Solvent extraction and antibacterial potential from bioactive metabolite of *Ophiocordyceps sinensis*: A soft gold mushroom from Himalaya

Loknath Deshmukh and Sardul Singh Sandhu

Abstract: *Ophio*cordyceps *sinensis* commonly known as soft gold mushroom is highly valuable medicinal mushroom in Asian medicine. The bio-metabolites obtained from this fungus have potent activities with far-ranging capacities. In the present research solvent extracted fractions of secondary metabolites were checked for their far ranging anti bacterial potentiality. The entomopathogenic fungus was procured from its habitat, isolated and identified microscopically. The solvent-solvent extraction method was performed to extract bio-active compounds from fermented broth. Various solvent fractions were tested against six bacterial strains (*Vibrio cholerae, Salmonella typhimurium, Escherichia coli, Bacillus subtilis, Klebsiella pneumoniae* and *Staphylococcus aureus*) for examining the antibacterial activity. The butanolic fraction showed maximum zone of inhibition against all six bacterial strains ranges from 15mm to 24mm as compared to positive control. Maximum standard error percentage was ±3.464102 and minimum standard error percentage was ±0.57735. *Ophio*cordyceps *sinensis* is well recognised fungi but its cultivation is challengeable but their vegetative cultivation and secondary metabolite production and their uses are convenient and approachable. Therefore the extracted and fractioned compounds containing bioactive metabolites can be used against deadly disease causing pathogens. Statistical analysis authenticated the outcomes for the acceptance of this eco-friendly and pharmaceutically important approach.

Key Words: Entomopathogenic fungi, Antibacterial activity, Novel drug, *Ophiocordyceps sinensis*, Secondary metabolite, Solvent extraction.

1. INTRODUCTION:

The study of fungi has been of great interest to science till the date. Fungi are of great importance and use in the group of eukaryotic organisms. There are few miraculous organisms alive in the amazing kingdom fungi (Kharkwal, 2016). The very famous combination between insect and fungi known as Yartsa-Gumba, Dong Chong Xia Cao and winter warm summer grass, Tibetan Chinese medicinal entomopathogenic higher fungi, has diversified name as well as bioactive compounds also. Natural fruiting body of *Ophio*cordyceps *sinensis* is infrequent and not easy to collect because it's existing in nasty environment, location and season specific efforts engrosses a high collection cost from its natural territory as wild yield. With these circumstances, in vitro culture of *O. sinensis* is the only way out to fulfill the necessity of such a towering value therapeutic and well priced higher fungi (Siddique et al., 2011). The macro fungi *O. sinensis* commonly known as "Himalayan Viagra" or "Himalayan Gold" is parasitic on larvae and pupae of insects in nature (Tuli et al., 2014a). Major emphasize of this caterpillar fungi is to achieve numerous antibiotics from their secondary metabolites. One of the most potent bioactive compound is cordycepin that is highly effective against several pathogenic bacteria those are having resistance with pre-existing antibiotics (Seth et al., 2014). Bio-metabolites obtained from medicinal mushrooms are known since thousands of years for treatment of diseases and can be used to develop novel drug molecules. The multi drug resistance in bacteria compels the research in development of new antibacterial. compounds

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Though *O. sinensis* is having tremendous pharmacological properties, the studies on antimicrobial effect of their extracts are reported very less (Liu et al., 2015). Bioactive compounds isolated from *O. sinensis* grown in in-vitro condition possess wide range of biological activity like naturally existing *Cordyceps*. Extracellular enzymes of *O. sinensis* have potentiality as antibacterial, larvacidal, immunomodulatory, antidiabetic, prosexual, antioxidant, antitumor, antifungal, apoptotic and anti-inflammatory activities (Lee et al., 2011). In relevance to this need, hence in present study bioactive metabolites from *O. sinensis* was extracted by using solvents-solvent extraction and tested against pathogenic bacteria. The inhibition in growth of bacteria may lead to characterize and develop new drug molecule from this important fungus against frequent disease causing pathogenic bacteria.

2. Materials and Methods:

2.1 Sample Collection: The fungus is abundant in tropical forests and humid temperate in Asian countries including India. Hence the collection of insect cadaver infected with *O. sinensis* was done from Pithoragarh District, Uttarakhand, India (Fruiting body depicted in Fig.01).

Figure 01: *Fruiting body; Cadaver infected by Cordyceps sinensis.*

2.2 Isolation, optimization and culture maintenance: Sample was aseptically brought to the FBIPL, Department of Biological Science, R.D. University, Jabalpur (M.P.) India. Surface sterilization using 70% ethanol in aseptic condition was the first step toward vegetative mycelia isolation from the collected sample. Small piece of caterpillar was used to follow serial dilution and different dilutions were spreaded on general purpose solid agar medium. Ten different weak, general purpose and specific media were used to find out the best media for the vigorous vegetative culture. Likewise different pH, incubation temperature and incubation period were optimised for the optimal growth of the sample culture.

