Effect of *Cordyceps militaris* on glucose homeostasis and wound healing under hyperglycemic conditions

Project report submitted in partial fulfillment of the requirement for the

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Master of Science

In

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By

Sefali Bhakuni

Under the supervision of

Dr. Udayabanu

to

Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology Waknaghat, Solan-

173234, Himachal Pradesh

DECLARATION

I hereby declare that the work presented in this project entitled "**Effect of *Cordyceps militaris* on glucose homeostasis and wound healing under hyperglycemic conditions**" in partial fulfillment of the requirements for the award of the degree of Master of Technology in Biotechnology submitted in the Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology Waknaghat is an authentic record of my own work carried out over a period from August 2022 to December 20222 under the supervision of **Dr. Udayabanu** Associate Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, Himachal Pradesh.

I also authenticate that I have carried out the above-mentioned project work under the proficiency stream.

The matter embodied in the report has not been submitted for the award of any other degree or Diploma.

Sefali Bhakuni, 217826

This is to certify that the above statement made by the candidate is true to the best of my knowledge.

Dr. Udayabanu

Associate Professor,

Department of Biotechnology and Bioinformatics,

Jaypee University of Information Technology, Solan, Himachal Pradesh

Dated:

CERTIFICATE

This is to certify that the project was carried out by Sefali Bhakuni student of M.Sc. Biotechnology, IV semester, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, Himachal Pradesh, during January to May 2023 as presented in the report was under the guidance and supervision of **Dr. Udayabanu** Associate Professor, Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, Himachal Pradesh. The project entitled "**Effect of *Cordyceps militaris* on glucose homeostasis and wound healing under hyperglycemic conditions**" is therefore being forwarded for acceptance in partial fulfillment of the requirements for the award of M.Sc. Biotechnology of Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Solan, Himachal Pradesh.

Dr. Udayabanu

Associate Professor

Department of Biotechnology and Bioinformatics,

Jaypee University of Information Technology,

Solan, Himachal Pradesh.

Place: Himachal Pradesh

Date:

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List of Abbreviations

Glc	Glucose
GLUT	Glucose transporters
DB	Diabetes mellitus
HyperGM	Hyperglycemic
AQ	Aqueous
MoH	Methanolic
INS	Insulin
GLgn	Glucagon
Na	Sodium
Ox-phosphorylation	Oxidative phosphorylation

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// ABSTRACT:

The surge in metabolic syndrome symptoms, including DB and obesity, is being caused by recent improvements in living conditions, dietary changes, and reductions in physical activity. Humanity is continually exposed to metabolic disorders like DB as a result of such socioeconomic trends, and the number of patients is growing. This study examined how glucose absorption in yeast cells was stimulated by *Cordyceps militaris* extract and how these extracts treatment affected glucose metabolism and wound healing under hypoglycemic conditions.
AQ and MoH extracts were made from the dried fruiting body of *Cordyceps militaris* using the boiling method and soxhlet extraction, respectively. The quantitative analysis of antioxidant, antimicrobial, percentage proliferation, glucose absorption, and wound healing under hyperglycemic circumstances of these extracts revealed that they displayed the aforementioned activities quite effectively. When used to treat hyperglycemia-induced wounds in zebrafish, the *Cordyceps militaris* extract improved glucose absorption in yeast cells, demonstrating its anti-diabetic effect.

Overall, the use of *Cordyceps militaris* increased glucose metabolism by enhancing INS sensitivity and glucose adsorption. These results lay the groundwork for potential *Cordyceps militaris* usage in hyperglycemic wound healing.

Key words: ***Cordyceps militaris,*** **glucose metabolism, glucose uptake, GLUT, hyperglycemic condition, wound healing.**

CHAPTER: 1

INTRODUCTION

One of the primary sources of energy in mammals is glucose, which has ATP as the main product through the mechanisms of oxidative phosphorylation and glycolysis. Cells take up glucose from the bloodstream and use it for energy production, and the efficient functioning of several bodily organs depends on this intake. INS, a pancreatic hormone, plays a crucial role in glucose absorption by aiding transport of glucose from circulation into cells, indicating a glucose-depositing nature, whereas GLgn serves as a glucose-mobilizing hormone. Glucose uptake is an intricate process that involves some steps and mechanistic operations. To enter cells, glucose requires specific transport proteins that act as gatekeepers. These transport proteins are called glucose transporters, or GLUTs. There are several GLUT types, and every type has expression in various tissues and cells. The primary function of the GLUTs is to regulate the flow of glucose compartments intracellularly and extracellularly, ensuring that there is always glucose homeostasis.

Then, in glucose metabolism, once glucose enters the cell, it undergoes several metabolic processes to produce energy. Glucose is first converted into glucose-6-phosphate, a process that requires the enzyme hexokinase. Glucose-6-phosphate can then be used in various metabolic pathways to produce ATP, the primary energy currency of cells. Cells can also store excess glucose as glycogen, a complex carbohydrate that can be broken down into glucose when needed through glycogen synthesis. Glycogen synthesis occurs in the liver and muscles, and it is regulated by INS. When glucose enters the cell via a GLUT transporter, it is transformed to glucose-6-phosphate via a process known as phosphorylation. A variety of metabolic processes, including glycolysis, the citric acid cycle, and oxidative phosphorylation involve in the breakdown of glucose is a complex process.

Breakdown of glucose is an intricate process that is required for the proper working of body. The dysregulation of glucose metabolism can have significant effects on glucose metabolism and contribute to the development of metabolic disorders. DB, heart disease, and obesity can all be exacerbated by impaired glucose absorption. DB is a metabolic condition characterised by elevated BGL (hyperGM), which is due to inefficient glucose metabolism. Glucose uptake measurement is a useful technique for monitoring metabolic

health and identifying metabolic diseases. Elevated BGLs can harm the body's organs and tissues, resulting in consequences such as wound healing neuropathy, retinopathy, nephropathy, and cardiovascular disease [18]. Controlling BGLs, treating any underlying illnesses that may be contributing to the wound, and providing appropriate wound care are all necessary components of a multidisciplinary strategy to improve diabetic wound healing.

Traditional medical practices include a number of naturally derived treatments that can enhance glucose absorption and promote normal blood sugar levels. The mushroom *Cordyceps militaris* is a key component of traditional Chinese medicine. Polysaccharides, nucleosides, organic acids, and amino acids are abundant in *Cordyceps militaris* and are thought to provide a variety of possible health advantages. Research on both humans and animals has revealed that *Cordyceps militaris* increases glucose absorption and INS sensitivity. This is most likely caused by the presence of bioactive compounds such polysaccharides, cordycepin, and adenosine, which have been shown to have hypoglycemic and INS-sensitizing effects.

In this study two model organisms is used. First, saccharomyces cerevisiae, or bakers yeast, is a common model organism for studying glucose metabolism. Yeast cells have a highly efficient glucose uptake system and are able to rapidly metabolize glucose to generate energy for growth and reproduction. Saccharomyces cerevisiae contains glucose transporters that share many characteristics with the glucose transporter proteins (GLUTs) present in other animals, including humans. GLUTs and yeast glucose transporters are both membrane-integral proteins that help move glucose across a cell's plasma membrane. Second, zebrafish have been shown to be a useful model organism for research on tissue regeneration and wound healing. Zebrafish have been shown to be a useful model organism for research on tissue regeneration and wound healing. The genetic and molecular processes enabling zebrafish wound healing have been the subject of substantial investigation as a result.

OBJECTIVES:

1. **Analysis of primary and secondary metabolites in AQ and MoH extract of *Cordyceps militaris*.**
2. **Effect of AQ and MoH extract of *Cordyceps militaris* on cytotoxicity and cell viability.**
3. **Evaluati0n of the effect of AQ and MoH extracts of *Cordyceps militaris* on uptake of glucose and wound healing under hyperglycemic conditions.**

CHAPTER – 2

REVIEW OF LITERATURE

2.1 GLUCOSE UPTAKE:

One of the primary sources of energy in mammals is glucose, which has ATP as the main product through the mechanisms of oxidative phosphorylation and glycolysis. Glucose, composed of a 6-carbon structure, is a precursor of biomolecules(nucleotides,amino acids,lipids) [1]. For all living things, it serves as a universal source of energy and is necessary for both aerobic and anaerobic cellular respiration. [2]. Cells take up glucose from the bloodstream and use it for energy production which is important for the proper functioning of various organs in the body, including the brain, heart, and muscles. INS, a pancreatic hormone, aids the transport of glucose from the circulation into cells, indicating a glucose-depositing nature. Whereas GLgn serves as a glucose-mobilizing hormone, by doing so, the blood glucose level is maintained. This is done by the balancing- opposing activities of GLgn and INS:glucose homeostasis. In the pancreatic islet of Langerhans both GLgn and INS are generated;GLgn produced by alpha(A)-cells and INS by beta(B)-cells. Based on the metabolic state of the body (fasting or energy-rich) there is a balance between these two hormones, with INS being more concentrated during energy-rich states and GLgn being more concentrated during fasting. Glycogen is catabolized, releasing glucose (during fasting) or synthesized further, absorbing surplus glucose (facilitated by INS during energy-rich times). Along with other hormones, INS and GLgn control the expression of a particular kind of glucose transporter, GLUT4, which in turn controls how much glucose enters and exits cells. [3, 5, 6].

Glucose uptake is an intricate process that involves some steps and mechanistic operations. To enter cells, glucose requires specific transport proteins that act as gatekeepers. These transport proteins are called glucose transporters, or GLUTs. There are several types of GLUTs, and each type is expressed in different tissues and cells. INS signaling is also one of the important mechanisms involved in glucose uptake. INS binds to specific receptors on the surface of cells and triggers a

series of events that promote glucose uptake. One of the primary actions of INS is to increase the number of GLUTs on the cell surface, allowing more glucose to enter the cell. Then, in glucose metabolism, once glucose enters the cell, it undergoes several metabolic processes to produce energy. Glucose is first converted into glucose-6-phosphate, a process that requires the enzyme hexokinase. Glucose-6-phosphate can then be used in various metabolic pathways to produce ATP, the primary energy currency of cells. Cells can also store excess glucose as glycogen, a complex carbohydrate that can be broken down into glucose when needed through glycogen synthesis. Glycogen synthesis occurs in the liver and muscles, and it is regulated by INS [3, 4].Glucose passes through the lipid bilayer through a unique membrane transporter, the sugar transporter. The main types of sugar transporters are GLUT (sodium-independent transporters), SGLT (sodium-dependent transporters), and SWEET. [7-11]. Coupled with the active transport of sodium through the cell membrane, the sodium-dependent transporters distribute the glucose molecule along the concentration gradient. SGLTs are vital for glucose reabsorption and absorption and are found largely in the renal tubules and intestinal epithelium. Because it takes ATP to actively transport sodium outside of the cell-lumen, this transporter promotes co-transport of glc while Na passively moves through the cell wall down its conc-gradient. Sodium-independent transporters do not require sodium to transfer glucose and instead use facilitated diffusion [8, 9].

2.2 GLUCOSE TRANSPORTERS:

The primary function of the GLUTs is to regulate the flow of glucose compartments intracellularly and extracellularly, ensuring that there is always glucose homeostasis. The gene symbol for the members of the GLUT family is SLC2A, and they are members of the 2A solute carrier family [9]. According to their basic sequences, the GLUT genes (14 isoforms) can be divided into three classes on the basis of selectivity of substrate, expression of tissue, and subcellular localization. Each GLUT has a unique expression pattern and kinetic properties that allow for efficient glucose transport in different tissues and under various physiological conditions. GLUT 1-4 and GLUT 14 make up the Ist class of genes (class I), which is referred to as the classical sugar conveyor. Sugar

transporters five, seven, nine, and eleven are included in Class II, and in Class III, six, eight, ten, and twelve. Another name for GLUT 13 is the H+ myoinositol transporter. Their dispersion across tissues reveals that GLUT expression is tissue-dependent. For example, the brain expresses GLUT1 and GLUT3 to meet the high glucose demand. Some tissues have multiple GLUTs, such as muscle, which has been shown to express GLUTs 3-5 and 10–11 [9-11].

