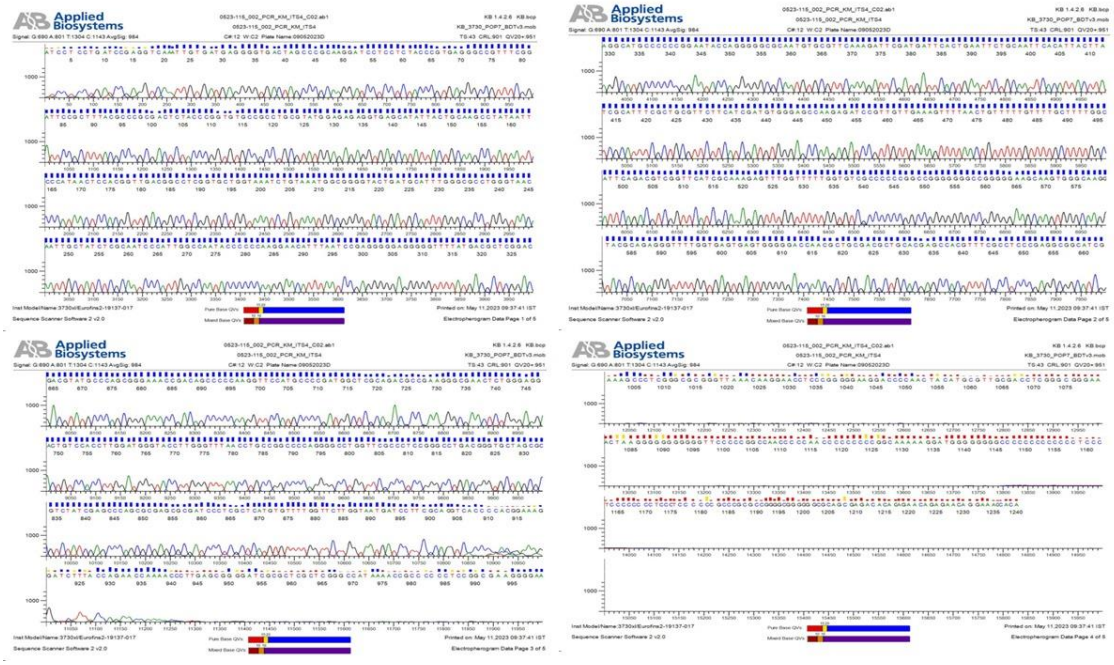


Fig. 6.4. Blast report ITS4

The above results indicate that the 1543bp consensus sequence obtained upon initial analysis using NCBI- nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi#) belonged to ***M. esculenta*** with 100% similarity.

6.1.2 Preparation of the mushroom extract

The mushroom mycelia dried, powdered and extracted in different solvents were analysed for the total obtained yields of the soluble components. Table.6.1 shows the yields obtained with different solvents.

Table 6.1. Extraction yields obtained with different solvents

Solvent	Dried extraction (gm)	Taken powder sample (gm)	Yield (%)
Methanol	3.369	25	13.47
Ethanol	5.632	25	22.52
Chloroform	0.653	25	2.61

The above results indicate that 13.47 % of the total weight of the dried extraction was composed of the bioactive compounds extracted from the mycelia using methanol. The extraction yield using ethanol was higher compared to methanol with a yield of 22.52

%, indicating that more bioactive compounds were extracted using ethanol as the solvent. The extraction yield using chloroform was the lowest among the three solvents with a yield of 2.61 %, suggesting that it was less efficient in extracting the bioactive compounds from the mycelia.

6.1.3 Qualitative phytochemical analysis

Table 6.2. Qualitative phytochemical analysis of Chloroform, Ethanolic and Methanolic extracts

Phytoconstituents	Chloroform extract	Ethanolic extract	Methanolic extract
Saponin	+	-	+
Tannin	-	-	+
Terpenoids	+	+	-
Steroids	-	-	-
Glycosides	+	+	-
Flavonoids	-	+	+
Alkaloids	+	-	+
Quinone	+	+	-
Phenol	+	+	+
Coumarin	-	+	-
Carbohydrate	+	+	+

The above table suggest that saponins, terpenoids, glycosides, alkaloids, phenol, quinine and carbohydrates were detected in the chloroform extract. Terpenoids, glycosides, flavonoids, quinine, phenol, coumarin and carbohydrates were detected in the ethanolic extract while saponin, tannin, flavonoids, alkaloids, phenol and carbohydrates were detected in the methanolic extract.

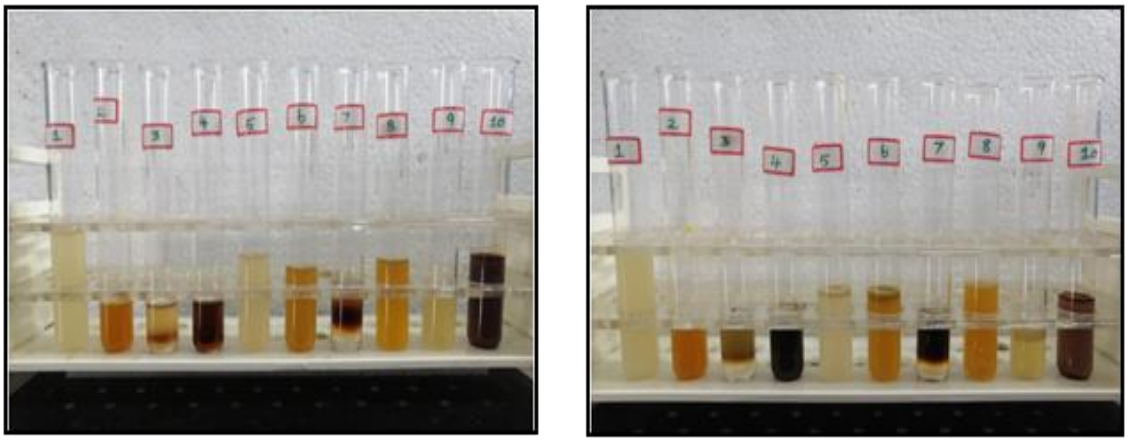


Fig. 6.5. Phytochemical analysis of ethanolic extract (left) and chloroform extract (right) of the *M. esculenta* sample

Saponins were detected in the chloroform and methanol extracts, indicating their presence in *M. esculenta*. Saponins are known for their foaming properties and have been associated with antimicrobial and anti-inflammatory activities [220]. However, saponins were absent in the ethanol extract, suggesting that ethanol may not be as efficient in extracting these specific compounds. Tannins were detected in the methanol extract, indicating their presence in *M. esculenta*. Both chloroform and ethanol extracts of *M. esculenta* showed the absence of tannins. Tannins are astringent compounds with antioxidant and antimicrobial properties, commonly found in plant materials [221]. Terpenoids were present in both chloroform and ethanol extracts. Terpenoids are a diverse group of compounds with various biological activities, including antimicrobial, antioxidant, and anti-inflammatory properties [222]. The presence of terpenoids in *M. esculenta* suggests that these compounds may contribute to its potential bioactivities. The absence of steroids was observed in chloroform, methanol and ethanol extracts. Steroids can have various physiological effects and are often associated with anti-inflammatory and hormone-like activities. The absence of steroids suggests that *M. esculenta* may not contain these specific compounds. Glycosides were present in both chloroform and ethanol extracts of *M. esculenta* . Glycosides are compounds formed by the combination of a sugar molecule with a non-sugar molecule and can have diverse biological activities, including cardiovascular effects and antimicrobial properties [223]. The presence of glycosides suggests their potential contribution to the bioactivity of *M. esculenta*. Flavonoids were absent in the chloroform extract but present in the methanol

and ethanol extracts. Flavonoids are known for their antioxidant, anti-inflammatory, and anticancer activities [224]. The presence of flavonoids in the methanol and ethanol extracts indicates its potential as a source of these bioactive compounds. Alkaloids were present in the chloroform and methanol extracts but absent in the ethanol extract. Alkaloids have diverse pharmacological activities, including analgesic and antimicrobial properties [224]. The presence of alkaloids in *M. esculenta* suggests their potential contribution to its bioactivity. Both chloroform and ethanol extracts of *M. esculenta* exhibited the presence of quinones. Quinones have diverse biological activities, including antimicrobial and antitumor properties [225]. The presence of quinones suggests their potential contribution to the bioactivity of *M. esculenta*.

Coumarins were absent in both chloroform and ethanol extracts but present in the methanol extract suggesting that *M. esculenta* contains these specific compounds. Phenols were present in all three extracts suggesting their potential role in antioxidant effect, oxidative stress and health benefits of *M. esculenta* [226]. Coumarins are known for their anticoagulant, antimicrobial, and anticancer properties [227] and their presence suggests such properties of the mushroom. Carbohydrates were also present in all the three extracts. We are also found that polysaccharides were reported to show the antidiabetic activity [228,229].

6.1.4 Quantitative phenol estimation

The total phenol extraction yield was maximum for the ethanol extract; likewise, it was quite significant for all of the solvents used. The Folin-Ciocalteu method was utilized to calculate the total phenolic content of the sample. This was accomplished by developing a standard curve using gallic acid (GAE) while taking into account the correlation between absorbance and concentration. The calibration curve is created from the analysis of the standard (gallic acid) was linear with $\mathbf{y=0.0417x + 0.128; R^2=0.995}$

The quantitative phenol estimation result for *M. esculenta* indicates a phenol content of 587.5 mg GAE/g DE (gallic acid equivalent per gram of dry extract), 588.33 mg GAE/g DE and 780 mg GAE/g DE in methanol, ethanol and chloroform extracts respectively. This value represents the concentration of phenolic compounds present in the extract of *M. esculenta* (**Fig 6.6.**).

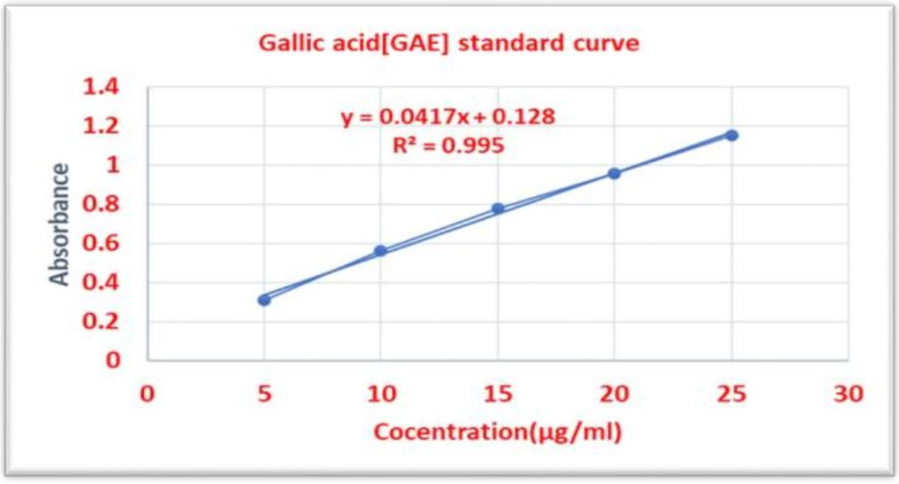


Fig 6.6. Calibration curve of gallic acid

6.1.5 Determination of antioxidants

6.1.5.1. ABTS (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay

The results of the ABTS assay performed for the three different solvent extracts of *Morchella esculents* are provided in Table 6.3. The standard compound i.e., ascorbic acid had shown 64.71% of free radical scavenging activity in 1 mg/ml concentration. Comparing the solvents, ethanol consistently exhibits higher percentage inhibition values than chloroform and methanol across all concentrations. This indicates that ethanol is more effective in extracting antioxidant compounds from *M. esculenta* compared to the other solvents tested. At the highest concentration (1.0 mg), ethanol shows the highest percentage inhibition, with values of 50.28%. This suggests that the ethanol extract of *M. esculenta* at this concentration possesses significant antioxidant activity, potentially due to the presence of phenolic compounds, flavonoids, or other bioactive components known for their antioxidant properties. The chloroform, ethanol, and methanol extracts all had IC50 values of 1.24 mg/ml, 1.04 mg/ml, and 1.48 mg/ml, respectively, according to the ABTS assay. Stronger antioxidant activity is indicated by lower IC50 values in this experiment. Thus, out of the three extracts tested, the ethanol one shows the strongest antioxidant activity, surpassing both the chloroform and methanol ones, with an IC50 of 1.04 mg/ml.These results are coherent with the earlier

reports of high ABTS scavenging activity in natural products, including plant extracts [230].

Table 6.3. ABTS assay

Concentration	Standard	Percentage inhibition		
mg/ml	Ascorbic acid	Chloroform extract	Ethanolic extract	Methanolic extract
0.2	39.24 ± 1.2	8.83±1.07	7.64±0.81	2.42±0.65
0.4	42.36 ± 0.62	13.2±0.6	16.19±0.81	10.92±1
0.6	46.6 ± 0.91	22.36±0.83	24.98±0.78	16.67±1.16
0.8	59.84 ± 0.88	30.58±1.11	37.75±0.95	25.12±1.12
1.0	64.71 ± 0.66	42.31±0.74	50.38±0.65	35.09±1.2

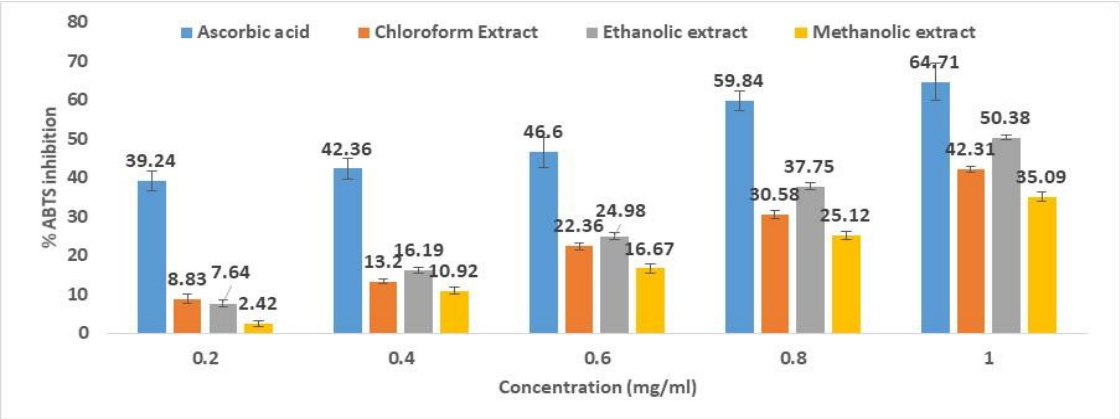


Fig 6.7. Graphical representation ABTS assay performed with chloroform ethanolic and methanolic extracts respectively; Ascorbic acid used as standard

