THE IMPACT OF *CORDYCEPS MILITARIS* ON WOUND HEALING

Dissertation submitted in partial fulfilment of the requirement for the degree of

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IN

BIOTECHNOLOGY

By

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DECLARATION

I hereby declare that the work reported in the M.Sc. dissertation entitle "**The Impact of *Cordyceps militaris* on Wound Healing.**" submitted at Jaypee University ofInformation Technology, Waknaghat, Solan, Himachal Pradesh, India, isa authentic record of my work carried out under the supervision of Dr. Udaybanu M, Dept. of Biotechnology and Bioinformatics, Jaypee University of InformationTechnology, Waknaghat, Solan, Himachal Pradesh-173234, India. I have not submitted this work elsewhere for any other degree or diploma.

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SUPERVISOR'S CERTIFICATE

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LIST OF ABBREVIATIONS

DHEA- Dehydroepiandrosterone

GC - Gas chromatography

ICP - Intra-cellular polysaccharides

EPSs -Extracellular polysaccharides

Mn- mannose

Glu- glucose

Gal- Galactose

Xyl- Xylose

Rha- Rhamnose

Ara-Arabinose

Cvd- Cardiovascular diseases

Ups - ubiquitin proteasome system

Cox2 -cyclooxygenase-2

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Abstract

Human health is preserved in big extent by the skin. The spread of wound disease occurs when bacteria enter the body through the skin. Effective wound management is difficult because wound healing is a challenging process which is influenced by various internal and external factors. Due to the side effects of modern medicine and the lower cost of herbal products, natural herbal remedies have now become essential for the management of skin disorders and the treatment of wounds. Ayurveda texts have described the potential of *Cordyceps militaris*. The objective of the current study was to evaluate the wound healing ability of the C. militaris by using in vitro wound healing assays. As scientific evidence of their efficacy is limited therefore it is important to introduce a scientific validation for the medicinal effect of plants used in traditional medicine. MTT assay was performed which revealed that there was 3-fold increase in the cell proliferation when McCoy fibroblast cell line was treated with the cordyceps extracts. Further the extracts are used on zebra fish which shows enhanced wound healing effect.

CHAPTER 1: INTRODUCTION

1. INTRODUCTION

From ancient times, people have used herbs and their products to boost immunity against colds, joint or muscle pains, fevers, and many other illnesses. Although synthetic drugs and antibiotics have advanced, herbs continue to hold a significant role in both modern and traditional medicine worldwide. Herbal treatments are heavily relied upon by people living in villages in developing countries. People in both developing and superpower nations are recognizing the significant advantages of herbs products, leading to an increased importance of herbal medicines and health foods derived from herbs. Herbal medicines have shown promise in treating allergies, metabolic disorders, and age-related degenerative disorders after being tested. [1]

For centuries, traditional Chinese medicine has utilized Cordyceps, a parasitic fungus that grows on specific caterpillars in the Himalayas. The existence of bio-active compounds as such polysaccharides, adenosine , and cordycapin in cordyceps has been shown by recent studies to possess wound healing properties. Promoting the production of collagen and angiogenesis, cordyceps has been shown to accelerate wound healing when applied tipically. [2]

1.1Wound

The continuity and integrity of the skin are disrupted by a wound.During the course of our lives, most people are likely to experience various types of wounds, which can be caused by mild or severe trauma, pathological processes, bruising and abrasions, burns, or even during surgical treatment. Lesions that disrupt the skin's normal function and structure can be caused by injuries, cuts, bruises, burns, poor circulation, ulcers, and diseases such as diabetes. Extensive treatment may be required for chronic, non-healing wounds in the elderly, diabetics, or those with circulatory problems resulting from simple cuts and bruises. [3]

1.2Classification of wound

There are two types of wound: **Acute wound and Chronic wound.**

Injuries or traumas that heal within a relatively short period of time, usually less than six weeks, are referred to as acute wounds. Cuts, abrasions, surgical incisions, and burns are all examples of acute wounds. The healing process of these wounds usually consists of four stages: hemostasis, inflammation, proliferation, and remodeling, which follow a predictable pattern.

On the other hand, wounds that are often associated with underlying medical conditions such as diabetes, peripheral vascular disease, and pressure ulcers are called chronic wounds and fail to heal within the expected timeframe. Managing these wounds can be challenging as they can persist for months or even years. Chronic wounds such as pressure ulcers, diabatic foot ulcer, venous ulcers, and arterial ulcers are among the examples. [4]

1.3 WOUND HEALING PROCESS

Wound healing process is the term used to describe the process by which tissue repair takes place. Right after the injury, this process begins. Although the healing process of wounds is similar, the time and pathway for complete restoration differ among different tissues. [5]

It has been stated that the process of healing wounds is dynamic and intricate. The wound region undergoes an initial inflammatory phase, followed by re-epithelialization, production of granulation tissue, neovascularization, and wound contraction. Temporal and spatial control is allowed during the wound healing process by the coordination of individual events through interaction among different cell types. [6]

1.3.1 STAGES OF WOUND RECOVERY PROCESS

The wound recovery procedure has four phases. However, recovery is not always sequential, and wounds can frequently shuffle between stages depending on external as well as internal variables. [7]

The following are the stages of the wound healing process:

A. Coagulation & hemostasis stage.

The first of the four stages of normal wound healing is commonly referred to as the stage of hemostasis and coagulation, which occurs immediately after the injury and before the inflammatory phase. To maintain vital organ functions, protecting the vascular system is a method that can be used. At the site of injury, plasma proteins are usually secreted, coagulating and forming a robust fibrin blood clot. The clot will limit the blood loss and seal the wound site as a barrier.

B. **Inflammatory stage.**

The inflammatory stage follows the hemostasis and coagulation stage. Reactive oxygen species, produced by phagocytes, can persist for a week in acute wounds, and for a much longer time in chronic wounds. White blood cells and certain enzymes invade the wound site during this phase to remove pathogens and debris, minimize the risk of infection, and prepare the wound site for tissue regeneration. Redness, heat, swelling, pain, and functional impairment - these are the normal characteristics of inflammation that can be observed during this stage. [8]

C. Proliferative stage.

During the proliferative phase, epithelial cells, dermal fibroblasts, vascular epithelial cells, and progenitor cells that originate in the bone marrow and travel to the wound site undergo diffusion, proliferation, and maturation. During the proliferation stage, fresh granulation tissue composed of collagen and extracellular matrix (ECM) is used to reconstruct the wound, allowing for the growth of new blood capillaries. [9]

Due to the integration of newly formed capillaries into a weakly laid collagen matrix, granulation tissue appears uneven and granular. Granulation tissue that is healthy has an irregular and granular structure, appears red in color, and is not prone to easy bleeding. In many cases, the healing process of the wound can be predicted by

observing the color and appearance of the granulation tissue. Dark granulation tissue can be caused by poor perfusion, ischemia, or infection. [10]

D. **Maturation or remodeling stage**

Maturation, also known as remodelling, happens after the wound has healed. Throughout the maturation of granulation tissue, changes in the structure, quantity, and arrangement of collagen occur, resulting in an increase in the tissue's strength properties. This step involves the transformation of collagen from type 3 to type I, the predominant fibrillar collagen found in the skin. Collagen, typically secreted during the first stage, thickens and disorganizes the wound. However, during this phase, collagen fibres align in a regular order, resulting in collagen fibres that are closer to one another and cross-links. Crosslinking is an essential procedure because it reduces scar thickness and improves skin condition at the wounded site.[11]

