Evaluation of Phytochemical Composition, Antioxidant Activities and Cytotoxicity on Hep-G2 cancer cell line in vitro from *Cordyceps militaris* Medicinal Mushroom

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Abstract
Background
There are many different biofunctionalities found in the medicinal fungus known as C*ordyceps militaris*(CM). It contains polysaccharides and other components with biological significance. Because of its wide range of pharmacological applications, *C. militaris* has recently been the subject of a research, with a special emphasis on its role in the prevention of and associated molecular pathways in liver disorders. *C. militaris* extract were analyzed for their total phenolic content and total flavonoids, in ethanol and aqueous extracts of mushroom. Two assays (DPPH, and hydrogen peroxide) were used to determine the antioxidant activity.The IC_{50} value of CM extract was determine by NRU assay. Ethanolic extract of *C. militaris* extract shows highest antioxidant activity at $CM_{80\%}$ as compare aqueous extract. Total flavonoids and phenolics of extract calculated as gallic acid equivalent and quercetin equivalent. Resultant shows 11.40 mg gallic acid equivalent/g of ethanolic extract and 7.64mg gallic acid equivalent/g of aqueous extract powder respectively. Whereas total flavonoid in ethanolic extract was calculates as 4.135mg Querecetin/g and 2.725mg Querecetin/g in aqueous extract. The NRU study revealed that *C. militaris* extract reduced the cell viability of Hep-G2 cancer cell line in a dose dependent manner.
Keyword- C*ordyceps militaris,* phenolic content, flavonoids content and antioxidant activities

1.1 Introduction

Human civilization has traditionally regarded medicinal mushrooms, and species in the genus Cordyceps are particularly prized.For centuries, medicinal mushrooms have been recognised for their ability to generate biometabolites, which are being explored as potential remedies for various illnesses. Improving our nutrition, mushrooms which are rich in antioxidants, could prevent or reduce more than two-thirds of cancer-related fatalities.Cordyceps is mostly distributed in North America, Europe, and Asia. In India, it is predominantly found in the subalpine areas of grassy fields in the Himalayas, colloquially referred to as "Keera Ghas". News has emerged from the villages of Sutol and Kanol in the Chamoli district of Uttarakhand.In order to generate functional foods and discover new medications, cordyceps, a macrofungus that parasitizes insects, is exploited as a source. It is closely related to mushrooms and belongs to the same subphylum Ascomycotina, class Pyrenomycetes, order Clavicipitales, and family Clavieipitaceae. Despite not being a mushroom in the taxonomic sense, it has always been referred to as and dubbed a medical mushroom. The Latin words co-d and caps, which translate to "club" and "head," respectively, are where the name originates.In Chinese *Cordyceps, Cordyceps militaris* is a rare and valuable medical fungus that has been utilized for ages in China as a source of traditional remedies or as a biocontrol agent against pests. One of the *Cordyceps militaris'* bioactive components, polysaccharides exhibit a range of biological actions, like immunomodulation, anticancer effectsetc. Most literature indicates that polysaccharides are mostly derived from artificially cultivated mushroom fruiting bodies (intracellular polysaccharides) or mycelium fermented broths (extracellular polysaccharides), due to the scarcity and

