

### ***Cordyceps militaris* on Silkworm Pupae: A Potential Platform for Synthesis of Cordycepin with Anti-Cancer Properties**

#### **Abstract:**

*Cordyceps militaris* are a class of entomopathogenic fungi which are currently in the limelight for its synthesis of Cordycepin – an anti-cancer component. The host range of *Cordyceps* is extremely broad, with predominance on the orders Lepidoptera, Coleoptera, and Hymenoptera. Although this fungus is extremely rare in nature, its artificial culture is becoming more and more popular on the global market. However, the commercial culture of this fungus has been hampered by the difficult to get seeding material and the complex developmental technique. For ages, the silkworm (Order: Lepidoptera) has played a significant economic role in the food and silk industries of Asian nations. The waste product of the silkworm rearing process is the dead silkworm pupae, which are generated in large quantities after stifling of the mulberry cocoons for the purpose of producing raw silk. Waste from sericulture in India has the potential to be used as a potential culture media for the growth of the *Cordyceps*-related sector, helping the silk farmers in their quest for future financial gain and the social advancement of the tribal society.

**Keywords:** *Cordyceps*, cordycepin, anti-cancer, silkworm pupa, sericulture waste, additional income, COVID-19 treatment

#### **Introduction**

Numerous insects in the orders Coleoptera, Hymenoptera, and Lepidoptera are hosts of the entomopathogen *Cordyceps* fungus (Aramwit, 2014; Hywel-Jones and Evans, 1996). *Cordyceps militaris* is one of the *Cordyceps* that is used in herbal medicine and as food. Cordycepin (Radwan and Wilson, 1980; Huang *et al.*, 2009) and a polysaccharide component (Yan *et al.*, 2008) are found in *C. militaris*, and these substances are thought to have anti-aging and cancer-treating properties (Jin *et al.*, 2008; Jeong *et al.*, 2013). According to Zhou *et al.* (2009), cordycepin has the ability to activate the immune system and has an impact on the gut immune system in addition to innate and adaptive immunity. It is produced by *Cordyceps* both in its fruiting body and in its mycelium (Xie *et al.*, 2009).

About 400 species of *cordyceps* are present worldwide and are abundant and dominating in wet temperate and tropical woods (El- Gharabawy, 2023). With only a few observations on pupae and adults, *Cordyceps* primarily targets insect larvae. The fungus infiltrates the host tissue, gradually replacing it with its mycelium. The key active components that have been uncovered include nucleosides, polysaccharides, sterols, cordycepin, and cordycepic acid. In a variety of

solvents, more than 20 pharmacologically bioactive chemicals have been identified, extracted, and applied to a range of medical conditions. In addition to their many medicinal uses for immune modulation, anti-oxidants, anti-tumor, anti-cancer, anti-metastatic, anti-inflammatory, anti-oxidative, antibiotics, hepatoprotective, nephroprotective, hypoglycemic, and hypocholesterolemic effects in humans, cordyceps are also known to lengthen life and reduce fatigue (Chaubey *et al.*, 2019).

A genus of ascomycete fungi called *Cordyceps mycelia* grows on Lepidoptera larvae. It has been used to treat a variety of illnesses, such as respiratory and pulmonary disorders, hyperlipidemia and hyposexuality, as well as liver, kidney, and cardiovascular disorders (Zhu *et al.*, 1998). Additionally, immunological diseases and cancer are treated with it (Holliday and Cleaver, 2008). As one of the most significant species that has been utilised as a traditional tonic food and crude medication, *Cordyceps militaris* is a member of the Ascomycetes class (Patel and Ingahlalli, 2013). Cordycepin (3'-deoxyadenosine) and its derivatives, ergosterol, polysaccharides, a glycoprotein, and peptides containing  $\alpha$ -aminoisobutyric acid are among the chemical components isolated from *Cordyceps mycelia* (Aramwit, 2014). Important bioactive components such cordycepin, adenosine, and N<sup>6</sup> - (2-hydroxyethyl) adenosine are also detected to be present.