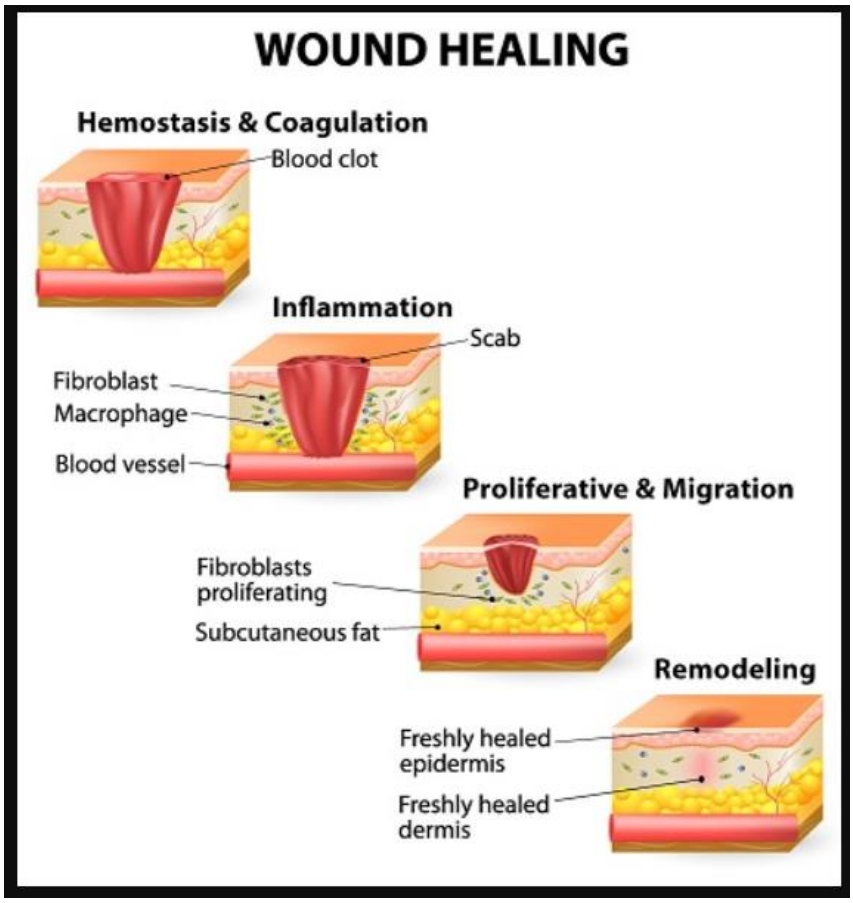

Fig-1 Stages of Wound healing[12]

1.4 FACTORS AFFECTING WOUND HEALING

A range of factors can impact wound healing and lead to poor healing of wounds. There are two types of elements that can help in wound healing: local and systemic.

1. Local Factors

Oxygenation. Almost all wound-healing activities, as well as cell metabolism, including energy generation via ATP, require oxygen. It stimulates wound contraction, keratianocyte maturation, migration, and re-epithelialization, and inhibits contamination. It also promotes fibroblast growth and collagen production. When there is a lack of oxygen, wound healing is hindered. Although severe hypoxia after an injury can stimulate the healing process, going on or prolonged hypoxia can delay it.

Infections. Microorganisms that are typically confined to the skin's surface gain internal access to the body and infect the area when a wound occurs on the skin. The wound can be categorized as contaminated, colonized, limited infection, or spreading invasive infection based on the level of contamination and microbe replication. The presence of microorganisms that do not replicate at the site of injury is referred to as contamination, while the presence of microorganisms that replicate but do not harm the tissue is referred to as colonization. Reproducing microorganisms present at the site of injury cause harm to the host in invasive infections.[11][12]

2. Systemic Factors

The person's age. Several studies have been carried out to investigate the delay in healing wounds in people as they get older. It has been shown that in healthy older persons, ageing can cause a delay in the healing process but not a decrease in healing efficacy. Wound healing delays in the old ones are asociated with a unique inflammatory reaction, include delay T-cell swelling into the affected area, change in chemo-kine assembly, and reduced macro-phage phagocytics capabilities.

Hormones of Sex. Sex-related hormones are also one of those factors impacting wound healing in the elderly. Female oestrogen, male androgen hormone, and their steroid precursor DHEA have all been found to improve wound healing.[13]

Stress. Stress has been linked to a range of disorders, including heart disease, poor wound healing, an increased chance of getting cancer, and diabetes. According to several research, stress causes a significant delay in both human and animal wound healing.[14]

Diabetes . Diabetes affects tens of millions of people throughout the world. Diabetic wounds include several dysregulated cellular processes, including reduced T cells immunity, phagocytosis, and bactericidal activity, as well as fibroblast and epidermal cell dysfunction. Hypoxia occurs in diabetic individuals as a result of poor perfusion and angiogenesis at the location of the wound. This can prolong the duration of the inflammatory response by increasing oxygen radical concentrations.[15]

Objectives:

a. Preparation of Methanolic extracts of *Cordyceps militaris.*
b. Quantitative Analysis & evolution of biological activity of prepared extracts.
c. Evaluation of wound healing potential of prepared extract in Zebra fishes.

CHAPTER 2: REVIEW OF LITERATURE

2. INTRODUCTION

In general, cordyceps and other natural bioactive substances have the potential to be a safer and more effective alternative to conventional medications for wound healing. Cordyceps wound healing effects have been ascribed to the detection of bioactive chemicals in it such as polysaccharides, adenosine, and cordycepin, which induce collagen formation and angiogenesis. Incorporating cordyceps into bioactive dressings and bioactive compound-loaded hydrogels has demonstrated promising outcomes in delivering wound-healing chemicals. Furthermore, the use of these natural compounds can meet the ongoing need for efficient wound healing bioactive compounds of natural origin that are safe, cost-effective, and have a long track record of safety and efficacy in traditional medicine. Cordyceps and other bioactive substances can be included into wound dressings manufactured from biopolymers such as proteoglycans, collagen, alginates, and chitosan to provide an ideal atmosphere for wound healing. Furthermore, the use of electrospun materials to create moisture-regulating and oxygen-transporting wound dressings improves their wound healing potential. To completely comprehend the potential of cordyceps and other bioactive chemicals in wound healing, more study is needed. Finally, the use of natural bioactive compounds such as cordyceps represents a promising alternative to conventional wound healing drugs. Researchers want to transmit cordyceps' wound healing qualities more efficiently by putting it into bioactive dressings and hydrogels. Cordycepin, the major active ingredient of C. militaris fruit bodies, was discovered to be found in Cordyceps sinensis and Cordyceps kyushuensis after being isolated from C. militaris. The significant bioactive compound cordycepin (3′-deoxyadenosine), a nucleoside analogue, is regarded as a nucleic acid antibiotic that may block cell canceration while also helping to cancer cell normalisation as one of the elements of gene DNA. Furthermore, cordycepin has been shown to be a substance that is antineoplastic, proliferation-resistant, inhibiting metastasis, insecticidal and microbicide. As a result, the medicinal fungus C. militaris is one of the most significant prospects for future usage as herbal medicinal basis for the benefit of humanity. [16]

*2.1*Taxonomical classification of *Cordyceps militaris*

Scientific classification of *Cordyceps militaris* (Table 1)

Kingdom	Fungi
Phylum	Ascomycoa
Sub-	Ascomycotina
Phylum	Ascomycetes/Pyrenomycetes
Class	Hypocreales
Order	Clavicipataceae
Family	*Cordyceps*
Genus	*Cordyceps*
Species	*militaris*

Table 1 Taxonomical classification

Fig 2 *C.militaris* [17]

Common name	Caterpillar fungus, *Cordyceps*, Cetepiller mushroom
Latin/English name	*Cordyceps militaris*, *Cordyceps* mushroom, Deer fungus, Caterpillar fungus
Chinese name	Dong Chong Xia Cao, Summer grass-winter worm, Hia tsao tong tchong
Japanese name	Tochukaso/Tochukasu, Totsu kasu
Korean name	Tong ch'ug ha ch'o
Nepali name	Yarsagumba, Jeebanbuti, Sanjivani, Kiraghans
Tibetian name	Yarchakunbu
Other names	Chong cao, Dong chong cao, Aweto

Table 2: Common names of *C.militaris*.

