Journal of Ethnopharmacology 214 (2018) 106–112

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Extracts of *Cordyceps sinensis* inhibit breast cancer cell metastasis *via* down-regulation of metastasis-related cytokines expression

Hongwei Cai[a], Jing Li[a], Baohua Gu[a], Ying Xiao[a], Rongsheng Chen[a], Xiaoyu Liu[a], Xiaomin Xie[a], Li Cao[b,*]

[a] Key Laboratory of State Administration of Traditional Chinese Medicine, Sunshine Lake Pharma Co., LTD, Dongguan 523850, China
[b] Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100193, China

ARTICLE INFO

Keywords:
Cordyceps sinensis
Breast cancer
Metastasis
Cytokines

ABSTRACT

Ethnopharmacological relevance: *Cordyceps sinensis* is a traditional Chinese medicine and has been used as adjuvant treatments for cancer and it has been also demonstrated to be effective in cancer patients.
Aim of the study: The objective of the present study is to investigate the anti-metastasis effects of water extracts of *Cordyceps sinensis* (WECS) in breast cancer and the potential mechanisms.
Materials and methods: The cytotoxicity of WECS on 4T1 breast cancer cells was evaluated *in vitro* using cell counting kit-8 (CCK8) assay. The *in vivo* anti-metastatic activity of intraperitoneally administered WECS and its effect on animal survival were measured in a mouse breast cancer metastasis model. To explore the molecular mechanisms of the anti-metastasis effect of WECS, the expression of matrix metalloprotein-9 (MMP-9) in serum was determined by enzyme-linked immunosorbent assay (ELISA). In addition, a protein array was used to examine the cytokine expression profiles in lung homogenates.
Results: Treatment with WECS (0.10–0.40 mg/ml) significantly inhibited 4T1 cell viability *in vitro*. In animal studies, 50 mg/kg WECS significantly reduced the number of metastatic lung nodules and the weight of lung, without affecting body weight of mice. Furthermore, WECS increased the survival rate of 4T1 tumor bearing mice in a dose dependent manner, and at high dose, WECS (50 mg/kg) significantly increased the life span of the mice compared to untreated control group. The expression level of MMP-9 in serum was decreased about 50% in 50 mg/kg WECS treated group compared to control group. The results of protein array showed that the expression of CC chemokine ligand 17 (CCL17), MMP-9, osteopontin (OPN), interleukin-33 (IL-33), CC chemokine ligand 12 (CCL12) and CC chemokine ligand 6 (CCL6) in the lungs of 4T1 tumor bearing mice was increased more than two fold compared with normal mice. Among them, the expression of CCL17, MMP-9, OPN, IL-33 was significantly reduced by treatment of 50 mg/kg WECS.
Conclusion: Our results demonstrated that WECS has potent anti-metastasis activity in a mouse breast cancer metastasis model possibly by down-regulation the expression of several metastasis-related cytokines.

1. Introduction

Cordyceps sinensis (Berk.) Sacc. is a well known Chinese herb medicine, generally called “Dong Chong Xia Cao”, because it consists of a fungus growing out of a worm. *Cordyceps sinensis* has been used for treatment of a wide range of diseases for hundreds of years in China and the Asia region. It has been also used as an adjuvant in cancer therapy (Zhu et al., 1998). In recent years, clinical trials had demonstrated the efficacy of *Cordyceps sinensis* in treating patients with hepatocellular carcinoma (Niwa et al., 2013). *Cordyceps sinensis* has also been shown to have antioxidant (Li et al., 2001) and immunomodulatory (Wang et al., 2017) activities among others.

