Lomatium dissectum Inhibits Secretion of CXCL10, a Chemokine Associated with Poor Prognosis in Highly Pathogenic Influenza A Infection

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ABSTRACT

Objective: *Lomatium dissectum* is a plant native to the Western US traditionally used in the Native American culture to treat influenza, which remains a persistent threat to human health. Evidence suggests that dysregulation of cytokines and chemokines, including CXCL10, is the primary factor leading to poor prognosis in highly pathogenic influenza infection. This study was conducted to address the hypothesis that an aqueous extract of *L. dissectum* root inhibits CXCL10 secretion by human bronchial epithelial cells stimulated with polyinosinic:polycytidylic acid (poly i:c), a synthetic analog of viral dsRNA.

Design: BEAS-2B cells treated with poly i:c were exposed to *L. dissectum* root aqueous extract simultaneously or at 2 h intervals up to 8 h post-stimulation. Supernatants were harvested at 24 h and enzyme-linked immunosorbent assay (ELISA) performed to determine CXCL10 concentrations.

Results: *L. dissectum* root aqueous extract at 1 µg/mL significantly inhibited CXCL10 secretion (P=0.043, Anova, Tukey HSD) and demonstrated maximal inhibition 6 h post poly i:c exposure. MTT cytotoxicity assay results suggest that this inhibitory effect was not due to extract-induced cytotoxicity.

Conclusion: The observation that *L. dissectum* extract inhibits CXCL10 secretion provides a plausible mechanism for the efficacy of *L. dissectum* in influenza treatment reported in ethnobotanical studies and case reports. *L. dissectum* may reduce morbidity and mortality associated with influenza and merits further research.

Keywords: *Lomatium dissectum*; CXCL10; Influenza; Immune modulation

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INTRODUCTION

Highly pathogenic influenza A remains a persistent threat to human health.[1, 2] Evidence suggests that vaccine coverage would likely be inadequate during the first six months of a pandemic.[3–5] Vaccine efficacy is limited in pandemics, as the causative strain of virus cannot be known until the pandemic begins and according to the WHO, "Global manufacturing capacity for influenza vaccines is limited, inadequate and not readily augmented".[6] This leaves antiviral therapy as a critical treatment needed for effective response to highly pathogenic influenza pandemics.[7–9]

There are currently four antiviral drugs licensed for use to treat influenza, which include the adamantanes, adamantadin and rimantadin, and the neuraminidase inhibitors, oseltamivir and zanimivir.[10] Resistance of influenza viruses to these agents has proven problematic. Since 2006, the CDC has recommended against the use of adamantanes due to resistance. Furthermore, resistance to oseltamivir between the 2007–2008 and 2008–2009 influenza seasons increased from 19.9% to 99.6%.[11] As resistance to these antiviral medications limits their usefulness, novel treatment approaches, such as those that modulate immune responses to influenza, need to be explored.[1, 7, 12]

The morbidity and mortality from highly pathogenic influenza infection is primarily caused by cytokine and chemokine dysregulation that may occur during the immune response to influenza and not by the virus itself.[1] In recent studies of humans infected with highly pathogenic H5N1 influenza, high levels of the chemokine CXCL10 were correlated with poor prognosis and high viral load. CXCL10 is a chemo-attractant for T lymphocytes, NK cells, and macrophages that express its ligand, CXCR3. High levels of CXCL10 may be responsible for excessive pulmonary macrophage infiltration.[13, 14] Overproduction of CXCL10 has also been demonstrated in animal and cell culture models of influenza.[15–17] Thus, therapies that target the immune response and inhibit CXCL10 production are ideal candidates for investigation.[1, 18, 19] One potential phytotherapeutic agent with ethnobotanical evidence of use in the treatment of influenza is *Lomatium dissectum*.

