Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 6, Issue 7, 2014

Original Article

ANTI-ANGIOGENIC ACTIVITY OF THE EXTRACTED FERMENTATION BROTH OF AN ENTOMOPATHOGENIC FUNGUS, *CORDYCEPS MILITARIS* 3936

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Received: 26 May 2014 Revised and Accepted: 08 Jul 2014

ABSTRACT

Objective: *Cordyceps militaris* is an entomopathogen and known to exhibit significant therapeutic potential. In the present study, we aimed to extract various fractions (aqueous; hexane; chloroform & butanol) including active ingredient cordycepin from fermented broth of *Cordyceps militaris* followed by their evaluation as anti-angiogenic agents.

Methods: The bioactive metabolite, cordycepin and various *Cordyceps* derived fractions were isolated from liquid culture of *Cordyceps militaris* using solvent-solvent extraction method followed by purification on silica gel column chromatography. Furthermore anti-angiogenic properties of extracted fermentation broth were also investigated using chorioallantoic membrane (CAM) assay.

Results: Butanolic fractions, demonstrated the highest anti-angiogenic activity followed by chloroform, hexane and aqueous fractions of extracted fermentation broth. Anti-angiogenic studies for extracted cordycepin showed that 40 µg/egg dosage of cordycepin was sufficient to inhibit the branching of blood vessels significantly (~50%) in a CAM assay.

Conclusion: It is concluded that butanolic extract/cordycepin from fermented broth of *Cordyceps militaris* potentially inhibits the angiogenesis and suggests that the inhibition of angiogenesis is one of the mechanisms by which *Cordyceps militaris* can mediate an anti-cancer effect.

Keywords: *Cordyceps militaris*, Solvent-solvent extraction, Cordycepin, Chorioallantoic membrane, Anti-angiogenic.

INTRODUCTION

Angiogenesis is a complex and tightly controlled physiological process by which new blood vessels are originated from pre-existing capillaries. Angiogenesis plays an important role in physiological and pathological processes such as wound healing, placentation, embryo-genesis, diabetic retinopathy, inflammatory disorders and tumor growth [1, 2]. Tumor cells can induce angiogenesis through the activation of endothelial cells followed by pro-angiogenic factors such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and epidermal growth factor (EGF) [3-5]. These factors are highly expressed and associated with growth of various types of human tumors [6, 7]. Therefore, compounds with anti-angiogenic properties have been identified as an attractive strategy for the treatment and prevention of cancer. Researchers have been trying to screen novel herbal preparations with anti angiogenic potential.

Cordyceps militaris is an entomopathogenic fungus, with variety of clinical health effects including immunomodulatory, anticancer, antioxidant, anti-inflammatory and anti-microbial activities [8, 9]. Due to specific distribution and potent medicinal value, the market price of *Cordcyeps* species costs about US$ 12000 kg^{-1} [10]. *Cordyceps militaris* have been known to produce a variety of pharmacologically active compounds like cordycepin, adenosine, cordymin and exo-polysaccharides [8, 11, 12].

Cordycepin is a kind of nucleoside analogue having structural similarity with adenosine, except that it lacks 3' hydroxyl group which makes it more potent and known to interfere with various biochemical and molecular processes including purine biosynthesis [13, 14], DNA/RNA synthesis [15] and mTOR (Mammalian Target of Rapamycin) signaling transduction etc. [16, 17]. Efforts have been made to artificially cultivate this mushroom by solid and submerged fermentation techniques. The solid culture of mushroom takes long time to complete fruiting body whereas liquid submerge culture is preferred to produce desired bio-metabolite (cordycepin). To overcome such limitations, researchers tried submerged fermentation for the production of cordycepin on commercial scale. In this study, the extracted fermentation broth of *Cordyceps militaris* was used to investigate the anti-angiogenic activity.

MATERIALS AND METHODS

Chemicals and microbial strain

Standard Cordycepin (3'-deoxyadenosine) was purchased from Sigma Chemical Corporation (St. Louis, MO, USA). Other chemicals and nutrient medium ingredients of analytical grade were purchased from E-Merck India and Hi media Ltd (India) respectively. *Cordyceps militaris* 3936 was procured from microbial type culture collection (MTCC) IMTECH, Chandigarh, India which was regularly maintained on potato dextrose agar (PDA) slants and stored at 4°C.

Inoculum preparation and fermentation

Cordyceps militaris 3936 was initially grown on PDA medium in a petri dish and then transferred to the seed culture medium by punching out 5 mm of the agar plate culture with a sterilized cork borer. The seed culture was grown in a 250-mL flask containing 50 mL of basal medium (glucose 15 g L^{-1}, peptone 5 g L^{-1}, KH_2PO_4 3 g L^{-1}, K_2HPO_4 1 g L^{-1}) at 25 °C on a rotary shaker incubator at 150 rpm for 4 days. The batch mode fermentation experiments were performed in a 1000-mL flask containing 500-mL of the media with 4% (v/v) inoculums of the seed culture. The inoculated bottles were initially incubated at 25°C for 7 days in stationary phase then transferred to shaking mode for 10 days followed by again stationary condition for 7 days.