high cost of real *Cordyceps militaris*. (Qin et al.,2019).Internally, spores are created in a sac known as the ascus. It typically lives at pupal surfaces of insects in winter, causing the development of fruiting bodies in summer, therefore earning the name "winter-worm summer-grass."We can isolate bioactive components from CM and convert it to powdered and capsule form (for example, Didanosine) because to recent breakthroughs in pharmaceutical bio-techniques(Phull et al.,2022).The entomopathogenic mycelia develop within the intestines of pupae or larvae during the autumn season. The following summer, a mature structure known as a stroma emerges from the body and produces sexual spores. Stromas, whether or not they include larvae, have long been utilised as medicinal substances in Asian nations for many ages. Thus, Cordyceps, which is commonly used as a nourishing food, has gained recognition in Asia as nutraceuticals or health-promoting foods. There are around 40 varieties of products that undergo fermentation with Cordyceps-related fungi, resulting in an enhanced value of the items. *Cordyceps militaris*, a fungus has gained significant interest in recent times because of its acknowledged biological qualities that promote health and possess medical effects. The inclusion of *C. militaris* in chicken essence products enhances their commercial value and confers health benefits to consumers.Furthermore, both the reproductive structures and the vegetative part of *C. militaris*are found in Taiwan and are utilised in the production of nutraceuticals and functional meals (Wang et al.,2012).The growth of Cordyceps is contingent upon certain environmental conditions and its small size, making the large-scale gathering of this mushroom a formidable undertaking. Individuals between the ages of 15 and 65, encompassing both males and females, as well as young boys and girls, are the primary gatherers of this fungus. The market price for 1 kilogramme of wild-collected mushroom in Nepal ranges from 30,000 to 60,000 Nepali Rupees, whilst in India it is priced at over 100,000 Rupees.Over the last five years, there has been extensive exploitation of Cordyceps, resulting in a dramatic decrease in its natural presence. Attempts have been attempted to artificially culture this fungus using surface and submerged fermentation methods.Cordyceps mycelium has the ability to thrive on diverse nutrient-rich substances. However, in the context of industrial fermentation and culture, insect larvae (namely silkworm leftovers) and a variety of cereal grains have historically been employed. Consistently, it has been observed that both insect larvae and cereal grains yield fruiting bodies of fungus with nearly equivalent therapeutic characteristics. There are two primary methods for cultivating mycelium biomass of Cordyceps: surface fermentation and submerged fermentation.Surface fermentation involves the growth of microbial biomass on the surface of either a liquid or solid substrate.Nevertheless, this approach is highly burdensome, costly, requires a significant amount of manual labour, and is seldom employed on a large industrial level. During submerged fermentation, microorganisms are cultured in a liquid medium under aerobic conditions with appropriate agitation to achieve uniform growth of cells and media constituents.Nevertheless, the extraction of extracellular compounds from the broth is diminished following the collection of mycelia. This necessitates the enhancement of both the composition of the culture medium and the technology used in downstream processing in order to achieve the large-scale production of secondary bio-metabolites. Repeated batch culture technique has been found to yield the maximum productivity by removing waste medium at the conclusion of the process and then refreshing the medium, resulting in increased production of cells and bio metabolites (Tuli et al.,2014).

The biologically active substances found in cordyceps, particularly its extract, includecordycepicacid, cordycepin,adenosine and exo-polysaccharides. Cordycepin, also known as 3′-deoxyadenosine (9-(3-deoxy-D-ribofuranosyl)adenine), is the primary active ingredient that has received the most attention due to its potential medical benefits and wide range of biological activity (Das et al.,2021). Cordycepin functions as a nucleoside analogue and shares a lot of structural similarities with the cellular nucleotide adenosine. The chemical name for cordycepin is$C_{10}H_{13}N_5O_3$, and its molecular weight is 251.24. The melting point of cordycepin is 228°C and 231°C, and its maximum absorption wavelength is 259nm (Sangwannaet al., 2023).

In vitro studies have demonstrated the antioxidant activity of *C. militaris*. The extract *of C. militaris* has antioxidant properties by scavenging free radicals and has been observed to possess hepatoprotective action in laboratory settings. The extract of *C. militaris* has mitigated oxidative damage in HepG2 cells caused by tert-butyl hydroperoxide (t-BHP) by the reduction of reactive oxygen species (ROS) production

and the development of thiobarbituric acid reactive substances (TBARS) (Choi et al.,2014).Free radicals are crucial to many biological processes, including host defence and the excessive creation of hydrogen peroxidealso the hydroxyl radical (OH), which are linked to diseases like cancer, AIDS, diabetes, and hypertension. However, antioxidants prevented an oxidative chain reaction from occurring, reducing the oxidation of other molecules such as proteins and lipids. Food breakdown occurs when lipids oxidize in the presence of free radicals, and this can be reduced by antioxidants (More and Makola, 2020). The food business and free radical pathology have shown plant phytochemicals to be helpful alternatives to natural antioxidants. Due to its significant role in metabolism and drug biotransformation, the liver is a suitable option for toxicological and pharmaceutical testing.Undoubtedly, in vivo liver toxicity testing forms a significant component of in vitro toxicology. (Sharma et al.,2020). Implementing high-capacity in vitro liver models could serve as a valuable asset and facilitate the transition towards increased utilisation of alternative toxicity assessment methodologies(Sharma et al.,2019).