Lomatium dissectum aqueous root preparations have been traditionally used in the treatment of respiratory tract infections.[20, 21] This Apiaceae species is native to western North America and was an important medicine for the peoples within its range.[20, 22–25] During the 1918 influenza pandemic, *L. dissectum* was hailed as curative of influenza and influenza-associated pneumonia.[26] Currently used by naturopathic physicians to treat respiratory tract infections, root extracts from this plant merit further study.[20, 27]

Because an aqueous extract of *L. dissectum* root was the form traditionally used to prevent influenza-associated pneumonia, and CXCL10 has been shown to correlate with poor prognosis in influenza, this study examined the hypothesis that *L. dissectum* inhibits CXCL10 in an *in vitro* model of viral infection. BEAS-2B human bronchial epithelial cells treated with the synthetic double stranded RNA (dsRNA) polyinosinic:polycytidylic acid (poly i:c) is an accepted model for influenza A infection.[28, 29] Poly i:c triggers the immune response by binding to the toll-like receptor 3 (TLR3), a cytoplasmic receptor that binds dsRNA ligands not regularly expressed in mammalian cytoplasm.[30–32] In response to poly i:c, the BEAS-2B human bronchial cell line secretes CXCL10.[33] Inhibition of CXCL10 would suggest a novel action of *L. dissectum* in preventing influenza-related pathogenesis.

METHODS

CELLS AND CELL CULTURE

BEAS-2B human bronchial epithelial cells originally from the American Type Culture Collection (Manassas, VA) were maintained in a humidified incubator at 37°C and 5% CO_2 in DMEM-F12 media (Invitrogen, Grand Island, NY) supplemented with 5% heat-inactivated FBS, 100 U/mL penicillin (Corning Life Sciences, Tewksbury MA),

100 µg/mL streptomycin (Corning), and 2 mM L-glutamine (Corning).[34]

LOMATIUM DISSECTUM ROOT AQUEOUS EXTRACT

L. dissectum root (25 g) obtained from Oregon's Wild Harvest (Sandy, OR; lot number LOM-03073w-WMJ) was decocted in 500 mL distilled water, which was reduced by 50%, and an additional 250 mL of distilled water was added. This decoction was reduced to 375 mL. The final aqueous extract was filter sterilized, and aliquots frozen at −20°C until time of assay. A final concentration of 0.16 µg/µL was determined using a Mettler Toledo HR 73 Moisture Analyzer.

MTT ASSAY

To assess the cytotoxicity of the *L. dissectum* aqueous extract, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was conducted. BEAS-2B cells were seeded in inner wells of 96 well plates. A standard curve of BEAS-2B cells in serial dilution from 30,000 to 469 cells/mL was included. The remaining wells were seeded at a density of 30,000 cells/mL and plates incubated for 24 h. Media was removed and fresh media (100 µL) added to cell standard wells. Test wells (six replicates per treatment) were treated with a serial dilution of 1:1 *L. dissectum* extract:media resulting in final concentrations ranging from 80 µg/mL to 1.25 µg/mL. After 24 h incubation, wells were aspirated and washed twice with 100 µL/well PBS. MTT solution was added and plates incubated for 3 h. Wells were aspirated and 100 µL/well dimethly sulfoxide (DMSO) added. Absorbance at 540 nm (OD_{540}) was determined using a Molecular Devices Spectra Max Plus 384 plate reader.[35]

L. DISSECTUM TREATMENT OF BEAS-2B CELLS

BEAS-2B cells seeded in 24 well plates at a density of 15,000 cells/mL were incubated for 24 h. Media was aspirated and replaced with either media alone or media with 1 µg/mL poly i:c (Sigma Aldrich). Treatment with *L. dissectum* root aqueous extract was either immediately after poly i:c exposure or at various time points thereafter (timed response). Cells were incubated for 24 h after stimulation and the supernatant collected and centrifuged at 1400 rpm for 5 min. Samples were used immediately in an enzyme-linked immunosorbent assay (ELISA) or stored at −20°C until ELISA, conducted within 30 days of supernatant harvest.

L. dissectum extract was tested at concentrations ranging from 1 to 10 µg/mL and for timed response assay, at 1 µg/mL. Sterile PBS (Invitrogen) was used as a control. Triplicate wells of poly i:c stimulated BEAS-2B cells were treated with *L. dissectum* aqueous extract either concurrently with poly i:c stimulation, or post poly i:c stimulation (timed response) at 2 h intervals up to 8 h.