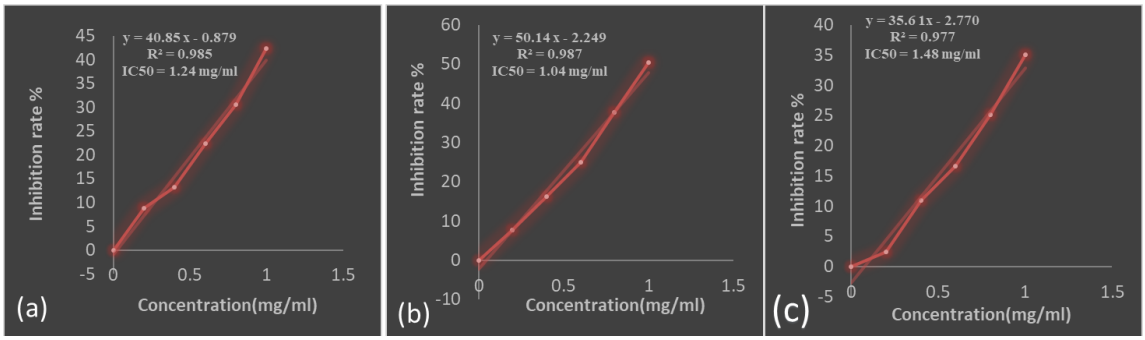


Fig 6.8. Inhibition rates and calculation of IC_{50} values for ABTS assay performed with chloroform (a), ethanolic (b) and methanolic (c) extracts respectively

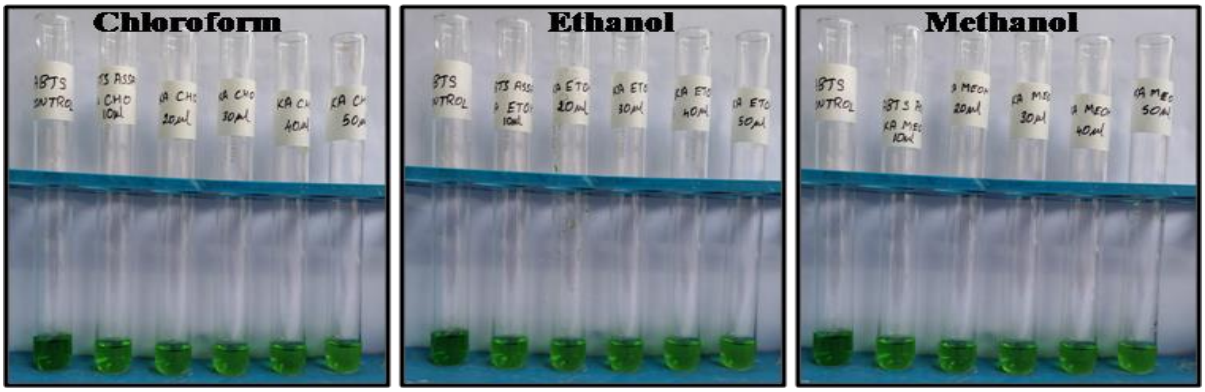


Fig 6.9. ABTS assay

6.1.5.2. DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical Scavenging assay

The percentage inhibition of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical by *M. esculenta* extracts of chloroform, ethanol, and methanol at different concentrations is provided in Table 6.4. The standard compound i.e., ascorbic acid had shown 80.71% of free radical scavenging activity in 1 mg/ml concentration. From the results, it can be observed that the percentage inhibition of the DPPH radical increases as the concentration of the *M. esculenta* samples rises for all three solvents. This suggests that the samples possess antioxidant properties and can effectively neutralize the DPPH radical. The standard had shown highest inhibition rate in 1mg/ml concentration of 80.61 %. When comparing the different extracts, chloroform extracts consistently demonstrates higher percentage inhibition values than ethanol and methanol extracts respectively, across most concentrations. However, at the highest concentration (1.0 mg), ethanol extract displays the highest percentage inhibition values among the three solvents, with values of 18.06 %. The chloroform, ethanol, and methanol extracts had IC50 values of 0.93 mg/ml, 0.78 mg/ml, and 2.62 mg/ml, respectively, according to the DPPH assay. Stronger antioxidant activity is indicated by lower IC50 values in this experiment. Thus, out of the three extracts tested, the ethanol one shows the strongest antioxidant activity, followed by the chloroform and methanol ones, with an IC50 of 0.78 mg/ml. These results are coherent with the earlier reports of higher scavenging activities in chloroform and ethanol extracts of mushrooms [231].

Table 6.4. DPPH assay

Concentration	Standard	Percentage of Inhibition		
mg/ml	Ascorbic acid	Chloroform extract	Ethanolic extract	Methanolic extract
0.2	35.24 ± 1.02	16.51 ± 0.74	6.74 ± 1.13	1.85 ± 0.38
0.4	48.36 ± 0.57	22.66 ± 0.71	21.14 ± 0.84	5.2 ± 0.32
0.6	52.6 ± 0.71	30.35 ± 0.88	39.76 ± 0.47	8.64 ± 0.5
0.8	66.84 ± 0.63	38.58 ± 1.02	50.46 ± 0.84	14.45 ± 0.32
1.0	80.71 ± 0.42	57.67 ± 0.95	65.74 ± 0.91	19.24 ± 0.49

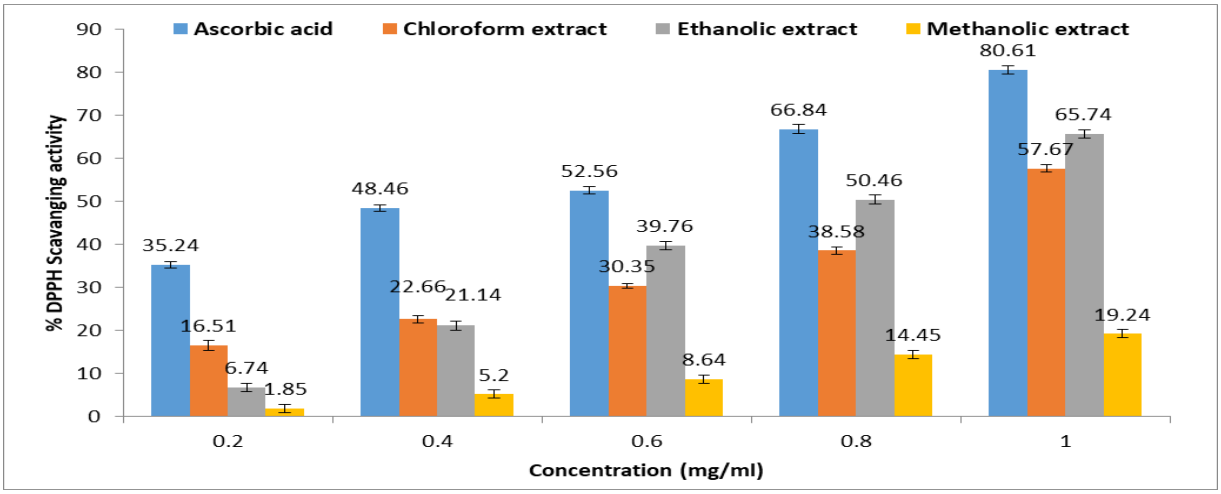


Fig 6.10. Graphical representation DPPH assay performed with chloroform ethanolic and methanolic extracts respectively; Ascorbic acid used as standard

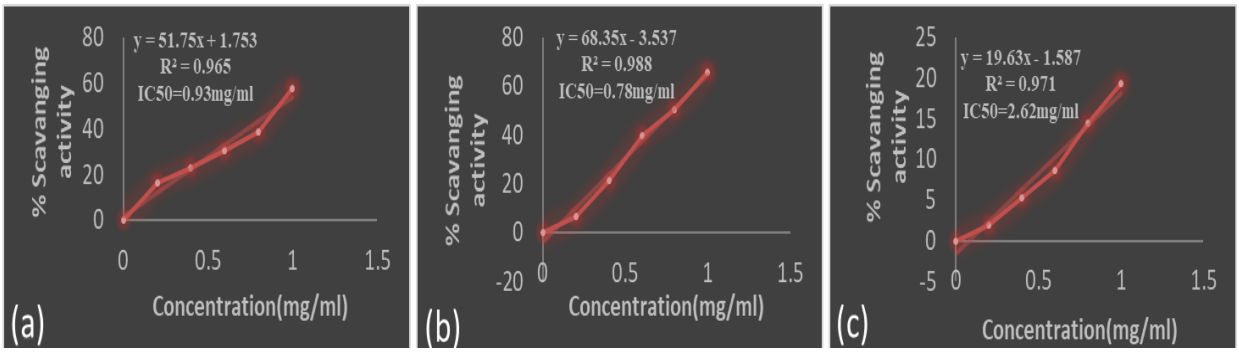


Fig 6.11. Scavenging activity and calculation of IC_{50} values for DPPH assay performed with chloroform (a), ethanolic (b) and methanolic (c) extracts respectively

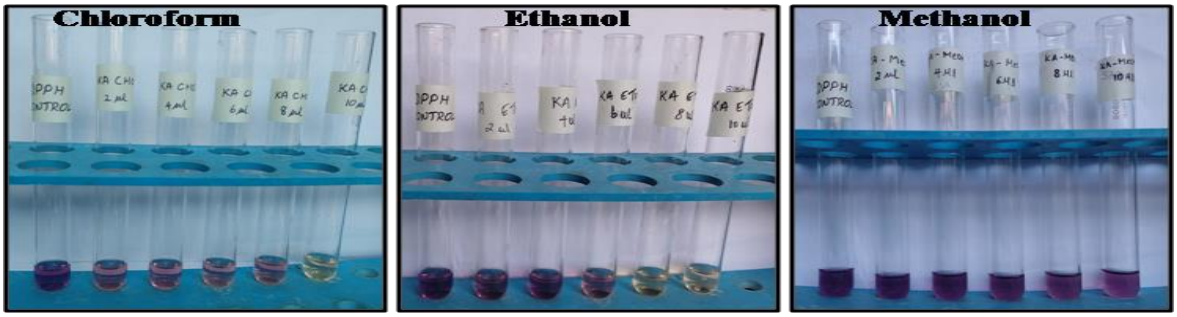


Fig 6.12. DPPH assay

6.1.5.3. Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging activity of various extracts of *M. esculenta* at different concentrations are represented in Table 6.5. The standard compound i.e., ascorbic acid had shown 70.1% of free radical scavenging activity in 1 mg/ml concentration. The percentage inhibition of hydrogen peroxide is used as an indicator of the antioxidant activity of the extracts. H2O2 scavenging ability exhibited the order (ethanol > chloroform > methanol).The results demonstrate that as the concentration of the *M. esculenta* extracts increases, the percentage inhibition of hydrogen peroxide also increases for all three solvents. This indicates that the extracts possess the ability to scavenge hydrogen peroxide and act as antioxidants. Comparing the solvents ethanol consistently shows higher percentage inhibition values than chloroform and methanol at most concentrations. This suggests that the ethanol extract of *M. esculenta* has stronger hydrogen peroxide scavenging activity compared to the other solvents. This finding is supported by a previous study conducted by Zhang et al. [232], where ethanol extracts of *M. esculenta* displayed significant hydrogen peroxide scavenging activity.

At the highest concentration tested (1.0 mg), the ethanol extract exhibits the highest percentage inhibition of hydrogen peroxide, with a value of 46.15%. This indicates that the ethanol extract contains bioactive components that possess potent hydrogen peroxide scavenging capabilities.

In the hydrogen peroxide scavenging assay, the chloroform, ethanol, and methanol extracts respectively had IC50 values of 1.26 mg/ml, 1.09 mg/ml, and 3.27 mg/ml. More efficient hydrogen peroxide scavenging is indicated by lower IC50 values in this experiment. Thus, out of the three extracts tested, the ethanol one shows the strongest

hydrogen peroxide scavenging activity, followed by the chloroform one and the methanol one, with an IC50 of 1.09 mg/ml.

These findings align with the study conducted by Nithi et al. [233], highlighting the potential antioxidant properties of *M. esculenta* extracts. However, further research is required to identify and characterize the specific antioxidant compounds responsible for the observed activity.

Table 6.5. Hydrogen peroxide scavenging activity

Concentration	Standard	Percentage of Inhibition		
mg/ml	Ascorbic acid	Chloroform extract	Ethanolic extract	Methanolic extract
0.2	39.24 ± 0.91	16.03 ± 0.6	15.17 ± 1.67	3.85 ± 0.61
0.4	44.36 ± 0.32	20.3 ± 1.36	18.59 ± 1.51	5.13 ± 0.3
0.6	51.6 ± 0.42	26.92 ± 1.21	26.92 ± 1.81	9.83 ± 0.46
0.8	57.84 ± 0.17	32.69 ± 1.51	37.18 ± 1.81	11.54 ± 0.3
1.0	70.1 ± 0.51	38.89 ± 1.52	46.15 ± 1.21	15.81 ± 0.46

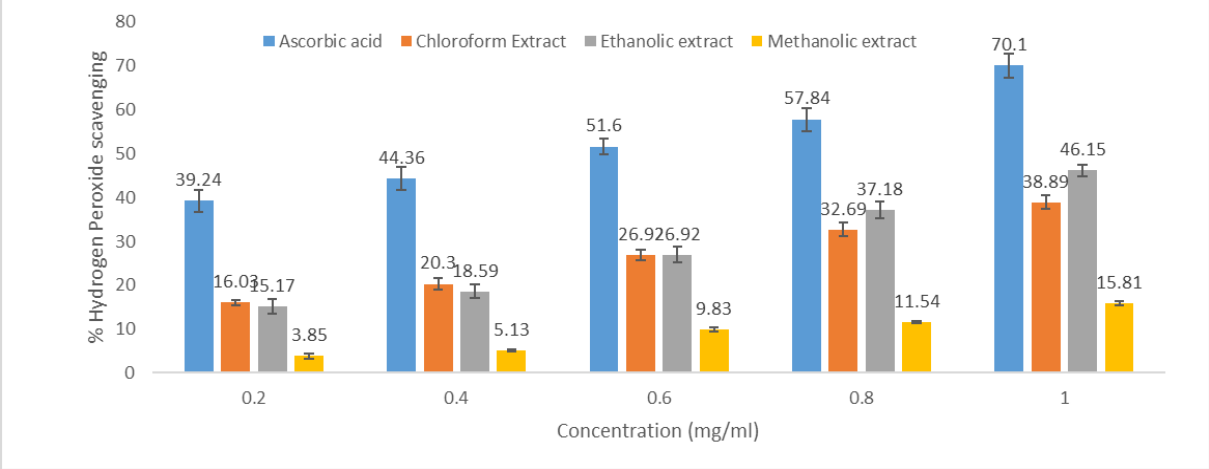


Fig 6.13. Graphical representation hydrogen peroxide scavenging assay performed with chloroform ethanolic and methanolic extracts respectively; Ascorbic acid used as standard