Plants with bioactive elements have been discovered to modulate oxidative stress, a crucial factor in liver illnesses, and may provide benefits in treating such conditions. Consequently, exploring biological substances that can decrease oxidative stress can be a potent therapeutic approach for the prevention and treatment of hepatotoxicity.

2. Material and Methods

2.1 Preparation of ethanol extract

Fresh fruiting bodies were removed from a cultivation medium and cut into little pieces ranging in size from 2 to 4 mm. In a hot air dryer, these pieces were dried at 40°C for 48 hours before being pulverized into powder. 10g of powder were macerated in 100ml of 70% ethanol and was kept at rotor shaker for 72 hours at room temperature. Mixture was then filtered using Whatmann paper 1 and kept at 4° C for a subsequent test.

2.2 Preparation of Aqueous extract

10g powders extract of *C. militaris* was mixed in 100ml of distilled water and was kept at rotor shaker for 72h. at room temperature, further the solution was filtered using whatmann filter paper 1 and stored at 4°C for different assays (Tran, 2022).

2.3Qualitative phytochemical analysis

Methods from earlier papers were used to determine the bioactive components in the ethanol extract from *C. militaris* fruiting bodies, including the Biuret test for proteins, Molisch's tests for carbohydrates, the lead acetate and ferric chloride tests for phenolics, the lead acetate tests for tannins, the alkaline reagent test for flavonoids (Agidew, 2022).

2.4 Quantitative phytochemical analysis

2.4.1 Folin–Ciocalteu Technique for Calculating Total Phenolic Concentration

The antioxidant, anticarcinogenic, antimutagenic, and gene-expression-altering capabilities of phenolics are just the tip of the iceberg. Maximumscavenging effects in plantsrely on phytochemicals, the most important class of phenolics. The GAE (gallic acid equivalence) method, also known as the FCR (Folin-Ciocalteureagent) were used to calculate the Total phenolic content, the study was carried out in 'Parul Institute of Applied Science' in the month of February, 2023 (Baliyanet al., 2022).

As instructed, standardized gallic acid was dispensed into sterile test tubes at concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 g/mL. DL was added to bring the volume up to a convenient 500 µL. After carefully mixing the solutions, add 2.5 mL of Folin-Ciocalteu solution to each test tube and incubate it for 5 minutes at room temperature. Two milliliters (7.5% Na_2CO_3) should be mixed to respective test tube and let to sit at room temperature for one hour. Each specimen's absorbance at 765 nm was measured with a spectrophotometer

2.4.2 Total flavonoid Content

The flavonoids in an isolated crude extract were measured using a slightly modified technique at 'Parul Institute of Applied Science'. Take 1.25ml of distilled water and 0.5ml of the sample (Extract). The 5% sodium nitrite solution was then added, and the mixture was permitted to settle for 5 minutes. 0.15 ml of 10% aluminium chloride was mixed, followed by 500µL of 1.0 M sodium hydroxide and 0.275 ml more distilled water to dilute the liquid after 6 minutes. The solutions absorbance was then measured at 510nm. The amount of flavonoid in each gram was measured in mg of quercetin equivalents (Mohammed et al.,2022).

2.5 Antioxidant Analysis

2.5.1 DPPH Assay

Based on the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH-0.002% in methanol) technique's ability to scavenge free radical. Using the appropriate solvents, the sample's working solution (10 mg/ml) was made. Ascorbic acid 10mg/ml was used as a standard to compare sample findings to. 2ml of sample/standard solution (200-1000 µg/ml) and 1.0ml of DPPH were combined in 3ml of solution,further incubate it at 37°C for 30 min. After that, 520 nm absorbance values were calculated into an antioxidant activity percentage(Kumar et al.,2020).