Extraction, isolation and identification of cordycepin

Fermented broth filtered and filtrate was concentrated up to $1/20^{th}$ volume in vacuum evaporator at a temperature below 50°C, which resulted in dark brown syrup. The crude material was extracted sequentially from non-polar to polar solvents by hexane, chloroform and n-butanol. The presence of cordycepin was confirmed in butanolic fraction by TLC, Spectrophotometric and HPLC studies. Further the butanol soluble fraction was subjected to repeated silica

gel column chromatography to get purify cordycepin. The obtained cordycepin was confirmed using UV, HPLC and NMR techniques.

Thin Layer Chromatography for cordycepin detection

Thin layer plates (10 × 20 cm) of silica gel GF 254, thickness 0.25 mm were prepared and placed under hot air oven to evaporate solvent. TLC plates were developed in chloroform/ methanol/water (64:14:1) and compounds were visualized in UV chamber at 260nm or by spraying 10 % H_2SO_4 in ethanol followed by heating on hot plates [18].

Spectro photometric assay for quantitative estimation of cordycepin

The compound was scratched out from TLC plates and quantified using spectrophotometric assay at 460nm which is based on cordycepin reaction with anthrone, resulted in production of cherry red color [19]. Reagent was prepared by dissolving 0.2 gm anthrone in 100 ml of 90 % H_2SO_4.

Chorioallantoic membrane assay

Anti-angiogenic properties of *Cordyceps* derived fractions and cordycepin were evaluated using modified Chorioallantoic membrane assay (CAM) [20]. The fertilized chicken egg was collected on 0^{th} day, cleaned with 70% ethanol to avoid infections and kept in a humidified (70%) chamber at 37°C. After 48 h, 2 ml of albumin was taken with a syringe at the lower side of the egg and the pierced hole was sealed with a sterilized laboratory tape (Fig. 1). This procedure allows separation of the intact, non injured CAM from the shell during the embryo development which is an important issue because any injury to the CAM may alter the potency of test sample. At 72 h of incubation, a small window was created by removing the egg shell at the blunt end. After confirmation normal and viable development of the embryo, various fractions (Hex, chl, BuOH and aq) of *Cordyceps* and cordycepin were introduced on 5 mm sterile filter discs and placed over the surface of extra embryonic membrane i.e. CAM. The window was sealed with sterile laboratory tape so as to prevent external environmental contact. On completion of another 48 h, potency of *Cordyceps* fractions and isolated cordycepin, as anti angiogenic agents were calculated in terms of inhibition of blood vessels branch points with comparison to control. All experiments were conducted at least in triplicate to confirm the results.

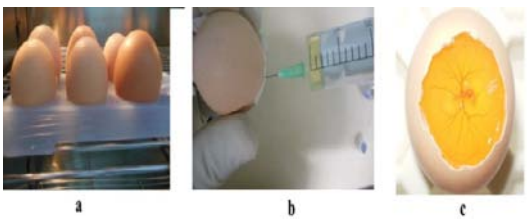

Fig. 1: (a) procurement of 0^{th} day eggs and incubated in BOD incubator. (b) Aspiration of 2 ml albumin from lower end after 24 h incubation. (c) Development of CAM with visible blood vessels after 72 h of incubation.

Quantification of angiogenesis

The CAM at the site of application for angiogenesis was examined with comparison to control. After 48h of treatment, anti angiogenic effect of *Cordyceps* fractions was manually counted in terms of branch points over CAM and calculated the percent inhibition as follows.

% Inhibition = Data of control – Data of treated X 100 / Data of control

RESULTS

In this study, the fermented broth of *Cordyceps militaris* was extracted with various solvents such as hexane, chloroform and butanol. Cordycepin was detected in butanolic fractions using various identification tools such as TLC, spectrophotometer and HPLC.

Purification of cordycepin

The butanolic portion was eluted on silica gel column with stepwise gradient of methanol: chloroform (5:95, 15:85, 25:75, 35:65, 45:55 and 55:45) and six fractions were obtained (F1-F6). Presence of cordycepin was found in fraction F2 by TLC which was sub-fractionated into four fractions (S1-S4) using elution gradient methanol: chloroform (15:85). Sub fraction S3 was found to carry cordycepin which was further vacuum dried and recrystallized in methanol giving a white/creamy powdery product. Spectral analysis of purified cordycepin was performed using UV, HPLC and NMR spectroscopy and the data is consistent with our earlier published values [21]. We performed the CAM assay to examine the effect of cordycepin/ *Cordyceps* fractions on angiogenesis.