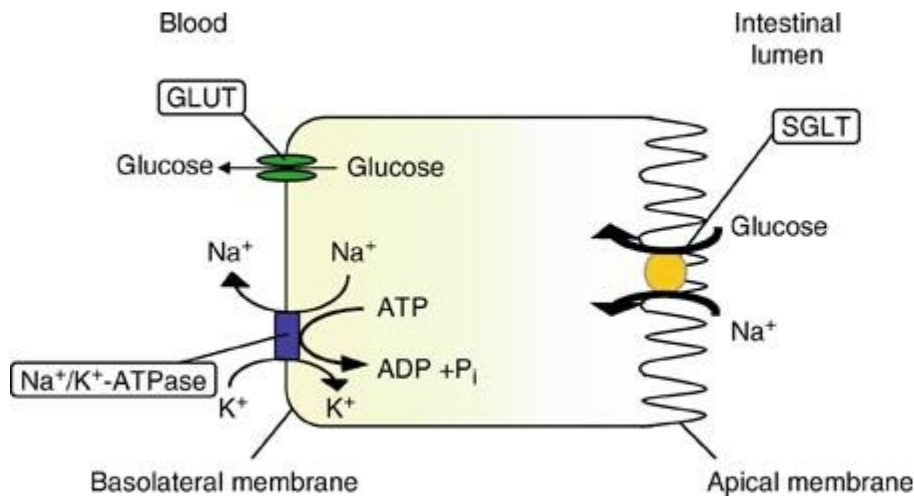


Figure 2.1: Glucose transporters

GLUT1 is widely expressed in many tissues, including the brain, red blood cells, and endothelial cells. It has a high-affinity for glc and is responsible for basal glucose uptake. GLUT2 is found largely in the liver, pancreas, and small intestine. It transports excess glucose from the circulation into the liver and pancreas and has a lower affinity for glucose than GLUT1. GLUT3 is found largely in the brain and CNS. The presence of GLUT-3 has been found in high-glucose-claiming tissues, such as the placenta and testes. This transporter has a high-affinity for glc, is in charge of glucose absorption in neurons, and is required for optimal brain function. GLUT4 is predominantly expressed in muscle and adipose tissue and is INS-regulated. INS signalling promotes GLUT4 to be translocated from intracellular vesicles to the cell surface, allowing for greater glucose absorption. When INS is released, these transporters suffer a 10 to 20-fold increase in density, resulting in a drop in BGL (glc will more readily goes inside of cells with GLUT4 on it). GLUT5 is located largely in the small intestine and is in charge of transporting fructose, a simple sugar present in fruits and vegetables [2, 12, 13].

2.3 GLUCOSE METABOLISM

When glucose enters the cell via a GLUT transporter, it is transformed to glucose-6-phosphate via a process known as phosphorylation. Glucose breakdown is an intricate process that involves numerous reactions of metabolism, like glycolysis, the citric-acid-cycle, and ox-phosphorylation. Glycolysis is initial stage in glc metabolism. Depending on the tissue type, the enzyme hexokinase or glucokinase catalyzes this process. Glucose-6-phosphate can then be utilized to create energy in various metabolic pathways or stored as glycogen for later use. During glycolysis, glucose turns into pyruvate. This process happens in the cytoplasm of cells and is oxygen-free. It generates a modest quantity of ATP, the cell's principal energy currency. Pyruvate enters the mitochondria for pyruvate oxidation and is transformed to acetyl-CoA. This mechanism generates NADH, a chemical required for the next stage of glucose metabolism. The citric-acid-cycle, frequently referred to as the Krebs-cycle, is a chain of biochemical activity that happens in the mitochondria. Acetyl-CoA joins the process and is transformed into a number of intermediate molecules, resulting in the production of ATP and NADH. The last step of glucose metabolism is oxidative phosphorylation, in which NADH is oxidized to form ATP. This process takes place in the mitochondria and necessitates the use of oxygen.

A number of factors, like INS levels, exercise, and dietary factors, influence glucose uptake. INS resistance can hinder glucose metabolism and result in higher BGLs. Obesity and metabolic syndrome are frequently related with this illness. Exercise promotes the translocation of glucose transporters to the cell surface, which increases glucose metabolism in muscles. A high-sugar, high-carbohydrate diet can result in higher BGLs and decreased glucose metabolism over time. Some medications like metformin can enhance glucose metabolism by decreasing INS resistance and boosting glucose absorption.

2.4 DYREGULATION OF GLUCOSE METABOLISM:

2.4.1 FACTOR AFFECTING GLUCOSE METABOLISM

Glucose transporter (GLUT) dysregulation can be caused by a variety of reasons, including genetic, environmental, and lifestyle variables. Here are a couple such examples: Genetic influences, mutations or polymorphisms in the genes that code for GLUTs can influence their expression or function, resulting in glucose transport dysregulation. For example, mutations in the GLUT1 gene have been linked to GLUT1 deficiency syndrome, a rare genetic condition characterized by seizures and developmental delay. Environmental pollutants such as bisphenol A (BPA) and phthalates have been found in animal studies to change the expression of GLUTs and impair glucose metabolism.

A sedentary lifestyle, a poor diet, and obesity can all affect glucose metabolism and result in GLUT dysregulation. A diet heavy in simple sugars and refined carbohydrates, for example, can result in higher BGLs and INS resistance, which can down regulate GLUT4 expression and decrease glucose absorption. Hormones such as INS, GLgn, and cortisol are major regulators of glucose metabolism and GLUT expression. Changes in hormone levels or signaling pathways can affect glucose transport and lead to metabolic diseases.

Glucose transporter (GLUT) dysregulation can have a major impact on glucose metabolism, which leads to the development of metabolic diseases such as DB.

In type 1 DB, for example, INS-producing beta cells in the pancreas are lost, resulting in decreased INS output. INS regulates glucose uptake by increasing GLUT4 translocation to the cell surface in muscle and adipose tissue. GLUT4 translocation is hindered in the absence of INS, resulting in decreased glucose absorption and increased BGLs. INS sensitivity, also known as INS resistance, is reduced in type 2 DB [14]. This can result in decreased glucose transport and higher BGLs. INS resistance is associated with decreased GLUT4 expression and decreased glucose absorption in muscle and adipose tissue. Other variables can potentially lead to GLUT dysfunction. High-fat diets, for example, can affect glucose metabolism by lowering GLUT4 expression in muscle and adipose tissue. Exercise, on the other hand, can boost GLUT4 expression and enhance glucose metabolism in INS-resistant people.

2.4.2 GLUCOSE METABOLISM DYSREGULATION: DIABETES

DB is caused by an imbalance in glucose metabolism. DB is a metabolic disorder characterized by high BGLs (hyg) caused by defective glucose metabolism. DB is classified into two types: type-1 DB and type-2 DB [15, 16, 17]. Type 1 DB is an autoimmune condition in which the immune system assaults and kills the pancreas' INS-producing beta cells. INS is essential for glucose absorption and use by cells in the body. Glucose cannot enter cells without INS, resulting in hyperglycemia. Type 1 DB patients require INS injections or INS pumps to control their BGLs [17]. Type 2 DB is a metabolic disorder characterized by INS resistance, which means that the cells of the body are less receptive to the effects of INS.

The pancreas first adjusts by making more INS, but over time, the beta cells get fatigued and are unable to keep up with the demand. This results in hyperglycemia. Glucose metabolism is reduced in type 2 DB due to a combination of variables such as INS resistance, decreased INS production, and dysregulation of glucose transporters [1].

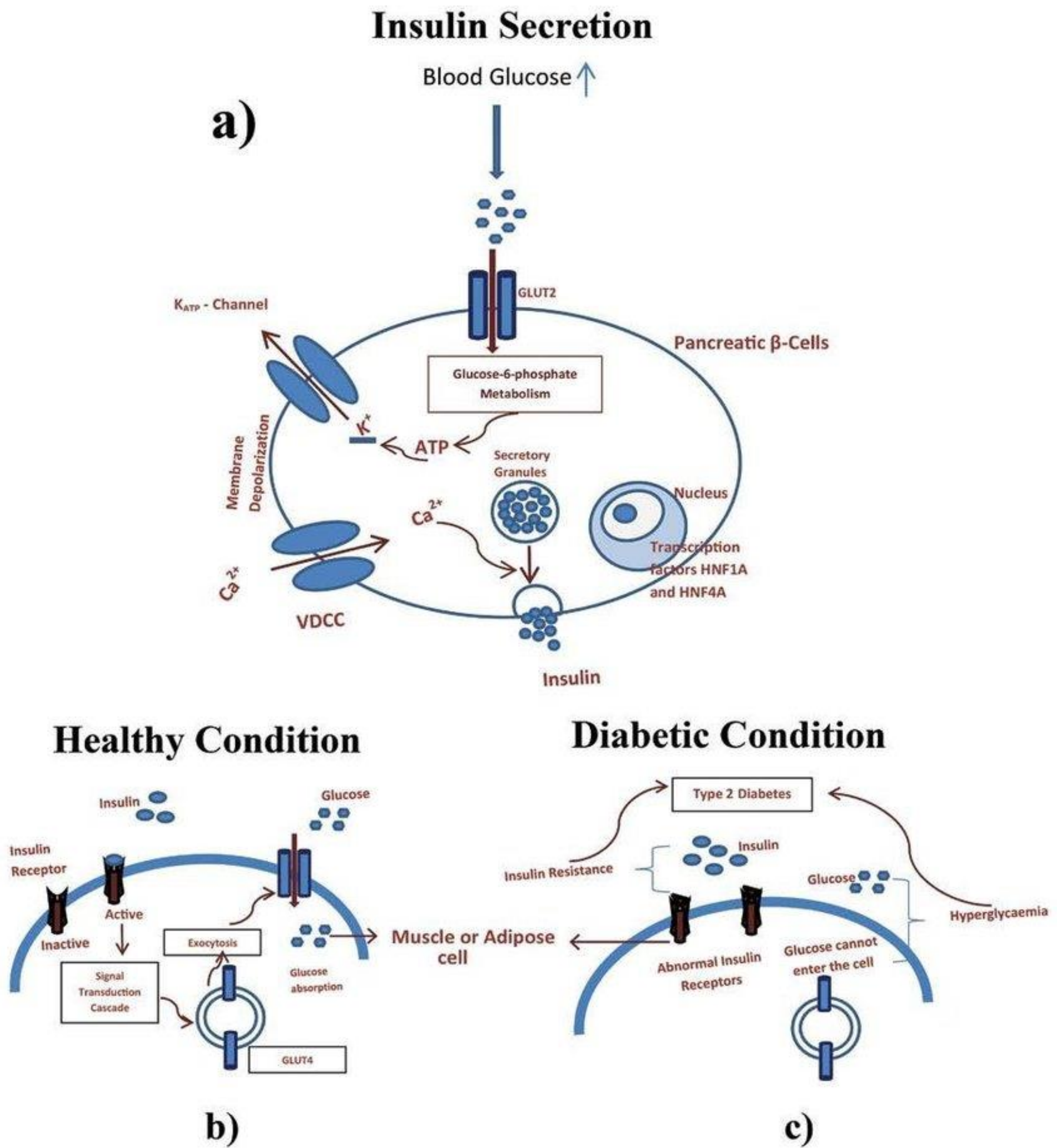


Figure 2.2: Representation of DB

2.5 DIABETES AND WOUND HEALING:

Dysregulation of glucose metabolism contributes to the development of hyperglycemia in both kinds of DB. Elevated BGLs can harm the body's organs and tissues, resulting in consequences such as wound healing neuropathy, retinopathy, nephropathy, and cardiovascular disease [18]. BGLs and wound healing exhibit a definite connection, according to many studies. DB affects the body's capacity to create or react to INS, a hormone that enables cells to absorb and utilize blood glucose for energy. It is more difficult for the body to control BGLs as a result of this INS disturbance. White blood cell functionality is hampered when BGLs are chronically elevated. In order for the immune system to function, white blood cells are essential. The body's capacity to combat infections and heal wounds is decreased when white blood cells are unable to operate properly. Reduced blood flow and altered circulation caused by uncontrolled DB which in turn makes it difficult for the wounds to get nourishment. As an outcome, there is rather a slow healing or no healing at all [27].

DB can affect wound healing for a variety of reasons, such as reduced immune function, altered angiogenesis, and inadequate circulation. Diabetic wounds can range in severity from minor nicks and scratches to serious ulcers and infections. Elevated BGLs can also cause damage to bloodvessels and nerves, which can slow wound healing. This is associated with poor circulation and nerve damage in the feet and lower extremities, which increases the risk of foot ulcers and lower limb amputations. The persistent hyperglycemia and associated diabetic complications, contribute to the exceptionally complex pathology of the diabetic wound, such as barrier disruption and infection, microvascular complications, neuropathy, high oxidative stress, and suboptimal chronic inflammatory reactions, as well as psychological issues involving the patient's mental health, self-esteem [18,19].