Percent inhibition of DPPH activity = (A – B)/A *100

Where A = Absorbance of the blank and B = Absorbance of the sample

2.5.2 Hydrogen Peroxide Assay

Hydrogen peroxide (H_2O_2) assay was conducted with slight modification. 0.1 mL sample of extracts was transferred into an eppendorf tube. The tube was then filled completely with 50 mM phosphate buffer (pH 7.4) and 600µ L of a 2 mM H_2O_2 solution. Following a reaction time of 10 minutes, the reaction mixture was vigorously mixed using a vortex, and the absorbance at a wavelength of 230 nm was measured (Tan et al.,2020). The utilised positive control was ascorbic acid(Li et al.,2021).

2.5 Test for Cytotoxicity

The extract sample were tested for cytotoxicity using the NRU (Neutral Red Uptake) Assay on the HepG2 cell line was carried out in Parul University, cell culture laboratory in the month of June, 2023. In a 24-hour experiment, cells (5000-8000 cells/well) were grown in 96-well plates at 37 degrees Celsius with 5% CO_2 in DMEM medium supplemented with 10% fetal bovine serum (HIMEDIA-RM 10432) and 1% antibiotic solution. The next day, the culture media was changed out for a new batch in each well. Following a 24-hour incubation period, 5 µl of Treatment dilutions (at varying concentrations) were applied to the specified wells of the treated plates. Following a one-hour incubation in a Heal Force-Smartcell CO_2 Incubator-Hf-90, 100 µl of NRU (SRL Chem-36248) (40 g/ml in PBS - phosphate buffered saline) was applied to the specified wells. After removing the medium, 100 µl of NRU Destain solution was used to dissolve the NRU. The plates were then read using an Elisa Plate Reader (iMarkBioRad-USA) at 550/660 nm. IC-50 Graph Pad Prism -6 was used for the computations (Rodrigueset al., 2023).

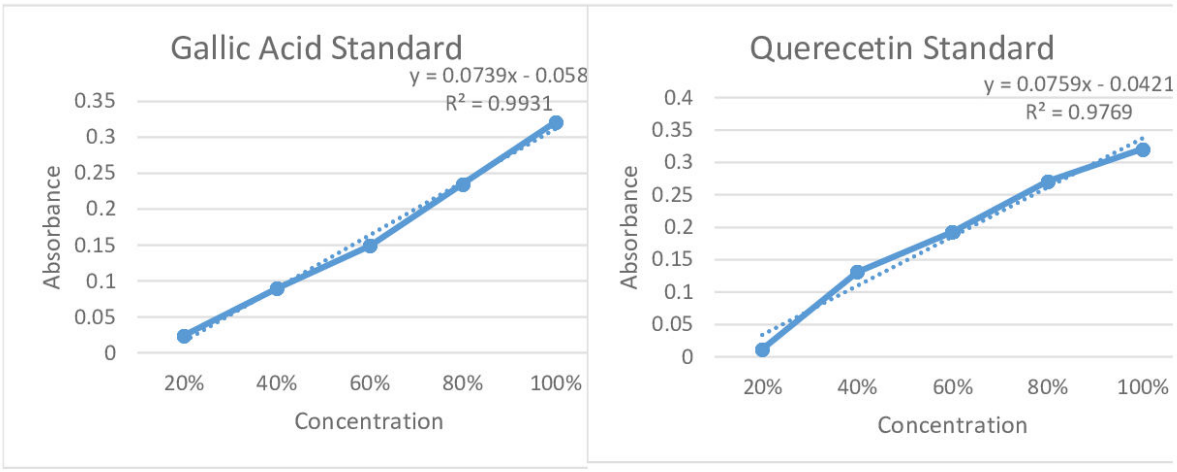
3. Results

3.1Phytochemical Analysis

The ethanol and aqueous extract of *C. militaris* had secondary metabolites that contained proteins, carbohydrates, phenolics, tannins, flavonoids, and saponins, according to the results of the qualitative phytochemical examination. Additionally, research showed that ethanol's strong polarity made it the best solvent for isolating secondary metabolites. Therefore, many nutritive components and environmental conditions connected to the growing process may result in the existence of bioactive substances in *C. militaris* extract (Oncho et al.,2021).