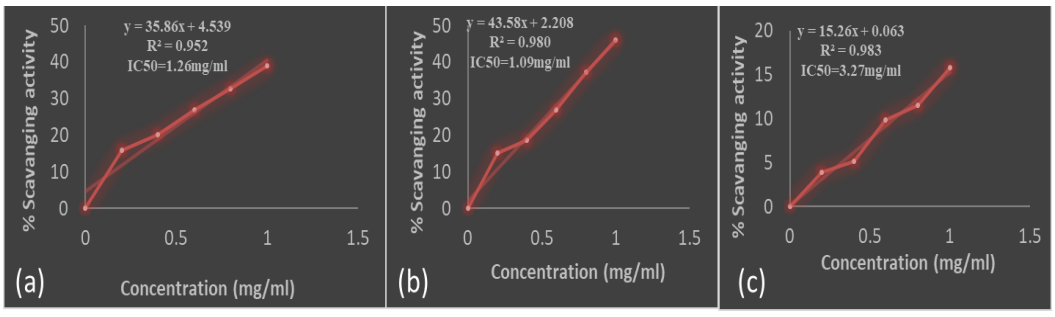


Fig 6.14. Scavenging activity and calculation of IC_{50} values for hydrogen peroxide scavenging assay performed with chloroform (a), ethanolic (b) and methanolic (c) extracts respectively

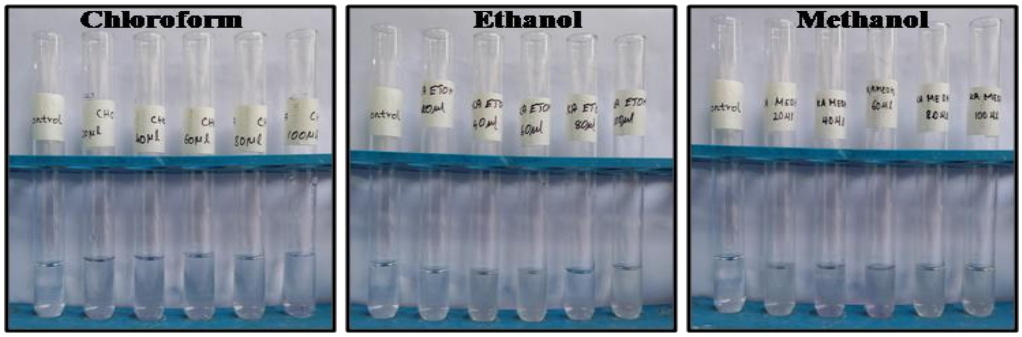


Fig 6.15. Hydrogen peroxide scavenging assay

6.1.5. Lipid peroxidation Assay

The lipid peroxidation assay of various extracts of *M. esculenta* at different concentrations are represented in Table 6.6. The standard compound i.e., ascorbic acid had shown 75.76% inhibition activity in 1 mg/ml concentration. The percentage inhibition values indicate the ability of the extracts to inhibit lipid peroxidation, which is a process that leads to the generation of reactive oxygen species and can cause damage to cellular membranes. Lipid peroxidation inhibition ability exhibited the order (ethanol> chloroform >methanol). The results demonstrate that as the concentration of the *M. esculenta* extracts increases, the percentage inhibition of lipid peroxidation also increases for all three solvents. This suggests that the extracts possess antioxidant properties and can effectively inhibit lipid peroxidation. Comparing the solvents, ethanol consistently shows higher percentage inhibition values than chloroform and methanol at most concentrations. This indicates that the ethanol extract of *M. esculenta* exhibits stronger inhibitory activity against lipid peroxidation compared to the other solvents. At the highest concentration tested (1.0 mg), the ethanol extract demonstrates the highest percentage inhibition of lipid peroxidation, with a value of 43.18 %.

The chloroform, ethanol, and methanol extracts each have an IC50 value of 1.37 mg/ml, 1.18 mg/ml, and 1.74 mg/ml, respectively, according to the results of the lipid peroxidation assay. In this particular assay, IC50 values that are lower indicate that the suppression of lipid peroxidation is more efficient. In light of this, the ethanol extract,

which has an IC50 value of 1.18 mg/ml, exhibits the most robust prevention of lipid peroxidation when compared to the chloroform and methanol extracts.

This indicates that the ethanol extract contains bioactive components that are particularly effective in inhibiting lipid peroxidation and protecting against oxidative damage to cell membranes. These results highlight the potential of *M. esculenta* as a natural source of antioxidants.

Table 6.6. Lipid Peroxidation Assay

Concentration	Standard	Percentage of Inhibition		
mg/ml	Ascorbic acid	Chloroform extract	Ethanolic extract	Methanolic extract
0.2	40.25 ± 1.02	10.12 ± 0.81	7.83 ± 0.55	6.82 ± 1.07
0.4	52.41 ± 0.68	13.58 ± 1.23	13.64 ± 0.36	13.38 ± 0.55
0.6	55.63 ± 0.79	22.22 ± 1.4	21.46 ± 0.89	17.42 ± 0.36
0.8	67.79 ± 0.64	28.64 ± 0.88	33.59 ± 0.74	22.73 ± 0.71
1.0	75.76 ± 0.58	37.53 ± 1.32	43.94 ± 0.71	29.04 ± 0.55

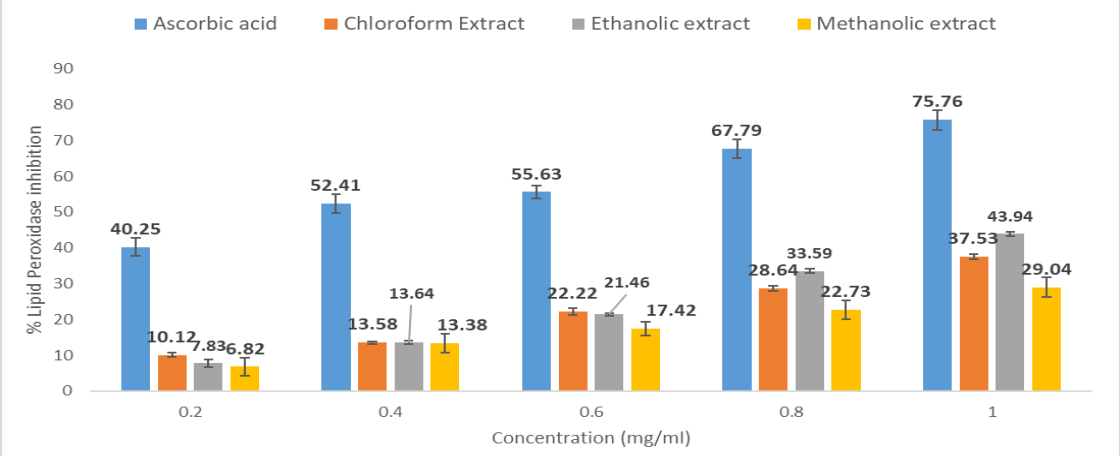


Fig 6.16. Graphical representation lipid peroxidation assay performed with chloroform ethanolic and methanolic extracts respectively; Ascorbic acid used as standard

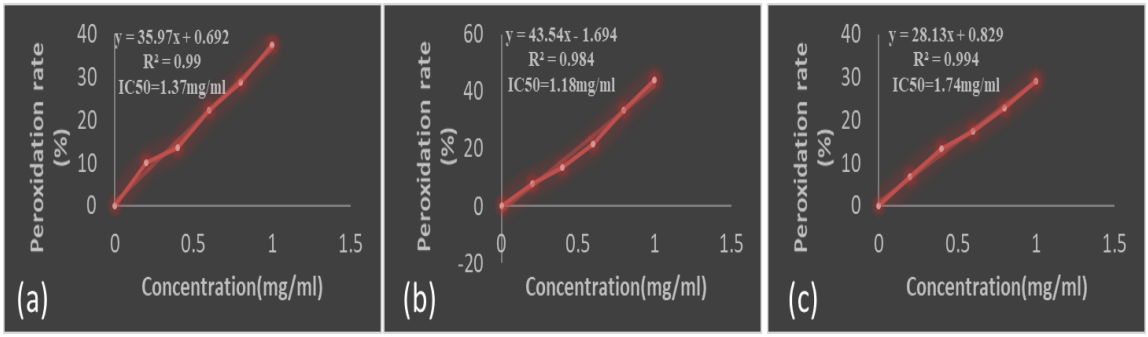


Fig 6.17. Peroxidation rates and calculation of IC_{50} values for lipid peroxidation performed with chloroform (a), ethanolic (b) and methanolic (c) extracts respectively

Fig 6.18. lipid peroxidation assay

6.1.5.5. Nitric Oxide Scavenging Assay

The nitric Oxide Scavenging assay of various extracts of *M. esculenta* at different concentrations are represented in table 6.7. The standard compound i.e., ascorbic acid had shown 66.13 % inhibition activity in 1 mg/ml concentration. The percentage inhibition values indicate the ability of the extracts to scavenge or neutralize nitric oxide, which is a reactive nitrogen species involved in various physiological processes and can contribute to oxidative stress and inflammation when present in excess. Nitric oxide scavenging ability exhibited the order (ethanol> chloroform >methanol).The results demonstrate that as the concentration of *M. esculenta* extracts increases, the percentage inhibition of nitric oxide scavenging also increases for all three extracts. This suggests that the extracts possess nitric oxide scavenging activity and can potentially mitigate the harmful effects of nitric oxide. Comparing the extracts, ethanol consistently shows higher percentage inhibition values compared to chloroform and methanol at most concentrations. This indicates that the ethanol extract of *M. esculenta* exhibits stronger

nitric oxide scavenging activity compared to the other solvents. This finding suggests that the ethanol extract contains bioactive compounds that are effective in neutralizing nitric oxide and reducing its potential damaging effects. At the highest concentration tested (1.0 mg), the ethanol extract demonstrates the highest percentage inhibition of nitric oxide, with a value of 25.34%.

According to the results of the nitric oxide scavenging assay, the chloroform, ethanol, and methanol extracts each had an IC50 value of 2.01 mg/ml, 1.47 mg/ml, and 3.2 mg/ml, respectively. In this particular assay, IC50 values that are lower imply that nitric oxide is being scavenged more effectively. As a result, the ethanol extract, which has an IC50 value of 1.47 mg/ml, demonstrates the most powerful nitric oxide scavenging action among the three extracts.

This indicates that the ethanol extract of *M. esculenta*, when used at this concentration, exhibits significant nitric oxide scavenging activity.

Table 6.7. Nitric Oxide Scavenging Assay

Concentration	Standard	Percentage of Inhibition		
mg/ml	Ascorbic acid	Chloroform extract	Ethanolic extract	Methanolic extract
0.2	27.29 ± 1.4	5.14 ± 0.32	6.68 ± 1.09	1.66 ± 0.73
0.4	34.31 ± 0.51	9.53 ± 0.56	11.19 ± 0.81	6.96 ± 0.57
0.6	41.46 ± 0.78	12.69 ± 0.52	15.18 ± 0.77	8.9 ± 0.37
0.8	57.72 ± 0.67	17.69 ± 0.57	27.28 ± 1.21	11.87 ± 0.4
1.0	66.13 ± 0.74	27.05 ± 0.85	35.5 ± 0.96	14.84 ± 0.81

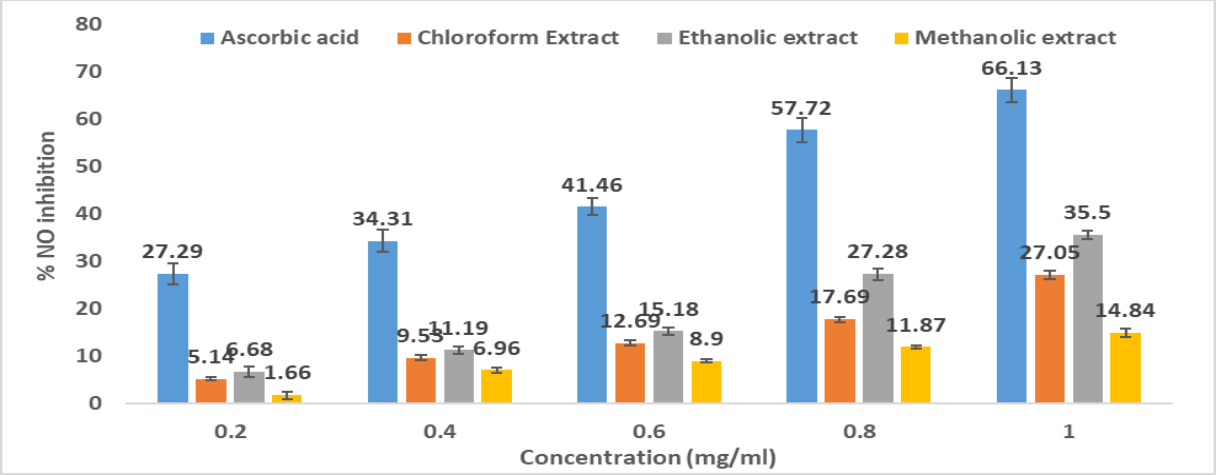


Fig 6.19. Graphical representation nitric oxide scavenging assay performed with chloroform ethanolic and methanolic extracts respectively; Ascorbic acid used as standard

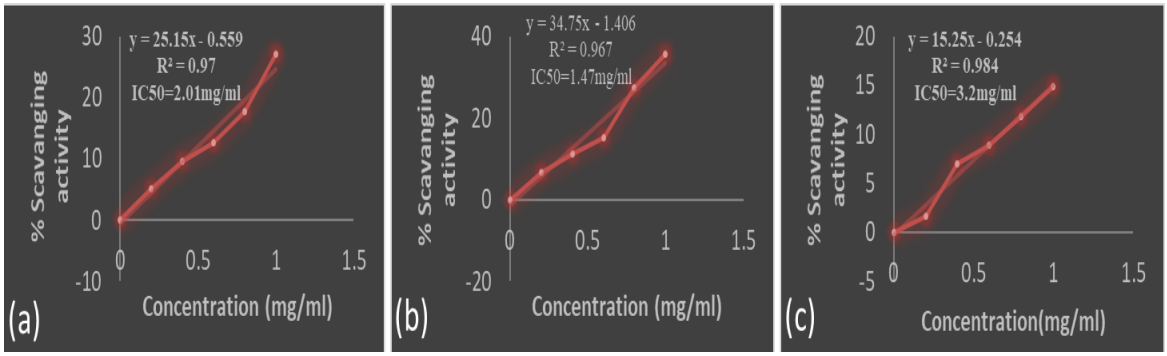


Fig 6.20. Scavenging activity and calculation of IC_{50} values for nitric oxide scavenging assay performed with chloroform (a), ethanolic (b) and methanolic (c) extracts respectively