As a result of a biological reaction to damage keratinocytes, fibroblasts, endothelial-cells, macrophages, and platelets are all activated during the healing process. These cells emit a lot of cytokines and growth factors. The following table outlines the known physiological variables that contribute to DB patients' wound healing impairments [19].

Table 2.1: Physiological variables contributing to DB patients' wound healing impairments

Growth factor production:reduced or impaired	19-21
Epidermal barrier function	21
Angiogenic response	21,22
Macrophage function	23
Collagen accumulation	21
Granulation tissue qty	21
Migration and proliferation:keratinocytes and fibroblast Number of epidermal nerves	24
Balance b/w ECM components and their remodeling by MMPs Healing of bones	27

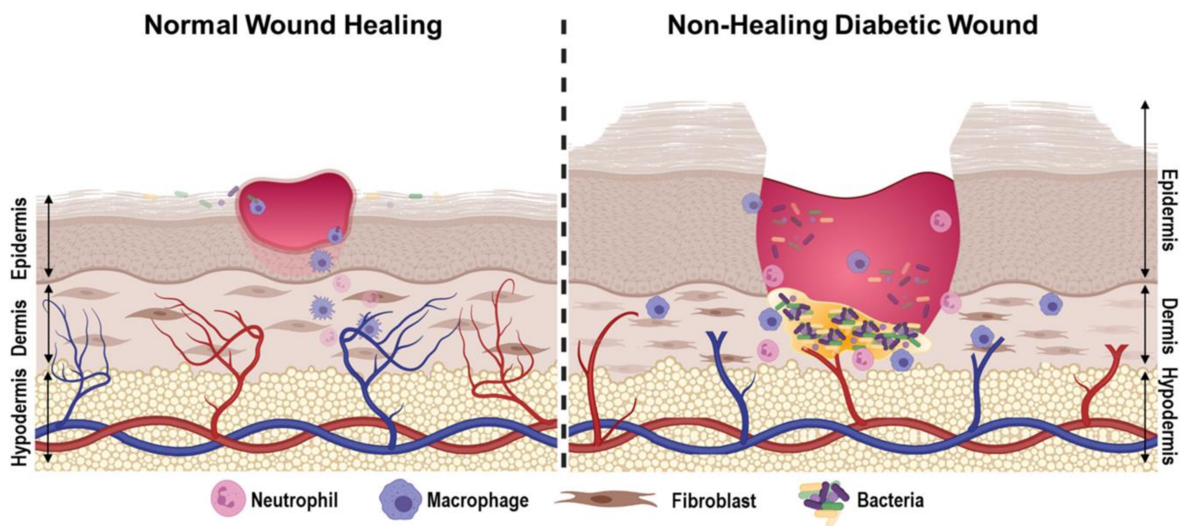


Figure 2.3: Wounds caused by DB: pathophysiology. Chronically maintained sub-optimal inflammation, elevated levels of ROS, and colonization and transformation of bacteria biofilm(difficult to treat) are all present in diabetic wounds [18].

2.6 WOUND HEALING MECHANISM: NORMAL WOUND VERSUS DIABETIC WOUND

The fusion of several signals regulates and maintains the acute wound healing process. In an individual(normal), these signals in the form of cytokines and chemokines are produced by keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets. This generation of signals gets disrupted and cannot be maintained in DB to heal the wound.

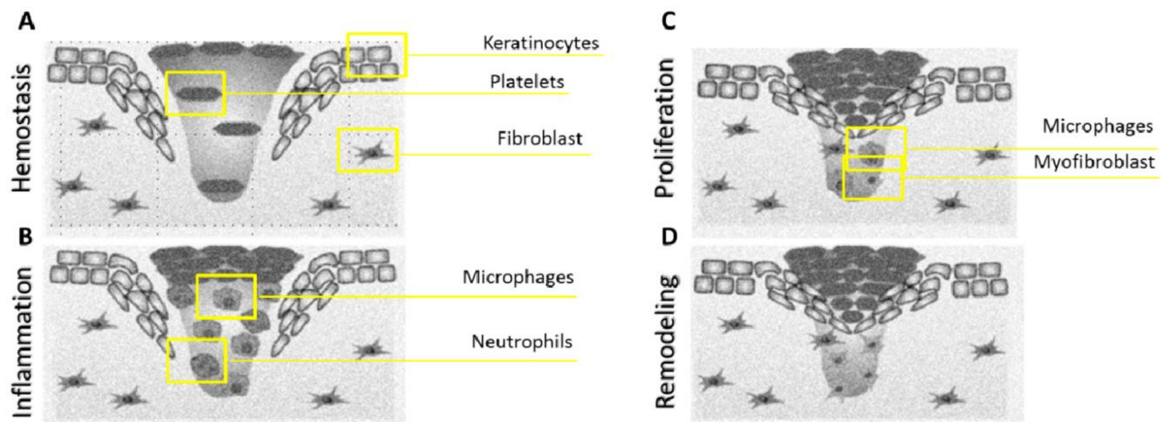


Figure 2.4: Phases of wound healing and related events [21].

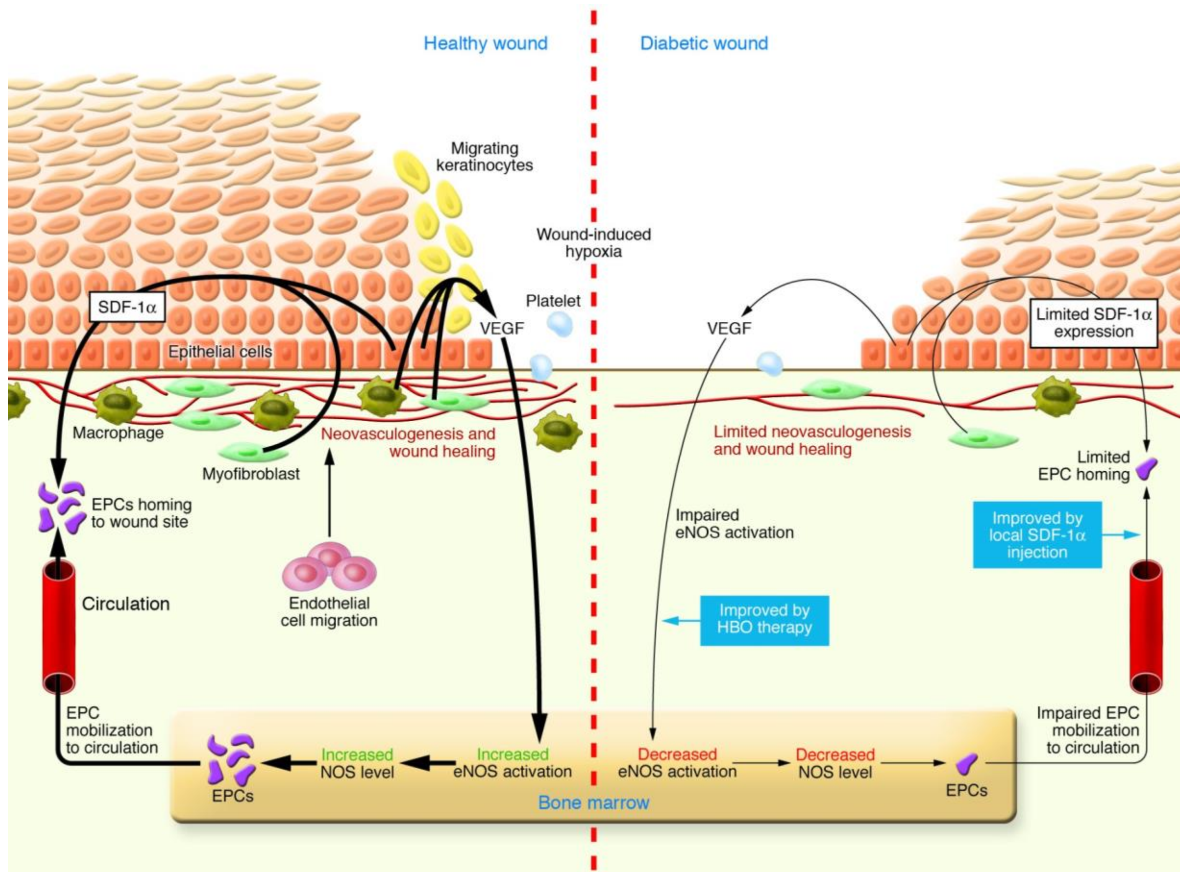


Figure 2.5: Wound healing mechanism (normal vs. diabetic)

According to studies, pathogenic markers such as β-catenin nuclear localization and overexpression of c-myc are associated with slower wound healing and have been discovered through patient biopsies molecular analysis taken from their epidermis [26].

Along w/ decrease in and aberrant localization of EGFR and glucocorticoid pathway activation the migration of keratinocytes becomes restricted. The absence of migration of keratinocytes, hyper proliferation, and incomplete differentiation can be found at the nonhealing edge of a diabetic wound. Fibroblasts demonstrate phenotypic change, a decrease in migration-proliferation. The cells from the adjacent areas display the appearance of physiologically impaired but normal phenotypic characteristics [26].

The first step in diabetic wound healing is to control BGLs through lifestyle changes, medications, and, if necessary, INS therapy. This can help reduce the risk of infection and promote wound healing. To encourage healing and prevent infection, wounds should be kept clean and moist. Advanced wound care treatments may be required in some circumstances for diabetic wound healing.

Negative pressure wound treatment, which includes applying negative pressure to the wound to facilitate healing, or the use of growth factors or stem cells to stimulate tissue regeneration, are two examples. Hydrogel dressings, for example, are frequently used to establish a moist wound environment. Debridement:removal of dead tissue from a wound, may also be required to assist healing. Hyperbaric oxygen treatment (HBOT) may be used to enhance diabetic wound healing in some circumstances. This involves subjecting the body to high quantities of oxygen in a pressurized chamber, which can aid with circulation and tissue repair [18].

2.7 DIABETIC WOUND HEALING IMPROVEMENT: IMPROVING GLUCOSE UPTAKE, THUS HEALING THE WOUNDS

Controlling BGLs, treating any underlying illnesses that may be contributing to the wound, and providing appropriate wound care are all necessary components of a multidisciplinary strategy to improve diabetic wound healing. Wound healing is hampered by high BGLs. Controlling BGLs through lifestyle changes, drugs, and, if necessary, INS treatment is crucial for enhancing diabetic wound healing. Since ancient times, traditional medicines have been used to treat a variety of illnesses, including DB. Improving the absorption of glucose in cells, which is essential for preserving stable blood sugar levels, is one of the fundamental issues in the management of DB.

2.8 TRADITIONAL MEDICINE AND DIABETES

Traditional medical practices include a number of naturally derived treatments that can enhance glucose absorption and promote normal blood sugar levels. Traditional Asian medicine has a long history of employing herbs and formulas to maintain a balanced glucose metabolism, including Ayurveda and Traditional Chinese Medicine (TCM). These treatments function in a variety of ways, including enhancing INS sensitivity (low INS sensitivity causes cells to become resistant to INS, which can develop high blood sugar levels), promoting INS secretion can help increase glucose uptake in the cells by promoting the uptake of glucose into the

cells, lowering INS resistance(cells stop responding to INS) can boost cellular absorption of glucose, and decreasing oxidative stress and inflammation can enhance INS sensitivity and boost cellular absorption of glucose, both can hinder the absorption of glucose by cells by interfering with INS signaling pathways. All of this aids in enhancing glucose absorption in cells.

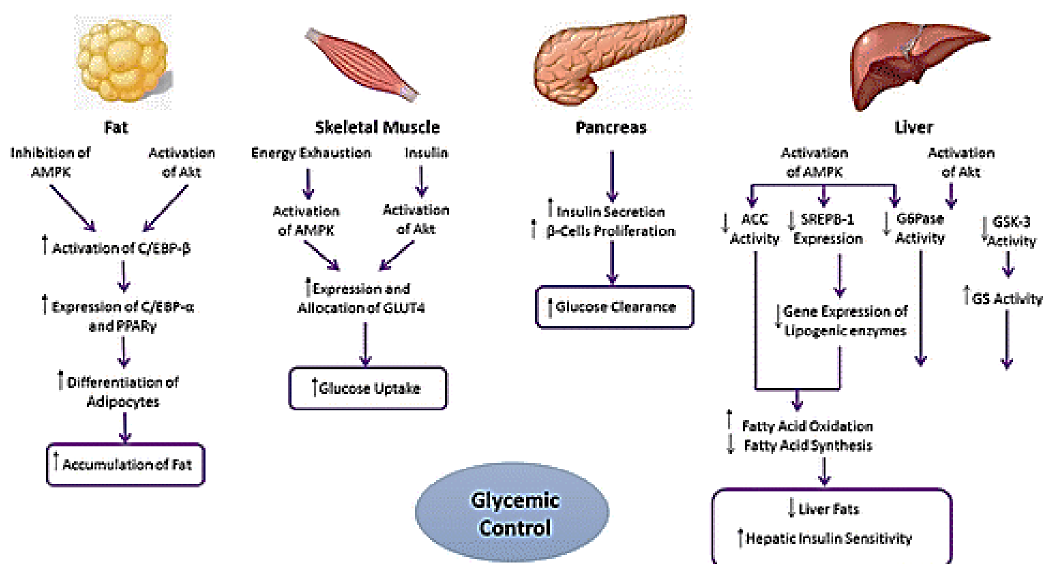


Figure 2.6: Traditional medicines mechanism of action.