3.2 Phytochemical Screening

Qualitative phytochemical screening carried on extracts showed the presence of phenolic compounds and flavonoids. The results revealed the presence of medicinal as well as physiological active compounds. Quantitative analysis of total phenolic content in ethanolic and aqueous extract was 11.40 mg gallic acid equivalent/g of ethanolic extract and 7.64mg gallic acid equivalent/g of aqueous extract powder respectively with reference of standardcurve (R^2 =0.9931). Whereas total flavonoid in ethanolic extract was calculates as 4.135mg Querecetin/g and 2.725mg Querecetin/g (R^2=0.9769) in aqueous extract as illustrated in Figure 1 (a,b,c) (He at al.,2020).



(a) **(b)**

(c)

Sample	Parameter	Quantity Ethanolic Extract (percentage inhibition)	Quantity Aqueous Extract (percentage inhibition)
1.	Total Phenolic Content	11.40 (57%)	7.64 (38%)
2.	Total Flavonoids content	4.135 (20%)	2.75 (13%)

Figure 1- Phytochemical analysis of ethanolic and aqueous extract of *Cordyceps militaris*

a. **Figure (a)illustrate the standard curve of gallic acid**
b. **Figure (b) illustrate the standard curve for quercetin**
c. **Table(c)shoes the percentage inhibition of the ethanolic and aqueous extract**

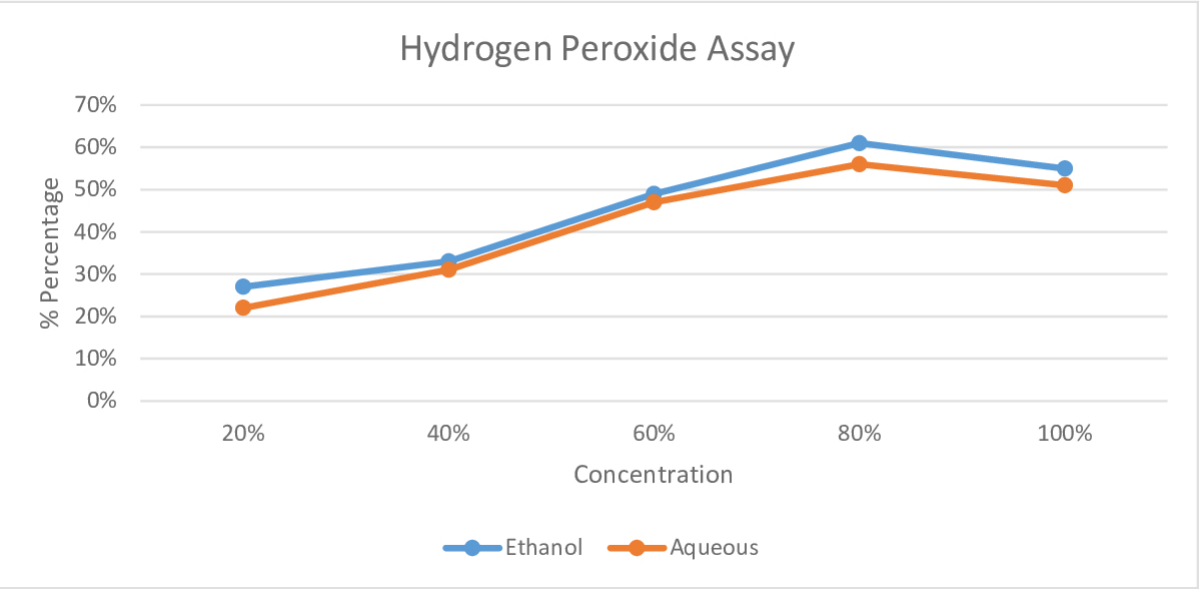
3.3 Antioxidant Activity

Antioxidants are utilised as food preservatives to stabilise the composition and ensure the safety of food by protecting it from the detrimental effects of free oxygen and other important substances that can compromise its quality. The current study focused on investigating the free radical scavenging activity derived from *Cordyceps militaris* using the DPPH scavenging assay. This test involves the use of a stable free radical that is counteracted by antioxidants. The DPPH molecule possesses an unpaired electron, which is accountable for its absorption at a wavelength of 520nm, resulting in a visible purple colour. The antioxidant activityfrom *Cordyceps militaris* shown a substantial similarity to ascorbic acid, which was used as the reference, in an in vitro setting. As illustrated in the figure 2 (a,b,c) the highest percentage inhibition of ethanol extract is at 80% concentration where as in aqueous extract the highest antioxidant activity was shown at 60% concentration (Eiamthaworn et al.,2022).

a.