ELISA

ELISA for CXCL10 was performed using the human CXCL10 ELISA kit (R&D Systems, Minneapolis, MN) following manufacturer's instructions. CXCL10 concentrations of triplicate supernatant samples were determined by extrapolation from a standard curve after determining absorbance (OD_{450} – OD_{570}) using a Molecular Devices Spectra Max Plus 384 plate reader and SoftMaxPro 5.4 software.

STATISTICS

Data were reported as mean ± standard deviation. Comparisons among treatment conditions were done using analysis of variance (ANOVA) followed by a post hoc test for multiple comparisons (Tukey Honest Significant Difference). All statistical analyses were performed using PASW statistics software. A P-value <0.05 was considered significant.

RESULTS

MTT ASSAY

No significant enhancing or inhibitory effect on proliferation of BEAS-2B cells was observed with treatment of *L. dissectum* at 40–1.25 µg/mL. A cytotoxic effect was observed at the highest *L. dissectum* concentration tested, 80 µg/mL (Figure 1).

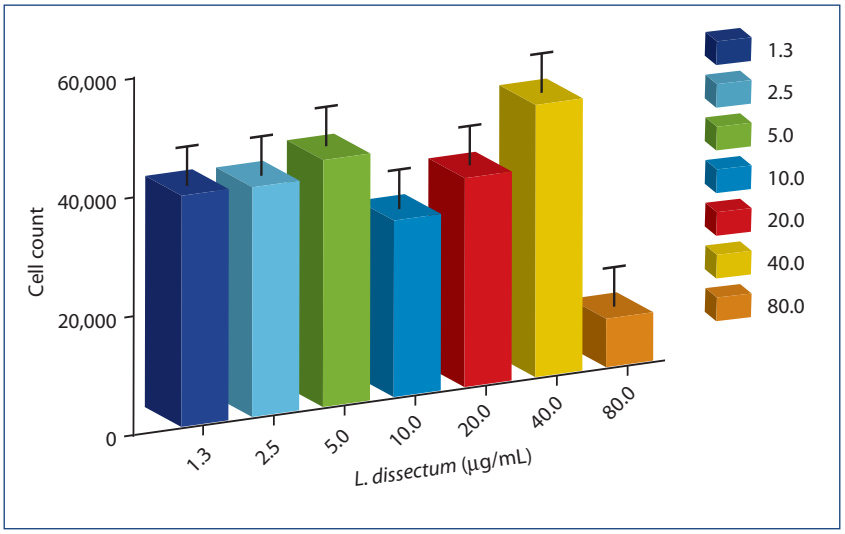

Figure 1: The cytotoxic effect of *L. dissectum* extract on poly i:c stimulated BEAS-2B human bronchial epithelial cells.

BEAS-2B cells were seeded at 30,000 cells/mL for 24 h. Media was replaced with media containing 1 µg/mL poly i:c and *L. dissectum* extract (1.3–80 µg/mL) in 6 wells per treatment condition. After 24 h incubation, MTT was added, cells incubated for another 3 h and MTT solvent DMSO added. Plates were shaken for 15 min and read on a microplate reader at OD_{540}. No cytotoxic effect was observed at 40 µg/mL or lower *L. dissectum* concentration.

EFFECT OF *L. DISSECTUM* ON CXCL10 SECRETION

Poly i:c stimulation of BEAS-2B cells induced significant CXCL10 secretion above constitutively expressed levels observed in the media control (Figure 2). *L. dissectum* (1 µg/mL) treatment concurrent with poly i:c stimulation resulted in significant inhibition (P=0.043) of CXCL10 secretion by BEAS-2B cells compared to poly i:c stimulation alone. This treatment decreased CXCL10 to a concentration that was not significantly different than that of the media control. Concentrations of 5 and 10 µg/mL *L. dissectum* led to a non-statistically significant trend in decreased CXCL10 secretion compared to poly i:c stimulation. Furthermore,