2.2Chemical and structural characteristics

The compound composition of fungal polysaccharides is diverse. Polysaccharides derived from the same parent material may have distinct structural and biological properties. Polysaccharide chemical structural characteristics include, among other things, monosaccharide composition, glycosidic bond configuration, glycosidic linkage location, monounsaturated sequence, solubility, and rheological properties. The monosaccharide content and chemical composition of C. militaris polymers from free and artificially farmed C. militaris varied greatly.EPSs or IPSs extracted from C. militaris have previously been examined. The primary techniques used for polysaccharide characterisation were liquids nuclear magnet resonance (one and two dimensions), a solid-state NMR analysis, methylation evaluation, infrared spectroscopy, and GC. [17]

2.2.1 Mono-saccharide

The analysis of monosaccharide constituent typically involves breaking glycosidic linkage through acid hydrolysis, followed by derivatization and quantitative detection through gas chromatography. However, a newer method involving high-performance anion exchange chromatography with pulsed amperometric detection has emerged as a replacement for traditional methods since it does not require derivatization. Another recent approach involves using a 1-phenyl-3-methyl-5-pyrazolone pre-column derivatization method for monosaccharide composition analysis. While a variety of polysaccharides have been extracted from IPSs and EPSs, the monosaccharide composition is largely comprised of Mn, Glu, and galactose in varying molar ratios. Other mono-saccharides, such as Rha, Xyl, and Ara, have also been identified in some extracted polysaccharides. The different monosaccharide components and molar ratios of polysaccharides may be influenced by factors such as raw materials, separation and purification methods, and other variables.[18]

2.2.2 Chemical structure

C. militaris can be used to extract polysaccharides with heated water (62-72°C). These polysaccharides may have Man, Xyl, Rho, and Gal linked together in various ways. Another watersoluble poly-saccharide (CPS-3) found in cultured C. militaris contains glucose and mannose linked together in a specific way. Yet another purified C. militaris polysaccharide is composed of galactose and mannose linked together in a specific way. A unique polysaccharide (CBP-1) was also extracted from fruiting body of cultured C. militaris, which has a backbone of mannose with occasional branches of glucose and galactose. The subcritical water extraction meathead was used to get an acidic and a neutral poly-saccharide from extracted C. militaris, which were consist of various linked sugar residues. LCMPs-II, a low molecular weight polysaccharide, was found in crude C. militaris polysaccharides and is a branched-rhamnoxyloglucan. Finally, the water-soluble polysaccharide (P70-1) has a backbone of linked mannopyranosyl residues with occasional chains of glucopyranosyl and galactopyranosyl residues, and it terminates with galactopyranosyl and glucopyranosyl residues. [18]

3. Bioactive component present in *Cordyceps militaris*

Scientists found out that C. militaris has special things called bioactive compounds in it, which are made up of things called nucleosides and polysaccharides. They found two specific nucleosides called cordycepin and adenosine in C. militaris, and they have more of these things than another similar mushroom called C. sinensis. They also found some other special things like GABA, ergothioneine, and vitamins and minerals in C. militaris. Previous study showed that the level of concentration and spatial distribution of bioactive chemicals in fruiting bodies is not homogeneous. The outermost regions of C. militaris fruit body contain the most nucleosides , poly-saccharides, carotenoids and selenium chemicals. Table 3 shows a comparison of the bioactive component composition of C. militaris mycelium and fruiting bodies. C. militaris prefers a drying temperature of 60 °C. The amount of cordycepin and phenolic substances decreases with increasing temperature. [19]

Bioactive Compound	**Mycelium**	**Fruiting Bodies**
Cordycepin	1.82 mg/g	1.10 mg/g
D-manitol	5.2 mg/kg	4.7 mg/kg
Ergothionein	130.6 mg/kg	782.3 mg/kg
GAbA	68.6–180.1 mg/kg	756.30 µg/g
Lovastatiin	37.7–57.3 mg/kg	2.76 µg/g
Vit. A	100 mg/kg	96 mg/kg
Vit. E (tocopherols)	1.3 mg/kg	3.6 mg/kg
Vit. B2 (riboflavin)	0.32 mg/kg	0.16 mg/kg
Vit. B3 (niacin)	15.2 mg/kg	4.9 mg/kg

Table 3: Bioactive compund Compositio

3.1Nucleosides

Cordyceepin (3-deoxyadenosine) is a molecule of organic material that is hydrophobic in water and is an analogue in structure of the nucleoside adenosine.

In 1950, Cordyceepin had been separated from C. militaris. Based on in vitro and in vivo research, this chemical has been shown to have the following properties: immunostimulant, anti-inflammatory , antiviral ,anticancer, ergogenic, hypolipidemic, hypoglycemia, and steroidogenesis and spermatogenesis regulation. Researchers found increase in the amount of the interleukins IL-4, IL-10, and IL-12, as well as the Th2- and Th1 cytokines, a drop in the concentrations of IL-2 and TGF-, and a rise in the level of T cells (CD4 and CD8). Fig 3 shows the chemical structure of the Cordyceepin.[19]

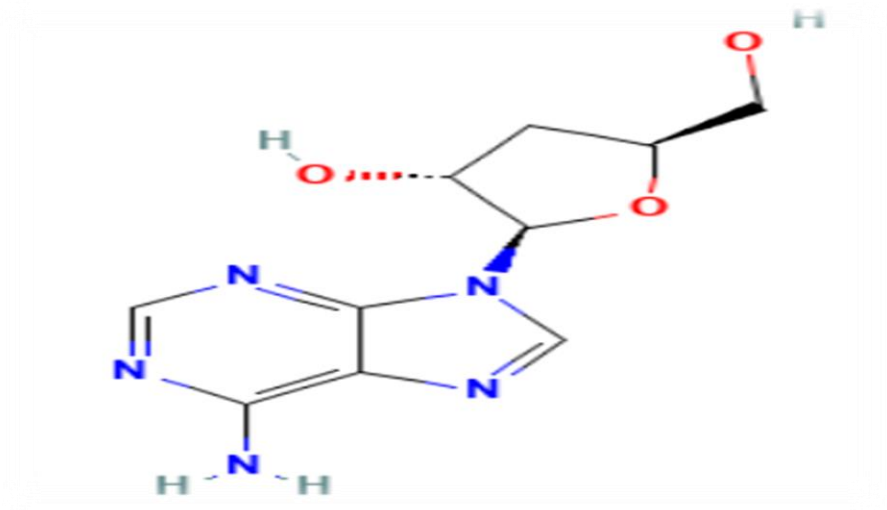

Fig 3: Chemical structure of Cordyceepin.