Sno.	Concentration	Ethanol % Inhibition of DPPH assay	Aqueous % Inhibition of DPPH assay	Ethanol % Inhibition of Hydrogen peroxide assay	Aqueous % Inhibition of Hydrogen peroxide assay
1.	**20%**	20%	19.2%	27%	22%
2.	**40%**	47%	38%	33%	31%
3.	**60%**	52%	49.5%	49%	47%
4.	**80%**	55%	39.6%	61%	56%
5.	**100%**	35.2%	22%	55%	51%

b.



c.

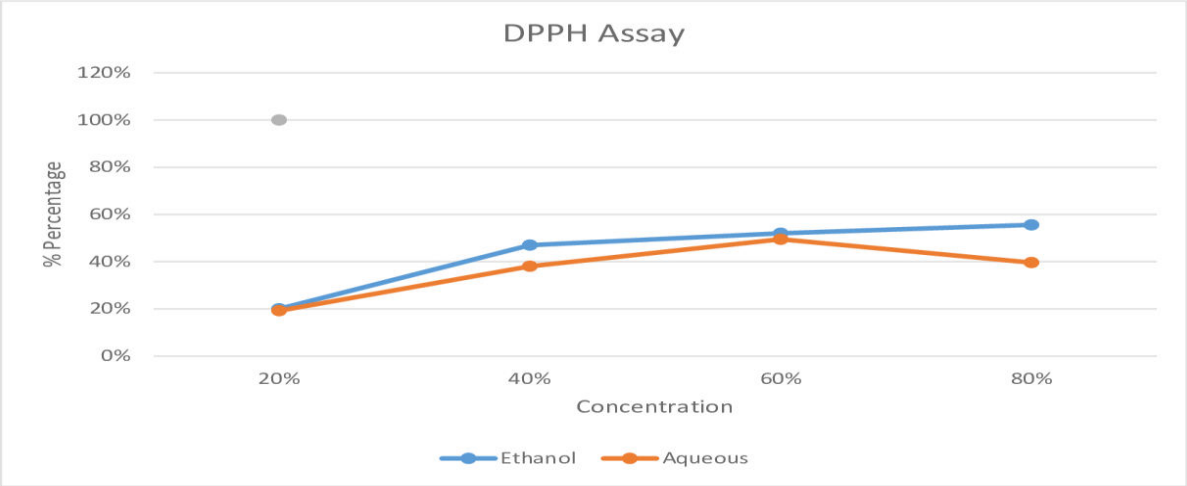


Figure 2 – Antioxidant analysis of ethanolic and aqueous extract of *Cordyceps militaris*

Figure (b) **illustrate the graph for concentration v/s percentage inhibition for Hydrogen peroxide Assay**

Figure (c) **illustrate the graph for concentration v/s percentage inhibition for DPPH Assay**

Cytotoxicity Analysis

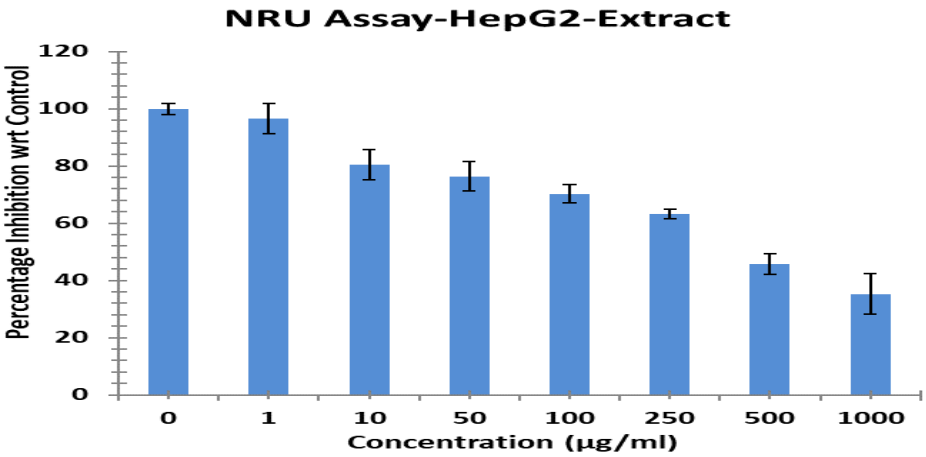
The NRU cytotoxicity data indicated that CM exhibited the most potent dose-dependent inhibitory effects on HepG2 cells. The CM compound exhibited a significant cytotoxic effect on Hep-G2 cells, with an IC50 value of 373.8 µg/ml as shown in figure-3. This indicates that even at the lowest concentration of the extract, the compound demonstrated a high level of cytotoxicity (Hassan et al.,2019).