Chorioallantoic membrane assay

We prepared various *Cordyceps* fractions by partitioning crude extract, sequentially from non polar to polar solvents such as hexane, chloroform, n-butanol. The collected fractions were dried under vacuum and studied for their anti-angiogenic activity at a dose of 100µg/disc. The significant differences were found among the various fractions (Hex, Ch, BuOH) and BuOH fraction comes out to be potent inhibitor of angiogenesis in the chicken embryos with comparison to untreated control. Furthermore, the anti-angiogenic effect of extracted cordycepin was also studied at various concentrations such as 10, 20, 40, 60 & 80µg/egg. Our results revealed that, 40 µg/egg doses was sufficient to inhibit branching of blood vessel up to 50% and found to be more potent than any extracted fractions of *Cordyceps* (Fig. 2 &3).

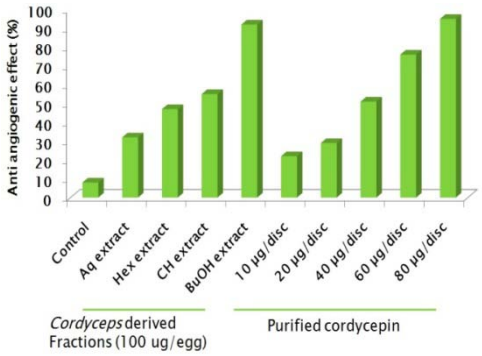

Fig. 2: Effect of *Cordyceps* derived fractions and cordycepin on angiogenesis. CAM were treated with various fractions Aq, Hex, Ch & BuOH at 100µg/egg and also with different concentrations of purified cordycepin. Control CAM treated with papper disc without drug. All the experiment was conducted in triplicates and data represented the average of three replicate.

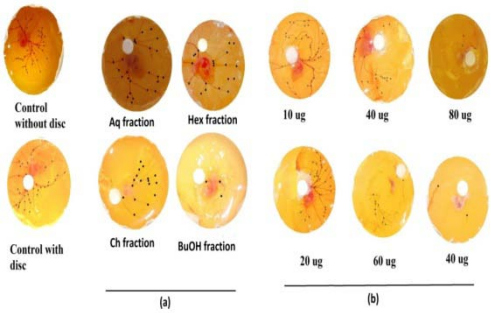

Fig. 3: (a) The photograph showed the anti-angiogenic activity of Cordyceps militaris extracted fraction (Aq = Aqueous; Hex = Hexane; Ch = Chloroform and BuOH = Butanol). b) The effect of purified cordycepin on angiogenesis at various concentrations (10, 20, 40, 60 and 80 µg/egg).

DISCUSSION

Cordyceps militaris has been used as a traditional Chinese medicine mushroom for the treatment of many diseases including cancer complications. On the other hand, cancer growth and metastasis are intimately related with angiogenesis [17, 22]. Due to this reason, anti-angiogenesis has been a focus in cancer research, and several clinical drugs have been used with satisfactory effects [23]. However some of antitumor and antiangiogenis activities of *Cordyceps militaris* crude extracts have been studied [24-28]. Though, its active compound, cordycepin have not been fully explored from the anti-angiogenic point of view. In this report, we investigated first time the inhibitory effects of fermentation broth of *C. militaris* 3936 on angiogenesis using CAM assay. CAM is one of the most widely used in vivo vessel development model [29]. The result of CAM assay showed that *Cordyceps* had the potential anti-angiogenic function. We isolated and identified a bioactive metabolite, cordycepin from *Cordyceps militaris* 3936. The *Cordyceps* derived fractions and cordycepin displayed anti-angiogenic and antimicrobial activity. Out of four *Cordyceps* derived fractions (Aq, Hex, Ch and BuOH), the BuOH fraction showed the highest inhibitory activity in angiogenic response, followed by Ch, Hex and then aqueous. However the active ingredient, cordycepin was even more effective than other fractions as anti-angiogenic agent.

CONCLUSION

This study found the anti-angiogenic activity of extracted fermentation broth of *Cordyceps militaris*. We observed that anti-angiogenic activity is directly correlated with the content of cordycepin in the *Cordyceps* derived fractions. We suggest that the inhibition of angiogenesis is one of the mechanisms by which *Cordyceps militaris* can mediate an anti-cancer effect. However, further studies are required to elucidate the exact mechanism underlying the anti-angiogenic property of *Cordyceps*. Further investigation is in progress in our laboratory to isolate other individual components which are present in *Cordyceps* extracts using modern chromatography techniques.

CONFLICT OF INTEREST STATEMENT

There are no potential conflicts of interest among the authors regarding the publication of this manuscript.

ACKNOWLEDGMENTS

The authors would like to acknowledge M.M. University Mullana (Ambala) for providing the requisite facilities to perform this study

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