Fenugreek, bitter melon, cinnamon, ginseng, mulberry, and Gymnema sylvestre are a few examples of traditional medicines used for glucose absorption in Ayurvedic medicine, while Huang Qi, Shan Yao, Wu Wei Zi, Jin Ying Zi, and Gui Zhi Fu Ling Wan are examples in TCM.

Table 2.2: Traditional medicines and their mechanism

Traditional medicines	Their mechanism	References
1. *Cinnamomum verum* 2. *Trigonella foenum-graecum* 3. *Momordica charantia* 4. *Panax* 5. *Aloe vera*	Increase INS sensitivity and improve glucose uptake in cells	28 31 30 32 29 33

6. *Gynostemma pentaphyllum* 7. *Astragalus membranaceus* 8. *Dioscorea opposita* 9. *Schisandra chinensis* 10. *Rosae Laevigatae Fructus* 11. *Gui Zhi Fu Ling Wan*		34 35 36 37 38
1. *Gymnema sylvestre* 2. *Morus alba*	Stimulate INS secretion and reduce INS resistance.	31 39
1. *Phyllanthus emblica*	Increase INS sensitivity and reduce oxidative stress	40
1. *Ganoderma lingzhi*	Increase INS sensitivity and reduce inflammation	41

2.9 *CORDYCEPS MILITARIS*: TRADITIONAL CHINESE MEDICINE

A prominent ingredient in traditional Chinese medicine is a fungus species called *Cordyceps militaris* , which is an entomopathogenic fungi; family: Clavicipitaceae, class:Pyrenomycetes; order:Hypocreales (ascomycetous-fungi). Since ancient times, "Winter-Worm-Summer-Grass" (Cordyceps), also known as "DongChungHaCao" in Korea and "DongChongXiaCao" in China, has been utilized as a traditional medicine in Asia. It parasitizes insects and colonizes live or dead caterpillar:Hepialus(Lepidoptera). By yeast-like budding, during autumn the fungus develops and multiplies spontaneously inside the host, destroying it. After spending the winters, the fungus ruptures the host body and creates a stroma, a sexually sporulating structure that develops upward and is attached to the dead larva. The stroma then emerges above the earth the next summer [42, 44].

Figure 2.7: ***Cordyceps militaris*** **grown on wheat, sclerotium and pupae**

Carl Linnaeus first referred to *Cordyceps militaris* as Clavaria militaris in his description of it in 1753. This fungus is the species of the genus Cordyceps, which belongs to the Cordycipitaceae family [42]. Various names for *Cordyceps militaris* are Caterpillar Fungus, Caterpillar Mushroom, Ophiocordyceps, Tochukaso, Vegetable Caterpillar, Cs-4, Champignon Chenille, ChineseCaterpillar Fungus, DongChongXiaCao, DongChongZia Cao, HsiaTs'Ao TungCh'Ung[42-55]. It is called a "caterpillar fungus" due to the fact that it thrives on the larvae of several insects, notably moths. Usually, rice or soybeans are used as the substrate for growing *Cordyceps militaris* . Since laboratory cultivation of *Cordyceps militaris* allows for exact control of growth conditions and lessens the environmental impact of collecting *Cordyceps militaris* from the wild, it may be a more environmentally friendly and economically advantageous method of producing the fungus for medicinal and culinary uses [43, 44]. It is grown on a variety of mediums like potato dextrose agar (PDA), malt extract agar (MEA) or corn steep liquor (CSL) agar. Incubated at optimal temperature and humidity that is 20 to 28 degree celsius with relative humidity

of 50 to 80%. Growth of culture is monitored and the mature fruiting body is harvested by cutting it from the growth medium. Then it is dried and stored in a cool, dry place until ready to be used.

According to traditional Chinese medicine, *Cordyceps militaris* has a number of health advantages, including enhancing athletic performance, lowering weariness, and strengthening the immune system. Although additional study is required to validate these possible advantages, several studies have also shown that it may have anticancer and antiviral activities. Additionally, *Cordyceps militaris* is employed in food preparation, especially Chinese cuisine. It may be used in soups and stews and has a mildly sweet and nutty flavor. Several substances found in the fungus *Cordyceps militaris* have been reported to offer potential health advantages[46-50]

2.10 COMPOUNDS PRESENT IN *CORDYCEPS MILITARIS* :

Polysaccharides, nucleosides, organic acids, and amino acids are abundant in *Cordyceps militaris* and have been reported to provide a variety of possible health advantages [47,50-53].

The following are some of the primary *Cordyceps militaris* ingredients:

1. Polysaccharides: Polysaccharides, complex carbohydrates with immune-stimulating characteristics, are abundant in *Cordyceps militaris* . These polysaccharides have been demonstrated to boost the generation of white blood cells and enhance immune system performance.

2. Cordycepin: Research has demonstrated the antiviral and anticancer effects of the nucleoside cordycepin. Additionally, it has demonstrated potential as an anti-fungal and anti-inflammatory agent.

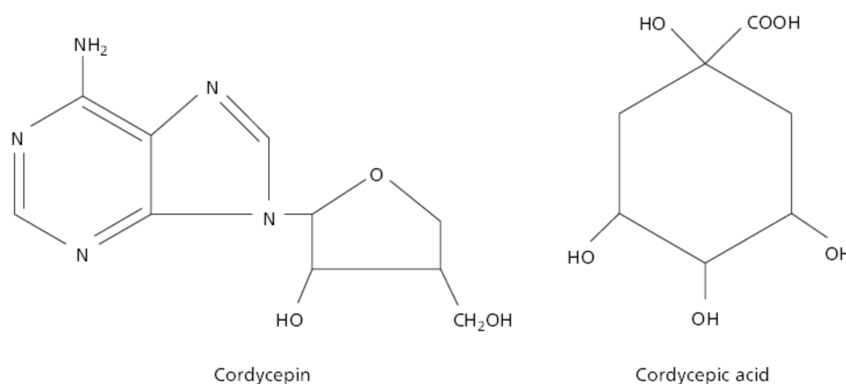


Figure 2.8: Cordycepin and cordycepic acid

3. Adenosine: Research on this nucleoside has revealed a number of health advantages. It has been demonstrated to enhance blood flow, reduce swelling, and guard against oxidative stress.
4. Cordycepic acid: This type of organic acid has been shown to have potential antiviral, antifungal, anti-inflammatory, and antioxidant properties
5. Ergosterol. Fungi contain ergosterol, a kind of sterol. It has been demonstrated that it may possess anti-inflammatory and antioxidant qualities, and it is a precursor of vitamin D.
6. Amino acids: *Cordyceps militaris* contains a range of aminoacids: the building blocks of proteins, including essential aminoacids like val, leucine, and isoleu, as well as non-essential aminoacids like glutamine [45-54].

There have been various activities associated with *Cordyceps militaris* , including:
1. Immune-stimulating properties: Polysaccharides, which are abundant in *Cordyceps militaris* , have been found to improve immunity by promoting the generation and functionality of white blood cells.
2. Antioxidant activity: Ergosterol and cordycepic acid, two antioxidants found in *Cordyceps militaris* , have been demonstrated to scavenge free radicals and decrease oxidative stress.
3. Anti-inflammatory properties: Adenosine and cordycepic acid, which are found in *Cordyceps militaris* shows anti-inflammation properties through the prevention of synthesis:pro-inflammatory cyto-kines.
4. Antiviral activity: Hepatitis B, herpes simplex, and influenza viruses have all been demonstrated to be susceptible to the antiviral effects of the cordycepin and cordycepic acid found in *Cordyceps militaris* .
5. Anti-tumor activity: Some studies have suggested that *Cordyceps militaris* may have potential anti-tumor effects due to its polysaccharides and cordycepin content.
6. Anti-fatigue activity: *Cordyceps militaris* has been reported to have anti-fatigue activity by improving exercise endurance and reducing fatigue [48, 50-52]

Table 2.3: Different extract and their activity

Extract	Activity	Mechanism	Reference
Water and AQ	Anti cancer	Cell cycle arrest	46
Cordycepin	suppress viral replication (inhibiting RNA and DNA synthesis)	Cytotoxic inhibition of nucleosides	43
Ethanolic	Inhibit: human colorectal carcinoma:RKO cells and xenograft mice.	Apoptosis(p53- dependent, mitochondrial mediated) Arrest of cell cycle	44
Hot water and alcoholic extract	tonic for longevity, endurance and vitality for thousands of years	Not known	48
Water extract	Anti tumor activity(xenografts in mice)	Not known	49
MoH extract	inhibitor of tumor cell growth	Not known	49
MoH extract	Inhibition : MCF-7 cells HCT-15 cells HeLa cells NCI-H460 cells	Not known	50
Water extract	inhibit telomerase activity; apoptosis induction in A549 cells	Not known	50
	Apoptosis: K562 cells in vitro		50
Water extract	Increase glucose metabolism	the expression of several proteins was related to the	55

		increase in glucose metabolism	
Alcoholic extract	ability to down-regulate the expression of the mecA gene in MRSA		45
	Activation:macrophage-mediated immune response	promote macrophages phagocytic ability:increase ROS/NO production:TNF-α release	51
Ethanolic extract	Inhibit:human glomerular mesangial cell proliferation	Not known	52
	anti-fibrotic	Not known	52
	anti-angiogenetic	Not known	52
	Improvement:INS resistance and INS secretion	Not known	52
	Anti inflammatory	Not known	52
	Growth inhibition:U937 leukemia cells	Not known	52
Water extract	strong growth-inhibiting activity towards Clostridium species	Not known	54
C.militaris protein (CMP)	to inhibit the growth of F. oxysporum	Not known	53

2.11 GLUCOSE METABOLISM AND *CORDYCEPS MILITARIS*

In both animal and human research, *Cordyceps militaris* has been found to improve INS sensitivity and glucose absorption. The presence of bioactive substances such polysaccharides, cordycepin, and adenosine, which have been demonstrated to have hypoglycemic and INS-sensitizing properties, is probably responsible for this. Extract from *Cordyceps militaris* has been demonstrated in studies on animals to enhance INS sensitivity in diabetic mice and boost glucose absorption in muscle cells. Additionally, it has been demonstrated to lower fasting BGLs and enhance glucose tolerance in diabetic rats. Studies on humans have demonstrated that *Cordyceps militaris* helps type 2 diabetics by improving their glucose metabolism. In comparison to a placebo, one study indicated that taking a *Cordyceps militaris* dose for 12 weeks reduced fasting blood sugar levels and increased INS sensitivity in people with type-2 DB. In a different study, it was shown that the extract of *Cordyceps militaris* lowered INS resistance and improved glycemic management in people with impaired glucose tolerance. There have also been several reports of the activities of C. militaris, including the improvement of liver functions, the hypoglycemic impact in diabetic mice, and the production of the glycometabolism-related enzyme glucokinase (GK) in liver cells. However, there hasn't been much information on the antidiabetic impact of C. militaris in the research that has been published [44, 55].

Mechanism of cordyceps on glucose metabolism:

The effects of *Cordyceps militaris* on glucose absorption in muscle cells were explored in research that was published in the 2014 issue of the journal Nutrients. The signaling system which is crucial for controlling energy metabolism, was shown to be activated by treatment with *Cordyceps militaris* extract, increasing glucose absorption in muscle cells. Additionally, the research revealed that *Cordyceps militaris* extract boosted the expression of the glucose transporter 4 (GLUT4), which helps glucose enter cells. Another 2017 study examined the effects of *Cordyceps militaris* on glucose metabolism in diabetic rats and found that treatment with *Cordyceps militaris* extract decreased fasting BGLs and enhanced glucose tolerance wrt a control group. The study also discovered that *Cordyceps militaris* extract elevated the expression of INS receptor substrate 1 (IRS-1) and uptake of glc. Adiponectin, a hormone that controls the metabolism of glucose and lipids, was

discovered to be expressed in greater quantities in the *Cordyceps militaris* extract, according to the study.

