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Antibacterial and fibrinolytic potential of Himalayan soft gold mushroom *Cordyceps sinensis*

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The medicinal mushroom *Cordyceps sinensis* collected from Himalayan region of India was found to have bioactive compounds. The mycelia free culture filtrate obtained from fermentation of *Cordyceps* mycelia were partially purified by solvent-solvent extractions. The subsequent fractions were tested for their potential regarding antibacterial and fibrinolytic activity. The butanolic fraction and aqueous layer showed significant antibacterial activity against six bacterial strains.While the final aqueous layer and hexane fraction showed partial fibrin lytic capacity in comparison with commercially available streptokinase as a positive control. It was assumed that the metabolite fractions when purified further can act as good antibacterial and fibrinolytic agents.

Keywords: *Cordyceps sinensis*, bioactive compounds, solvent fractions, antibacterial activity, fibrinolytic activity

Introduction

Fungi are found to be one of the best sources of alternative medicines apart from plants and bacteria. They have the potential to produce novel compounds which are medicinally very important[1]. Higher fungi especially medicinal mushrooms are being studied since ages for their bio-metabolites and are renowned for the treatment of various diseases[2]. One of the most important and demanding medicinal mushroom belongs to class ascomycetes is *Cordyceps* a soft gold, having a valuable source of natural products with diverse biological activities. This is entomopathogenic in nature and exists asgrowth from the body of infected insect[3]. Fungus *C. sinensis* is endemic to the grasslands and shrubs of Central Asia and grows in a frigid and arid environment at the elevations of 3500–4500 m[4,5].

This medicinal mushroom is considered as therapeutic bio-factory, which possesses anti-cancer, anti-metastatic, anti-microbial, anti-inflammatory, anti-oxidant and immuno-stimulating properties[6-7]. The cultivation and collection of *Cordyceps* from its habitat is challenging; because it is rare and scarce. However, the ever increasing demand of this medicinal mushroom and its metabolites leaves us with little choice but to cultivate it artificially[8-9]. Today *C. sinensis* becomes very prominent mushroom and cultivated from anamorphic mycelia for its medicinal and pharmaceutical properties[10].

The resistance in pathogenic organisms against various available drugs leads to necessitate the new drugs from new sources[11]. The disorders in fibrin network and blood clotting result in thrombosis, which induces deadly diseases like myocardial, cerebral and pulmonary infarction. Hence, the present study is based on the artificial cultivation of anamorphic mycelia of *C. sinensis* collected from Indian Himalayas. The metabolites obtained were then subjected to test for their antibacterial and fibrinolytic potential.

Materials and Methods

Collection and Isolation of Culture

The *Cordyceps sinensis* fruting body was collected from Pithoragarh District of eastern Uttaranchal, India in the month of June-July 2014. The specimen was stored in a sterilized plastic bag and brought to the laboratory (Fungal Biotechnology and Invertebrate Pathology Laboratory) where it was grown on selected and optimized PPDA (potato dextrose agar with peptone) media plates at 20 ± 2°C for 10 days. Initially, small pieces were cut and taken out from the specimen and were surface sterilized by sodium hypochlorite and 70% ethanol in order to grow

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specimen sample on media plates.The sterilized sample pieces were then placed on PPDA media plates. Culture growing from the edges of the samples was then sub-cultured to the fresh PPDA plates. The pure cultures were maintained on PPDA slants at 4°C for further use.

Cultivation of Fungus for Metabolite Production

The anamorphic mycelium of *C. sinensis* from culture plate was transferred aseptically to 250 ml flask containing 100 ml media (15 g glucose, 5 g peptone, 3 g KH_2PO_4, 1 g K_2HPO_4, 0.5g $MgSO_4$, 0.5 g NaCl in 1000 ml distilled water) and incubated at 20±2°C for 15-20 days. The extracellular metabolite was filtered through Whatman filter (paper no.1) (Thermo Fischer) and obtained filtrate was known as mycelial free culture filtrate (MFCF). The solvent-solvent extraction was performed by dissolving equal amount of MFCF and solvent in a separating funnel and was shaken for a particular time. Upon shaking the organic layer was separated and aqueous layer was continued with another solvent. This results in getting the solvents fraction one after the other.

