Antitumor Activity of Water Extracts From 
*Cordyceps Militaris* in NCI-H460 Cell 
Xenografted Nude Mice

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**Abstract**

This experimental study investigated the antitumor effect of *Cordyceps militaris* in NCI-H460 cell transplanted nude mice. After feeding an aqueous solution of *C. militaris* extracts in NCI-H460 cell xenografted nude mice for 4 weeks, we measured the size of a tumor mass and calculated the inhibition rate. We also estimated survival time and calculated mean survival time and percent increase in lifespan. Results showed that the inhibition rate of water extract of the 150 mg/kg/day *C. militaris*-administered group was 94.73−75.08% and that of the 300 mg/kg/day *C. militaris*-administered group was 85.81−73.81%. The tumor weights and volumes decreased in a dose-dependent manner. Mean survival time of the 150 mg/kg/day *C. militaris*-administered group was extended to 19.43±2.44 days and 5.42% increased in lifespan (ILS) and that of the 300 mg/kg/day *C. militaris*-administered group was 21.86±3.53 days and 18.61% ILS. The relative liver weight was significantly increased in 300 mg/kg/day *C. militaris*-administered group, but there was no histopathological difference. In conclusion, *C. militaris*, shrunk tumors and increased mouse lifespan, suggesting that *C. militaris* was effective in treating tumors in nude mice.

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lines: stomachic adenocarcinoma, colorectal adenocarcinoma, and hepatocellular carcinoma. The presumed active component in the extract was cordycepin [5]. One study, however, has suggested that many ingredients in *C. militaris* have a stronger antitumor effect compared with cordycepin [2].

Recently, an *in vitro* assay to determine the antitumor mechanism of *C. militaris* reported that apoptotic events in A549 cells due to water extract of *C. militaris* were mediated via diminished telomerase activity through the inhibition of human telomerase reverse transcriptase transcriptional activity [6].

Based on these results, we performed an *in vivo* trial to evaluate the antitumor effect of administration of water extracts from *C. militaris* to lung cancer xenografted mice. We measured the volume of xenografted tumor, weight of each organ and survival time of mice. Cells were also inspected to determine if there was any kind of pathologic change.

2. Materials and Methods

2.1. Cell line and culture conditions

Human lung cancer (NCI-H460) cells were obtained from the Korean Cell Line Bank (Seoul, Korea). The cells were grown in RPMI-1640 medium (Gibco, USA) supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin in an incubator with 5% CO₂ at 37°C. When the cells became confluent, they were washed twice with Hank’s balanced salt solution (HBSS), trypsinized with 0.25% trypsin in HBSS and subsequently washed twice with fresh culture medium [7].

2.2. Experimental animals

Balb/c nude mice (male, 9–11 weeks, n=21) weighing 21–25g were purchased from Japan SLC, Inc. (Shizuoka, Japan) and were housed under specific pathogen-free conditions according to the guidelines of Chungbuk National University Animal Care and Use Committee. The animal room was controlled for temperature (22±2°C), light (12 hour light/dark cycle) and humidity (50±10%). All laboratory feed pellets and bedding was autoclaved.

2.3. Experimental design

The tumor regression model in nude mice has been successfully applied to evaluate antitumor activity [8]. This model was therefore used to evaluate suppression of solid tumor growth in water extracts of *C. militaris* (WECM). When the tumor volume reached 100 mm³ in the nude mice, xenografted tumor fragments were randomly distributed into three groups: NCI-H460+saline only (control group), NCI-H460+150 mg/kg/day WECM-treated group, and NCI-H460+300 mg/kg/day WECM-treated group. Seven mice were included in each group. WECM was orally administered daily for 4 weeks.

2.4. Xenografts

A total of 1×10⁶ NCI-H460 cells in 0.1 mL HBSS were injected subcutaneously into the flank of each mouse using a 26-gauge needle. After 16 days observation, an apparently solid tumor mass was excised from three of five mice inoculated with NCI-H460 cells. Tumor fragments (3×3×3 mm) were created by trimming with a knife, and xenografted into the flank of new mice using a trocar. The suppressive effect of anticancer agents on solid tumor was evaluated in a tumor-regression model. In brief, from the day tumor volume reached 100 mm³, the mice xenografted with a tumor fragment received orally administered WECM (saline, 150 mg or 300 mg/kg) every day.

2.5. Changes in tumor volume

The sizes of tumor mass were recorded twice a week following measurement with a digital caliper. The tumor volume was estimated according to the formula:

\[ V_{\text{tumor}} = \frac{(A \times B^2)}{2} \]

\( V_{\text{mean}} \) is the mean tumor volume in mm³, A is the largest tumor diameter and B is the smallest tumor diameter in mm.