Asia's most well-known traditional edible and medicinal mushroom is *Cordyceps militaris*. Its fruiting body resists influenza viruses (Lee *et al.*, 2014), induces apoptosis in cancer cells (Jo *et al.*, 2020), and has anti-tumor properties (Liu *et al.*, 2017). It also protects the liver and kidney. Techniques for producing *C. militaris* fruiting bodies on a large scale for commercial use are progressively being developed using artificial medium like rice (Long *et al.*, 2020) or insects like silkworm pupae of the species *Bombyx mori* (Song and Zhu, 2020). Nevertheless, *C. militaris* has a low yield in industrial settings, in part because the regulation of fruiting body growth is a poorly understood process (Lu *et al.*, 2019). In light of this, it has become popular to study how the growth of fruiting bodies is regulated in *C. militaris*.

For centuries, Far East Asian countries like Korea, Japan, and China have utilised cordyceps mushrooms, also known as "winter-worm summer-grass," as a food ingredient or traditional folk medicine to boost the immune system and restore energy, much like ginseng (*Panax spp.*). Only a few numbers are gathered for their therapeutic qualities, though. *Ophiocordyceps sinensis* (previously *Cordyceps sinensis*), *C. militaris*, *C. ophioglossoides*, *C. sobolifera*, *C. liangshanensis*, and *C. cicadicola* are the species known as pharmaceutical Cordyceps mushrooms (Ying *et al.*, 1987). *O. sinensis* and *C. militaris* are generally the two species of Cordyceps mushrooms that are highly esteemed and used in traditional Asian medical treatment. While *C. militaris* is a global fungus that forms fruiting bodies on unconverted rice grain, *O. sinensis* is rather rare and difficult to cultivate in culture, while being the most well-known and costly fungus (Sung *et al.*, 1999).

### **What is Cordycepin?**

The primary active ingredient, cordycepin, was initially isolated from *Cordyceps militaris* (Mao *et al.*, 2005; Masuda *et al.*, 2006; Das *et al.*, 2010; Masuda *et al.*, 2011; Jeong *et al.*, 2012). It was later discovered to be present in *Cordyceps sinensis* and *Cordyceps kyushuensis* (Li *et al.*, 2002; Ling *et al.*, 2009). As a nucleoside analogue, cordycepin is a nucleic acid antibiotic that prevents cell cancer and helps normalise cancer cells by being a component of gene DNA. Moreover, cordycepin's anti-cancer action can be enhanced by its incorporation into RNA molecules, which results in the early halting of RNA dyssynthesis (Paterson, 2008). The anti-inflammatory impact of cordycepin on the generation of inflammatory mediators in lipopolysaccharide (LPS)-stimulated murine BV2 microglia was demonstrated by Jeong *et al.* (2010). The inhibition of the NF- $\kappa$ B, Akt, and MAPK signalling pathways is linked to its impact. According to Lee *et al.* (2011), cordycepin exhibited dose-dependent anti-proliferative and apoptotic effects on cancer cell lines in vitro.

In addition to having strong antiviral efficacy against a variety of human viruses, cordycepin from medicinal fungi (*Cordyceps* spp.) and its nucleoside analogues that target viral RNAdependent RNA polymerase and human RNase L also have immunomodulatory and anti-inflammatory properties. Along with antivirals, anti-inflammatory therapy is crucial and needs to be customized for each patient on a timely basis. Cordycepin and its analogues' combination antiviral and anti-inflammatory properties points to them as novel therapies for the management of systemic COVID-19 infection (Chellapandi and Saranya, 2022).