Antibacterial Activity

The antibacterial activity of extracts from *C. sinensis* was carried out by using agar well diffusion assay[12]. In order to examine the positive control, highly concentrated antibiotic discs (HiMedia Laboratories, India) were tested along with solvent extracts in a bacterial plate. Diluted culture of six pathogenic bacteria *Vibrio cholerae, Salmonella typhimurium* (MTCC#3904), *Escherichia coli* (MTCC#82), *Klebsiella pneumonia* (MTCC#109), *Bacillus subtilis* (MTCC#441) and *Staphylococcus aureus* (MTCC#96) were made separately in test tubes. Eighty µl of bacterial culture was spread on the surface of nutrient agar plates. Twenty µl of solvent extracted sample (all organic layers; hexane, chloroform, n-butanol and final aqueous layer fractions of *C. sinensis*) was loaded into the wells (6 mm diameter) made in media plates and incubated at 37°C for 24hrs. Zone of inhibition were measured (in mm) with the help of zone measuring scale (HiMedia Laboratories, India). Experiment was performed in triplicate against each organism, a positive control and negative control for each plate was also made separately.

Antibacterial activity of all the solvent fractions from *C. sinensis* metabolite along with standard antibiotic was statistically analyzed. Here, Student t-test was performed by using Microsoft Office Excel's two-sample assuming unequal variances data analysis tool.

Fibrinolytic Activity

The best way to test the activity of fibrinolytic/thrombolytic compounds is via *in vitro* clot lysis method[13]. Venous blood from healthy human volunteers (without any previous anti-coagulant therapy) was brought safely from University's Health Centre with due permission and help of medical practitioner. Icikinase (Abbott, India) a recombinant streptokinase enzyme (15,00,000 IU/vial) was used as positive control. The different concentrations (100%, 75%, 50% and 25%) of Icikinase were added to blood clot as positive control. Stock solution of Icikinase was made by dissolving 5 ml phosphate buffer saline to an Icikinase vial. Following procedure was used for *in vitro* clot lysis method:

Five hundred µl of venous blood was taken in all pre-weighed sterilized Eppendorf tubes and were incubated at 37°C for 45 min for clot formation. Serum was completely removed carefully without disturbing the clot. Weight of clot was taken by subtracting from the weight of empty tubes. Two hundred fifty µl of test samples (hexane, chloroform, n-butanol and final aqueous layer fractions of *C. sinensis*) and different concentrations of positive control were added to the clot and incubated at 37°C for 90 min for clot lysis. Water was used as negative control. Fluid obtained after incubation was removed and tubes were weighed again. Difference obtained in weight taken before and after clot disruption was expressed as percentage of clot lysis.

Results and Discussion

Isolation of *Cordyceps*

The insect-borne fungus *Cordyceps sinensis* (*Ophiocordyceps sinensis*) is a well-recognized medicinal mushroom[14]. The traditional medicine system and modern ethno-medicine exploits majority of its bioactive compounds but the most used one is cordycepin (3' deoxyadenosine)[10]. Apart from cordycepin other bioactive compounds are adenosine[15], ergosterol, polysaccharides[16], a glycoprotein, and peptides containing α-aminoisobutyric acid[17]. Genus *Cordyceps* has anti-tumour[17], anti-viral, anti-leukemia[18], anti-cancerous[2], anti-microbial[19], anti-oxidant[20], fibrinolytic[21] and hypolipidemic activity[22]. In present study, fruiting body of the specimen collected was found

to divide into two part *viz*. stipe (the fruiting body, 1.5–6 cm in length) and stroma (insect tissue infected with fungal mycelia, 3–5 cm in length). The colour of stroma was yellowish brown while stipe was dark brown to black in colour. Fruiting body with insect cadaver was 4–12 cm in length while 3–4 mm in diameter. The fruiting body of *Cordyceps* sp. collected from Uttaranchal, India was grown vegetatively in solid and liquid media both (Fig. 1).