2.3 Pure culture and identification: Pure culture was maintained on optimised agar media with storage culture tube of vegetative mycelia were grown and stored at 4ºC for further use. Isolated pure mycelia culture was microscopically identified by using literatures (Pure culture and Microscopic observation depicted in Fig. 02). Purified fungal culture was confirmed up to genus level by molecular sequencing.

Figure 02: *Pure culture agar plate and microscopic visualisation of fungal spores.*

2.4 Mass cultivation and extracellular metabolite production: Broth medium of optimized media was used to produce extracellular metabolite. Three discs (8mm) of inoculums were aseptically inoculated in liquid medium for the mass cultivation and incubated for different incubation periods. Mycelia were filtered and filtrate used for secondary metabolites using whatman filter paper No. 01 after the completion of designed incubation period. Mycelia free culture filtrates (MFCF) were stored at 4ºC for additional test.

2.5 Solvent-Solvent Extraction: Desired compound extraction from MFCF was performed (Kredich and Guarino, 1960) in a range of polar: non polar solvent ratio including 1:1, 1:2, 1:3 and 1:4 (v/v) with solvents hexane; chloroform; n-butenol (Butanol) and water (Schematic representation depicted in Fig. 03). Different fractions were obtained after solvent-solvent extraction of fermented broth and kept in sterilised screw cap bottles at 4ºC for antibacterial activity test.

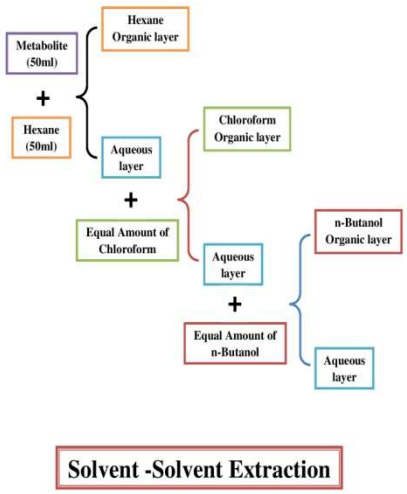


Figure 03: *Solvent extraction by compatible solvents*

2.6 Procurement of test microbial culture: In the present study antibacterial activities of different fractions was measured alongside six pathogenic bacteria namely *Bacillus subtilis, Klebsiella pneumonia, Vibrio cholerae, Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus* and were procured from IMTECH Chandigarh. All pathogenic bacteria were cultivated on Nutrient Agar Media at 37ºC.

2.7 Antibacterial activity by agar well diffusion method: In vitro antibacterial activity carried out to analyse the biologically active potent compounds derived from solvent-solvent extraction using agar well diffusion method with slight modification. Butanol fraction and aqueous layer were used to check antibacterial potentiality. Where pre-existing antibiotic was used as a positive control and pure solvents were used as a reference. Sterilised distilled water was negative control in this experiment. 8mm diameter wells were prepared by sterilised cork-borer and filled with different test fractions. Plates were observed for the clear zone of inhibition and zone diameters were measured by using Hi-media antibiotic zone scale and compared with positive control and reference.

3. RESULTS:

3.1 Sample collection and culture maintenance: A fresh and mature cadaver infected fruiting was collected and measured their length. The length of fungal fruiting body was 2.4 in centimetre and the length of larvae was 5.7 in centimetre. Vegetative mycelia culture was isolated and cultivated on optimised Potato Dextrose Agar (PPDA) media with peptone supplemented media. Subculture and pure culture were maintained in tube. The optimised pH was 6.2±1 and optimised temperature was 25ºC with incubation period of 7±1 days.

3.2 Microscopic identification: The vegetative mycelium of sample was identified by using slide culture technique. Spores and mycelia growth pattern was observed and capture in light microscope provide by the Metzer Pvt. Ltd., for the identification of sample fungi.

3.3 Extracellular metabolite production and solvent-solvent extraction: 500mL Erlenmeyer flask was used to make 250mL broth media of peptone potato dextrose and MFCF was achieved with spore seeded liquid medium. Further MFCF was mixed with different selected polar and non-polar solvents and fractions hexane to chloroform and butenol with aqueous fractions were accomplished by using separation funnel. Butanol and aqueous layer were tested for the antibacterial activity.

3.4 Screening of antibacterial activity: In the present study various solvent fractions were tested against three Gram negative and three Gram positive pathogenic bacteria (*Bacillus subtilis, Klebsiella pneumonia, Vibrio cholerae, Escherichia coli, Salmonella typhimurium* and *Staphylococcus aureus*). Butanol fraction showed maximum zone of inhibition against all used test bacterial strains ranges from 15mm to 24mm as compared to positive control and reference solvent (Graph 01). Butanol fraction of MFCF was more effective against *Staphylococcus aureus* and *Bacillus subtilis*. Where *Escherichia coli* was least effected by Butanol fraction. Other fractions showed very inconsequential results against test bacterial strains. Hence Butanol fraction was more competitive and effective fraction than positive control (Fig 04).