Research has shown that cordycepin, a primary chemical found in CM, exhibits chemopreventive effects against various types of cancer cells, including those found in the colon, liver, bladder, kidney, lung, and breast.

a.

Sample code	IC50 value (µg/ml)
Extract	373.8

b.



c.

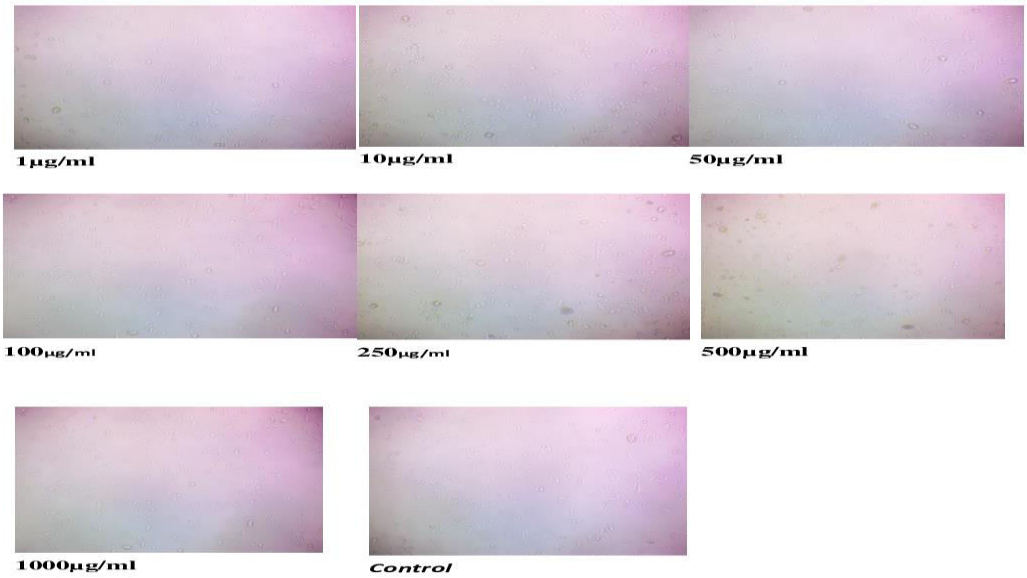


Figure 3- The cell viability by NRU assay was conducted on Hep- G2 cell line which was treated with *Cordyceps militaris* extract for 24Hrs, the presented data shows the result of cytotoxicity of the extract at different doses

Discussion and Conclusion

This study explored the potential anticancer effects of CM on HepG2 cells and evaluated the underlying processes. When compared to other bioactive extracts that have been published, the crude character of CM implies that itmay contain multiple beneficial components that can target different molecules. As a result of the systematic targeting, fewer negative side effects are anticipated. For centuries, CM has served as a basic remedy and a conventional treatment in East Asia, underscoring its safety and limited adverse reactions.

Also, the study demonstrated that the extract of *C. militaris* included a diverse range of bioactive compounds, including proteins, carbohydrates, phenolics, tannins, flavonoids, and saponins, with a particular emphasis on cordycepin.The ethanol extract had the greatest efficacy in terms of total phenol and total flavonoid content. Moreover, this extract exhibited antioxidant properties as well. It exhibited low cytotoxicity against Hep-G2 cell lines. Overall, this study suggests that the *C. militaris* extract derived from fruiting bodies is a favourable option for herbal supplements and pharmaceutical uses in the field of pharmacy.

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Conflicts of Interest

The authors report no financial or any other conflicts of interest in this work.

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