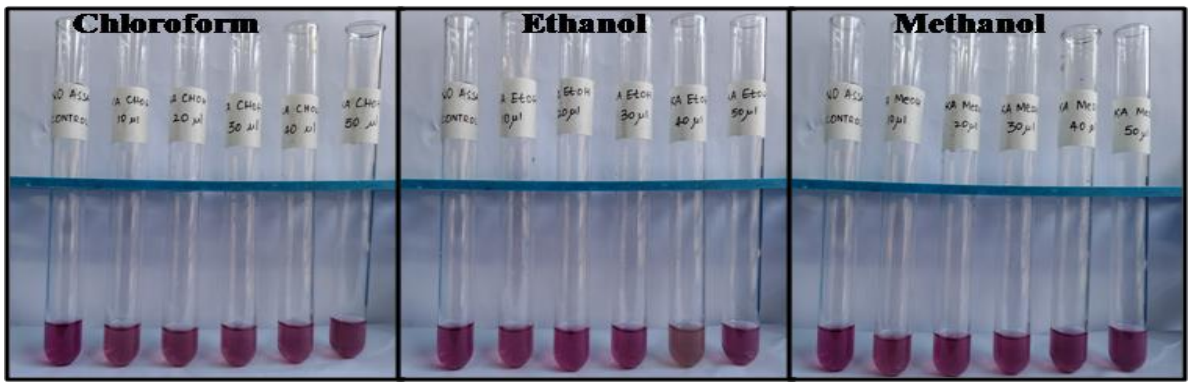


Fig 6.21. nitric oxide scavenging assay

6.1.6. α -glucosidase inhibition assay

The α -glucosidase inhibition assay of various extracts of *M. esculenta* at different concentrations were carried out to the results are shown in table 6.8. The standard i.e., acarbose had shown highest inhibition rate of 76.79 % in 1 mg/ml concentration. The percentage inhibition values indicate the ability of the extracts to inhibit the activity of the α glucosidase enzyme, which is responsible for the breakdown of complex carbohydrates into glucose. α-glucosidase inhibition ability exhibited the order (ethanol > methanol > chloroform). The results show that as the concentration of *M. esculenta* extracts increases, the percentage inhibition of α glucosidase activity also increases for all three solvents. This suggests that the extracts possess inhibitory activity against the α-glucosidase enzyme and have the potential to modulate carbohydrate digestion and glucose absorption. Comparing the extracts, ethanol consistently demonstrates higher percentage inhibition values compared to chloroform and methanol at most concentrations. This indicates that the ethanol extract of *M. esculenta* exhibits stronger inhibitory activity against α -glucosidase compared to the other solvents. This finding suggests that the ethanol extract contains bioactive compounds that are effective in inhibiting the activity of the α -glucosidase enzyme. At the highest concentration tested (1.0 mg), the ethanol extract exhibits the highest percentage inhibition of α glucosidase activity, with a value of 49.06%. This indicates that the ethanol extract of *M. esculenta*, when used at this concentration, shows significant inhibitory activity against the α glucosidase enzyme.

As a result of conducting the α-glucosidase inhibition assay, the chloroform, ethanol, and methanol extracts were found to have IC50 values of 1.61 mg/ml, 1.06 mg/ml, and 1.31 mg/ml, respectively. In this particular assay, IC50 values that are lower imply that the inhibition of α-glucosidase activity is very effective. As a result, the ethanol extract, which has an IC50 value of 1.06 mg/ml, demonstrates the most powerful inhibition of α-glucosidase among the three extracts.

Inhibition of α-glucosidase has been known to slow down carbohydrate metabolism and thus manage diabetes [234]. These findings indicate the potential of *M. esculenta* as a natural source of compounds with potential antidiabetic properties by modulating carbohydrate digestion and glucose absorption.

Table 6.8. α -glucosidase inhibition assay

Concentration	Standard	Percentage of Inhibition		
mg/ml	Acarbose	Chloroform extract	Ethanolic extract	Methanolic extract
0.2	31.29 ± 0.33	5.24 ± 0.53	10.48 ± 0.53	6.99 ± 0.44
0.4	48.23 ± 0.74	9.36 ± 0.64	17.23 ± 0.53	14.36 ± 0.44
0.6	64.57 ± 0.89	18.6 ± 0.83	27.34 ± 0.35	21.47 ± 0.83
0.8	70.75 ± 0.48	24.84 ± 0.62	34.96 ± 0.56	31.21 ± 0.56
1.0	49.43 ± 0.69	30.71 ± 0.76	49.43 ± 0.63	37.83 ± 0.35

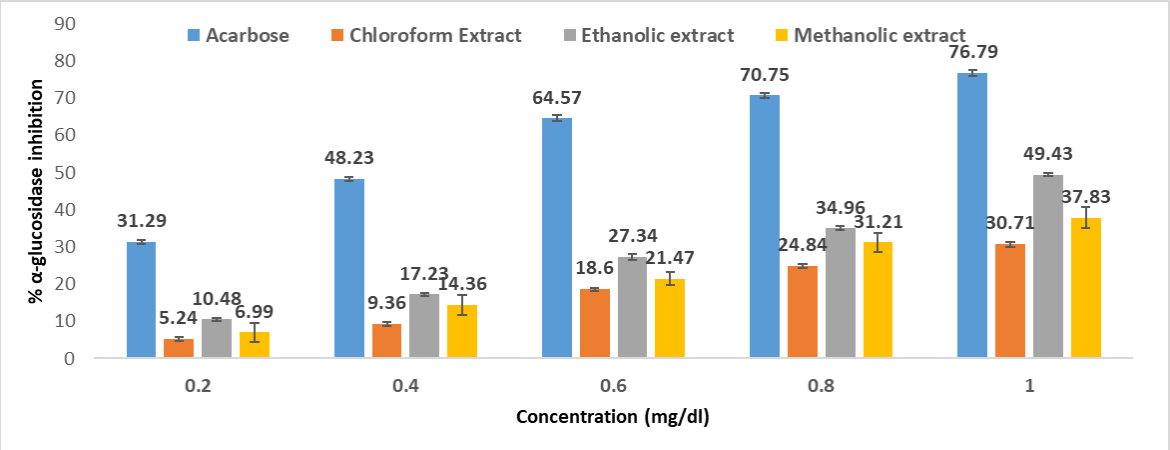


Fig 6.22. Graphical representation α -glucosidase inhibition assay performed with chloroform ethanolic and methanolic extracts respectively; Acarbose acid used as standard

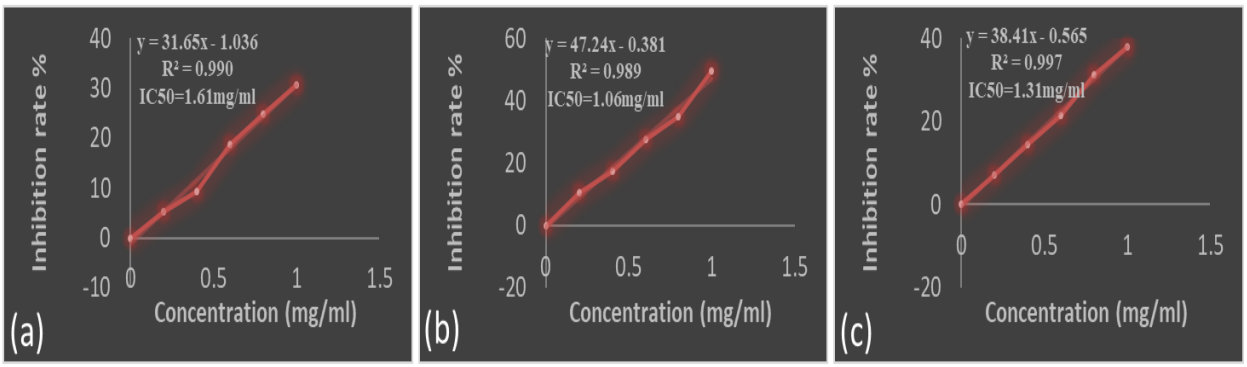


Fig 6.23. Inhibition rates and calculation of IC_{50} values for α -glucosidase inhibition assay performed with chloroform (a), ethanolic (b) and methanolic (c) extracts respectively

6.1.7. α -Amylase inhibitory assay

The α-amylase inhibitory assay of various extracts of *M. esculenta* at different concentrations were calculated are shown in Table 6.9. Acarbose has used as standard and it show the highest inhibition rate of 68.71%. However, Highest inhibition rate had been shown in ethanolic extract (50.4%±1.14, IC_{50} =1.0384mg/ml), followed by

chloroform extract (39.67%±1.44, IC_{50}=1.33 mg/ml), and methanolic extract (33.95%±1.12, IC_{50}=1.416 mg/ml), in a concentration dependent manner. The percentage inhibition values indicate the ability of the extracts to inhibit the activity of the α amylase enzyme, which is responsible for the breakdown of starch into simpler sugars. Comparing the extracts, ethanol consistently demonstrates higher percentage inhibition values compared to chloroform and methanol at most concentrations. This indicates that the ethanol extract of *M. esculenta* exhibits stronger inhibitory activity against α-amylase compared to the other solvents. This finding suggests that the ethanol extract contains bioactive compounds that are effective in inhibiting the activity of the α-amylase enzyme. At the highest concentration tested (1.0 mg), the ethanol extract exhibits the highest percentage inhibition of α amylase activity, with a value of 50.4%. This indicates that the ethanol extract of *M. esculenta* , when used at this concentration, shows significant inhibitory activity against the α amylase enzyme.

The α-amylase inhibition experiment yielded IC50 values of 1.33 mg/ml for the chloroform extract, 1.03 mg/ml for the ethanol extract, and 1.41 mg/ml for the methanol extract, respectively. More efficient suppression of α-amylase activity is indicated by lower IC50 values in this experiment. The ethanol extract shows the strongest inhibition of α-amylase among the three extracts, with an IC50 of 1.03 mg/ml.

The results suggest that *M. esculenta* extracts possess α-amylase inhibitory activity, with the ethanol extract showing the highest activity among the solvents tested. A significant reduction in the postprandial increase in blood glucose can be achieved by inhibiting the activity of the α-amylase enzymes involved in the digestion of carbohydrates; this may be an essential technique in the control of blood glucose level in type 2 diabetic and borderline patients. These findings indicate the potential of *M. esculenta* as a natural source of compounds with potential anti-diabetic properties by modulating carbohydrate digestion and glucose release. Further research is required to identify and characterize the specific bioactive compounds responsible for the observed activity.

Table 6.9. α -amylase inhibitory assay

Concentration	Standard	Percentage of Inhibition		
mg/ml	Acarbose	Chloroform extract	Ethanolic extract	Methanolic extract
0.2	38.24 ± 1.12	9.94 ± 1.78	13.81 ± 1.31	6.42 ± 1.82
0.4	44.36 ± 0.57	15.22 ± 1.16	21.46 ± 0.94	12.58 ± 1.09
0.6	51.6 ± 0.89	22.34 ± 1.72	28.94 ± 1.27	19.17 ± 2.03
0.8	57.84 ± 0.54	29.02 ± 2.2	37.55 ± 1.28	26.73 ± 1
1.0	68.71 ± 1.36	39.67 ± 1.44	50.4 ± 1.14	33.95 ± 1.12

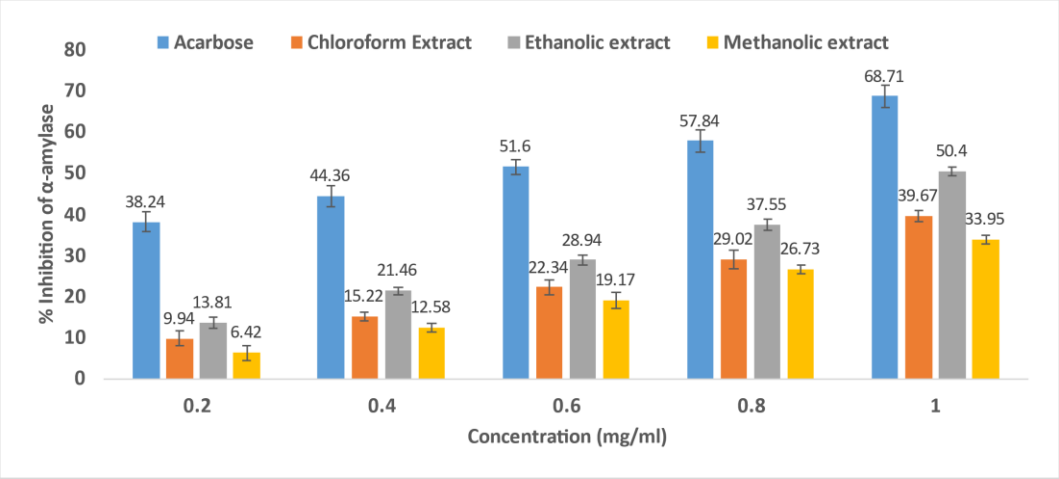


Fig 6.24. Graphical representation α -amylase inhibition assay performed with chloroform ethanolic and methanolic extracts respectively; Acarbose acid used as standard

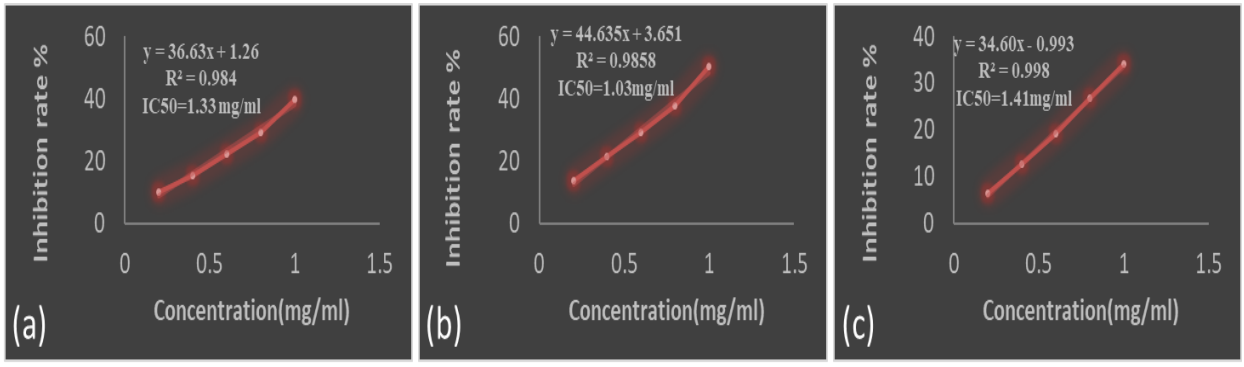


Fig 6.25. Inhibition rates and calculation of IC_{50} values for α -amylase inhibition assay performed with chloroform (a), ethanolic (b) and methanolic (c) extracts respectively

6.1.8. Glucose uptake assay

The glucose uptake assay was performed on L6 myotubes cell lines using various extracts of *M. esculenta* at different concentrations (Table 6.10). The results also include

a positive control group treated with insulin, a known stimulator of glucose uptake. The percentage of glucose uptake represents the amount of glucose taken up by the L6 myotubes cell lines under different treatment conditions. The control group (untreated) exhibited a baseline glucose uptake of approximately 33.70%, which was comparable across all three solvent extracts. When the L6 myotubes cell lines were treated with *Morchella* extracts at concentrations of 25 µg/ml, 50 µg/ml, and 100 µg/ml, an increase in glucose uptake was observed compared to the control group. This indicates that the *Morchella* extracts have the potential to enhance glucose uptake in the L6 myotubes cell lines. Comparing the three solvent extracts, the ethanol and methanol extracts consistently demonstrated higher percentages of glucose uptake compared to the chloroform extract at all concentrations tested. This suggests that the ethanol and methanol extracts contain bioactive compounds that can stimulate glucose uptake in the L6 myotubes cell lines more effectively.