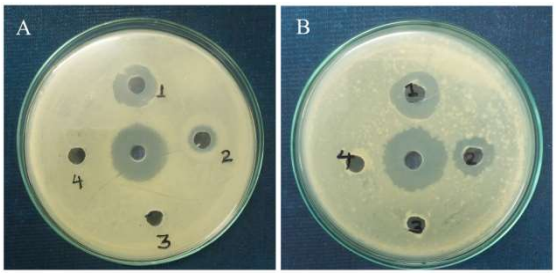
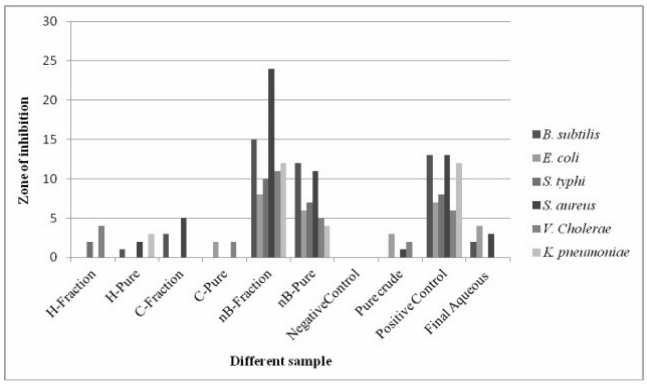


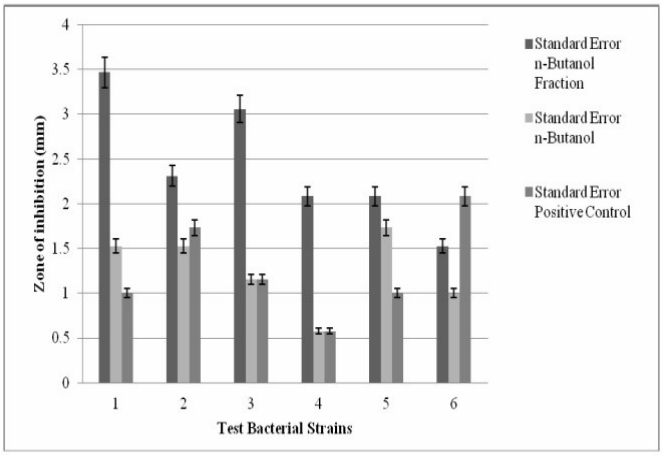
Figure 04: *Agar well diffusion method: showing potent antibacterial activity.*



Graph 01: Showing different fractional potentiality against six pathogenic bacteria. Where H= Hexane, C= Chloroform and nB= n Butanol.

Graph 01: *Broad spectrum antibacterial activity*

3.5 Statistical Analysis: The result output of n-Butanol fraction, Crude Butanol and Positive controls were analysed for their possible standard error percentage with the help of standard deviation within potent fraction and respective comparatives. Maximum standard error percentage was ±3.464102 and minimum standard error percentage was ±0.57735. Standard error depicted in the form of percentage error bar in Graph 02.



Graph 02: Showing possible standard error with potent antibacterial activity.

Graph 02: *Possible standard error*

4. DISCUSSION:

The natural ecosystem and habitats are scarred to be endangered and they need to get new advanced alternate that should be analogous with beneficial side. Ecofriendly products are not only source of bioactive compounds but also it is safe than the utilization of chemically synthesised artificial merchandise (Deshmukh et al., 2014). Uttarakhand state is very good source place for this medicinal fungus. *Ophio*cordyceps *sinensis* was collected from Pithoragarh district at steeped region of western Himalaya, Uttarakhand, India (Rakhee et al., 2016). *Ophio*cordyceps *sinensis* is a biologically, pharmaceutically and economically important higher fungi having marvellous potency to battle against chronic diseases causing bacteria. There are several studies as Hleba et al., (2016) worked with cure ethanolic extract of *Cordyceps sinensis* to determine antimicrobial activity against Gram negative and Gram Positive bacteria by disc diffusion methods. He mentioned the need of further study for improved data towards antimicrobial activity. Likewise, an antibacterial protein was isolated from *Cordyceps sinensis* and that was strongly inhibited the growth of *Staphylococcus aureus* and showed very least activity against *Bacillus subtilis* (Zheng et al., 2006). Butanol extract of *Cordyceps militaris* was tested for antibacterial activity, the TLC study and spectrometric assay showed the presence of cordycepin that was responsible for the broad spectrum antibacterial activity (Tuli et al., 2014b).

5. CONCLUSION:

Ophiocordyceps sinensis is very well known for its invigorating effects in strengthening the body and restoring energy. Few covered literature says that an artificially cultured and fermented mycelial products are found to have similar pharmacological activities as compared to wild ones. In present work, the butanolic fraction of metabolites showed highest inhibition zone against all six pathogenic bacterial strains and their data was statistically analysed by using percentage standard error. This can be purified and identified for further studies to make novel drug against

disease causing bacteria. In research, cordycepin is found to be a broad spectrum antibiotic but the mechanism of antibacterial activity including probable targets are still lacking. The ecological production of antibacterial compounds from this fungus may revolutionize the pharmaceutical industry and further research leads to reduce the price as well as broaden its usage for curing various diseases.

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8. CONFLICT OF INTEREST:

There is no eventual conflict of interest with reference to the current research. Authors have read the manuscript and agreed to submit for the publication.

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