3.2Carbohydrates

D-Manitol is a poly-hydric OH also one of the most important products of C. militaris metabolism. Cordycepiic is also known for D-Manitol from C. militaris. C. militaris uses it as a carbs resource as well as for the transportation of some other substances in osmo-regulation and metabolic pathway control . D-mannitol has osmotic action, hence can utilised as a diuretics and antiedematous medication in therapeutic use. [20]

C. militaris also includes poly-saccharides, which are saccharides with more complicated structures than D-mannitol. These can occur as secondary intra-cellular or extra-cellular metabolic products depending on the site of polysaccharide biosynthesis

C. militaris hyphae. Poly-saccharides extracted from C. militaris mycelium exhibit a distinct chem structure. The chem structure of a polysaccharide is dictated by the kind of monosaccharide that forms it, its linear sequence, spatial arrangement, placement of glyosidic linkages, and degre of branch of the chain.[19][20]

3.3Amino Acid

GABA is an amino acid that is not a protein and that body produces of glutamic acid. GABA is an in-hibitory neuro-transmitter that affects different regions of the neurological system, include the cerebellum, hippocampus, the hypothalamus striatum, and spin cord. GABA inhibits the function of GABA ergic receptor subtypes a, b, and c. It is in charge of sleep, memory, and studying, as well as mental functions such as anxiety and stress.

The amino acid content of C. militaris fruiting bodies is 56.40 mg per gramme weight dry. Non-protein amino acid compounds, such as GABA and ergothioneine present in the fruit bodies. [20]

Ergothioneine is a soluble in water sulphur analogue of the amino acid L-histidine with a betaine molecule attached. Ergothioneine is an amino acid that doesn't belong to proteins generated by a number of bacteria, herbs, and fungal organisms, but not by mammals, hence it must be obtained from food. Ergothioneine cannot be biosynthesized by the human body. This chemical has a particular transporter cation transporter 1 (OTcN1), and a significant concentration of ergothionein has been proven in several organs and cells, including erythrocyte the spleen liver, and eyes. Ergothioneine's an antioxidant cytoprotective, and radioprotective effects have been proven in animal models testing both invitro and invivo. Fig 4 shows chemical structure of ergothionein.[21]

Fig 4: Chemical structure of Ergothionein.

3.4Carotenoid

Carotenoids, particularly xanthophyll derivatives, has found in the fruit body of several fungus, including C. militaris. The bright yellow-orange colour of C. militaris fruit bodies is due to carotenoids. The primary xanthophylls found in C. militaris fruiting bodies are -carotine, lycopine, lutin, and zeaxanthins. Lutin and zeaxanhin may be present in the eye's macula, that consist a collection of cons which is necessary for colour full eyesight [22]. Along with to studies saving effects of lutin and zea-xanthin on eyes frameworks, Earlier studies has shown carotenoid pigment products improves cognitive abilities, lowers cortisol levels and symptoms of stress in adult and young people & leads to anti-oxidant activity. Lycopene supplementation has been shown to improve the functioning of the vasculars endothellium in individuals with CVDs. Moreover, there was no discernible effect on endothelial functions in healthy volunters. The link in lycopine intake & metabolic syndrome has been shown [23]. Lycopene has been found in vivo and in vitro to suppress prostate cancer cell progression and cause apoptosis. Lycopene is an auxiliary drug used to augment basic chemotherapeutic and hormone treatment in prostate cancer patients.

3.5 Statins

C. militaris fruiting bodies are an important lovastatin source, which belongs to a class off chemicals known as statin and is extensively used as a cholesterol-lowering medication. Lovastatin is an organic substance that preferentially inhibits endogenous cholesterol production. Merck launched it as a pharmaceutical chemical in 1987 after it was discovered in Aspergillus terreus in 1978. Lovastatin is made up of a 6-lactone ring with a –OH & a partly hydrolyzed naphtha-lene along with –OH substituent esterified by 2methylbutyric acid residue. As a result, the transformation of HMG-CoA to mevalonate, a critical step in cholesterol production, is inhibited.[24] Fig 5 shows the chemical structure of the lovastatin.[25]

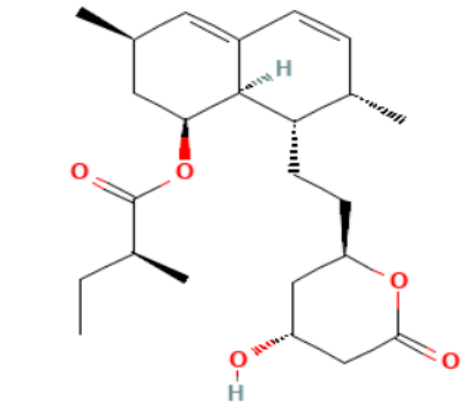

Fig 5: Chemical structure of lovastatin.

3.6Phenolic compound

Phenolic acid and flavonoids are two types of phenolic chemicals found in mushrooms. Their therapeutic value is similar with an intense anti-oxidant activity & their capacity of protecting vital compositions from oxidative damage, as lipids, enzymes, proteins, or nucleic acids. Phenolic acids, which include vanillic and caffeic acids, have the greatest antioxidant effects. In-vitro and in-vivo research has defined the antioxidants p-hydroxy-benzoic, gallic, and proto-catechuic acids present in edible mushrooms also have anti-bacterial, anti-viral, anti-fungal & antiinfalmmatory activity. [26]

3.7 Other Bioactive Compound

Lectins, which were discovered in C. militaris fruiting bodies based on their chemical makeup, are molecules of protein with associated polymers pieces (glycoproteins). Lectins has shown too exhibit mito-genic action. That attaches fragment of sugar at cells surface, causing cell clustering— agglutination in cells. Beauveriolides, which have a complicated chemical structure cyclodepsipeptides—are another type of chemicals identified in this species. Beauveriolides showed antiatherosclerotic action as well as the capacity to lower -amyloid concentration. Militarinones is a family of chem substances known as alkaloids. These are pyridine derivatives products or tetramic acids (pyrrolidine-2,4-dione) in structure and have been shown to have anti-microbial & cyto-toxic properties.[27]

C. militaris fruiting bodies have been found to contain bio elements mainly magnesium, potassium, selenium, and Sulphur, Because of the substantial amount of these bio-elements in C. militaris, it can be used as an alternate supply of these minerals in the human being's meal. [28]

Selenium is found in organic form in the fruiting structures of C. militaris, coupled with amino-acids or proteins. Selenium can form a ring like structure with either L-methionine (selenomethionine) or L-cysteine . Methylselenocysteine is formed when it mixes with proteins. The addition of selenium (sodium selenate) to the C. militaris region raises the amount of the bioactive chemicals in the fruitting bodies, including nucleo-sides, polysaccharides, amino acids, and natural selenium. The content of cordycepin and adenosine in fruiting bodies rises when the *C.militaris* substrate is enriched with organic or inorganic selenium (sodium selenate(IV); sodium selenite. C. militaris mycelium was used to produce a selenium-fortified polysaccharide (SeCSP - I). An in vitro investigation revealed SeCSP-I's antioxidant activity.[29]

4. Bioactivity

4.1 Anti-Fatigue Activity

Cordycepin, like creatine, is an indirect ATP precursor. A lot of scientific evidence support creatine's ergogenic activit. Cordyceps militaris has been demonstrated in studies to aid boost physical endurance and minimise tiredness. This is assumed to be owing to its capacity to boost the generation of adenosine triphosphate, also known as

ATP, which is the body's primary source of energy. Based on one research published in the Journal of Medicinal Food, treatment with Cordyceps militaris extract improved fatigue resistance and exercise performance in mice. The Cordyceps militaris extract raised the amounts of ATP and glycogen in the muscles, which are both vital sources of energy for physical activity, according to the researchers.[30]

4.2Immunostimulant Action

Cordyceps militaris have immunostimulant characteristics, which means it may assist enhance the body's defences. **T**here are two ways to increase immune system activity, depending on what solvent is used and hence the chemicals that are active in the C. militaris extracts. Aqua or a poly-saccharide-containing ethanol extract (50%) of C. militaris causes type 1 immune response, whereas cordycepin-containing ethanolic extracts (70-80%) increases type 2 immune response**.** Natural killer cells, a kind of WBC that plays a crucial part in the immune system's defence against viruses and cancer cells, were activated by Cordyceps militaris extract. The scientists also saw an increase in the production of interferon-gamma, a signalling molecule that aids in the activation of the immunological response.