Concentration	**Standard**	**Percentage**		
µg/ml	**Insulin**	**Chloroform extract**	**Ethanolic extract**	**Methanolic extract**
0	33.97 ± 1.46	33.67 ± 0.80	33.67 ± 0.80	33.67 ± 0.80
25	60.60 ± 0.92	37.67 ± 1.11	39.77 ± 0.85	39.90 ± 1.41
50	70.50 ± 0.80	41.93 ± 0.85	45.83 ± 1.22	46.37 ± 1.05
100	89.73 ± 0.91	46.80 ± 0.98	50.73 ± 0.80	51.50 ± 0.95

Table 6.10. Glucose Uptake assay

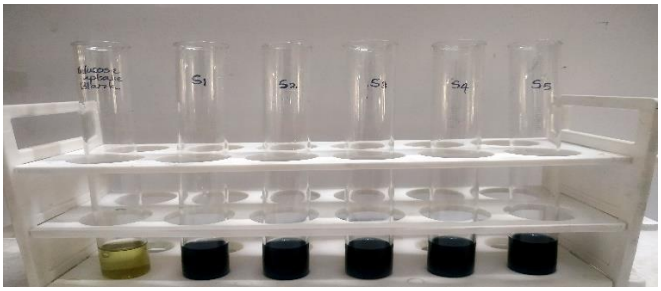


Fig 6.26. Glucose uptake assay-samples for standard curve

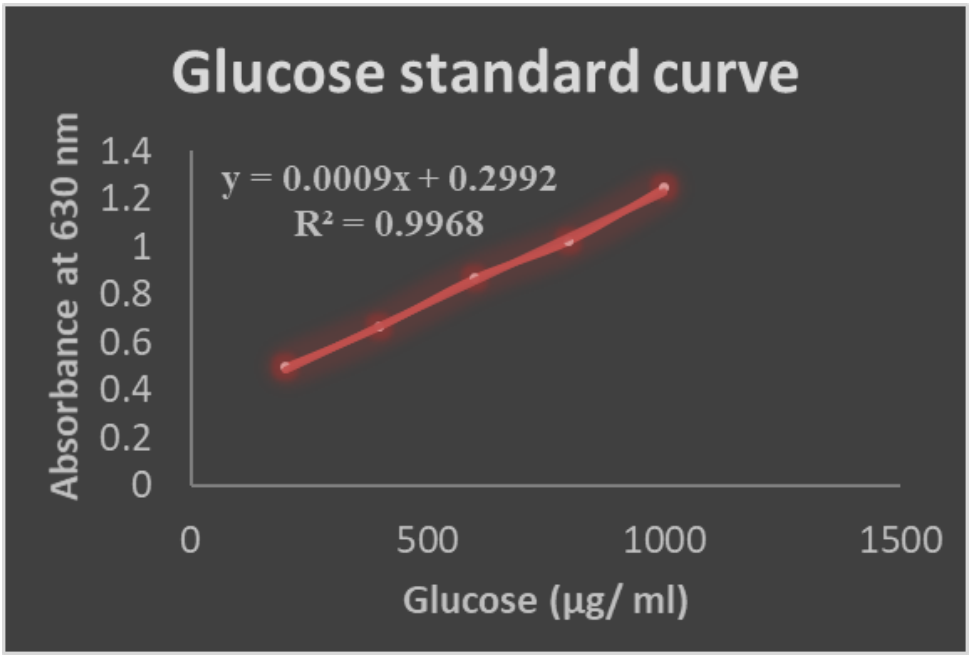


Fig 6.27. Standard curve for the glucose uptake assay

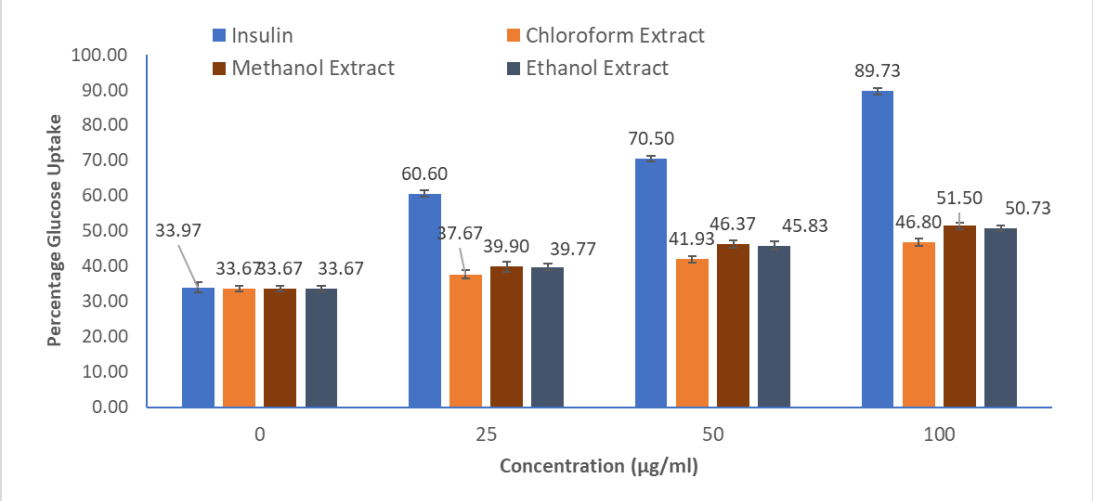


Fig 6.28. Graphical representation glucose uptake assay performed with chloroform ethanolic and methanolic extracts respectively

6.1.9. Isolation of pure molecules using column chromatography

The methanolic extract demonstrated the most significant glucose uptake activity. Subsequently, we proceeded to isolate a pure compound from the methanolic extract. Column chromatography is a common tool for fractionation. This technique allows for the isolation of individual compounds by sorting them into categories based on their binding strengths to the column's stationary phase. To achieve this, we employed column

chromatography. A total of 12 set of fractions were obtained after elution which were pooled based on the similarity of spots obtained on a TLC plate. These fractions were named as A, B, C, D, E, F, G, H, I, J, K, and L. Re-chromatography was performed on these fractions as they seemed to be a mixture of compounds. To verify the presence of polysaccharides, qualitative tests were carried out following the isolation process. The purpose of these assays was to offer preliminary proof that the isolated material contained the necessary components, specifically polysaccharide since polysaccharides/ carbohydrates are responsible for antidiabetic activity [229,235]. *M. esculenta* exopolysaccharide has been identified as an anti-diabetic agent [236]. Fractionation is frequently accomplished by column chromatography. Specific compounds can be isolated using this method, which divides substances according to how differently they bind to the stationary phase in the column. We continued with the 12 fractions, and the results of the polysaccharide quantitative test (Molish test) indicated that the tenth fraction was positive. The 10th (J) portion was then subjected to quantitative examination using Mass spectrometry, FTIR, and C-NMR for the identification of compound.

6.1.10. Spectral Analysis of the isolated compound

6.1.10.1. Fourier-transform infrared spectroscopy (FTIR)

The FTIR data obtained is represented in figure 6.29.

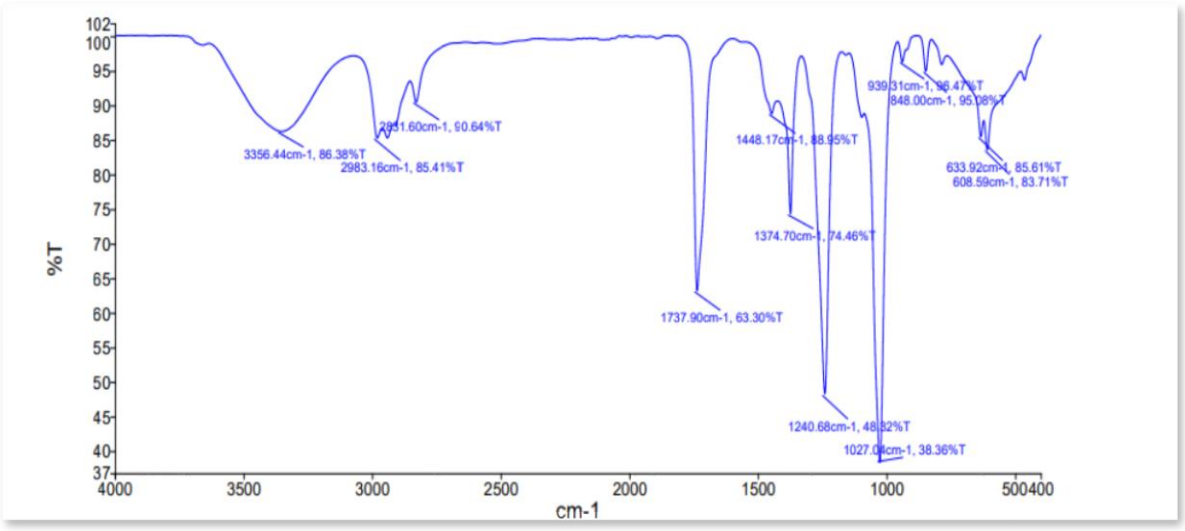


Fig. 6.29. Peaks obtained after FTIR of 10^{th} (J) fraction

Presence of OH Group: The FTIR spectrum showed a broad peak in the 3000-3500 cm^{-1} range, specifically around 3356.44 cm^{-1} due to hydrogen bond in hydroxyl group. This peak indicates the presence of hydroxyl (OH) groups in the compound. Hydroxyl groups are characteristic of compounds that contain alcohol or phenolic functional groups. In the context of polysaccharides, the presence of hydroxyl groups is expected because they are composed of sugar units, and sugars contain hydroxyl groups.

Absence of Aromatic Compounds: The FTIR analysis revealed that there were no peaks in the spectrum below 3000 cm^{-1} i.e., 2983.16 cm^{-1} and 2831.60 cm^{-1}. This indicates the absence of aromatic compounds, as the stretching vibrations of aromatic C-H bonds typically occur in the lower frequency range. Instead, the absence of such peaks suggests that the compound is primarily composed of aliphatic (straight or branched) and cyclic (ring-like) structures. Polysaccharides are typically composed of sugar units linked together in a linear or branched fashion, making them aliphatic.

Presence of Ester Group: Another significant finding from the FTIR analysis is the presence of a peak in the range of 1750-1700 cm^{-1} (specifically 1737.9 cm^{-1}). This range is characteristic of carbonyl (C=O) stretching vibrations, which are typically associated with ester groups. The presence of ester groups in the compound indicates that it might contain ester linkages, which are common in some polysaccharides. Ester linkages are formed by the reaction between a carboxylic acid and an alcohol group, and they are important in the structure of some polysaccharides.

Presence of Ether linkage: There is a peak in range of 1200- 1000 cm^{-1} (specifically 1027.04 cm^{-1}, which indicate the presence of ether linkage in the isolated compound.

6.1.10.2 Nuclear magnetic resonance (NMR)

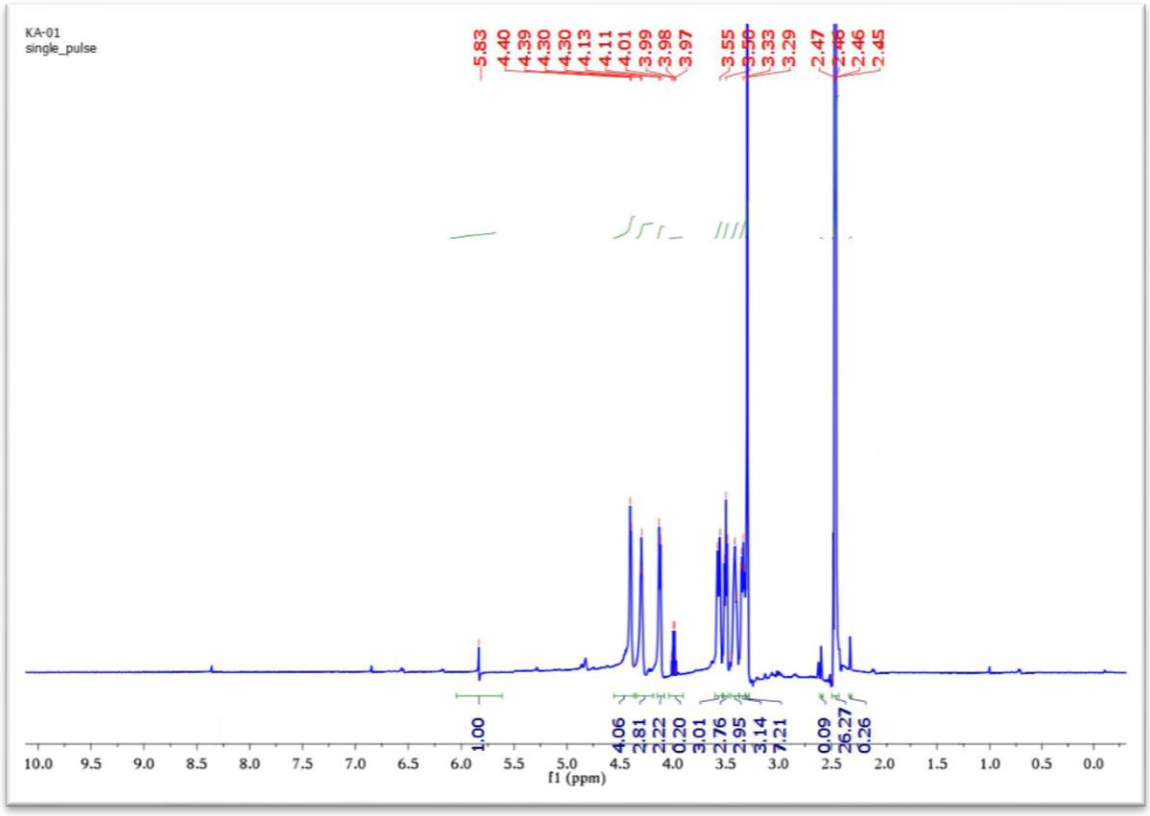


Fig. 6.30. H^1 NMR Spectrum of 10^{th} (J) fraction

H^1 NMR Spectrum of the tenth fraction reveals distinct signals corresponding to different protons within the molecule. A series of peaks is observed between 3.25 and 3.60 ppm, representing the protons (H1-H6) of the glucosyl moiety. The multiplet nature of the peaks suggests the presence of multiple coupled protons in this region. Peaks in the range of 4.392 to 4.88 ppm correspond to the hydroxyl protons (OH2, OH3, OH4, OH6) of the glucosyl group. The multiplet pattern indicates the complex coupling relationships between these protons. A singlet peak at 2.5 ppm is attributed to the acetyl group's proton, indicating a non-coupled environment for this particular proton.