Antibacterial Activity

The antibacterial activity was performed by agar well diffusion assay against six test bacterial strains. Among these bacterial strains, two are Gram-positive (*B. subtilis* and *S. aureus*) and rest four are Gram-negative (*V. cholerae, K. pnuemoniae, E. coli* and *S. typhi*) in nature. All six bacterial strains were tested against the *C. sinensis* metabolites which were extracted by solvents. The n-butanol, hexane and chloroform fractions were tested along with crude metabolite and positive control. The graph in figure Fig 1 showed, butanolic extract with maximum zone of inhibition against all the tested bacterial strains. Their zone of inhibitions are 16mm, 12mm, 17mm, 17mm, 14mm and 16mm for bacteria *B. subtilis, E. coli, S. typhi, S. aureus, V. cholerae*and *K. pnuemoniae*, respectively. Final aqueous layer also showed some amount of antibacterial potential against the three test bacterial strains. These three strains are *B. subtilis, E. coli* and *S. aureus* showing zone of inhibition 10mm, 12mm and 12mm respectively. For the confirmation of antibacterial activity by *C. sinensis* pure solvents were also tested which showed least or no activity against any of the test bacterial strains. The chloroform, hexane fraction and negative control (distilled water) didn't show any zone of inhibition against any of the test bacterial strains. On the other hand positive control antibiotic did show high zone of inhibition against all the test bacterial strains. An average mean of a zone of inhibition from all the tested plates was taken which showed a high antibacterial activity (Figs. 2 & 3).

The antibacterial activity of different solvent fractions of *C. sinensis* was analyzed statistically. It

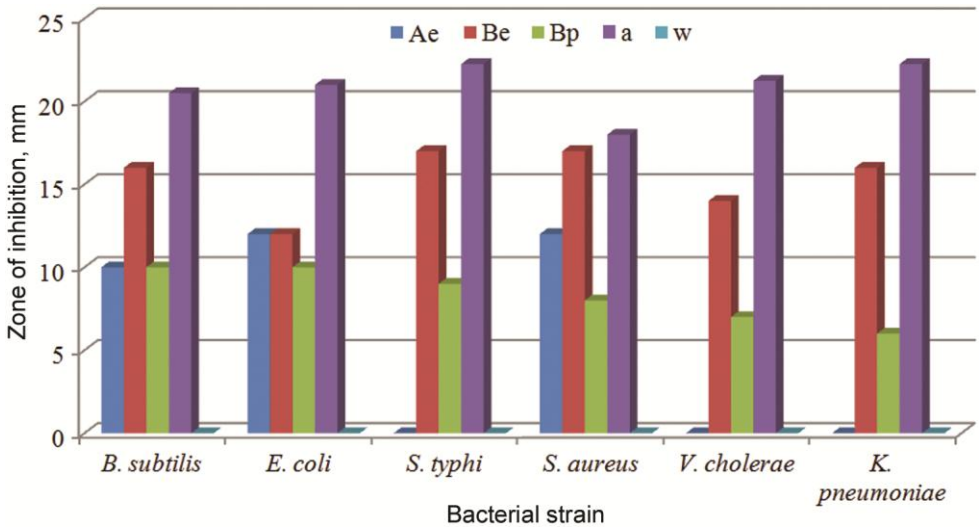

Fig. 1 — *Cordyceps sinensis*, fruiting body, mycelia and hyphae

Fig. 2 — Zone of inhibition against growth of test bacterial strains by solvent extracts of *C. sinensis*
Ae = aqueous layer, Be = n-butanol extract, Bp = n-butanol pure
Antibiotic (a) was used as positive control and distilled water (w) was used as negative control

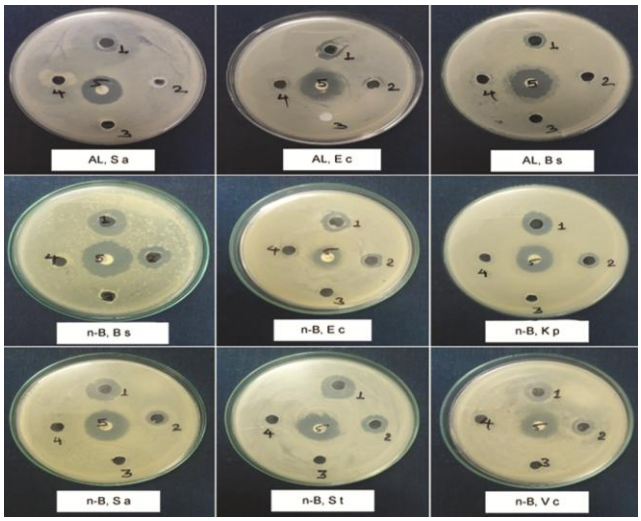

Fig. 3 — Antibacerial activity of plates showing zone of inhibition of extracts from *Cordyceps sinensis* with respect to positive and negative control
AL = aqueous layer, n-B = n-butanol, 1 = extract, 4 = crude, 3 = pure solvent, 5 = antibiotic (positive control), w = distilled water (negative control). Bs - *B. subtilis*, Ec - *E. coli*, St - *S. typhi*, Sa - *S. aureus*, Vc - *V. cholerae*, Kp - *K. pnuemoniae.*

was found that the butanolic and aqueous extract along with antibiotic standard was found significant at 5% significance level. The t-value for aqueous and butanolic extract was found 3.61 and that of butanolic and standard antibiotic was found 5.39. Both the values were found more than t-critical value hence, the results were found statistically significant as shown in Table 1.