### **Why to grow *C. militaris* on dead silkworm larvae and pupae?**

It is discovered that the activity of cordycepin isolated from various *Cordyceps* mycelia sources varies greatly, and controlling the natural sources of *Cordyceps* mycelia is challenging. For this reason, the quality of *Cordyceps* mycelia cultivated on artificial diets ought to be more constant. The dead pupa and larvae of the *B. mori* silkworm have been used in numerous researches as a growing substrate for a variety of fungi, such as *Cordyceps militaris*, *Isariatenuipes*, and *Isaria farinose*, which produce *Cordyceps* mycelia. They were introduced as hosts, which are thought of as artificial diet food for the creation of *Cordyceps* mycelia with more uniform quality, in order to boost the value of dead silkworms and pupae. The dead pupa and larva of *B. mori* silkworms were also studied for their proximate composition as growth media.

### **Tasar silkworm waste to wealth by *Cordyceps***

In 2020, Jena and Baig carried up an experiment to test the suitability of Tasar silkworm excrement as a *Cordyceps* fruiting body. On silk waste, *Cordyceps militaris* DMRO 1163 was grown. It was discovered that 20–22 °C and 80–85% RH were the appropriate temperatures and humidity. Furthermore, out of all the strains examined, rice has the highest biological efficiency percentage. In terms of biological efficiency percentage, rice is followed in descending order by wheat, ragi, barley, oats, and soybeans. Extract from cordyceps has the ability to scavenge free

radicals. After Cordyceps was subjected to phenolic and flavonoid profiling, the main phenolic components found were Gentistic acid, Coumaric acid, Ferulic acid, Cinnamic acid, and Gallic acid. Similarly, Naringenin, Catechin and Epicatechin were the major flavonoids observed in Cordyceps fruiting bodies (CTR&TI, Annual Report, 2022-23).

**Table.1: Phenoloic and flavonoid profiling of Cordyceps**

<b>Phenolic components (µg/gm)</b>	<b>Control</b>	<b>Eri pupae</b>	<b>Muga Pupae</b>	<b>Tasar Adult</b>	<b>Tasar Egg</b>	<b>Tasar Pupae</b>
Benzoic acid	31.06	36.30	28.60	43.47	24.41	63.08
p-hydroxy benzoic acid	0.25	0.35	0.48	1.03	0.47	0.95
Salicylic acid	0.19	0.23	0.33	2.66	0.16	0.45
3-Hydroxy benzoic acid	0.29	0.36	0.45	0.84	0.46	0.92
t-Cinnamic acid	4.62	3.27	8.63	70.88	115.18	24.44
2,4-dihydroxybenzoic acid	17.17	13.78	6.86	6.62	4.41	31.08
Gentistic acid	31.34	31.17	9.38	21.04	14.52	53.29
Protocatechuic acid	26.37	6.45	4.24	9.31	7.26	65.61
p- Coumaric acid	14.48	2.68	2.98	34.63	9.97	39.84
o-Coumaric acid	0.70	0.44	0.82	1576.71	2.22	13.42
Vanillic acid	2.41	4.44	3.71	3.16	1.95	5.17
Gallic acid	37.10	32.72	45.58	300.75	21.77	15.43
Caffeic acid	10.10	10.07	5.70	12.18	8.35	7.02
Ferulic acid	16.19	85.56	63.34	73.59	88.83	40.87
Syringic acid	0.89	1.11	0.66	0.47	0.31	1.08
Sinapic acid	0.31	0.35	0.18	0.34	0.32	0.58
Ellagic acid	0.30	0.03	0.05	0.10	0.03	0.10
Chlorogenic acid	0.01	0.00	0.00	0.00	0.00	0.00

Source: CTR&TI, Annual Report, 2022-23

<b>Flavonoids (µg/gm)</b>	<b>Control</b>	<b>Eri pupae</b>	<b>Muga Pupae</b>	<b>Tasar Adult</b>	<b>Tasar Egg</b>	<b>Tasar Pupae</b>
Umbelliferone	0.01	0.01	0.01	0.01	0.00	0.01
Apigenin	0.47	0.69	0.36	0.43	0.03	0.69