One of the key *Cordyceps militaris* constituents that has been demonstrated to have advantageous effects on glucose absorption is cordycepin. A nucleoside analogue known as cordycepin has been found to have hypoglycemic effects via triggering signaling pathway(AMPK), essential for controlling how much energy is used. The AMPK pathway triggered by cordycepin through elevation of the intracellular AMP:ATP ratio. Low energy status, oxidativestress, and inflammation are just a few examples of the several cellular stressors that cause AMPK to become active as crucial regulator of energy metabolism. The activation of AMPK and subsequent intracellular signaling processes that result in higher glucose absorption and enhanced INS sensitivity happen when the AMP:ATP ratio rises. In particular, AMPK stimulates the protein TBC1D1, which in turn increases the tra glc transporter translocation GLUT4 to the plasma membrane, increasing the absorption of glc by muscle cells. Adenosine, polysaccharides, and other bioactive substances that have been demonstrated to have hypoglycemic and INS-sensitizing effects are also present in *Cordyceps militaris* in addition to cordycepin. However, the precise processes behind these effects are still poorly known, and further investigation is required to establish the best dosages and delivery strategies for various medical problems.

2.12 YEAST: MODEL ORGANISM FOR GLUCOSE UPTAKE

Saccharomyces cerevisiae is a common organism usedas a biological model for studying glucose metabolism. Yeast cells have a highly efficient glucose uptake system and are able to rapidly metabolize glucose to generate energy for growth and reproduction. Yeast cells absorb glucose when it is present in the environment using specialized transporters found in the pm. The addition of phosphate group to glc to form glc-6-phosphate with the help of hexokinase and it is changed into fructose6phosphate by phosphohexose isomerase subsequently. Fructose6phosphate can go via the glycolytic route and undergo further metabolism to produce ATP and other metabolic intermediates. Hexokinase is a crucial regulator of yeast glucose metabolism and is inhibited by excess glucose-6-phosphate. This aids in preventing the buildup of glucose-6-phosphate, which can have detrimental consequences for metabolic and cell development. Yeast cells may metabolize glucose

using the pentose phosphate route in addition to glycolysis, which produces vital cellular building blocks such as nucleotides and amino acids. Additionally, extra glucose may be stored in yeast cells as glycogen, which can later be used as a source of energy [56-59].

A complicated network of signaling pathways that responds to variations in glucose availability and cellular energy status controls glucose metabolism in Saccharomyces cerevisiae. These pathways include post-transcriptional and transcriptional regulation of gene expression as well as post-translational changes that control the activity of enzymes and feedback inhibition of metabolic pathways. The cAMP-PKA pathway is a crucial regulatory route in yeast glucose metabolism. The glycolytic pathway breaks down glucose when it is present, producing ATP and lowering intracellular levels of AMP in the process. As AMP levels drop, the enzyme adenylate cyclase is activated, producing cyclic AMP (cAMP). The protein kinase A (PKA) enzyme controls the expressing nature of several genes involved in glucose metabolism and activated by increased amounts of cAMP. The Snf1/AMPK pathway is a crucial regulatory route in yeast glucose metabolism. The cell's energy status declines when glucose is limited, which causes a rise in intracellular levels of AMP. This rise in AMP levels activates the Snf1/AMPK protein kinase, which controls the genes activation involved in alternate metabolic pathways:the glyoxylate cycle and gluconeogenesis. Overall, Saccharomyces cerevisiae's intricate control of glucose metabolism enables the cell to adapt to shifting environmental conditions and use available energy sources most efficiently [56, 57].

Saccharomyces cerevisiae has a variety of distinct glucose transporters that are in charge of absorbing glucose from the environment. Hxt1, Hxt2, Hxt3, Hxt4, Hxt6, and Hxt7, all membrane proteins belonging to the sugar transporter family (STP), are the major glucose transporters in yeast. The affinity for glucose and the patterns of expression of these transporters under various growth circumstances vary. For instance, Hxt2, Hxt4, and Hxt7:low-affinity transporters and expressed under high glc settings whereas Hxt1 and Hxt:high-affinity glc transporters that are expressed under low glc situations. An intricate web of signalling channels that react to variations in glucose availability and cellular energy status controls the production of these transporters. For instance, the Rgt1-Snf3 pathway controls the expression of Hxt1 and Hxt3 by sensing extracellular glucose levels and turning on the production of high-affinity glucose transporters when glucose is limited. The Mig1 route, on the other hand, responds to intracellular glucose levels and promotes the production of low-affinity transporters when glucose is plentiful. This pathway controls the expression of Hxt2, Hxt4, and Hxt7 [58,59].

Saccharomyces cerevisiae contains glucose transporters that share many characteristics with the glucose transporter proteins (GLUTs) present in other animals, including humans. GLUTs and yeast glucose transporters are both membrane-integral proteins that help move glucose across a cell's plasma membrane. GLUTs and yeast glucose transporters both include 12 transmembrane domains in their structural makeup, with the transporter's central cavity housing the glucose-binding site [10-12]. The binding of glucose causes a change in the protein structure that enables the release of glucose on the other side of the membrane, and the transporters go through conformational changes to aid the transport of glucose across the membrane. GLUTs and yeast glucose transporters both have significant effects on glucose homeostasis, or the equilibrium of glucose levels in the blood or inside cells. Numerous hormonal and metabolic signaling mechanisms closely regulate their expression and function

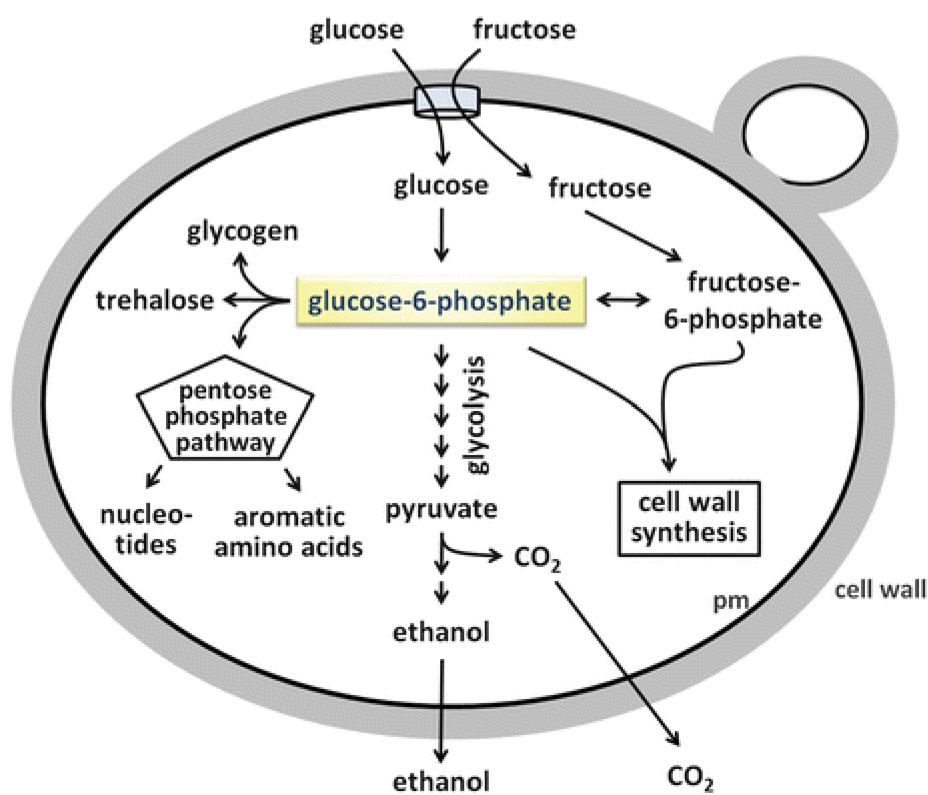


Figure 2.9: Glucose metabolism in yeast cell

2.13 ZEBRAFISH AS A MODEL FOR WOUND HEALING:

Small freshwater fish called zebrafish (Danio rerio) are indigenous to Southeast Asian streams and rivers. Due to their tiny size, quick reproduction, transparent embryos, and genetic resemblance to humans, they are frequently employed as model organisms in scientific studies. Due to their capacity for regeneration, zebrafish are frequently employed as model organisms in studies on the healing of wounds [60]. Zebrafish are an excellent model for researching tissue regeneration and wound healing because, unlike mammals, they have the capacity to regenerate tissue and organs, including the heart, spinal cord, and fins [61].

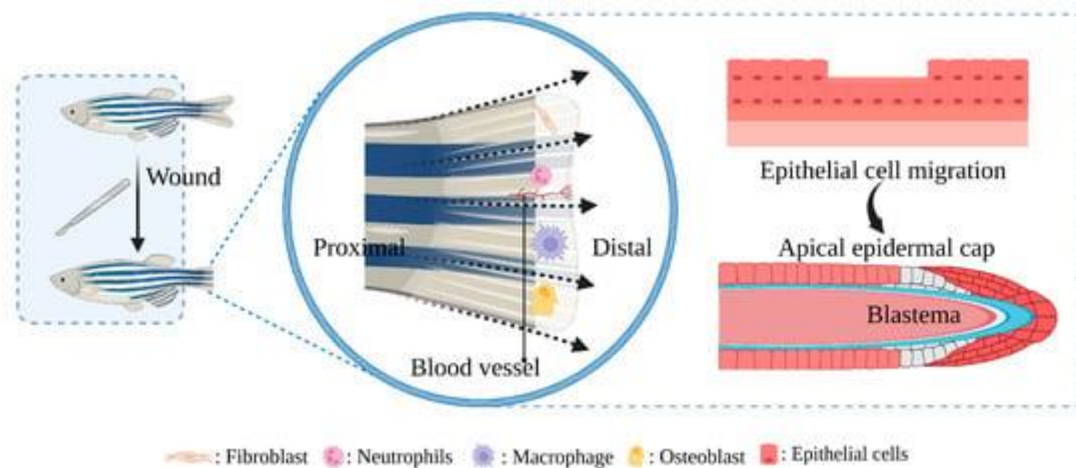


Figure 2.10: Zebrafish with wound

Zebrafish have a remarkable capacity to replace lost tissue and heal tissue injury fast. The genetic and molecular processes enabling zebrafish wound healing have been the subject of substantial investigation as a result. To find novel treatments to enhance wound healing in humans, researchers have studied the impact of numerous medicines and chemicals on wound healing in zebrafish. Additionally, the relevance of signalling pathways in wound healing; including the Wnt and Hedgehog pathways has been investigated using zebrafish.

Overall, zebrafish have shown to be a useful model organism for research on tissue regeneration and wound healing. They may develop new therapies for human wounds and traumas as a result of their capacity for regeneration, which provides a unique viewpoint on the molecular and genetic factors behind these processes [60-62].

CHAPTER-3

MATERIALS AND METHOD

3.1MATERIAL AND CHEMICAL REQUIRED:

Dried mushroom, pure methanol, muslin cloth, soxhlet extractor, isomantle heater, lyophilzer, rotary evaporator, anthrone reagent, glucose, sodium bicarbonate, sodium hydroxide, sodium potassium tartrate, copper sulphate, Folin reagent(FCR) , BSA, aluminium chloride, sodium nitrite, Gallic acid, quercetin, ABTS, DPPH, MHA, MHB, MTT dye, clove oil, PBS, DMEM, FBS, McCoy cell line(NCCS,PUNE).

3.2PREPARATION OF *CORDYCEPS MILITARIS* EXTRACT:

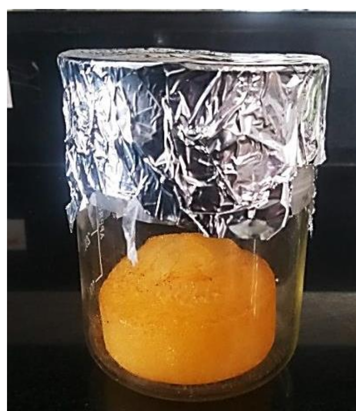
Two types of extraction were used for samples A and B.

3.2.1 PREARATION OF AQ EXTRACT

The AQ extract was made by boiling 2 grams of dried Cordyceps in autoclaved water till the water was evaporated. The concentrated extract was filtered using Whatman No. 1 filter paper. Lyophilisation of the crude extract was done overnight to obtained powdered extract and stored at 4 °C for further use.