Similarly, Dong *et al*[23]. used methanolic extract obtained from the fermentation of *C. militaris* mycelia was subjected to the test of antimicrobial (antibacterial and anti-fungal) potential by agar well diffusion method. The antibacterial activity of *C. sinensis* (CSEs) was also subjected to and determined by disc diffusion assay. Alcoholic extracts of various minimum inhibitory concentration (MIC) of CS25, CS50, CS75 and CS100 were tested against three bacterial strains. As a result the CS25, CS75 and CS100 of alcoholic extracts were found to be more effective against *E. coli*, *P. aeruginosa* and *B. subtilis* showing a zone of inhibition of 9 mm, 7 mm and 6.5 mm, respectively. Their concentrations were 93.75 µg, 93.75 µg and 45 µg, respectively. The aqueous extract showed minimum inhibition against all the three strains[24]. Tuli *et al*[25] tested various extracts obtained from *Cordyceps militaris* 3936 strain *via* agar well diffusion method. Their results shows that butanolic

Table 1 — Student t-Test statistics for antibacterial activity of potent *C. sinensis* solvent fractions:

	Ae	Be
Mean	5.666667	15.33333
Variance	39.06667	3.866667
Observations	6	6
Hypothesized mean difference	0	
Df	6	
t Stat	-3.61372	
P(T < = t) one-tail	0.00559	
t Critical one-tail	1.94318	
P(T < = t) two-tail	0.011181	
t Critical two-tail	2.446912	
	Be	a
Mean	15.33333	20.875
Variance	3.866667	2.46875
Observations	6	6
Hypothesized mean difference	0	
Df	10	
t Stat	-5.39298	
P(T < = t) one-tail	0.000152	
t Critical one-tail	1.812461	
P(T < = t) two-tail	0.000304	
t Critical two-tail	2.228139	

Be- Butanolic extract, Ae – aqueous extract, a – standard antibiotic

extract have maximum antimicrobial activity against all tested bacterial and fungal strains. The reason for antimicrobial activity was might be the presence of cordycepin or its derivative in *Cordyceps* derived extracts. Also Holbein *et al*[26]. investigated the toxicity of cordycepin in yeast cells by inhibiting RNA synthesis. In the present research work on antibacterial assay, results showed that the butanolic extract of *C. sinensis* metabolite can act as a promising antibacterial agent against all the tested bacterial strains. Further, the final aqueous layer could be taken as an antibacterial agent against only three tested bacterial strains *viz. B. subtilis, E. coli* and *S. aureus*. Hence, *C. sinensis* extracts might have cordycepin or its derivative as an active ingredient showing antibacterial activity. These extracts when further purified can be used for the development of drugs in pharmaceutical industries.

Fibrinolytic Activity

A novel fibrinolytic enzyme was produced by submerged fermentation of *C. militaris* culture. As the enzyme could degrade thrombin therefore it could be used as an anticoagulant to prevent thrombosis[27-28].

Here in this research work, the *in vitro* clot lysis activity of *C. sinensis* solvent fractions (butanolic, hexane, chloroform fraction and final aqueous layer) was performed with reference to streptokinase. The *C. sinens*is fraction and crude metabolite along with positive control (streptokinase) and negative control (water) was added in blood clot separately and was allowed to react. Figure 4 shows a clear representation of clot lysis by different samples. In case of positive control streptokinase, concentrations like 100%, 75%, 50% and 25% show significant clot lysis activity with percentage clot lysis of 78.26, 49.50, 45.83 and 37.82 respectively. This shows that with decrease in the percentage of streptokinase, clot lysis activity also decreases. On the other hand, the negative control water shows 1.03% of lysis which was considered negligible. In case of the test samples, the solvent fractions of *C. sinensis* metabolite also showed clot lysis activity. Crude metabolite showed 9.18% of clot lysis and hexane fraction showed 19.78% of clot lysis, while the final aqueous layer obtained in solvent-solvent extraction of *C. sinensis* metabolite showed 48.33% of clot lysis. In comparison with streptokinase tested concentrations, 48.33% lysis lie between 75% and 50% of concentration (Table 2). Rest of the fractions showed no dissolution of blood. The formula for clot lysis percentage was calculated as under:

Clot weight = weight of tube containing clot – weight of empty tube

$$\text{Clot lysis (\%)} = \frac{\text{weight of lysis}}{\text{weight of clot before lysis}} \times 100$$

Similarly, *in vitro* anti-coagulant capacity of enzyme purified in saline from *C. militaris* was also studied in coagulated blood. Sample was tested along with heparin and control saline separately. Apparently, no coagulation was observed either in heparin or in samples (purified enzyme) tube after 3 hrs but after 24 hrs blood was found to be partially coagulated in heparin tube and sample tube showed complete dissolution of the blood clot. It was because of an enzyme from *C. militaryis* which significantly acts as an anti-coagulant and is quite better than that of heparin. The enzyme isolated by Liu *et al*.

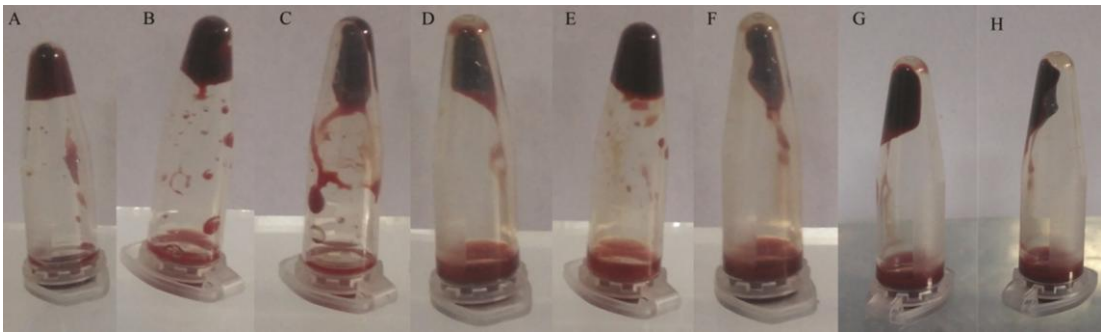

Fig. 4 — Fibrinolytic activity of *C. sinensis* extract with respect to control
A – water, B – MFCF, C – hexane extract, D – aqueous extract, E – 25% streptokinase, F – 50% streptokinase, G – 75% streptokinase, H – 100% streptokinase

Table 2 — Effect of *C. sinensis* extracts on *in vitro* clot lysis along with positive and negative control

S. No.	Type of sample	Weight of clot	Weight of lysis	Clot lysis %
1	S 100%	0.184	0.144	78.26
2	S 75%	0.204	0.101	49.50
3	S 50%	0.168	0.077	45.83
4	S 25%	0.193	0.073	37.82
5	Water	0.193	0.002	1.03
6	Crude	0.185	0.017	9.18
7	^{C}aqueous layer	0.180	0.087	48.33
8	^{C}butanol	0.176	−0.084	–
9	^{C}hexane	0.187	0.037	19.78
10	^{C}chloroform	0.189	−0.061	–
11	Butanol	0.171	−0.075	–

S = streptokinase, C = extract of *C. sinensis*

from *C. militaris* is a novel fibrinolytic enzyme whose molecular weight is 28 kDa. It can act as plasmin like protein as well as plasminogen activator which remains active at pH 7.2 and temperature 37°C. Here in the current study, the partial lysis of metabolite fractions on purification could give complete lysis of clot. From the above observations it can be assured that the fraction of *C. sinensis* metabolite may act as a good fibrin lytic drug after complete purification.

Conclusion

In the present research, the fruiting body of *Cordyceps sinensis* was collected from the Himalayan region, Uttarakhand, India. The metabolite obtained from anamorphic mycelia was subjected to the solvent fractions which showed characteristic anti-bacterial and fibrinolytic activities. The butanolic fraction was found to have a potential antibacterial activity against all the tested pathogenic bacterial strains, while that of the aqueous extract showed partial anti-coagulant property. After complete purification of both the extracts, obtained subsequent products could be estimated to be used as an antibiotic drug and fibrinolytic drug by pharmaceutical industries. The intravascular thrombosis is the main cause of cardiovascular diseases which may be treated by using these natural thrombolytic extracts after complete purification. Based on the facts, extracts from "Himalayan soft gold mushroom *Cordyceps sinensis*" can help in prevention of microbial infections as well as obstructions created by clots in blood streams.

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