Galangin	0.01	0.01	0.00	0.01	0.00	0.01
Naringenin	5.16	6.87	6.88	15.53	13.20	12.89
Kaempferol	0.02	0.02	0.02	0.02	0.01	0.01
Luteolin	0.72	0.96	0.74	1.12	0.68	1.54
Fisetin	0.00	0.00	0.00	0.00	0.00	0.00
Eriodictyol	0.00	0.00	0.00	0.00	0.00	0.00
Catechin	1.83	4.70	5.16	5.10	3.49	12.12
Epicatechin	1.03	1.82	1.19	1.28	0.48	2.65
Hesperetin	0.12	0.13	0.09	0.14	0.09	0.16
Quercetin	1.27	0.60	0.55	1.57	0.67	1.23
Epigallocatechin	0.40	1.07	0.43	0.74	0.23	0.61
Myricetin	0.83	0.91	1.11	0.74	1.58	3.44
Rutin	0.38	0.84	0.67	0.32	0.30	1.33

Source: CTR&TI, Annual Report, 2022-23

From the above table, Table 1, it can be seen that the major phenolic components, *viz.*, Gentistic acid, Coumaric acid, Ferulic acid, Cinnamic and Gallic acid, which are the precursor for fruiting body of *Cordyceps*, are present in the highest amount in Tasar refuses. The highest Gentistic acid content 53.29  $\mu\text{g/gm}$  and p- Coumaric acid content 39.84  $\mu\text{g/gm}$  was found in Tasar pupae. Maximum t-Cinnamic acid content 115.18  $\mu\text{g/gm}$  and Ferulic acid content 88.83  $\mu\text{g/gm}$  was found in Tasar egg. Highest Gallic acid content 300.75  $\mu\text{g/gm}$  and o-Coumaric acid content 1576.71  $\mu\text{g/gm}$  was estimated in Tasar adult.

The three main flavonoids found in *Cordyceps* fruiting bodies were epicatechin, catechin, and naringenin. The maximum Epicatechin concentration (2.65  $\mu\text{g/gm}$ ) and Catechin content (12.12  $\mu\text{g/gm}$ ) were assessed in Tasar pupae, while the highest Naringenin level (15.53  $\mu\text{g/gm}$ ) was discovered in Tasar adults. Tasar refuses appear to have the ability to act as a medium for *Cordyceps* fruiting bodies.

### Studies done so far:

Siripuk and Chandrasekhar (2016) investigated whether *B. mori* pupal powder was suitable for inducing the mycelial growth of *C. militaris* and its anamorph, *Paecilomyces tenuipes*, in culture media that included yolk and one of five types of Thai rice: plain rice, black rice, brown rice, glutinous rice, and rice bran. For all *C. militaris* strains, the most successful mycelial growth recipe was found to be glutinous rice + pupal powder + yolk.

In an experiment done in 2002, Chen and Ichida developed methods for large-scale production of *Cordyceps militaris* (L.) stromata. *Bombyx mori* L., the silkworm, was infected, and the development of the stroma was studied. The fifth instar larvae and pupae of silkworms raised on a synthetic diet in a sterile indoor environment were injected hypodermically, sprayed, or dipped in *C. militaris*. Each of the three techniques had a different infection rate, with the

hypodermic injection having the highest rate. Additionally, compared to the larvae, the pupae had a greater infection rate. The silkworm pupae had improved stromata development and growth. Several locations on the pupal body were injected with a conidial suspension. However, there was no discernible difference in infection rates between the sites. The injection site had no effect on the stromata's development or growth.

In an experiment, *Cordyceps militaris* was produced using the pupae of the silkworm *Bombyx mori* as the infecting host (Sung *et al.*, 2002). Against silkworm pupae, three different inoculation techniques— injection, spray, and immersion— were tested. The three inoculation techniques evaluated on silkworm pupae showed 100% infectivity. The injection method was the most successful of the three inoculation techniques in shortening the time needed for the endosclerotium and fruiting body production to be fully formed. These findings suggest that the pupae of silkworms are highly efficient host insects for the development of *C. militaris*.