Based on the regression of tumor volume, the antitumor activities of treatment were expressed by inhibition rate:

\[ \text{Inhibition rate (\%)} = \left(\frac{V_{\text{control}} - V_{\text{treatment}}}{V_{\text{treatment}}}\right) \times 100 \]

\( V_{\text{control}} \) and \( V_{\text{treatment}} \) are tumor volume of control group and treatment respectively. The tumor weights were also measured on the final day of the experiment after sacrifice of animals and excision of the tumor mass.

2.6. Mean survival time and percentage increase in lifespan

To compare the lifespan of mice xenografted with NCI-H460 tumor fragments, mean survival time was estimated from the day when the tumor volume reached 1000 mm³ as described previously, and
percentage increase in lifespan (%ILS) was calculated according to the equation:

\[ \%ILS = \left( \frac{D_{\text{treatment}} - D_{\text{control}}}{D_{\text{control}}} \right) \times 100 \]

\( D_{\text{control}} \) and \( D_{\text{treatment}} \) are the mean survival day of mice in control and treatment group, respectively [9,10].

2.7. Statistical analysis

The results are presented as mean±standard deviation, and the significance of difference between the mean of control and treatment groups was analyzed using one-way analysis of variance (ANOVA) followed by a Dunnett’s \( t \) test correction, paired \( t \) test, and linear regression analysis. Statistical significance was determined at the level of \( p < 0.05 \) and \( p < 0.01 \).

3. Results

3.1. Changes in body weight

Changes in body weight of each group are shown in Figure 1. The mean body weights were 23.67–25.89 g in the 150 mg/kg/day WECM-treated group and 24.24–25.99 g in the 300 mg/kg/day WECM-treated group. But there were no significant differences when compared with the control group (24.10–26.43 g).

3.2. Changes in tumor volume

As shown in Figure 2, WECM treatment (150 and 300 mg/kg/day) resulted in slight growth inhibition of NCI-H460 cell-transplanted solid tumor compared with the control group, but there were no significant differences. On day 22, the mean tumor volume of the 300 mg/kg/day WECM-treated group (1771.5 mm\(^3\)) was lower than that of control group (2150.81 mm\(^3\)), but there were no significant intergroup difference.

3.3. Inhibition rate (IR) on tumor volume

The IR of each group is shown in Table 1. In the whole experiment, the IR of the 150 mg/kg/day WECM-treated group was 94.73–75.08% and that of the 300 mg/kg/day WECM-treated group was 85.81–73.81%.

3.4. Final tumor weights and volume

Tumor weights and volumes of each group on day 22 are shown in Table 2. Tumor weight and volume of the control group was 3.046±0.793 g and 3.379±1.184 cm\(^3\), respectively. Tumor weight and volume of 150 mg/kg/day WECM-treated group were 2.435±0.586 g and 2.288±0.510 cm\(^3\), respectively. Tumor weight and volume of 300 mg/kg/day WECM-treated group were 2.175±0.475 g and 2.069±0.440 cm\(^3\), respectively. Compared with the control group, both tumor weight and volume of in two WECM-treated groups were decreased. The 300 mg/kg/day WECM-treated group showed significant decrease compared with control group (\( p < 0.05 \); Table 2).

3.5. Mean survival time and %ILS

Mean survival time and %ILS are shown in Figure 3 and Table 3. The mean survive time of control
Antitumor effect of *C. militaris* in vivo

The mean survival time of the 150 mg/kg/day WECM-treated group was extended to 19.43 ± 2.44 days, increasing %ILS by 5.42%. The mean survival time of 300 mg/kg/day WECM-treated group was 21.86 ± 3.53 days, increasing %ILS by 18.61%. However, the difference were not statistically significant.

### 3.6. Histopathological findings and organ weights

Absolute organ weights of the kidney, liver, spleen, heart and lung are shown Table 4. Both the 150 and 300 mg/kg/day WECM-treated groups showed a marked decrease in liver weights (*p* < 0.05). However, light microscopic histopathological examination showed no specific lesion in liver tissue of mice in neither WECM-treated groups nor control group (Figure 4).

### 4. Discussion

*C. militaris* is well known to contain cordycepin and ergosterol peroxide, two ingredients with antitumor effects [4]. Cordycepin was reported to be 3’-deoxyadenosine, a derivative of adenine, which could have antitumor effects by replacing adenine in DNA and RNA [10]. Ergosterol peroxide is reported to exhibit cytotoxic activities against tumor cells [11].