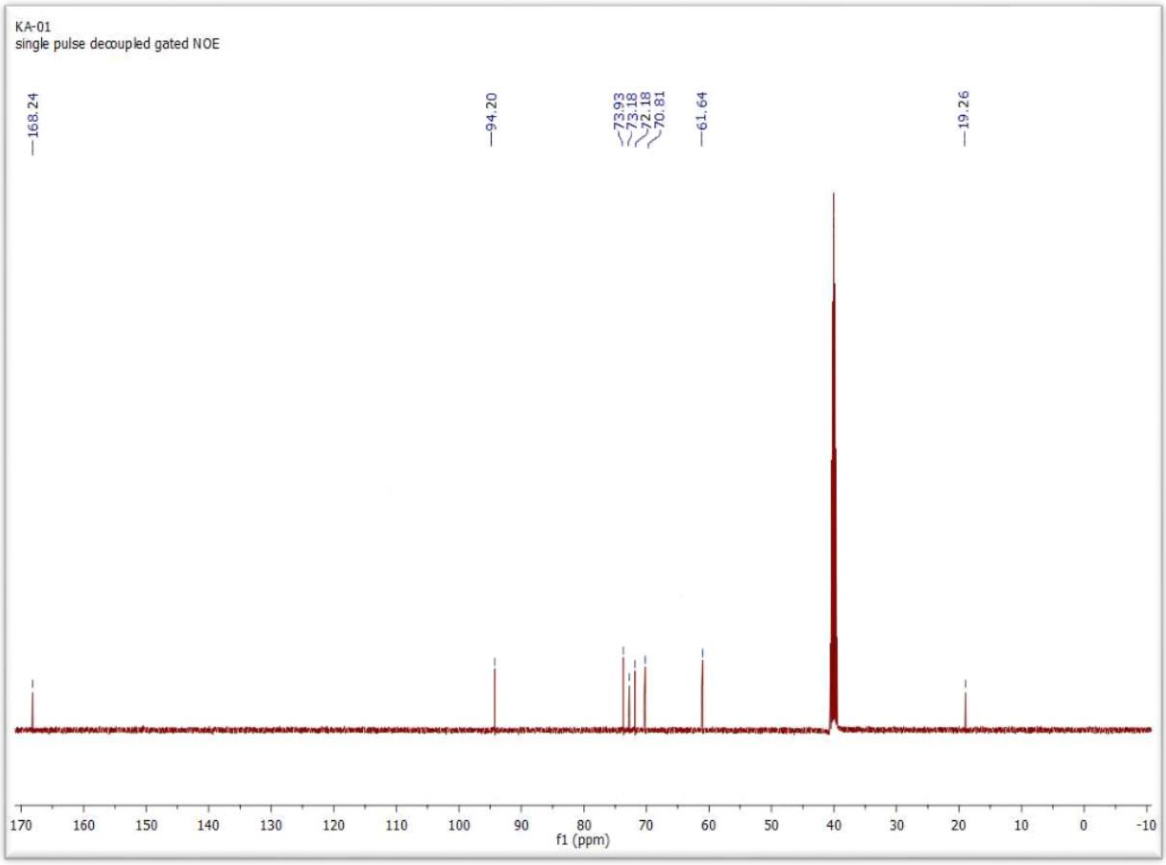


Fig. 6.31. C^{13} NMR spectrum of 10^{th} (J) fraction

The C^{13} NMR spectrum provides insights into the carbon environments within the tenth fraction. C1-C6 carbons of the glucosyl moiety exhibit distinct chemical shifts: C1 at 94.20 ppm, C2 at 72.18 ppm, C3 at 73.93 ppm, C4 at 70.81 ppm, C5 at 73.18 ppm, and C6 at 61.64 ppm. These shifts are indicative of the carbon environments within the glucose unit. The carbonyl carbon appears at 168.24 ppm, consistent with the expected shift for a carbonyl group. The acetyl carbon is observed at 19.26 ppm, confirming the presence of an acetyl group within the compound. These NMR results collectively provide valuable information about the molecular structure of tenth fraction, enabling further elucidation of its chemical composition and bonding patterns.

6.1.10.3 Liquid chromatography–mass spectrometry (LC–MS)

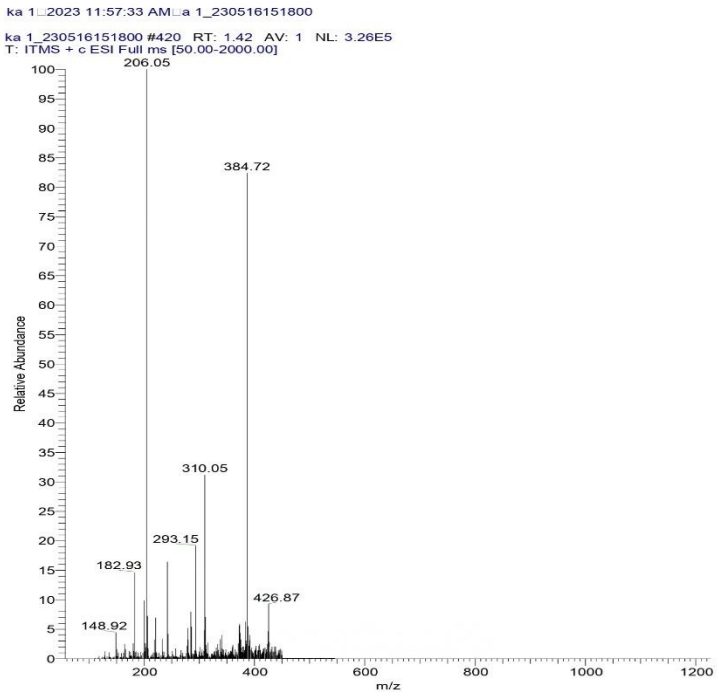


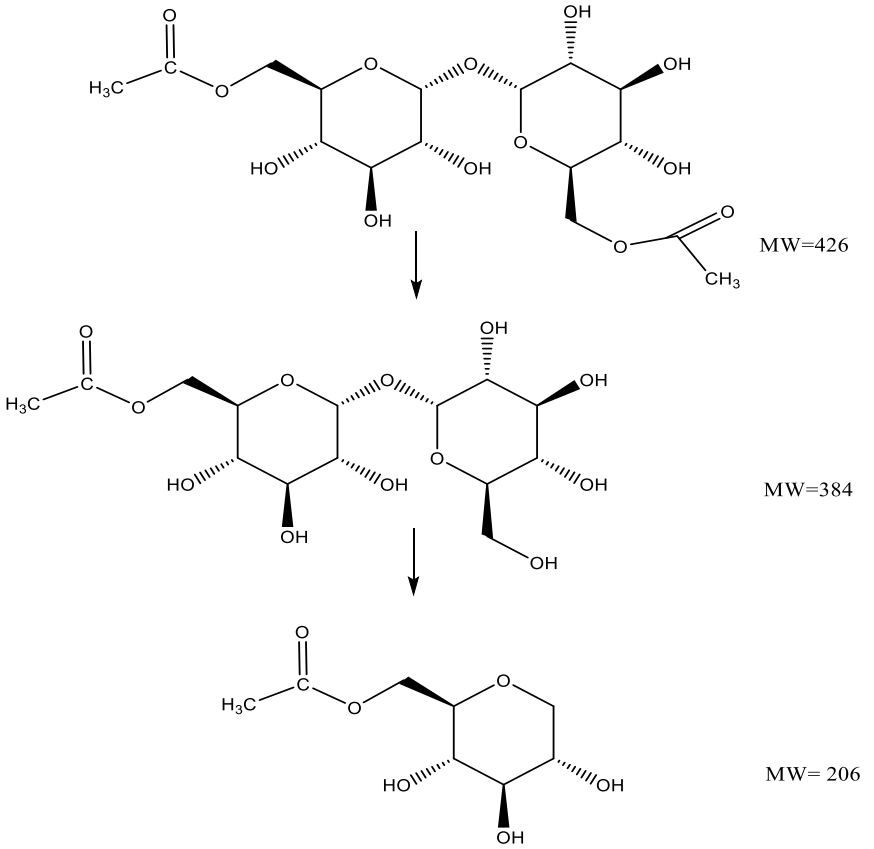
Fig. 6.32. Mass spectrum of 10th (J) fraction

Molecular Ion Peak: This is the molecular ion of the compound (diacetyl glucose ester), and its mass-to-charge ratio (m/z) corresponds to the compound's molecular weight or mass. It's often used to identify the molecular formula of the compound. The molecular ion peak at 426 m/z represents the intact or parent ion of the compound, which corresponds to the compound's molecular weight.

Base Peak: The base peak in a mass spectrum is the most intense peak, which means it represents the ion with the highest abundance in the sample. In this case, the base peak at 206 m/z indicates that a fragment or ion with a mass-to-charge ratio of 206 is the most abundant species in the analyzed sample. This fragment is likely a result of the compound's fragmentation during the mass spectrometry process. This base peak represents a fragment of the compound, which results from the breaking of an ether linkage. As mentioned earlier, it leads to the formation of two compounds: acetyl glucose ester (more stable) and another fragment.

Other Significant Peak: It has been identified that Acetyl Trehalose is another common disaccharide derivative for which a peak observed at 384 m/z on mass spectra.

Fragments of final compound



The final compound identified is diacetyl trehalose.

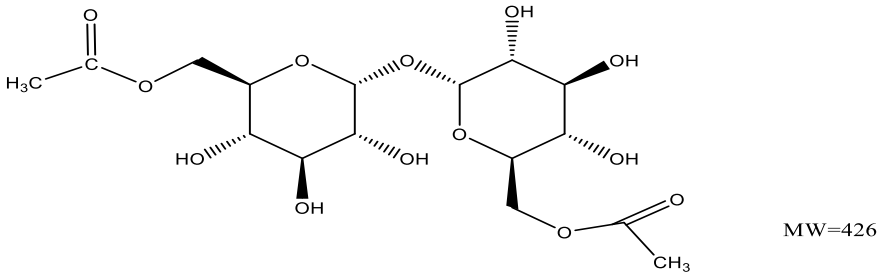


Fig. 6.33. Identified final compound Diacetyl trehalose

6.1.11. MTT Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay is a commonly used method to assess cell viability and proliferation. In this context, the results indicate the percentage of viable cells at various concentrations of a substance being tested. The concentrations are given in micrograms per milliliter (µg/ml), and the

corresponding percentages represent the relative viability of the cells compared to a control group (presumably untreated cells) (Table 6.11).

At a concentration of 6.25 µg/ml, the cells exhibit a high level of viability, with approximately 99.31% of the cells remaining viable compared to the control group. The viability remains high at 98.41% when the concentration is increased to 12.5 µg/ml, indicating a minimal impact on cell viability at this concentration. Even at 25 µg/ml, the cells maintain a relatively high viability of 97.47%, suggesting a mild decrease in viability compared to lower concentrations. At 50 µg/ml, there is a slight decrease in cell viability to 96.28%, indicating a dose-dependent response where higher concentrations start to impact cell viability to a small extent. The highest concentration tested, 100 µg/ml, still maintains a relatively high viability of 95.98% (Table 6.11.). While there is a further decrease compared to lower concentrations, the cells overall remain viable at this concentration. No IC_{50} value could be calculated as the minimum percentage of viability was found to be 95.98%. These results indicate that the compound diacetyl trehalose is not toxic to the cells.

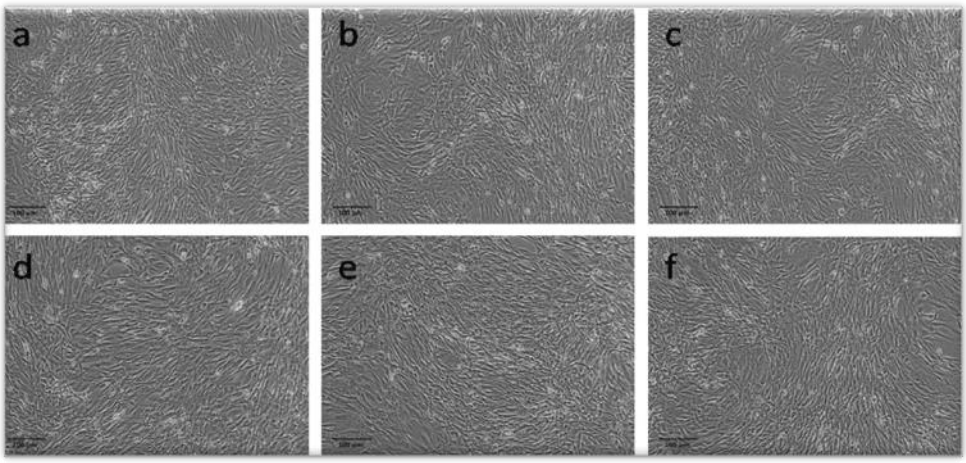


Fig. 6.34. Cell images obtained after the treatment of a) control, b) 6.25µg/ml, c)12.5µg/ml, d) 25µg/ml, e) 50µg/ml and f) 100µg/ml of the sample

Table 6.11. Percentage viability for varying concentrations of test sample

Concentration (µg/ml)	Percentage of viability
6.25	99.31 ± 0.73
12.5	98.41 ± 0.22
25	97.47 ± 1.02
50	96.28 ± 0.98
100	95.98 ± 1.2