### **Fruit Body Formation on Silkworm by *Cordyceps militaris***

Upon mounting, pupae between the ages of nine and eleven days exhibited the highest rate of infection with an injection volume of 100  $\mu$ L. When injected with a hyphal suspension concentration exceeding  $2 \times 10^5$  colony-forming units (cfu), silkworm pupae demonstrated an infection incidence of over 96%. Furthermore, *C. militaris* fruit bodies were generated and induced at light intensities ranging from 500 to 1,000 lux (Hong *et al.*, 2010).

### **Culture of *Cordyceps militaris* on synthetic substrates under artificial conditions**

At the moment, *Cordyceps militaris* is extremely rare due to overexploitation in the wild. Thus, it is necessary and has been effectively explored to cultivate *Cordyceps militaris* on artificial substrates under artificial conditions. Huy and Phuong (2021) discovered that the *Cordyceps militaris* fungus grew quickly (covering the medium's surface after six days of culture) and produced a large number of fruit bodies (an average of 60 fruitbodies per jar) when grown on a synthetic medium made of 35g brown rice per box, 60ml mineral solution, and 5% dry pupae powder.

### **Chitinase –A key factor in the fruiting body development of *Cordyceps militaris***

During the growth of *C. militaris* fruiting bodies, there is a considerable rise in both the transcription level and the enzyme activity of chitinase. In an experiment by Zhang *et al.* (2023), two chitinase genes (Chi1 and Chi4) were chosen to create a gene-silencing strain by RNA interference, in order to verify this finding. Upon knocking down the Chi1 and Chi4 genes, the primordium's ability to differentiate was hindered, resulting in a notable 50% reduction in fruiting bodies as compared to the wild-type (WT) strain. In strains silenced by Chi1 and Chi4, the length of the single mature fruiting body was reduced by 27% and 38%, respectively. On the other hand, strains that were silenced for Chi1 and Chi4 had far higher chitin contents and thicker cell walls. Chitin concentration ranges from 2.6% to 4.2% in *Bombyx mori* L. pupae

(Battampara *et al.*, 2020). The findings of this investigation shed fresh light on the biological roles of chitinase and the role that *Bombyx mori* L. pupae plays in the development of *C. militaris*' fruiting bodies.

### **Efforts of the Central Silk Board, Ministry of Textiles, Govt. of India in popularization of the concept:**

A project with Project ID: BPC 04005SI entitled “**Tasar waste to wealth by Cordyceps**” (March 2020- Feb 2023) with PI: K. Jena, Scientist- D, Central Tasar Research and Training Institute, (CTR&TI), Ranchi was launched with an aim to utilize the Tasar waste in the generation of additional revenue in the sector. Dr. P.K. Mishra Retd. Director (Tech), Central Silk Board, Bengaluru suggested for commercialization of the Cordyceps, as patent has already been filed. The Research Advisory Committee (RAC) of suggested Principal Investigator (PI) to submit the final report in RMIS10 during the 52<sup>nd</sup> meeting of Research Advisory Committee (RAC) of CTR&TI held on 20th - 21st June, 2023 at CTR&TI, Ranchi. Also, a patent *viz.*, ‘Process to Mass Produce *Cordyceps militaris* on Vanya Silkworm Refuses’ (NRDC/IPR/PC/22075/2022) was filed during the year 2020-23 (CTR&TI, Annual Report, 2022-23).

### **Conclusion:**

The main wastes and byproducts created during the rearing process are the dead and ill silkworm larvae during rearing and the dead pupae following stifling of cocoons. Through numerous repurposing of by-products that can also boost a farm's economy, the efficient use of these goods is crucial to establishing sericulture as a potential career option in farmers' minds. In an effort to increase the sustainability of sericulture domestically and internationally, this paper attempts to highlight the waste (dead pupae and larvae) generation in sericulture (both mulberry and non-mulberry) and their collection, consolidation, and reutilization in the agriculture and pharmaceutical industry as a potential media for cultivation of *Cordyceps militaris* fungi through the most recent research findings.

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