However, clinicians in the East have practiced using whole *C. militaris*, the evaluation of the antitumor effect of whole *C. militaris* would be more practical. To this end, an assay showing that water extract of *C. militaris* induced apoptosis and inhibited telomerase activity in human lung carcinoma cells has been published [6]. This activity occurred by activating the intrinsic caspase pathway along with the death receptor-mediated extrinsic pathway and was associated with a down regulation of telomerase due to a decrease of human telomerase reverse transcriptase expression [6].

Human tumor xenografts in immunodeficient animal models provided a means to evaluate potential antitumor drugs in preclinical studies and are applicable for studying many different types of human malignancies [12]. Thus we also conducted an *in vivo* experiment on nude mice with NCI-H460 cell-xenografted tumor for 28 days. These tumors...
**Table 4** Organ weights of NCI-H460 tumor-bearing mice on the final day

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Body weight</th>
<th>Absolute organ weights (g)</th>
<th>Relative organ weights (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Epididymis</td>
<td>Kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td>Saline</td>
<td>26.429 ± 1.149</td>
<td>0.036 ± 0.006</td>
<td>0.034 ± 0.005</td>
</tr>
<tr>
<td>WECM 150mg/kg/day</td>
<td>26.177 ± 1.535</td>
<td>0.035 ± 0.011</td>
<td>0.033 ± 0.005</td>
</tr>
<tr>
<td>WECM 300mg/kg/day</td>
<td>26.049 ± 1.010</td>
<td>0.043 ± 0.016</td>
<td>0.043 ± 0.016</td>
</tr>
<tr>
<td>WECM 150mg/kg/day</td>
<td>26.039 ± 0.945</td>
<td>0.163 ± 0.058</td>
<td>0.175 ± 0.065</td>
</tr>
</tbody>
</table>

*<p><0.05. WECM = water extracts of *Cordyceps militaris.*
were treated with WECM (150 and 300 mg/kg/day). Time-dependent changes of tumor volume were measured and excised tumor volume was measured on the final day. Results showed that the tumors were suppressed by WECM treatment but did not shrink in a dose-dependent manner.

WECM inhibited the NCI-H460 tumor growth in nude mice throughout the experimental period. WECM treatment (150 and 300 mg/kg/day) prolonged the mean survival time and increased %ILS compared with the control group. The survival time of the 150 mg/kg/day WECM treatment group was extended to an average of 19.43±2.44 days and increased %ILS by 5.42%. Also, the mean survival time of the 300 mg/kg/day WECM treatment group was 21.86±3.53 days, an 18.61% increase in %ILS. Intraperitoneal injection of Cordyceps militaris extracts into ICR mice bearing at least 180 sarcoma cells was shown to prolong the lifespan of mice from 52.4 to 132% [2]. Findings from this study and previous results are similar in that the lifespan of mice was increased even though the methods were different. However, the difference in lifespan extension between two studies may indicate that different administration methods could cause differences in efficacy.

The results showed that WECM inhibited the growth of NCI-H460 cell-transplanted solid tumor. Compared with the control group, the treatment group showed tumor growth inhibition from day 8 to day 22. However, this trend was not significant statistically. On day 22, the volume of the solid tumor was 2.069±0.440 cm³ in the 300 mg/kg/day WECM-treated group and 3.337±1.184 cm³ in the control group. Tumor weights were 2.175±0.475 g in the 300 mg/kg/day WECM-treated group, 2.435±0.58 g in the 150 mg/kg/day WECM-treated group and 3.046±0.793 g in the control group (Table 2). Previously, an in vitro study demonstrated that an ethanol extract of C. militaris produced an inhibitory effect of up to 65.5% in A549 human lung cancer cells [13], indicating that C. militaris may inhibit cancer growth. Numerical differences between this previous study and the present data may be due to the differences in methodology (in vitro vs. in vivo) and extraction technique (ethanol vs. water). The underlying reasons for these differences should be elucidated to allow further exploitation of the use of C. militaris in clinical practice.

There were no differences in body weight, gross toxicity and organ weight between WECM-treated group and control group. There was no significant change in absolute organ weights, except in the liver. The results of in vivo studies implied that WECM may have potential as a growth inhibitor of solid tumor induced NCI-H460 without marked side effects. These results suggest that WECM may have the possibility to be a useful anticancer agent for therapy in human lung cancer.

Most of the results of this study indicate that WECM shows an antitumor effect in animal models but some are not significant. Although no significant pathologic changes in liver tissue were observed, the reasons for increasing relative liver weight needs to be further investigated.

In summary, this study showed that WECM may suppress the growth of a human lung cancer cell mass and increase the lifespan of nude mice. Results of this trial may stimulate more studies into WECM to determine its clinical use in cancer patients.

References