A

B



C **D**

Figure 3.1: Extraction process of AQ extract: A is the dried *Cordyceps militaris* , B is the AQ extraction process, C is the extract after lyophilization, and D is the final extract after several lyophilizations.

3.2.2 MOH EXTRACT PREPARATION

The MoH extraction was done using a Soxhlet apparatus. 5 g of dried powder of *Cordyceps militaris* was loaded inside the thimble of the soxhlet apparatus. The solvent was loaded through the condenser and reached the flask, where it was heated to 60 °C. The vapor formed gets collected into the reservoir of the siphon, then it goes to the flask, and like this, one cycle gets completed. The process was conducted for 3 to 4 days (12 hours per day) until the solvent in the siphon was colourless. Then the extract was further concentrated using a rotary-evaporator. After evaporation, the extract was lyophilized and stored for further use at 4 °C.

Figure 3.2: MoH extraction through soxhlet apparatus.

$$\textbf{Percentage of yield} = (W_2/W_1) \times 100$$

Where, W_2 is the weight of the extract

W_1 is the dry weight of *Cordyceps militaris*

3.3QUANTITATIVE ANALYSIS:

3.3.1 CARBOHYDRATE CONTENT ESTIMATION:

Carbohydrate content in the extract was measured using an anthrone assay. Anthrone reagent was prepared by mixing 0.2 g of it in chilled concentrated sulphuric acid. 10 mg/ml glucose stock was prepared. One mL of both samples was mixed with the 4 mL anthrone reagent & incubation was given at 100 °C for 8 minutes, and then the ABS was taken at 630 nm [45,66]. The carbohydrate content in the different samples was expressed as glucose equivalent (GE) using a standard prepared from glucose solution (10 mg/mL).

3.3.2 PROTEIN CONTENT ESTIMATION:

The protein-content in the extract, estimated using the Folin-Ciocalteu assay. Reagent 1 was prepared by making 2% sodium bicarbonate in 0.1 N sodium hydroxide and adding 1% Na-K tartrate and 0.5% cu-sulphate. For reagent 2, FC reagent was mixed with d/w in a ratio 1:1, & BSA stock was made. The samples were mixed with reagent 1, followed by 10 minutes of incubation at 30 °C. After incubation, FC reagent was added and incubation was given in the dark for 30 minutes at rt. The ABS was taken at 750 nm [45,66]. The protein content in the different samples was expressed as BSA equivalent (BSAE) using a standard prepared from glucose solution (10 mg/mL).

3.3.3 TOTAL PHENOLIC CONTENT(PC) ESTIMATION

The total PC was estimated with the FCR-based assay. The samples mixed with the FCR and incubation was given in the dark for 5 minutes. After addition of 7.5% sodium bicarbonate, the sample with FCR was given incubation for 30 mins in the dark at room temperature. The ABS was taken at 765 nm [45,66]. Using a standard curve prepared from gallic acid solution the total PC in the samples was expressed as a gallic-acid equivalent (GAE) using a standard curve prepared from gallic acid solution.

3.3.4 FLAVONOID CONTENT(TFC) ESTIMATION

The TFC of the crude extract was estimated using the $Alcl^3$ colorimetric assay. 5% sodium nitrite was added to the samples, followed by a 5-minute incubation. 10% aluminium chloride was added to it and then incubated at rt for 5 mins. ABS was measured at 510 nm after addition of 4% sodium hydroxide [45,66]. The TFC in the samples was expressed using a standard curve, as quercetin equivalent (QE).

3.4 ANTIOXIDANT ACTIVITY ESTIMATION

3.4.1 ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ASSAY

A 7Mm 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonicacid) stock solution was made with 2.45 mM K-persulfate in methanol. After the components had been dissolved, the mixture was allowed to sit -16-24 hours at room temperature in the dark before being diluted with methanol to create a working solution with an ABS of 0.7 at 734 nm. The samples were mixed with the working solution of ABTS and incubated for 6 minutes in the

dark. The ABS was measured at 734 nm. A standard curve of gallic acid was prepared [45,66].

$$\textbf{Percentage of ABTS inhibition} = [(A_C - A_S) / A_C \times 100]$$

Where, A_C is the ABS of control and A_S is the ABS of sample

3.4.2 DPPH ASSAY

0.002% of the DPPH stock sol was made in methanol and ABS was taken at 517 nm for control. A standard curve of gallic acid was made. The DPPH solution was added to the samples followed by incubation for 30 minutes in the dark. the ABS was measured at 517 nm [45,66]. The results are expressed in GAE.

$$\textbf{Percentage DPPH inhibition} = [(A_C - A_S) / A_C \times 100]$$

Where, A_C is the ABS of control and A_S is the ABS of sample.

3.5 DETERMINATION OF ANTIMICROBIAL ACTIVITY

Antimicrobial activity was determined in both Gram-negative and Gram-positive bacteria through the well diffusion method. These strains were maintained by subculturing and grown in MHB overnight at 37 °C before use. The culture was compared with the 0.5 McFarland standard, and the ABS of the culture was taken, which should be between 0.1-0.2 which indicates that the culture has 10^6-10^8 CFU, which were further diluted 10 times. The diluted culture was spread over the MHA plates, and wells were punctured. Samples were loaded into the wells containing filter paper disc with positive and negative controls. The plates were kept in an incubator for 24 hours to allow diffusion of the samples, and a zone of inhibition was measured [65].

3.6 MTT ASSAY FOR CELL VIABILITY AND CYTOTOXICITY

The MTT sol was prepared by mixing MTT (5 mg/mL) in PBS. The cells were harvested from the T-flask after centrifugation and resuspended in the media. Seeding was done in a 96 well plate with media and cells having a count of 10^3 - 10^4 cells per well. The cells were incubated at appropriate conditions for 24 hours. After 24 hours, the media was changed, followed by washing with PBS and then the addition of new media with the samples. After 24 hours of incubation, the media was discarded, followed by PBS washing and the addition of new media with MTT solution. After incubation for 4 hours, the media was discarded and DMSO was added. The ABS was taken at 580 nm after 30 minutes [64].

3.7 GLUCOSE UPTAKE ESTIMATION IN YEAST CELLS

The yeast was inoculated in PDB and kept in an incubator shaker overnight. The yeast with media was centrifuged at 3500 x g for 5 minutes, and centrifugation was repeated until a clear supernatant was obtained. The pellet was dissolved in distilled water to prepare the suspension. A glucose solution of different concentrations (5 mM, 10 mM, and 25 mM) was made and incubated with samples of different concentrations for 10 minutes at 37 °C. After incubation, the yeast suspension was added and mixed by vortexing them for 2 minutes. Further incubation for 60 minutes was given at 37°C. The reaction tubes were centrifuged at 2500 x g for 5 minutes, and the ABS of the supernatant was measured at 540 nm to estimate glucose uptake [63]. The percentage increase in glucose uptake by yeast cells was calculated using the following formula:

$$\textbf{Percentage activity} = A_C – A_S / A_C \text{ x100}$$

Where, A_C is the ABS of control and A_S is the ABS of sample

3.8 DETERMINATION OF WOUND HEALING ON HYPOGLYCEMIC ZEBRAFISH AS A MODEL

Zebrafish were purchased from the market and made accustomed to the lab environment for a few days. Fish were divided into five groups; normal control, positive control, diabetic control, and samples. The fish were anesthetized with clove oil, and wuth the help of sterile scalpel the caudal fin was transected from the posterior. Photos of the fish before and after the transection were taken. The transected fish were transferred to the glucose water for a few days to induce hypoglycemic conditions. Then the hyperglycemic fish were transferred to the water treated with the samples for 5 minutes under aseptic conditions. After treatment, the fish were transferred back to their respective tanks. After 5 -7 days of post wounding the fins were measured and observed for fin regeneration and the results were recorded [62].

CHAPTER 4

RESULT AND DISCUSSION

4.1 RESULT OF EXTRACTION YIELD

The AQ extract yield of *Cordyceps militaris* obtained was 0.69 gm from the 1gm of dried *Cordyceps militaris* and the percentage yield was 69%.

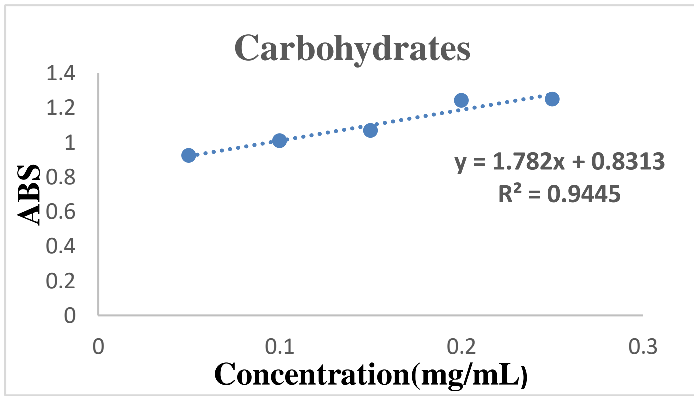
The MoH extract yield of *Cordyceps militaris* obtained was 0.8 gm from the 5gm of dried *Cordyceps militaris* and the percentage yield was 16%.

4.2 RESULT OF QUANTITATIVE ANALYSIS

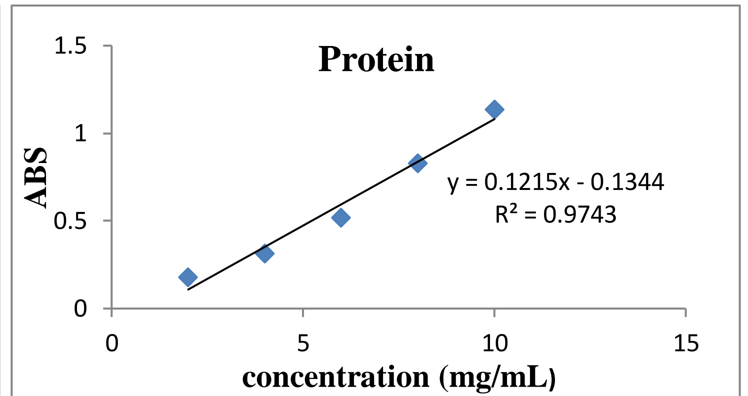
After quantitative analysis of *Cordyceps militaris* the extract showed carbohydrates 0.07 mg of GE/mg of DW extract, protein 0.028 mg of BSAE/mg of DW extract, 0.08 mg of GAE/mg of DW extract of total phenol and 0.05 mg of QE/mg of DW extract of total flavonoid. The obtained results are depicted in the following table:

Table 4.1: Result of quantitative analysis.

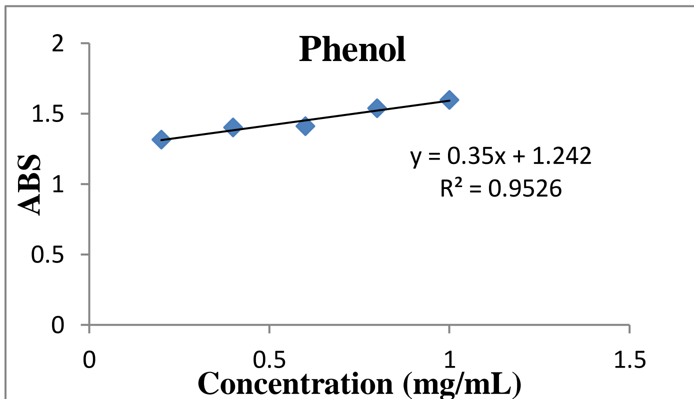
Metabolites	**Concentration**
Carbohydrates	0.07 mg of GE/mg of DW extract.
Protein	0.028 mg of BSAE/mg of DW extract.
Phenol	0.08 mg of GAE/mg of DW extract.
Flavonoid	0.05 mg of QE/mg of DW extract.



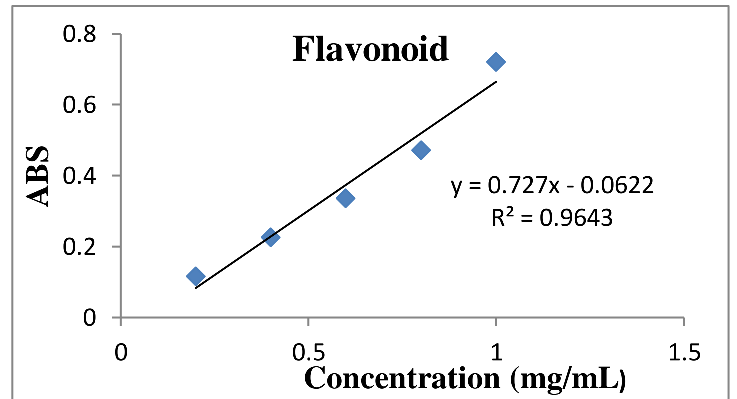
13(a)



13(b)



13(c)



13(d)

Figure 4.2: Graphical representation of quantitative analysis. a) carbohydrate estimation, b) protein estimation, c) phenol estimation and d) flavonoid estimation.