Cordycepin-containing C. militaris extract stimulates the immune system in mice macro-phages. The procedure of C. militaris and cordycepin immune-stimulatory activity is based on macrophage activation to produce NO and proinflammatory cytokines IL-1, IL-6, TNF-, and prostaglandin-2 (PGE2), as well as a rise in the expression of induced NO synthase and Cox2. The activation of the nuclear transcription factor by C. militaris/cordycepin caused macrophages to generate proinflammatory mediators. It has the potental to improve immune function by raising white blood cell numbers and immune cell activity.[31] In Vivo Research Rodent tests revealed an increase in phagocytosis and lymphocyte generation in spleen cells. The immunostimulatory effect of the investigated mushroom material can be understood by the action of polysaccharides. The polysaccharide portion of C. militaris fruiting bodies has found to have immune-stimulatory property via macrophage activation.[32]

4.3Antitumor Activity

Several investigations have been carried out to investigate Cordyceps militaris' antitumor activity and its potential as a natural cancer treatment. An extract from Cordyceps militaris revealed substantial anticancer activity against human liver cancer cells in one research published within the Global Journal of Medicinal Mushrooms in 2015. The extract also suppressed cancer cell development by causing apoptosis, a sort of programmed cell death, according to the study. C. militaris aqueous extract has been shown to have cytotoxic action against human breast cancer cells. The stimulation of caspase-3 resulted in the enhancement of apoptosis in tumour cells.[33]

The militaris-derived cordycepin and ergosterol exhibit antiproliferative action against human colon cancer cells. Anti-inflammatory action was linked to proliferative activity. Cordycepin isolated from C. militaris was investigated for its cytotoxic effect in vitro. Cordycepin repress the growth and movement of human beings bladder cancer cells, according to findings. Cordycepin's cytotoxic action has been connected to its influence on many signalling pathways, including metalloproteinase-9, TNF-, NF-B, and protein activator-1. The extract greatly suppressed the development of cancer cells and decreased tumour size. The extract was also discovered to have an immunomodulatory impact, which means it aided to improve the immune system's ability to fight cancer. Other research has suggested that Cordyceps militaris has the potential to be used as an adjuvant therapy for cancer, which means it could be used in conjunction with traditional cancer treatments like chemotherapy and radiation to improve their effectiveness and reduce side effects.[34]

In ovarian cancer cells, C. militaris induces apoptosis. The anticancer effect system appears to result in the activation of TNF-, TNFR1, NF-B, caspase-9, and caspase-3, as well as downregulation of Bcl-2 and BclxL levels. C. militaris has been shown to trigger apoptosis in non-small cell lung cancer. The suppression of tectonic-3 protein (TCTN3) expression is the cornerstone of apoptotic action. Reduced TCTN3 expression was associated with the inhibition of many signalling pathways, including the Smoothened , Patched1 , and glioma-associated pathways. The aforementioned processes are associated with reduced Bcl-2 and BclxL levels, as well as elevated Bak, cleaved caspase-3, and caspase-9 levels.[35]

4.4 Anti Inflammatory effect

In mice with produced acute lung damage, Cordyceps militaris demonstrated strong anti-inflammatory benefits. The extract decreased inflammation and oxidative stress in mouse lung tissue, showing that Cordyceps militaris has potential as a natural therapy for inflammatory lung illnesses. Cordycepin was shown to reduce TNF-, COX-2, NF-B & iNOS production in macrophages in a model of lipopolysaccharide -induced inflammation.C. militaris' anti-inflammatory activity was confirmed in subsequent in vitro investigations by suppressing the production of pro-inflammatory mediators, most notably IL-6 ,NO TNF-, and, by LPS in murine macrophages.[36]

Ergosterol and Cordycepin and from the C. militaris have been appear in vitro to decrease the production of inflammatory mediators such as IL 12 and NO which corresponds with anti-proliferative action in colon tumour cells. Cordyceps militaris has the potential to be used as a natural treatment for Rheumatoid arthritis and inflammatory bowel disease are examples of chronic inflammatory illnesses. chronic inflammatory disorders including rheumatoid arthritis and inflammatory bowel disease. However, more research is needed to fully understand Cordyceps militaris' anti-inflammatory properties and its potential as a natural remedy for these kinds of illnesses. Cordycepin, which is found in the mycelium of C. militaris, repress the action of iNOS and slowers the levels of NO during an inflammation reaction. Analgesic action was also observed in the research. [37]

4.5 Antioxidant Activity

Cordyceps militaris appears to have antioxidant qualities, which means it may help protect the body from oxidative stress, which may cause cell damage and a variety of ailments. This fungus includes a number of bioactive chemicals that are thought to contribute to its antioxidant effect, such as polysaccharides, nucleosides, and flavonoids. There is also evidence that polysaccharides isolated from cultured C. militaris contain antioxidant properties such as DPPH free radical scavenging, ferrous ion chelating capacity, and ferric reducing power as antioxidants.

Several in vitro studies for the elements, CMP, CMP-1, WCBP50and SeCSP-I shown the antioxidant activity of polysaccharide fractions. The addition of selenium to

Cordyceps militaris medium improved the polysaccharide fractions' antioxidant capacity. [38]

4.6 Hypoglycemic Activity

C. militaris extract was found to affect glucose utilization by tissues and reduce insulin resistance in rat studies. In another study on rats, polysaccharides including C. The hypoglycemic action of militaris extract was confirmed. Furthermore, the ability of LCMPs-II polysaccharide to inhibit the -glucosidase enzyme. The cerebroside fraction, on the other hand, inhibits PTP1B activity. In streptozotocin-induced diabetic rats, C. Exopolysaccharide (EPS III) obtained from militaris culture broth exhibits hypoglycemic action. With a dose-effect relationship, EPS III showed activity as an antagonist against the enzyme at -glucosidase. Effects of Cordyceps militaris on blood glucose levels in individuals with impaired fasting glucose levels. The study found that using Cordyceps militaris powder for 12 weeks significantly reduced fasting blood glucose levels compared to a placebo group.

Cordycepin has the potential to treat diabetes by regulating the expression of liver proteins such as Nfat3, Flcn, and Psma3. These proteins are linked to energy production, the AMPK signaling pathway and the UPS. [39]

4.7 Anti-microbial Activity

Cordymin is the peptide found in C. militaris. In vitro experiments revealed that it has antifungal and antiviral action. Cordymin prevented the development of many fungi. It also inhibited the human being’s immunodeficiency virus reverse transcriptase. Many scientific investigations been established cordymin's antinociceptive effects and anti-inflammatory. Cordycepin and its derivatives have been shown to have antiviral action in vitro. Cordycepin antiviral effect is mediated via the suppression of the virus's RT enzyme and ribonucleic acid polymerase.

Another nucleoside that has been demonstrated to have antibacterial action is

adenosine. It works by suppressing microbial development and decreasing inflammation. [40] In 2020, the FDA reapproved potential and experimental drug or chemical candidates in the United States, including cordycepin, as an antiviral agent. Cordycepin precursor research in India opened up new opportunities for the use of this drug in the treatment of Covid-19. Cordycepin was found to have significant chemical interactions with SARS-CoV-2, the receptor-binding domain (RBD) on the spike protein, and the primary protease (Mpro) of the virus in in silico studies. The anti-SARS-CoV-2 mechanisms of action of cordycepin were related to lower viral replication. [41]

Chapter:3 Methodology

MATERIAL AND METHODS

Chemicals and reagents used

Material(C.militaris), Methanol, muslincloth, rota-evaporater, isomantle condenser, $Fecl_3$, H_2so_4, Sodium nitrate, DPPH, Galic acid, Al2Cl3, KOH, H2O2, methyl red, DPPH, Galic acid, methanol, ABTS,DMSO,Muller-Hinton agar.