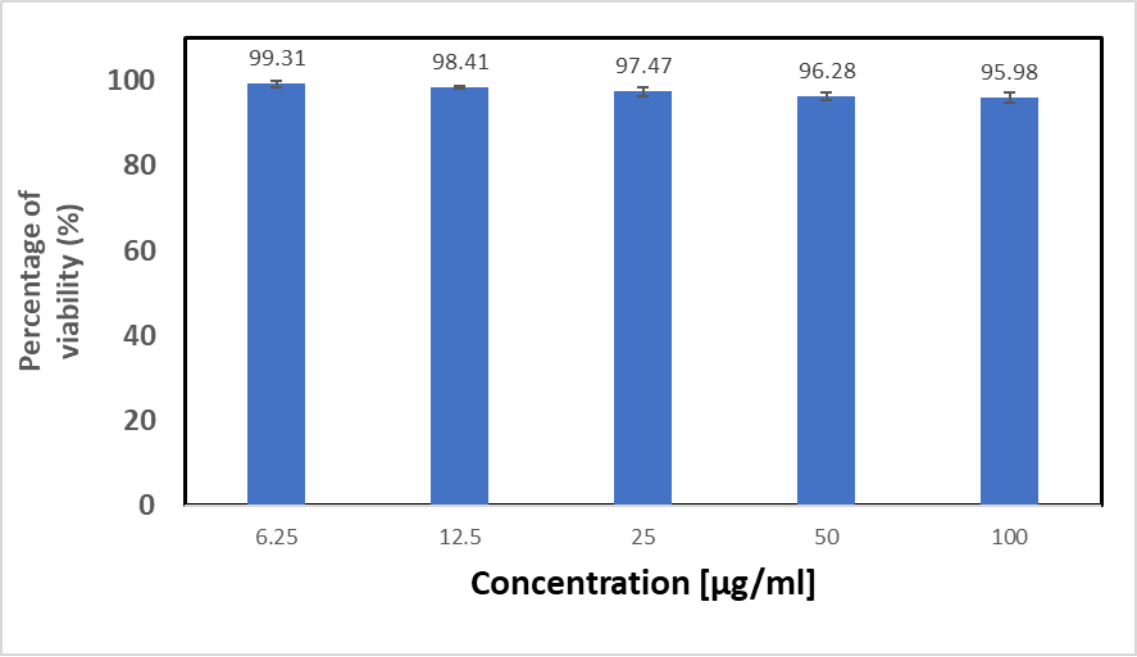


Fig. 6.35. Graphical representation of cell viability with varying concentrations of the compound diacetyl trehalose

6.1.12. Glucose Uptake assay for the compound diacetyl trehalose

The glucose uptake assay was repeated with the final compound diacetyl trehalose and was performed on L6 myotubes. The results also include a positive control group treated with insulin, a known stimulator of glucose uptake. The percentage of glucose uptake represents the amount of glucose taken up by the L6 myotubes cell lines under different treatment conditions. The control group (untreated) exhibited a baseline glucose uptake of approximately 30.0%, which was comparable across both the sample and insulin.

When the L6 myotubes were treated with isolated fraction at concentrations of 25 µg/ml, 50 µg/ml, and 100 µg/ml, an increase in glucose uptake was observed compared to the control group (Table 6.12.). This indicates that the fraction have the potential to enhance glucose uptake in the L6 myotubes cell lines. Furthermore, the percentage of glucose uptake does not show major changes in concentration-dependent manner. This indicates that the glucose uptake-enhancing effect of the isolated compound diacetyl trehalose is not dose-dependent. Importantly, when comparing the glucose uptake values of the compound diacetyl trehalose to the positive control group treated with insulin, it can be observed that the isolated compound diacetyl trehalose does not exceed the glucose uptake stimulation achieved by insulin. This suggests that the isolated fraction possess potent glucose uptake-enhancing properties, however, lower than that of insulin.

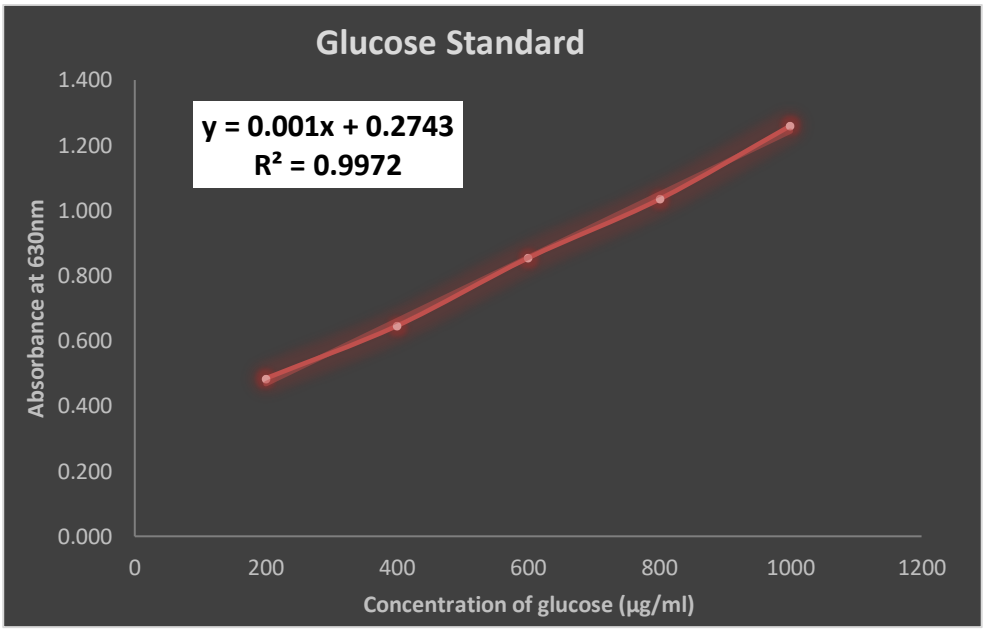


Fig. 6.36. Standard curve for the glucose uptake assay

Table 6.12. Glucose Uptake assay for the compound diacetyl trehalose

Concentration	Standard	% of Glucose Uptake
µg/ml	Insulin	KA-1 (Diacetyl Trehalose)
0	31.00 ± 0.15	30.16 ± 0.12
25	56.76 ± 0.21	31.13 ± 0.26
50	67.86 ± 0.12	32.1 ± 0.25
100	88.43 ± 0.20	33 ± 0.15

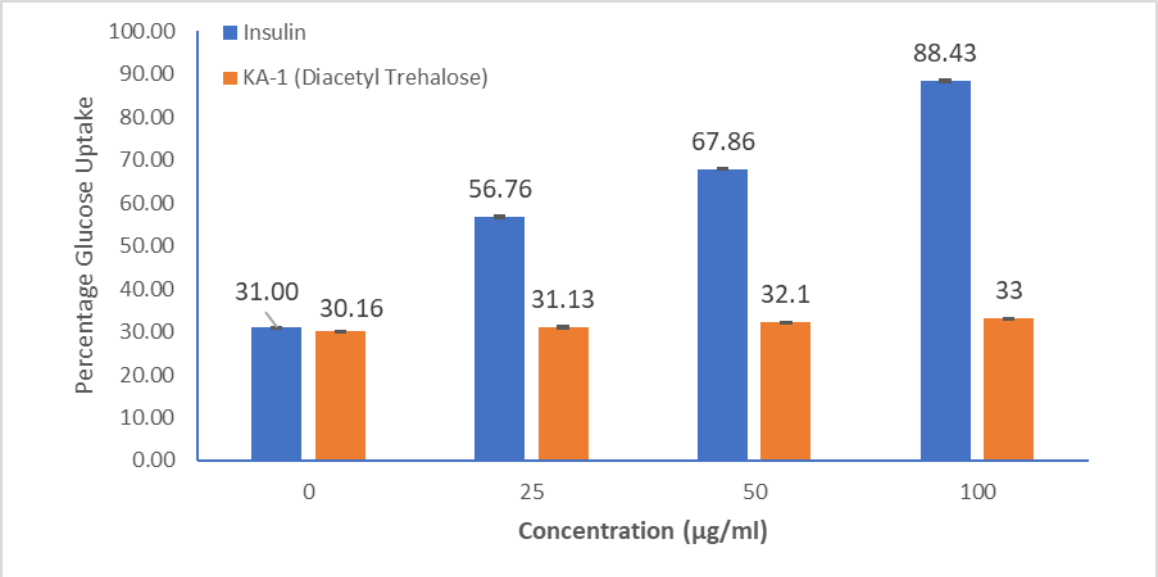


Fig. 6.37. Graphical representation glucose uptake assay of the compound diacetyl trehalose compared to insulin

6.2. Discussion

The current study suggests *M. esculenta* as an antidiabetic agent identifying the novel compound diacetyl trehalose responsible for the antidiabetic effect. *M. esculenta* has historically been valued for its culinary properties, but the transition from traditional usage to scientifically validated medicinal benefits is a recent development.

M. esculenta is a culinary mushroom renowned for its delectable flavor and rich nutritional content [195]. This mushroom has been known for its nutritional properties and medicinal value. The hepatoprotective potential of *M. esculenta* in mice has been assessed due to its antioxidative and anti-hyperlipidaemic properties [237]. The results obtained pertaining to the antioxidant assays (ABTS radical cation decolorization assay, DPPH radical scavenging assay, Hydrogen peroxide scavenging assay, Lipid peroxidation, Nitric oxide scavenging assay) in the current study clearly indicate towards the antioxidant abilities of the extracts equally establishing the previous studies hypothesizing the antioxidant properties of *M. esculenta*. *M. esculenta* is comprised of a range of bioactive components, including proteins, polysaccharides, vitamins, and dietary fiber [238]. Even the potential of the mushroom polysaccharide on obesity,

inflammation and gut microbiota has been studied [229]. The polysaccharides found in *M. esculenta* have been documented for their potential to inhibit the proliferation of human colon cancer [196]. The exopolysaccharides derived from *M. esculenta* exhibit remarkable tumor-suppressive effects and immunostimulatory activity [239]. They activate T-cells and promote the proliferation of splenocytes [240]. Additionally, the heteropolysaccharides demonstrate potent antioxidant activity, providing protection to zebrafish embryos against oxidative stress [241]. Numerous clinical ramifications of mushroom polysaccharides have been explored [239,242], with a focus on examining the positive impact of *M. esculenta* Polysaccharide (MEP) specifically on gut microbiota [229].

Lately, there has been growing interest in the use of polysaccharides obtained from natural sources due to their ability to regulate glucose and lipid metabolism. Certain polysaccharides have been explored for their potential anti-diabetic effects, focusing on improving insulin function, addressing β-cell dysfunction, and inhibiting α-glucosidase and α -amylase [243]. Various assays such as α-glucosidase inhibition, α-amylase inhibition and glucose uptake performed with the *M. esculenta* extracts in the current study indicate the potential of *M. esculenta* as a natural source of compounds with potential antidiabetic properties by modulating carbohydrate digestion and glucose absorption.

Polysaccharides show diverse therapeutic potential for addressing diabetes and associated metabolic disorders through various mechanisms. They can have hypoglycemic and hypolipidemic effects, inhibit β-cell apoptosis, and promote β-cell proliferation. Additionally, polysaccharides target β-cell dysfunction by providing antioxidative and anti-inflammatory effects. In carbohydrate metabolism, certain polysaccharides act as inhibitors of α -amylase and α -glucosidase, regulating glucose release [243]. Others enhance insulin function through pathways like PI3K/Akt, modulate the MAPK pathway, and inhibit protein-tyrosine phosphatase 1B, contributing to improved glucose homeostasis [243]. Polysaccharides also impact insulin-independent glucose metabolism by inhibiting glucose uptake, activating AMP-activated protein kinase, modulating pancreatic duodenal homeobox-1, and targeting hepatic processes. Their role as peroxisome proliferator-activated receptor modulators and influence on the

cAMP-PKA pathway highlight their complexity [243]. This comprehensive approach underscores polysaccharides' potential in diabetes management, but further research is needed to identify specific polysaccharides and understand their detailed mechanisms of action.

Several polysaccharides with potential therapeutic effects on diabetes have been investigated, each derived from different plant sources. *Anoectochilusroxburghii* polysaccharide (ARP), sourced from *Anoectochilusroxburghii*, consists of monosaccharides such as l-rhamnose, l-arabinose, d-xylose, d-mannose, d-glucose, and d-galactose in specific molar ratios. In a study on streptozotocin (STZ)-induced diabetic mice, ARP demonstrated the ability to decrease fasting blood glucose (FBG) and plasma lipid levels, increase superoxide dismutase (SOD) activity, cut-down malondialdehyde (MDA) levels, and attenuate pathologic lesions in the liver and pancreas [244]. *Artemisia sphaerocephala* Krasch (seed) polysaccharide (ASKP) derived from *Artemisia sphaerocephala* Krasch seeds comprises monosaccharides like d-mannose, d-glucuronic acid, d-galacturonic acid, d-glucose, d-xylose, d-galactose, and l-arabinose. In a study using high-fructose-induced hyperglycemic mice, ASKP showed the ability to reduce fasting serum glucose and insulin concentrations, reduce liver lipid levels, increase hepatic SOD and Glutathione peroxidase (GSH-Px) activities, decrease MDA levels, and ameliorate hepatic steatosis [245]. *Acanthopanax senticosus* polysaccharide (ASP), sourced from *Acanthopanax senticosus*, has a molecular weight of 59 kDa and contains glucose (Glc), galactose (Gal), and arabinose (Ara). In alloxan-induced diabetic mice, ASP demonstrated the ability to decrease FBG and blood lipid levels, increase serum insulin levels, enhance SOD, catalase (CAT), and GSH-Px activities, and decrease MDA levels [246]. *Coptis Chinensis* polysaccharide-1 (CCPW-1), derived from *Coptis Chinensis*, consists of glucose (Glc), arabinose (Ara), xylose (Xyl), galactose (Gal), and galacturonic acid (GalA) in specific ratios. In a study using a high-fat diet (HFD) with STZ-induced diabetic mice, CCPW-1 demonstrated the ability to decrease FBG and blood lipid levels, increase serum insulin levels, enhance SOD and CAT activities, and decrease MDA levels [124]. Polysaccharides from various species of Dendrobium, including *Dendrobium huoshanense* (DHP), *Dendrobium nobile* (DNP), and *Dendrobium officinale* (DOP), showed similar effects in alloxan-induced diabetic mice. These polysaccharides decreased FBG and glycosylated serum protein levels, increased

serum insulin levels, enhanced SOD and CAT activities, decreased MDA levels, and attenuated pathologic lesions of the pancreas [247]. Dioscorea opposite Thunb polysaccharide-80 (DOTP-80), sourced from Dioscorea opposite Thunb, has a molecular weight of 123 kDa and consists of glucose (Glc), galactose (Gal), mannose (Man), and arabinose (Ara) in specific molar ratios. In alloxan-induced diabetic rats and mice, DOTP-80 demonstrated the ability to decrease FBG levels and enhance SOD activity [243].