4.3 RESULT OF ANTIOXIDANT ACTIVITY

The half maximal inhibitory concentration IC50 of the sample are 0.025615785 and 0.106004406 for ABTS and DPPH respectively.

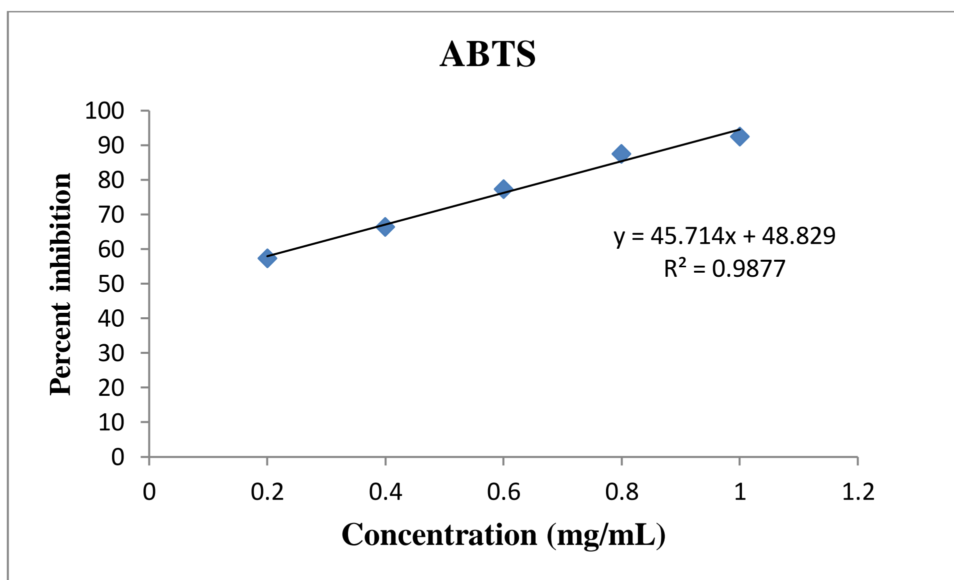


Figure 4.3: Antioxidant activity (a) ABTS assay for antioxidant activity of *Cordyceps militaris* .

Concentration (mg)	Inhibition
0.2	57.42857143
0.4	66.42857143
0.6	77.28571429
0.8	87.57142857
1	92.57142857
IC50	**0.025615785**

Table: 4.2: Percent inhibition for various concentrations of *Cordyceps militaris* (a) ABTS assay

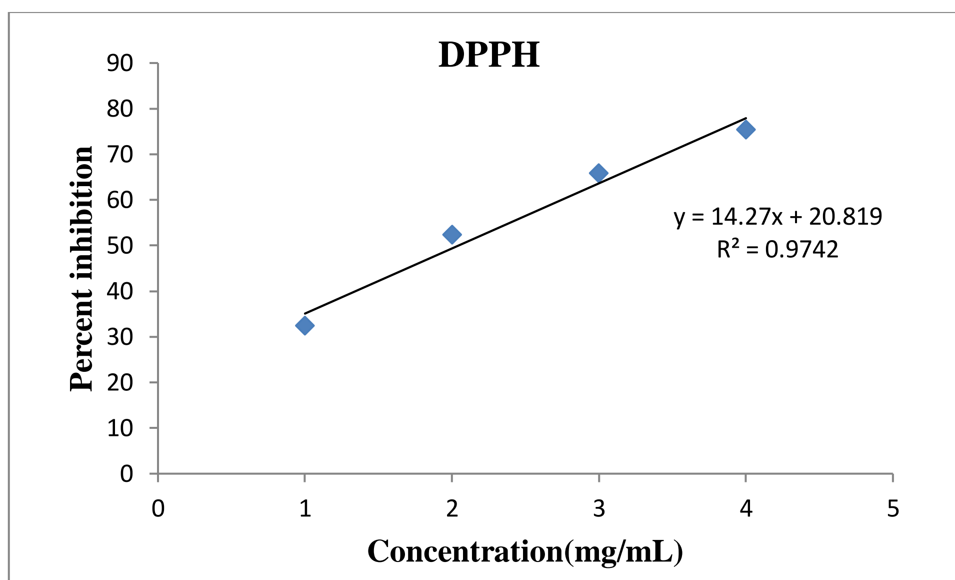


Figure 4.3(b): DPPH assay for antioxidant activity of *Cordyceps militaris* .

Concentration(mg)	**Inhibition**
1	**32.38434**
2	**52.31317**
3	**65.8363**
4	**75.44484**
IC50	**0.106004406**

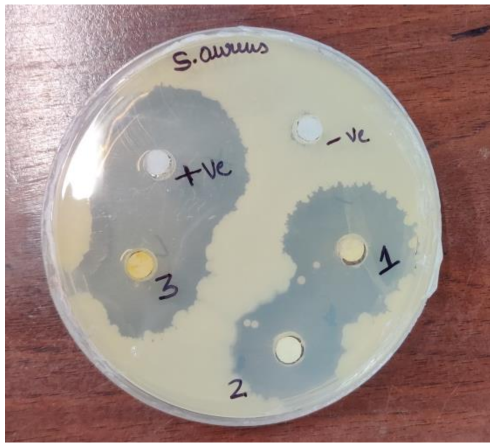
Table 4.2(b): Percent inhibition for various concentrations of *Cordyceps militaris*

4.4 RESULT OF ANTIMICROBIAL ACIVITY

The zone of inhibition was measured and depicted in following table:

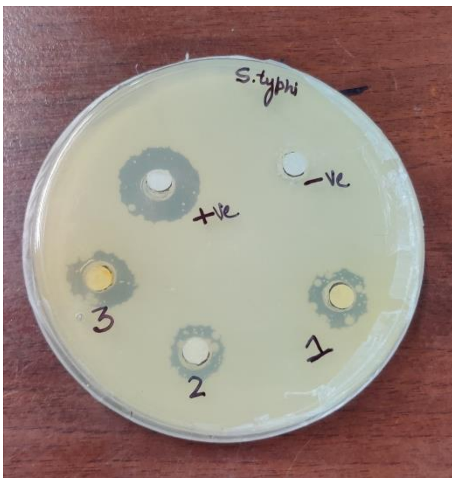
Table 4.3: Zone of inhibition of different bacterial strain.

Sample	*Staphylococcus aureus* MTCC3160(mm)	*Bacillus subtilis* MTCC121(mm)	*Salmonella typhi* MTCC98(mm)	*Escherichia coli* ATCC25922(mm)
Positive control (Ampicillin)	22 mm	19 mm	33 mm	41 mm
Negative control	Not detectable	Not detectable	Not detectable	Not detectable
AQ extract	16 mm	13 mm	25 mm	29 mm
MoH extract	17 mm	14 mm	28 mm	23 mm
Cordyceps militaris	18 mm	15 mm	26 mm	27 mm



(a)

(b)



(c)

(d)

Figure 4.4: Antimicrobial activity of *Cordyceps militaris* against the growth of a) *Staphylococcus aureus* b) *Bacillus subtilis* c) *Salmonella typhi* d) *Escherichia coli*

4.5 RESULT OF MTT ASSAY

MTT assay was performed for evaluation of the effect of *Cordyceps militaris* on McCoy cells. Cells evaluated at five different concentrations and cell proliferation was observed. Thus the results from the MTT assay revealed that the extract of *Cordyceps militaris* has a proliferative effect on the McCoy cell line.

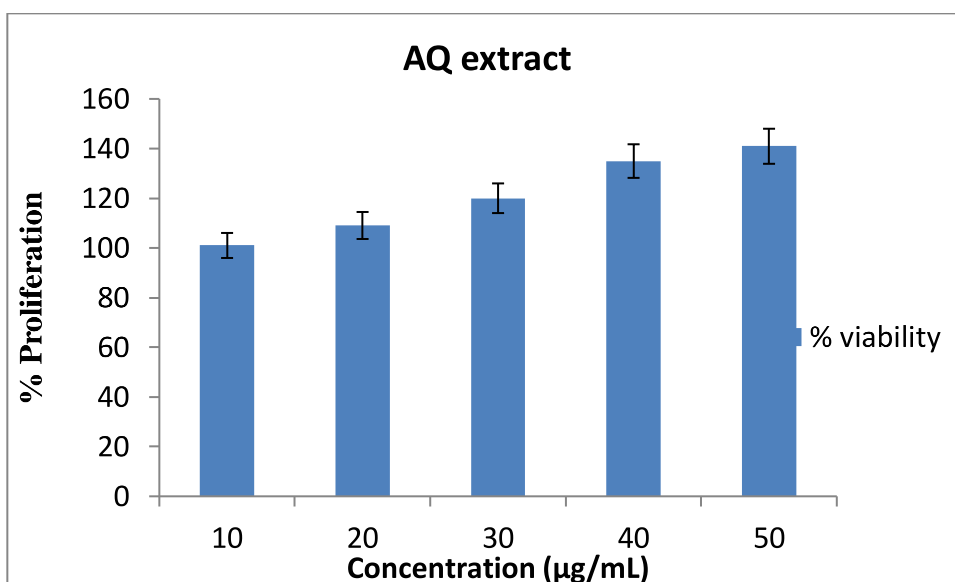


Figure 4.5: Percent proliferation at different concentration: (a)AQ extract of *Cordyceps militaris* .

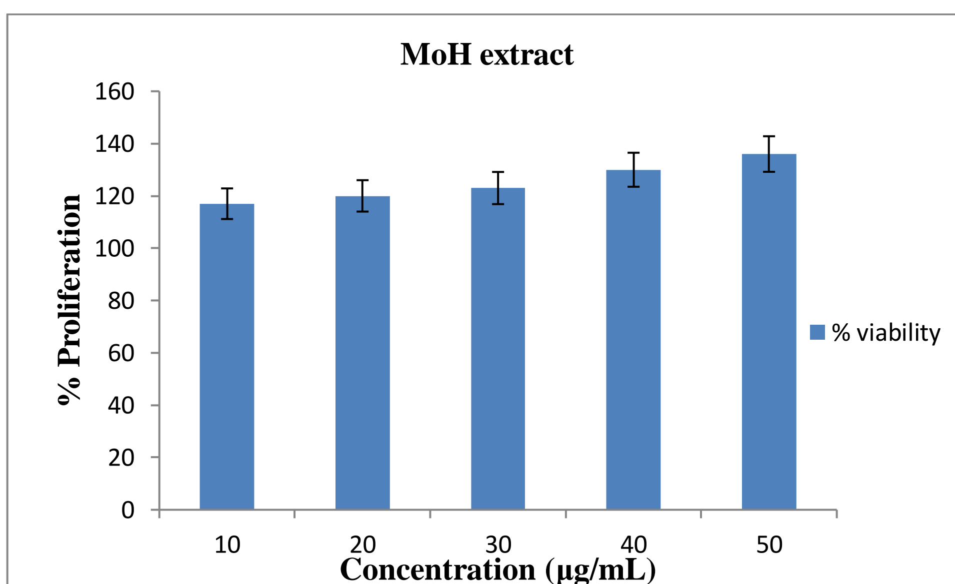


Figure 4.5: (b) MoH extract of *Cordyceps militaris* .

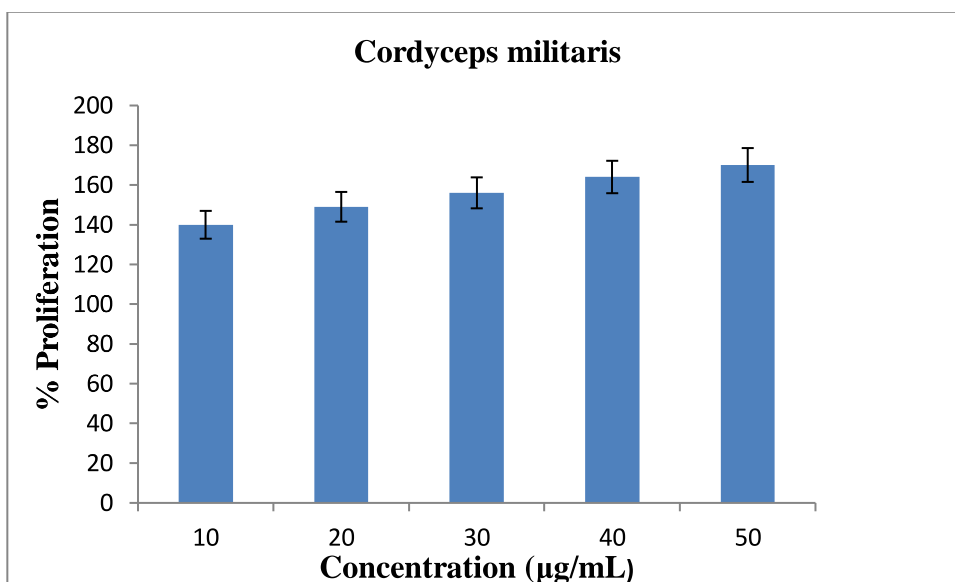


Figure 4.5(c): Percent proliferation at different concentration of *Cordyceps militaris* .