Process for Extraction

Extracts was prepared using fungus(*C.militaris*) and for the extraction two different concentration of methanol was used along with Rota evaporator .

General method for Solvent Extraction:

100.0 mL methanol (100%conc.) and (80%) Methanol was added to the flask. Then 2 gram of the Dried sample was added. Then the flasks are kept in the shacking incubator at room temperature for 3 days. Each day fresh methanol was added (supernatant was collected). The solvent was evaporated using a rotary evaporator. The fungus material was frozen at 4°C after evaporation for subsequent use) The Fungus yield was calculated using the following equation. [42]

$$\frac{\text{Weight of extract after evaporating solvent and drying}}{\text{Dry weight of the sample}} \times 100$$

Fig 6 Rota Evaporator

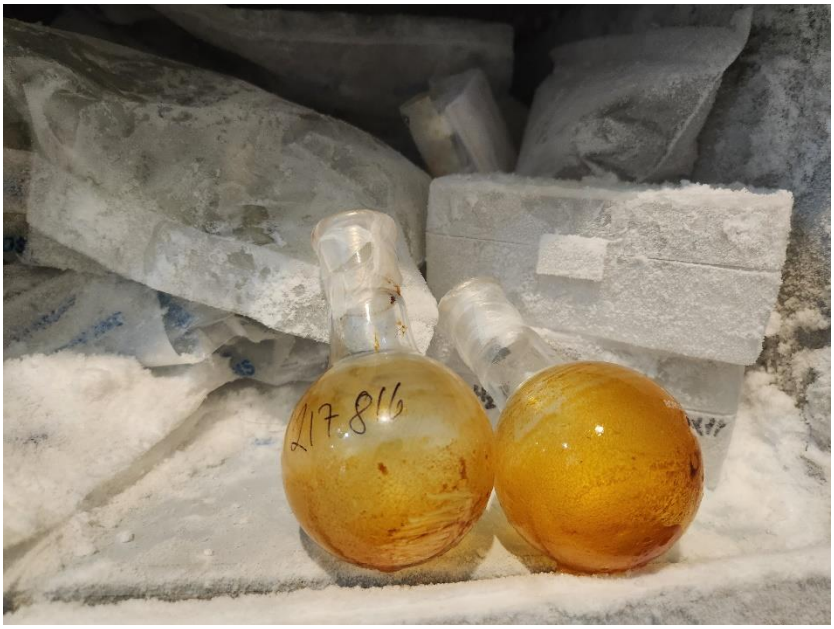

Fig 7 Sample Stored

Characterization of *C.militaris* Extract

Quantitative Screening of Phytochemicals

Estimation of Total Protein Content

Reagents:

Reagent A: 48 millilitres of 2% sodium carbonate mixed in 0.10 N sodium hydroxide and 1 millilitre of 0.5% CuSO4.5H20 in sodium potassium tartrate.

Reagent B: Folin Reagent Diluted (1:1)

Methodology:

Reagent A is made by combining 48 mL of Na2CO3 in 0.1N NaOH and 1 mL of sodium potassium tartrate in 1 mL of copper sulphate. Reagent B was made by diluting Folin reagent in distilled water in a 1:1 ratio. 1 mL of test sample and 4 mL of Reagent A need to be mixed together and kept at room temperature for 10 minutes. To the mixture, add 0.5 millilitre of Reagent B. After then, leave it in the dark for 30 minutes. Measure absorbance at 750nm in a microplate reader.[43]

Estimation of Total Flavonoid Content

To produce a 10% Quercetin solution, dissolve 1g in 100 mL methanol. To construct a standard gallic acid curve, prepare dilutions of standard quercetin solution in methanol at various concentrations (0.1, 0.5, 1.0, 2.5, and 5mg/ml). After combining 100 ml of each quercetin dilution with 500 ul of distilled water and 100 l of 5% sodium nitrate, let aside for 6 minutes. Then, add 150 ul of a 10% aluminium chloride solution and set aside for 5 minutes. Then, slowly add 200 ul of a 1M sodium hydroxide solution. Measure the absorbance at 510 nm with a microplate reader. Calculate flavonoid content in milligrammes of quercetin equivalents per gramme (mgQE/g).[44]

Evaluating biological Properties of extract.

ABTS Assay:

To make ABTS free radical solution, thoroughly combine ABTS (7 millimolar) and potassium persulfate (02.45 millimolar) solutions and incubate in the dark for 24 hours. Adjust the absorbance of this combination by adding methanol to 0.7 at 745nm. After combining 300mL extract working dilutions with 03.0mL ABTS solutions, incubate for 6 minutes. Finally, measure the absorbance at 745nm with a microplate reader. Gallic acid served as a positive control. The following formula was used to compute the ABTS scavenging potential in percent:

$$\frac{\text{C Absorbance} - \text{Sample absorbance}}{\text{C Absorbance}} \times 100$$

DPPH scavenging Assay:

Make a methanol solution of 0.002% DPPH and measure its absorbance at 520 NM. 50 ul fungal extract (1 mg/mL methanol) in 50 ul DPPH solution for 15 minutes in the dark. Record the OD again at 520 NM. Calculate the inhibition's % of DPPH by fungus extracts using the formule below.

$$\%Inhibition = \frac{A - B}{A} \times 100$$

Establish a percentage inhibition of Galic acid calibration curve for various concentrations in order to determine the IC50 values, which are the concentrations at which 50% of the DPPH solution is inhibited (A represents the absorbance of pure DPPH in its oxidised state, and B represents the OD of test sample after 15 minutes of DPPH reaction).[46]

Evaluation of Antimicrobial Activity of extracts

Well Diffusion Method:

Grown bacteria in Muller-Hinton broth to equal the viscosity of 0.5 McFarland standards (106 cells) before inoculating on Muller-Hinton agar. After inoculation, allow the plates to dry for 15 minutes before punching the wells with a sterile pipette tip. Fill the wells with 50 ul of

various concentrations of fungal extracts, as well as a positive control (antibiotic) and a blank control (DMSO). Incubate the plates for 24 hours (37°C) to allow fungal extracts to spread through the agar substrate and generate zones of inhibition. Calculate the widths of the zones of inhibition for various extracts when applied against various bacteria after incubation.[47]

Evaluation of Proliferation activity of extract.

MTT Assay:

NCCS, Pune, provided the Mccoy fibroblast cell line. These cells were kept in high glucose DMEM with 10% FBS and 1% penicillin-streptomycin at 37°C, 95% humidity, and 5% CO2.The MTT test was used to measure cell growth. Separate raw cells with a cell separating solution (trypsin in PBS). Centrifuge the cells for 5-7 minutes. Seed the cells on 96-well culture plates and incubate for 24 hours in an incubator containing carbon dioxide (at 37°C and 5% CO2). Fill 100 ul microtiter plates with various fungus extract concentrations (25, 50, 75, 100 mg/ml) and place in a CO2 incubator for 24 hours. Remove the test solutions after incubation and replace them with MTT (0.650 mg/ml MTT in PBS). In a 5% CO2 environment, incubated the plates at 37°C for 4 hours. Drain the supernatant and gently shake the plates with 100 ul of DMSO to dissolve the so-called formazan that has formed.