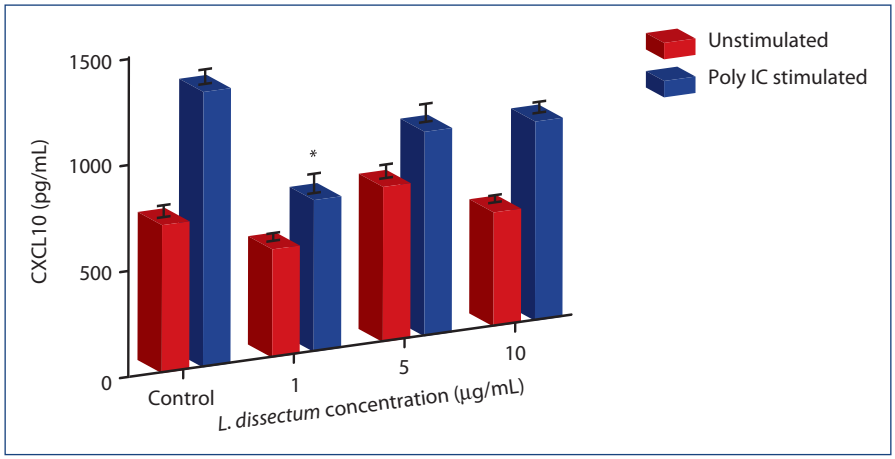

Figure 2: *L. dissectum* decreases CXCL10 production by poly i:c stimulated BEAS-2B human bronchial epithelial cells.

BEAS-2B cells were seeded at 15,000 cells/mL and incubated for 24 h. Media was replaced with media containing 1 µg/mL poly i:c and *L. dissectum* at 1, 5 or 10 µg/mL. Cells were incubated for 24 h, supernatants harvested and assayed for CXCL10 secretion by ELISA. CXCL10 secretion was significantly inhibited compared to poly i:c alone (P=0.043) at a concentration of 1 µg/mL. *Significantly different (P=0.043) than poly i:c stimulation

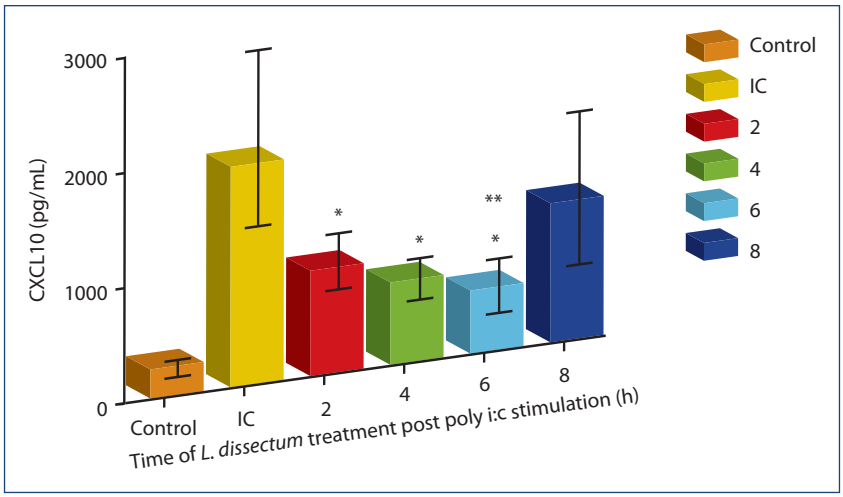

Figure 3: The effect of *L. dissectum* treatment time on CXCL10 secretion by BEAS-2B cells.

BEAS-2B cells were seeded at 15,000 cells/mL and incubated for 24 h. Media was replaced with media containing poly i:c (1 µg/mL). *L. dissectum* extract (1 µg/mL) was added at 2, 4, 6, or 8 h post poly i:c stimulation. Cells were incubated for 24 h post poly i:c addition, supernatants harvested and assayed for CXCL10 secretion by ELISA. *L. dissectum* treatment at 2, 4, and 6 h post poly i:c stimulation significantly inhibited CXCL10 secretion ($P<0.001$). *L. dissectum* treatment at 6 h resulted in CXCL10 secretion that was not significantly different than unstimulated media control. *Significantly different ($P<0.001$) than poly i:c stimulation; **Not significantly different than media control.

treatment of unstimulated BEAS-2B cells with *L. dissectum* (1 µg/mL) resulted in significant inhibition of CXCL10 compared to unstimulated media control (data not shown).