4.6 RESULT OF GLUCOSE UPTAKE

After the treatment of the yeast cells with the *Cordyceps militaris* (AQ and MoH extracts), the amount of glucose uptake increases with the concentration of the extract. At 5mM glucose concentration both the extract shows maximum activity, this indicates that the *Cordyceps militaris* has a strong anti-diabetic effect. The following graphs show the % increase in glc uptake by the yeast cell at various glc concentrations, including 25 mM, 10 mM, and 5 mM, respectively

Table 4.4(a): Glucose uptake (Percent activity) of different concentration of AQ extract.

Concentration (mg/mL)	Percent activity		
	25mM	10mM	5mM
250	66.55	67.53	68.18
500	67.20	68.18	70.77
750	69.48	71.10	73.70
1000	71.42	73.70	76.29

Table 4.4(b): Glucose uptake (Percent activity) of different concentration of MoH extract (10mg/mL).

Concentration (mg/mL)	Percent activity		
	25mM	10mM	5mM
250	25.35	36.17	50.00
500	42.00	42.00	63.72
750	57.84	57.84	67.6
1000	63.82	63.82	71.80

Table 4.4(b): Glucose uptake (Percent activity) of different concentration of MoH extract (20mg/mL).

Concentration (mg/mL)	Percent activity		
	25mM	10mM	5mM
250	33.79	50.6	72.5
500	31.00	44	49
750	22.00	35.4	37.34
1000	30.00	37	41.00

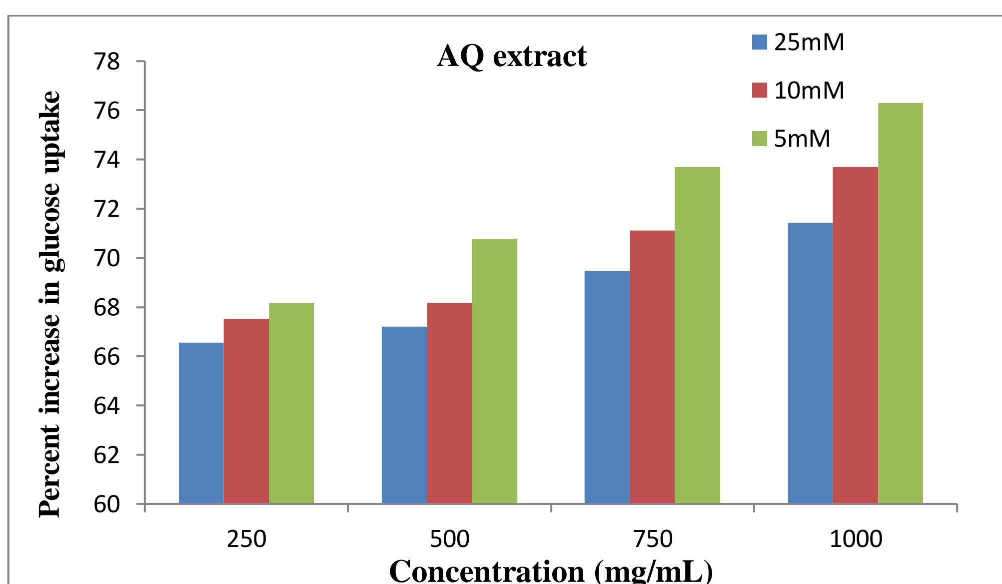


Figure 4.6(a): Graphical representation of glucose uptake (Percent activity) of different concentration of AQ extract.

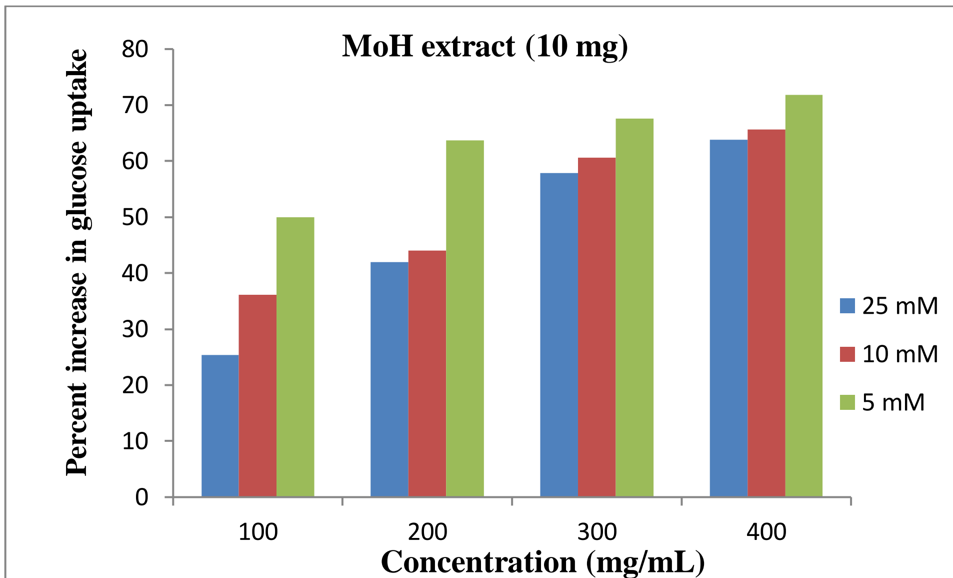


Figure 4.6(b): Graphical representation of glucose uptake (Percent activity) of different concentration of MoH extract (10mg/mL).

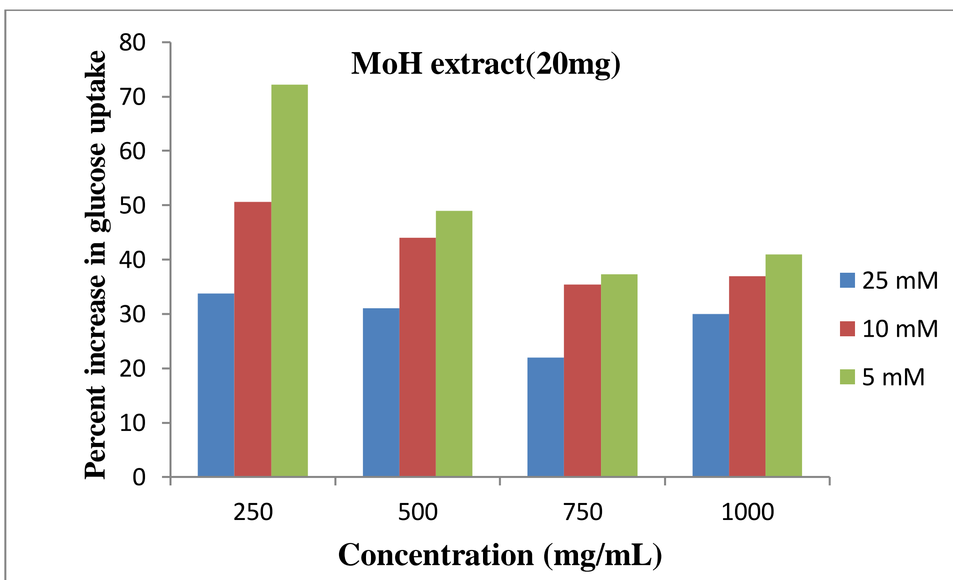


Figure 4.7(c): Graphical representation of glucose uptake (Percent activity) of different concentration of MoH extract(20mg/mL).

4.7 RESULT OF WOUND HEALING

On the eighth day, the fin growth measurement was compared to the control. When fish treated with the MoH extract to those treated with *Cordyceps militaris* showed the increased rate of regeneration. Thus the fish treated with *Cordyceps militaris* and MoH extract had the highest levels of fin regeneration in comparison to the control fish but less than the positive control. This indicated that the *Cordyceps militaris* has the similar wound healing effect as the commercial wound healing ointment.

Table 4.5: fin regeneration with respect to the treatment.

Treatment	Fin regeneration (mm)
Normal control	2.6
Diabetic control	1.75
Positive control	3
MoH extract	3
Cordyceps militaris	2.25

Figure 4.7: Zebrafish post transection (a) control.

Figure 4.7: (b) hyperglycemic control.

Figure 4.7: (c) positive control.

Figure 4.7: (d) MoH extract treated.

Figure 4.7: (e) Dried *Cordyceps militaris* treated zebrafish post transection.

DISCUSSION:

Traditional medical practices include a number of naturally derived treatments that can enhance glucose absorption and promote normal blood sugar levels [38]. These treatments function in a variety of ways, including enhancing INS sensitivity (low INS sensitivity causes cells to become resistant to INS, which can develop high blood sugar levels), promoting INS secretion can help increase glucose uptake in the cells by promoting the uptake of glucose into the cells, lowering INS resistance(cells stop responding to INS) can boost cellular absorption of glucose, and decreasing oxidative stress and inflammation can enhance INS sensitivity and boost cellular absorption of glucose, both can hinder the absorption of glucose by cells by interfering with INS signaling pathways. All of this aids in enhancing glucose absorption in cells [28,31].

In animal and human research, the traditional medicine; *Cordyceps militaris* has improved INS sensitivity and glucose absorption. The presence of bioactive substances such as polysaccharides, cordycepin, and adenosine, which have been demonstrated to have hypoglycemic and INS-sensitizing properties, is probably responsible for this [44]. Quantitative testing reveals the presence of phenol, flavonoids, proteins, carbohydrates, and proteins in the *Cordyceps militaris* extract, all of which are crucial for several biological processes. These metabolites enhance hyperglycemic wound healing by supporting tissue regeneration and blood sugar regulation. Proteins play a critical function in glucose absorption by aiding the transfer of glucose into the cells and can assist regulate blood sugar levels. They also enhance diabetic wound healing by encouraging tissue repair and regeneration. Carbohydrates have a supporting role in wound healing. Plant-based chemicals known as phenols and flavonoids have been proven to provide a variety of health advantages, including antioxidant and anti-inflammatory qualities. These substances have the potential to protect the body from the oxidative stress and inflammation that are two major contributors to the emergence of DB problems. By increasing INS sensitivity and decreasing INS resistance, they can aid in improving glucose absorption. They can also aid in promoting wound healing, fibroblast production, and capillary vessel growth. Additionally, antioxidants can aid in the regeneration and repair of damaged tissue in diabetic wounds. In both types of DB, dysregulation of glucose metabolism contributes to the development of hyperglycemia. High BGLs can injure the body's organs and tissues, resulting in neuropathy that hinders wound healing. Controlling BGLs will improve diabetic wound healing since uncontrolled DB lowers blood flow and changes circulation,

which causes wounds to heal more slowly. One of the key *Cordyceps militaris* constituents that have been demonstrated to have advantageous effects on glucose absorption is cordycepin. A nucleoside analogue known as cordycepin has been found to have hypoglycemic effects by triggering the AMPK signaling pathway, which is essential for controlling how much energy is used [53]. This study clearly demonstrated that yeast cells' ability to absorb glucose improved with extract concentration, demonstrating the anti-diabetic properties of *Cordyceps militaris* . Additionally, in contrast to controls or fish treated with commercial healing formulas, the fin regeneration in fish maintained under hyperglycemic circumstances and treated with *Cordyceps militaris* afterwards had the highest regeneration. Also, the anti-microbial properties shown by *Cordyceps militaris* could have played an essential role in avoiding prolonged inflammation by eliminating the microbial load at the wound site. As a result, the *Cordyceps militaris* extract examined demonstrates its ability to treat wounds by controlling glucose homeostasis. The yeast and adult Zebrafish fin regeneration system is a quick yet effective alternative model to represent molecules' capacity to absorb glucose and repair wounds under hyperglycemic conditions, two crucial steps in the hyperglycemic healing process.

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