Measure the OD at 570nm.Calcluate the percent proliferation and 50%IC_{50} value.This formula is used to calculate the percent proliferation.[48]

$$\%Proliferation = \frac{OD\ of\ Sample}{OD\ of\ Control} \times 100$$

Preparation of the wound

Fishes were allowed to acclimatize to the lab condition for one week before the experiment. The fishes were anaesthetized with clove oil for 4-5 mins. The caudal fin was transected from the posterior end with blade.The fishes was photographed before and after .

Treatment of the wound with the extract

Zebra fish are employed to collect experimental data. Groups were formed and treated with a 100% methanol extract.1mg extracts was carefully dissolved in water. Treatment was given for 4 mins.

The treated fish were transported to the recovery tank before being separated into separate tanks labelled with the concentration treated. Throughout the investigation, quadrubles were used. The control fish were not given any treatment. [49]

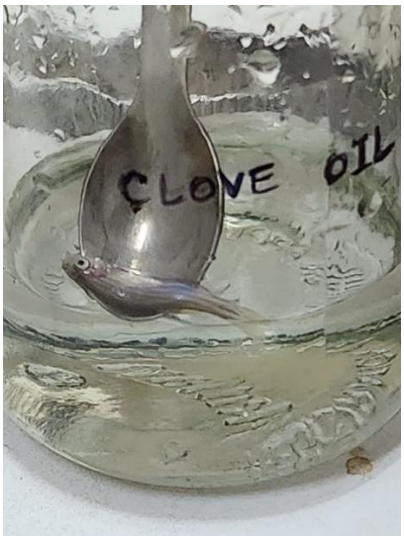

Fig 8 Treatment of fishes with clove oil

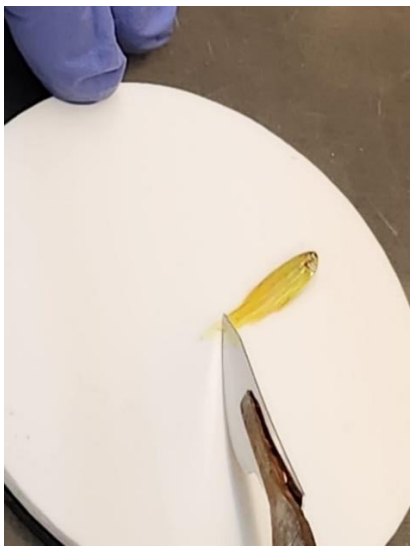

Fig 9 Tail was transacted.

CHAPTER 4: RESULTS

Percentage yield of extract

Dry weight of the *Cordyceps militaris* sample taken for solvent extraction was 2g.

The percentage yield of sample was calculated by following equation:

$$\frac{\text{Weight of extract after evaporating solvent and drying}}{\text{Dry weight of the sample}} \times 100$$

Obtained result for methanolic (100% & 80%) extraction method are 27.5% (0.55g) & 23.5% (0.47%), respectively.

Result of quantitative Screening of phytochemicals

Various quantitative test was done on both the extracted sample from the *Cordyceps militaris* different results were obtained and analysed. The result obtained were that the 100% methanol extract show more flavonoid content from the 80% methanolic extraction and give more evidence to support the use of the fungus in the wound healing results shows for 1ml/mg concentration of both the samples have flavonoid content 5.56 QE/g DW & 4.23 QE/g DW.

Protein content

It was observed that the protein content is much higher in the 100% methanol extracted sample as compare to the 80% methanol extracted sample. Results are as follow

100% methanol extraction (mgQ/g)	80% methanol extraction (mgQ/g)
3.18	2.92

Table 4 Result of the protein content present in the fungus.

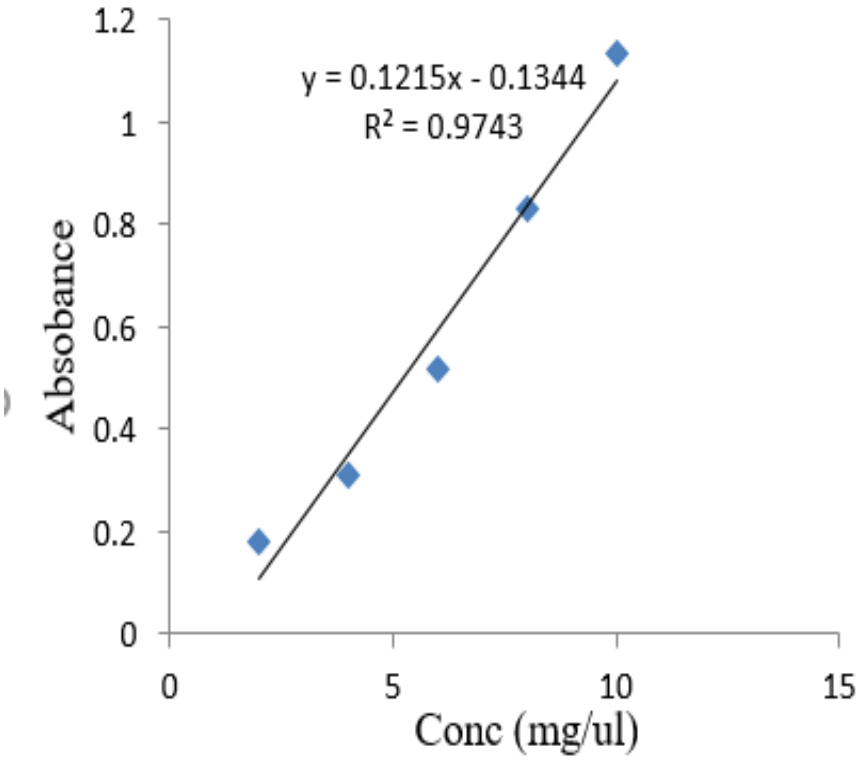

Fig 10 Concentration vs. Absorbance

Results of Antioxidant activity

On performing antioxidant activity on the fungus *Cordyceps militaris* the result was obtained

For DPPH Assay

Concentrations (mg)	Absorbance	Inhibition
1	0.19	32.38434
2	0.134	52.31317
3	0.096	65.8363
4	0.069	75.44484

Table 5 Result of DPPH.

For this IC50 value was calculated from the equation, (IC_{50} = Mx+N) where, IC_{50} = 14.27x + 20.819 and the value obtained is 2.7 mg for the 100% methanol extraction and for the 80% methanol extraction IC_{50} = 12.562x + 27.936 and the value obtained is 1.8 mg.

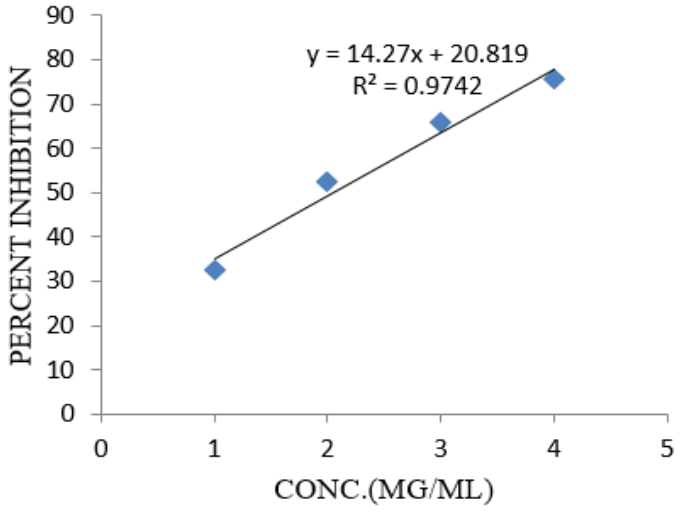

Fig 11 Concentration vs. Percent Inhibition.