TIMED RESPONSE OF *L. DISSECTUM* ON CXCL10 SECRETION

Cells were treated with *L. dissectum* (1 µg/mL) at either 2, 4, 6, or 8 h post stimulation with poly i:c (Figure 3). Treatment at 2, 4 and 6 h resulted in significant inhibition ($P<0.001$) of CXCL10 secretion compared to poly i:c stimulated BEAS-2B cells. Treatment at 6 h post-stimulation produced maximal inhibition, resulting in a CXCL10 concentration that was not significantly different than unstimulated media control. *L. dissectum* treatment at 8 h post poly i:c stimulation resulted in insignificant inhibition of CXCL10 compared to poly i:c stimulation alone.

DISCUSSION

L. dissectum root has traditionally been used in the treatment of respiratory tract infections, including influenza A infection. Despite its rich ethnobotanical heritage, *L. dissectum* has been insufficiently studied. This is the first report of an inhibitory effect of an aqueous *L. dissectum* root extract on CXCL10 secretion, a chemokine implicated in the pathogenesis of influenza A infection. This inhibitory effect was not due to cytotoxicity of the extract on BEAS-2B cells, since *L. dissectum* was not cytotoxic at the concentrations that decreased CXCL10 secretion. *L. dissectum* significantly inhibited CXCL10 secretion when added up to 6 h post poly i:c stimulation, suggesting that aqueous *L. dissectum* root extract may be useful as treatment during influenza virus infection. Early and excessive production of CXCL10 is also associated with poor prognosis in other conditions such as ARDS, hepatitis, SARS-CoV infection, inflammatory bowel disease, and other autoimmune conditions.[36–40] *L. dissectum* warrants further investigation as a novel treatment for the prevention of influenza-associated morbidity and mortality and for other diseases associated with CXCL10 dysregulation.

To date, there have been few investigations of the biological effects of *L. dissectum* extracts. The dichloromethane fraction contains water soluble tetronic acids, flavonoids and three coumarin glycosides, including an apiose glycoside.[41, 42] The tetronic acids are antimicrobial and both the aqueous and volatile oil fractions of *L. dissectum* root have demonstrated widespread antibacterial

activity.[42–44] The purported effect of *L. dissectum* in the treatment of respiratory tract infections may be due to a combination of direct antibacterial effects and immunomodulatory effects. Given that the effect of *L. dissectum* on CXCL10 was the focus of this study, more in-depth investigations of immunomodulatory effects of the aqueous root extract and its constituents are needed.

This preliminary investigation is limited in that the results may be due to unidentified microbially-derived elements or other impurities in the root extract. Although the raw *L. dissectum* starting material tested negative for *Escherichia coli*, the final extract was not tested for endotoxin or other microbial components. Further, because no information is available about the bioavailability of *L. dissectum* extracts, the physiological relevance of the concentrations tested is unknown. Although significant inhibition of CXCL10 at 1 µg/mL was observed up to 6 h post poly i:c stimulation, the higher concentrations tested showed an insignificant trend toward decreased CXCL10 secretion.

L. dissectum has been regarded as safe in the historic literature, with a rash being the main reported side effect and those allergic to plants in the Apiaceae family are advised against its use.[21, 45] Nausea from ingestion of large amounts has been reported, although one of its traditional uses was for stomach and other internal disorders.[46] *L. dissectum* is listed as "at risk" by United Plant Savers and although its ecological status is debated among experts, conscientious harvesting is necessary to ensure the survival of native stands of *L. dissectum.*[47] Establishment of *L. dissectum* plant fields is possible but extended seed stratification is required for successful propagation and established plants typically do not produce seeds and flowers until the fourth year of production.[46] Contacting local experts or visiting resources such as The Northeast School of Botanical Medicine website for wildcrafting references is recommended before harvesting.[48]

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