Trehalose, an FDA-approved naturally occurring disaccharide comprising two glucose molecules, is abundantly present in various organisms and plants, serving as a resilient energy source [248]. It forms a glycosidic bond with two D-glucose links at the reducing end α-1,1, rendering it more resistant to acid and α-glycosidase cleavage [249]. Widely employed in the food industry as a sweetener and additive, trehalose finds application as a moisturizing agent in cosmetics and a stabilizer in pharmaceutical formulations, including antibodies, enzymes, liposomes, and genomic material [249]. Recent findings indicate that trehalose induces a lower glycemic response and diminishes insulin resistance in comparison to other disaccharides [250]. Furthermore, research suggests its antioxidant, anti-inflammatory, and autophagy-enhancing properties, holding therapeutic promise for prevalent health issues such as cardiometabolic disorders and neurodegenerative diseases. Given the association of Type 2 Diabetes (T2D) with inflammation pathways, endoplasmic reticulum (ER) stress, oxidative stress, and compromised autophagy, trehalose may contribute to mitigating diabetes-related health complications [251].

The identification of diacetyl trehalose as a specific compound in the current study responsible for the antidiabetic effects adds a layer of specificity to the discussion. Diacetyl trehalose may possess unique properties that distinguish it from trehalose alone. This has been substantiated by the glucose uptake assay performed by the tenth fraction. However, research is needed to elucidate the exact mechanisms through which diacetyl trehalose exerts its antidiabetic effects and whether it acts synergistically with other compounds present in *M. esculenta*.

CHAPTER-7

SUMMARY AND CONCLUSIONS

Chapter-7

Summary & Conclusions

7.1. Conclusion

Type 2 Diabetes Mellitus is introduced as a complex metabolic disorder involving insulin resistance, compromised pancreatic β-cell function, and dysregulated glucose homeostasis. The escalating global prevalence of T2DM underscores the need for innovative therapeutic approaches. The study addresses a research gap by focusing on *M. esculenta*, an understudied culinary fungus, particularly in the context of its pharmacological properties and its potential in managing diabetes. Samples were collected from Jammu and Kashmir, India, in April 2021, and morphologically identified as *M. esculenta* using standard taxonomic keys. *M. esculenta* is highlighted as both pharmacologically and nutritionally important. While studies have explored the nutritional components of various edible mushrooms, the pharmacological aspects of *M. esculenta*, especially its hypoglycaemic activities, remain largely unexplored.

The study underscores the tremendous scope for investigating the medicinal properties of *M. esculenta*. The presence of saponins, tannins, terpenoids and steroids, glycosides, flavonoids, alkaloids, quinones, phenols, curcumins, and carbohydrates was determined through qualitative analysis using chloroform, methanol, and ethanol. In addition, the samples demonstrated free radical scavenging activity in DPPH, ABTS, Lipid peroxidation, Hydrogen peroxide scavenging activity, and the Nitric Oxide Scavenging Assay. The ABTS assay results for three solvent extracts of *M. esculenta* are presented in Table 6.3. Ascorbic acid, used as the standard compound at a concentration of 1 mg/ml, exhibited 64.71% free radical scavenging activity. Ethanol consistently demonstrated higher percentage inhibition values compared to chloroform and methanol across all concentrations, indicating its superior efficacy in extracting antioxidant compounds from *M. esculenta*. At the highest concentration (1.0 mg), ethanol displayed the highest percentage inhibition at 50.28%, suggesting significant antioxidant activity. This effectiveness may be attributed to the presence of phenolic compounds, flavonoids, or other bioactive components known for their antioxidant properties. Table 6.4 presents the percentage inhibition of the DPPH radical by *M.*

esculenta extracts in chloroform, ethanol, and methanol at varying concentrations. Ascorbic acid, the standard compound at 1 mg/ml, exhibited an 80.71% free radical scavenging activity. The results indicate a concentration-dependent increase in the percentage inhibition of the DPPH radical for all three solvents, suggesting the antioxidant properties of *M. esculenta* extracts. Comparing the extracts, chloroform consistently demonstrated higher percentage inhibition values than ethanol and methanol across most concentrations. Notably, at the highest concentration (1.0 mg), ethanolic extract exhibited the highest percentage inhibition among the solvents, reaching 18.06%. Table 6.5. presents hydrogen peroxide scavenging activity of *M. esculenta* extracts at various concentrations. Ascorbic acid, the standard compound at 1 mg/ml, exhibited 70.1% free radical scavenging activity. The order of H_2O_2 scavenging ability was found to be ethanol > chloroform > methanol. Results indicate an increase in percentage inhibition with rising concentrations for all solvents, demonstrating the extract's ability to scavenge hydrogen peroxide. Ethanol consistently displayed higher percentage inhibition values than chloroform and methanol, suggesting its stronger hydrogen peroxide scavenging activity. At the highest concentration (1.0 mg), the ethanol extract exhibited the highest percentage inhibition at 46.15%, indicating the presence of potent bioactive components with strong hydrogen peroxide scavenging capabilities. Table 6.6. outlines the lipid peroxidation assay results for *M. esculenta* extracts at various concentrations, with ascorbic acid as the standard compound showing 75.71% inhibition activity at 1 mg/ml. The order of lipid peroxidation inhibition ability was found to be ethanol > chloroform > methanol. Results indicate an increase in percentage inhibition with rising concentrations for all solvents, suggesting the extracts' antioxidant properties in inhibiting lipid peroxidation, a process that generates reactive oxygen species and poses a threat to cellular membranes. Ethanol consistently exhibited higher percentage inhibition values than chloroform and methanol at most concentrations, indicating its stronger inhibitory activity against lipid peroxidation. At the highest concentration tested (1.0 mg), the ethanol extract displayed the highest percentage inhibition at 43.18%, suggesting the presence of potent bioactive components effective in protecting against oxidative damage to cell membranes. Table 6.7. summarizes the nitric oxide scavenging assay results for *M. esculenta* extracts at various concentrations, with ascorbic acid as the standard compound displaying 66.1%

inhibition activity at 1 mg/ml. The order of nitric oxide scavenging ability was observed as ethanol > chloroform > methanol. The results show an increase in percentage inhibition with rising concentrations for all extracts, indicating their nitric oxide scavenging activity and potential to mitigate the harmful effects of excess nitric oxide, which is associated with oxidative stress and inflammation. Ethanol consistently exhibited higher percentage inhibition values compared to chloroform and methanol at most concentrations, suggesting its stronger nitric oxide scavenging activity. At the highest concentration tested (1.0 mg), the ethanol extract demonstrated the highest percentage inhibition of nitric oxide at 25.34%, highlighting its significant scavenging activity. Table 6.8. displays the α-glucosidase inhibition assay results for *M. esculenta* extracts at different concentrations, with acarbose as the standard exhibiting the highest inhibition rate of 76.71% at 1 mg/ml. The order of α-glucosidase inhibition ability was observed as ethanol > methanol > chloroform. Results indicate an increase in percentage inhibition with rising concentrations for all solvents, suggesting the extracts' ability to inhibit the α-glucosidase enzyme, which plays a role in carbohydrate digestion and glucose absorption. Ethanol consistently showed higher percentage inhibition values compared to chloroform and methanol at most concentrations, indicating its stronger inhibitory activity against α-glucosidase. This suggests that the ethanol extract of *M. esculenta* contains bioactive compounds effective in inhibiting the α-glucosidase enzyme. At the highest concentration tested (1.0 mg), the ethanol extract demonstrated the highest percentage inhibition of α-glucosidase activity at 49.06%, underscoring its potential in modulating carbohydrate digestion and glucose absorption. Table 6.9. outlines the α-amylase inhibitory assay results for *M. esculenta* extracts at various concentrations, with acarbose as the standard exhibiting the highest inhibition rate of 68.71%. Among the extracts, the ethanolic extract demonstrated the highest inhibition rate (50.4%±1.14, IC50 = 1.0384 mg/ml), followed by chloroform (39.67%±1.44, IC50=1.33 mg/ml), and methanolic (33.95%±1.12, IC50=1.416 mg/ml), in a concentration-dependent manner. Results indicate the extracts' ability to inhibit the α-amylase enzyme responsible for starch breakdown into simpler sugars. Ethanol consistently displayed higher percentage inhibition values compared to chloroform and methanol at most concentrations, suggesting its stronger inhibitory activity against α-amylase. This implies that the ethanol extract of *M. esculenta* contains bioactive

compounds effective in inhibiting α-amylase activity. At the highest concentration tested (1.0 mg), the ethanol extract exhibited the highest percentage inhibition of α-amylase activity at 50.4%, indicating significant inhibitory potential. Table 6.10. presents the results of the glucose uptake assay performed on L6 myotubes cell lines using various extracts of *M. esculenta* at different concentrations. The control group (untreated) exhibited a baseline glucose uptake of approximately 33.70%, consistent across all three solvent extracts. Treatment with *M. esculenta* extracts at concentrations of 25 µg/ml, 50 µg/ml, and 100 µg/ml led to an increase in glucose uptake compared to the control group, indicating the potential of *M. esculenta* extracts to enhance glucose uptake in L6 myotubes cell lines. Comparing the three solvent extracts, both the ethanol and methanol extracts consistently demonstrated higher percentages of glucose uptake compared to the chloroform extract at all concentrations tested. This suggests that the ethanol and methanol extracts contain bioactive compounds that can more effectively stimulate glucose uptake in L6 myotubes cell lines.

The methanolic extract demonstrated the most significant glucose uptake activity. Subsequently, we proceeded to isolate a pure compound from the methanolic extract. Column chromatography is a common tool for fractionation. This technique allows for the isolation of individual compounds by sorting them into categories based on their binding strengths to the column's stationary phase. To achieve this, we employed column chromatography, resulting in the separation of the compound into 12 fractions, each containing a single compound. These fractions, designated as A, B, C, D, E, F, G, H, I, J, K, and L, were then analysed on TLC plates for conformation. Qualitative tests were conducted after the extraction process to confirm the presence of polysaccharides. The tenth fraction (J) was found to be positive according to the results of the polysaccharide quantitative test (Molish test), thus we proceeded with the remaining eleven fractions. After that, the 10^{th} (J) part was quantitatively examined for compound identification using Mass spectrometry, FTIR, and NMR.

The FTIR data, along with NMR and Mass Spectroscopy analyses, collectively confirmed the presence of diacetyl trehalose in the isolated compound. Specifically, the FTIR analysis identified the presence of OH groups, ester groups, and ether linkages, which are consistent with the structural features of diacetyl trehalose. Moreover, the use

of complementary analytical techniques such as NMR and Mass Spectroscopy provided additional confirmation. The presence of diacetyl trehalose was further supported by its functional groups and structural characteristics identified through these techniques. The biological activity of diacetyl trehalose was evidenced by the significant glucose uptake, reaching around 33%. This finding suggests that diacetyl trehalose has the potential to enhance glucose uptake, which could be beneficial in various physiological contexts, particularly in the regulation of blood glucose levels.

Table (6.11.) showen the MTT assay results for diacetyl trehalose demonstrate a notable level of cell viability at various concentrations. At 6.25 µg/ml, the cells exhibit a high viability of approximately 99.31%, indicating minimal impact on cell health. This favourable trend continues with 98.41% viability at 12.5 µg/ml, emphasizing the compound's benign effect on cell viability. Even at a concentration of 25 µg/ml, the cells maintain a relatively high viability of 97.47%, suggesting a mild decrease compared to lower concentrations. As the concentration is further increased to 50 µg/ml, there is a slight reduction in cell viability to 96.28%. Although this indicates a dose-dependent response with a small impact on cell viability, the cells still remain viable at this concentration. The highest tested concentration, 100 µg/ml, maintains a relatively high viability of 95.98%, signifying that diacetyl trehalose is well-tolerated by the cells even at this upper limit. Notably, no IC50 value could be calculated, as the observed minimum viability was still high at 95.98%. Overall, these results collectively suggest that diacetyl trehalose does not exhibit toxicity towards the cells, indicating its favourable biocompatibility in the tested concentrations. The glucose uptake assay, repeated with the final compound diacetyl trehalose on L6 myotubes, revealed promising results. The control group (untreated) exhibited a baseline glucose uptake of approximately 30.0%, consistent across both the sample and insulin-treated groups. Treatment with diacetyl trehalose at concentrations of 25 µg/ml, 50 µg/ml, and 100 µg/ml resulted in an increase in glucose uptake compared to the control group (Table 6.12.). This indicates the compound's potential to enhance glucose uptake in L6 myotubes cell lines. Notably, the percentage of glucose uptake did not show significant changes in a concentration-dependent manner, suggesting that the glucose uptake-enhancing effect of diacetyl trehalose is not dose-dependent. Importantly, when comparing the glucose uptake values of diacetyl trehalose to the positive control group treated with insulin, it becomes evident

that diacetyl trehalose does not surpass the glucose uptake stimulation achieved by insulin. This implies that while diacetyl trehalose exhibits potent glucose uptake-enhancing properties, its efficacy is lower than that of insulin. The study highlights *M. esculenta* as a potential antidiabetic agent, identifying diacetyl trehalose as a novel compound responsible for this effect. Traditionally valued for its culinary properties, the mushroom's transition to scientifically validated medicinal benefits is a recent development.

M. esculenta, known for its rich nutritional content, has demonstrated hepatoprotective potential in mice due to its antioxidative and anti-hyperlipidaemic properties. The current study's antioxidant assays align with previous hypotheses about *M. esculenta's* antioxidant properties. The mushroom contains various bioactive components, including proteins, polysaccharides, vitamins, and dietary fiber. Polysaccharides from *M. esculenta* show potential in inhibiting colon cancer proliferation and exhibit tumor-suppressive and immunostimulatory effects. Research on mushroom polysaccharides, including those from *M. esculenta,* has focused on their impact on gut microbiota and their potential therapeutic effects on diabetes and associated metabolic disorders. The current study's assays, such as α-glucosidase inhibition, α-amylase inhibition, and glucose uptake, indicate *M. esculenta's* potential as a natural source for compounds with antidiabetic properties by modulating carbohydrate digestion and glucose absorption. Polysaccharides, in general, have diverse therapeutic potential for addressing diabetes through mechanisms like hypoglycaemic and hypolipidemic effects, inhibition of β-cell apoptosis, and promotion of β-cell proliferation.

The study emphasizes the need for further research to identify specific polysaccharides and understand their detailed mechanisms of action. Several polysaccharides from different plant sources have been investigated for their potential therapeutic effects on diabetes. Trehalose, a naturally occurring disaccharide, is noted for its lower glycaemic response and potential in mitigating insulin resistance. The study identifies diacetyl trehalose as a specific compound in *M. esculenta* responsible for antidiabetic effects, emphasizing the need for further research to elucidate its mechanisms of action and potential synergy with other compounds. The findings suggest

that *M. esculenta*, particularly diacetyl trehalose, holds promise as an antidiabetic agent, and further research is warranted to understand the specific mechanisms and potential synergies with other compounds present in the mushroom.

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