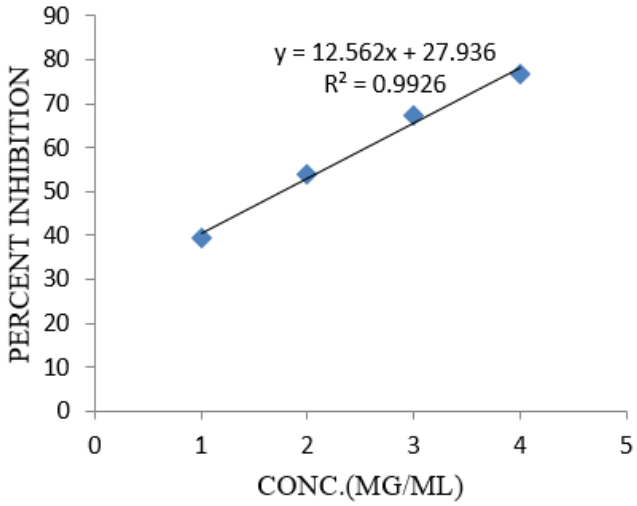

Fig 12 Concentration vs. Percent Inhibition.

For ABTS Assay

Concentration (mg)	Absorbance	Inhibition
0.2	0.568	18.8571429
0.4	0.522	25.4285714
0.6	0.438	37.4285714
0.8	0.412	41.1428571
1	0.382	45.4285714

Table 6 Result of ABTS assay showing percentage inhibition of 100% methanol extraction

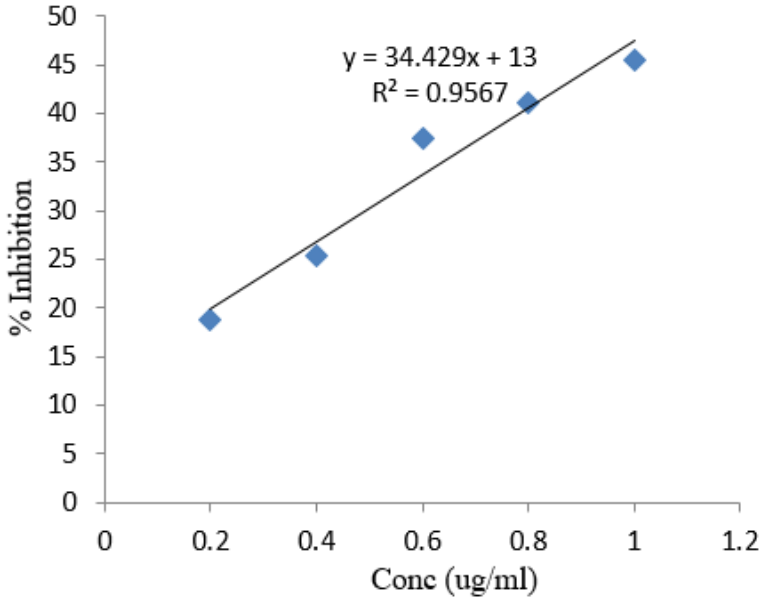

Fig 13 Graph of percentage inhibition vs. con. Of *C.militaris* in ABTS assay.

IC50 value was calculated using the equation y = 34.429x + 13

IC50 value for 100% methanol extraction was: 1.07 mg.

Concentration (mg)	Absorbance	inhibition
0.2	0.498	28.8571429
0.4	0.43	38.5714286
0.6	0.398	43.1428571
0.8	0.389	44.4285714
1	0.31	55.7142857

Table 7 Result of ABTS assay showing percentage inhibition of 80% methanol extraction

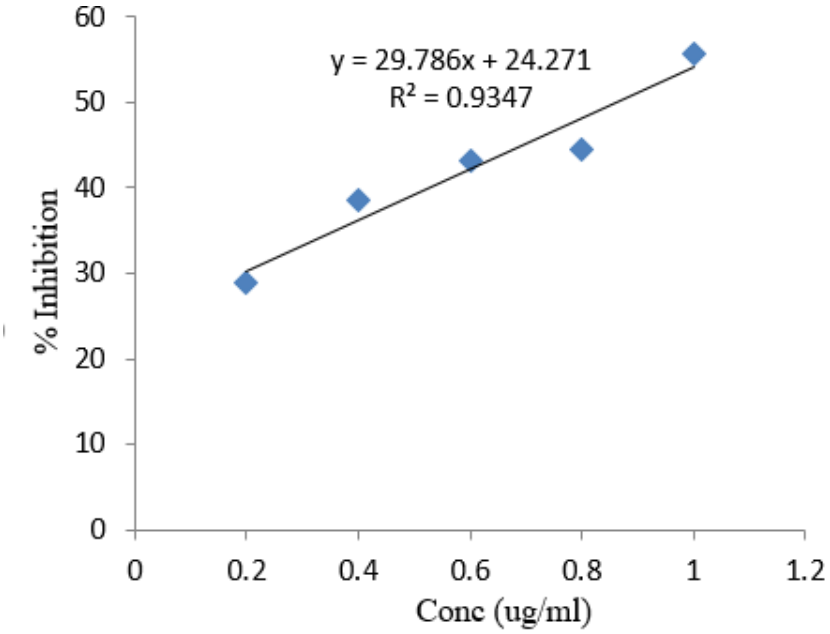

Fig 14 Graph of percentage inhibition vs. con. Of *C.militaris* in ABTS assay.

IC50 value was calculated using the equation y = 29.786x+24.271

IC50 value for 80% methanol extraction was: 0.86 mg.

Cell Proliferation Activity

MTT test results demonstrated that extract had a proliferative effect on Mccoy fibroblast cells.Cell proliferation was increased thrice by the extract.

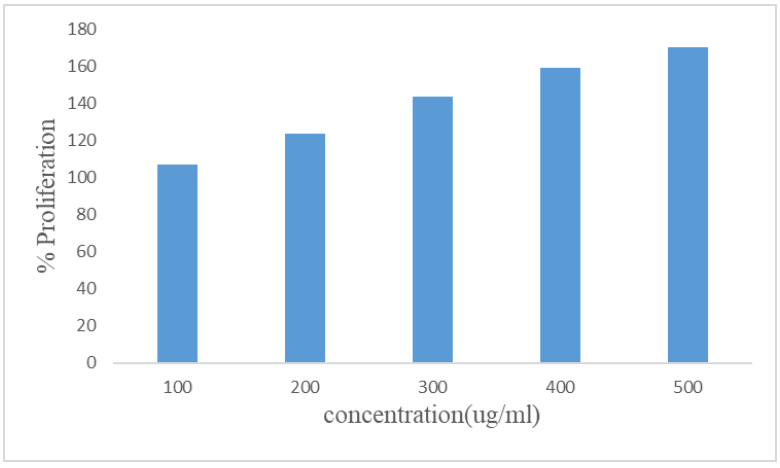

Fig 15 Graph showing cell proliferation activity

Results of Wound Healing

The C.militaris extract shows a great effect in regeneration of the fin. The fishes which are not treated with the extract shows slow generation rate.

Fig 16 Control.

Fig 17 After Treatment with extract.

Chapter 4-Conclusion

The purpose of the present study was to evaluate wound healing activity of the fungus. In present study, it has been found that the *Cordyceps militaris* have strong anti-microbial and anti-oxidant activity. An extensive review of literature had revealed that many varieties of cordyceps species where used traditionally in treatment of wound possess anti-microbial, anti-oxidant and wound healing phytochemicals which encourages blood clotting, fight infection and accelerate the healing of wounds. Cordyceps extracts also showed cell proliferation effect in cell line assay indicating that they could be a potent candidate for wound healing. So, it is good to assume that *Cordyceps militaris* can strongly influence the wounds and can further increase the healing process. Positive results were observed in the zebra fish.

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