Breast cancer is the most common cancer worldwide and is the leading cause of death in woman (Li et al., 2016). The lifetime probability of developing breast cancer is 12.3% (Stuckey, 2011). Breast cancer has poor prognosis due to its high rate of metastasis, which is the most common cause of mortality. There has been great progress in the early detection and treatment of breast cancer; breast cancer mortality

Abbreviations: WECS, water extracts of Cordyceps sinensis; MMP-9, matrix metalloproteinase-9; CCK8, cell counting kit-8; ELISA, enzyme-linked immunosorbent assay; CCL17, CC chemokine ligand 17; OPN, osteopontin; IL-33, interleukin-33; CCL12, CC chemokine ligand 12; CCL6, CC chemokine ligand 6; HPLC, high-performance liquid chromatography; H&E, haematoxylin and eosin; CCR4, chemokine receptor 4; DDR, DNA damage response

* Corresponding author.
E-mail addresses: caihongwei@hec.cn (H. Cai), lijing@hec.cn (J. Li), gubaohua@hec.cn (B. Gu), xiaoying@hec.cn (Y. Xiao), chenrongsheng@hec.cn (R. Chen), liuxiaoyu@hec.cn (X. Liu), xiexiaomin@hec.cn (X. Xie), lcao@implad.ac.cn (L. Cao).

https://doi.org/10.1016/j.jep.2017.12.012
Received 12 October 2017; Received in revised form 7 December 2017; Accepted 11 December 2017
Available online 15 December 2017
0378-8741/

rate has steadily decreased in recent years (Cardoso et al., 2012). However, not all patients have benefited from those progresses. A lack of effective treatments against metastasis has become the major obstacle to the survival and quality of life of patients with breast cancer (Smith et al., 2012).

Given that there are few therapies options for tumor metastasis, there is an interest to study the potential effects and mechanisms of *Cordyceps sinensis* against breast cancer metastasis. Animal experiments have already shown that water extract of cultured mycelium of *Cordyceps sinensis* is effective in reducing metastasis in experimental animal models of metastasis (Kubo et al., 2010; Nakamura et al., 2003), including breast cancer metastasis (Jordan et al., 2010). However, there is few report of anti-metastatic effect of *Cordyceps sinensis*, which is quite different from cultured mycelium in the chemical composition, bioactive components and pharmacology effect (Li et al., 2001; Wang et al., 2012, 2015). In addition, these earlier studies research the anti-metastatic mechanism of the mycelium of *Cordyceps sinensis* only in the *in vitro* experiments, and their research just focused on apoptosis or cell cycle arrest of tumor cell (Nakamura et al., 2003). Therefore, there is a need to further investigate the *in vivo* anti-metastatic effect and mechanism of *Cordyceps sinensis*. The present study evaluated the anti-metastatic effects of *Cordyceps sinensis* in a murine model of breast cancer metastasis and explored the potential molecular mechanisms by monitoring a number of cytokines.

2. Materials and methods

2.1. Preparation of Cordyceps sinensis extracts

The sample of *Cordyceps sinensis* (Berk.) Sacc. was cultivated at a manufactory facility at Yichang Hubei, China (GPS coordinates: latitude: 30.359155; longitude: 111.498733) in 2016 and were provided by Sunshine Lake Pharma Co., Ltd (Dongguan, China). The cultivation conditions mimic the dynamic growth environment of natural *Cordyceps sinensis*. The sample was deposited in our laboratory with voucher specimen number as 160225. 600 g of *Cordyceps sinensis* was cut into small pieces, and soaked with 6000 ml double distilled water. The mixture was homogenized with a homogenizer (IKA, Germany) at 16,400 rpm for 3 min in ice bath, and then sonicated for 30 min. The matrix was cooled at −20 °C for 40 min, and sonicated again for another 30 min. After sonication, the residues were removed by using suction filtration, and the filtrate was lyophilized to obtain water extracts of *Cordyceps sinensis* (WECS). The percentage yield of the extract was 6.635% (w/w). WECS was aliquoted and stored at −20 °C.

One of the major components of the WECS is polysaccharide. The content of the polysaccharide was analyzed by using the phenol sulfuric acid assay (Baharara and Amini, 2015). Other main components of the WECS are nucleosides. To analyze the nucleoside content of WECS, high-performance liquid chromatography (HPLC) was used as previous reported (Huo et al., 2017).

2.2. Laboratory animals and cell line

Adult female BALB/C mice weighing 18–20 g were provided by Hunan SJA Laboratory Animal Co., Ltd and were housed under pathogen-free conditions. Animal studies were performed in accordance with protocol procedures approved by the Institutional Animal Care and Use Committee of Sunshine Lake Pharma Co., LTD. The 4T1 cells were purchased from American Type Culture Collection, USA, and cultured according to provided protocols.

2.3. Cell culture

The murine cell line 4T1 was cultured in RPMI media (Hyclone, USA) containing 10% FBS (Hyclone, USA) at 37 °C with 5% CO_2 in humidified atmosphere. The medium was refreshed every other day.

2.3.1. In vitro cytotoxicity of WECS on 4T1 cells

4T1 cells were seeded in 96-well plate at a density of 4×10^4/well. After 24 h, 4T1 cells were treated with various concentrations (0.0063, 0.013, 0.025, 0.05, 0.10, 0.20, 0.40 mg/ml) of WECS for additional 72 h. The cell viability was measured by CCK8 assay (Dojindo Molecular Technologies Inc., Japan).

2.4. In vivo anti-metastatic activity of WECS

Intraperitoneal administration of drugs is a commonly used route in animal study. The bioavailability of Intraperitoneal injection is much higher than gavage, because of reduction of degradation by gastrointestinal tract (Kuttan, 2016).

1×10^6 4T1 cells in 100 μl RPMI media were injected into the tail vein of the mice. From the day of tumor inoculation, the mice were intraperitoneally injected with 50 mg/kg/day WECS or vehicle (normal saline) for 15 days. We choose the dose of this experiment based on the results of a related study (Kubo et al., 2010) and our unpublished data. On the 15th day after tumor inoculation, all of the mice were sacrificed. The lung of mice was excised, weighed and metastatic tumor nodules were counted. Lung tissues were fixed in 10% formaldehyde for haematoxylin and eosin (H&E) staining, and the stained sections were examined under microscope.

2.5. Mouse survival experiment

In the survival experiment, the way of tumor inoculation is the same as described above. From the date of 4T1 cell inoculation, the mice were intraperitoneally injected with different dose of WECS or vehicle once a day. The mice in each group were followed up for 33 days and the date of death was recorded. Survival data was analyzed with the Kaplan-Meyer method.

2.6. Matrix metalloproteinase 9 (MMP-9) in serum

On the 16th day after tumor inoculation, blood was collected from orbit. The blood serum was separated, and the levels of MMP-9 in blood serum were measured using ELISA kits (R&D, USA).

2.7. Protein array of the lung tissues

Lung tissue samples were homogenized in phosphate buffered solution with protease inhibitors. After homogenization, Triton X-100 was added to a final concentration of 1%. Samples were frozen at −70 °C, thawed and centrifuged at 10,000 g for 5 min to remove cellular debris. Cytokines in the sample were measured using a Mouse XL Cytokine Array (R&D, USA), a membrane-based antibody array for the determination of the relative levels of selected mouse cytokines. The chemoluminescent signals were detected using a LAS-4000 gel imaging system (General Electric Company, USA). The signal intensity of the blots was quantified by using Quantity One software (Bio-rad).

2.8. Statistical analysis

Statistical and graphical analyses were performed using GraphPad Prism 5 software. Results are expressed as means ± SD. P values were analyzed using two-sample Student's t-test. Survival curves were compared using the two-sided log-rank test. $P < 0.05$ or $P < 0.01$ was considered significant.

3. Results

3.1. Chemical analysis of WECS

Polysaccharides and nucleosides are main components of *Cordyceps sinensis* and their contents were used for quality control. Previous