Effects of *Ligusticum porteri* (Osha) Root Extract on Human Promyelocytic Leukemia Cells

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**ABSTRACT**

**Background:** *Ligusticum porteri* roots have been traditionally used in folk medicine, but the scientific basis is unclear. **Objective:** To investigate the cytotoxicity, antioxidant, and immunomodulatory effects of *L. porteri* root extract on human promyelocytic leukemia (HL-60) cells and H$_2$O$_2$-induced oxidative damaged HL-60 cells. **Materials and Methods:** HL-60 cells were incubated with different concentrations of root extract, and cells were harvested for viability assays on day 3 and 7. Cytokine levels (interferon-gamma [IFN-γ], interleukin-2 [IL-2], and interleukin-10 [IL-10]) and antioxidant indexes (malondialdehyde [MDA], reduced glutathione [GSH], superoxide dismutase [SOD], and catalase [CAT]) in H$_2$O$_2$-induced-stressed HL-60 were measured after 2 days. **Results:** The viability of HL-60 challenged with H$_2$O$_2$ declined by 42% compared to un-stressed cells. After 7 days of incubation with 200 or 400 µg/mL *L. porteri*, the viability of HL-60 cells was two-fold higher than the control. Stressed HL-60 cells treated with 100, 200, and 400 µg/mL *L. porteri* reduced the lipid peroxidation by 12%–13%. We noted an increase in GSH levels, SOD and CAT activities in stressed HL-60 supplemented with 400 µg/mL root extract. Treatment with 400 µg/mL *L. porteri* significantly (P < 0.05) increased IFN-γ and IL-2 in H$_2$O$_2$-challenged cells. **Conclusion:** Our data do not support the use of the extract as an antiproliferation and differentiation therapy for acute promyelocytic leukemia. The protective function of *L. porteri* root extract against oxidative stress could occur through increasing GSH and higher expression of antioxidant enzymes.

**Key words:** Antioxidative, cytotoxicity, human promyelocytic leukemia cells, immune-modulatory, *Ligusticum porteri*

**SUMMARY**

- Findings from this study may not support the use of *Ligusticum porteri* root extract as an antiproliferation and differentiation therapy for acute promyelocytic leukemia

- Our data suggest that *L. porteri* root extract may be a potential antioxidant with protective effect against the oxidation of reduced glutathione (GSH)

- Treatment with *L. porteri* root extract may be effective in preventing oxidative damage through increasing the activities of antioxidant enzymes (superoxide dismutase [SOD] and catalase [CAT]) in acute promyelocytic leukemia cells.

**INTRODUCTION**

Hispanics and Native Americans have used *Ligusticum porteri* preparations for many years for the treatment of many diseases.[1] *L. porteri* enhances the immune system, stimulates appetite, and improves gastrointestinal discomforts such as indigestion and stomach upset associated with vomiting.[2] *L. porteri* is used to treat acute influenza, acute bronchial pneumonia with dyspnea, and leukocytosis.[3,4] Phytochemical studies conducted identified butylidenephthalide and ligustilide as the most common constituents isolated from *Ligusticum*.[5] Butylidenephthalide is effective as an anti-angina, antihypertensive, antioxidative, antiplatelet, antispasmodic, and vasodilator.[6,7] Ligustilide has antimicrobial activities against Gram-positive, Gram-negative, and yeasts organisms. The essential oil (100 µL/mL) prepared from the roots increased the antimicrobial activity of antibiotic norfloxacin against the norfloxacin-resistant strain of *Staphylococcus aureus*. *Ligusticum* spp. is employed in traditional medicine to boost the immune system.[6,8,9] Z-ligustilide was reported to possess anti-inflammatory activity.[9] Doses of 20 mg/kg/day of Z-ligustilide reduced pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), vascular endothelial growth factor-α, and IL-17 in endotoxin-infected mice within 24 h. The suppression of these pro-inflammatory mediators has been found to reduce the severity of the inflammatory reaction.[10] Z-ligustilide and Senkyunolide A, which make up 50% of organic constituents in *Ligusticum* species, suppressed the production of TNF-α during inflammation.[11,12] Most inflammatory diseases are treated with conventional steroidal and nonsteroidal anti-inflammatory drugs.[13] The use of medicinal plant preparations in the management of diseases is on the increase. However, the scientific basis for the traditional use of many plant preparations in the treatment of many diseases including cancer is not clear. For

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example, it was reported that L. porteri extract does not exert anticancer activity on breast and colon cancer cells at a concentration of 50 µg/mL.[16] The HL-60 cells were established in 1977 from the peripheral blood of a patient with acute myeloid leukemia.[15] It has been used as a model in many studies of inflammatory cells because this cell line can be induced to differentiate into granulocytes in vitro.[16] We have previously reported the cytotoxicity, antioxidant and immune-modulatory effects of L. porteri root extract on human peripheral blood lymphocytes.[21] In this study, we used similar protocols to evaluate the effects of L. porteri root extract at a concentration range of 50–400 µg/mL on oxidative stress and inflammation in human promyelocytic leukemia (HL-60) cells and the probable mechanism of action.

MATERIALS AND METHODS

Preparation of Ligusticum porteri root extract

The extract was prepared by the following Beltran’s method with modifications.[20] About 30 g of dry L. porteri root was pulverized and mixed with 300 mL of 40% ethanol. The mixture was sieved through a cheese-cloth, followed by a final filtration with 0.2 µm membrane. The filtrate was dried under vacuum and stored at −20°C for further use. The dried sample was weighed and then dissolved in 0.1% dimethyl sulfoxide (DMSO) (Corning Cellogiro, Virginia, USA) at a concentration of 440 µg/mL (stock solution). Final working concentrations of the root extract at 50, 100, 200, 400, and 0 µg/mL (the control containing only DMSO) were prepared right before the experiments.[21,22]

Preparation of cultured human promyelocytic leukemia cells

HL-60 cells (ATCC, Virginia, USA) at the seeding concentration of 10^6 cells/mL were suspended in Iscove's Modified Dulbecco's Medium (ATCC, Virginia, USA) supplemented with 20% (v/v) fetal bovine serum (ATCC, Virginia, USA). The HL-60 cells were incubated at 37°C in humidified atmosphere containing 5% CO₂. When the cell concentration reached 10^6 cells/mL, the cell culture was diluted for subculturing according to the manufacturer's instruction (ATCC, Virginia, USA). Hydrogen peroxide (50 µM) [20] was used to induce oxidative stress in HL-60 cultures and cell concentration was determined by 0.4% Trypan blue as previously described.[17]

Cytotoxicity assays

One hundred microliters of cell suspension (10^6 cells/mL) was seeded in each well in a 96-well plate and preincubated for 24 h in a humidified incubator at 37°C with 5% CO₂. Subsequently, 10 µL of L. porteri was added to test for cytotoxicity (ratio of root extract and cell suspension is 1:10 v/v so that the final concentrations of L. porteri were 50, 100, 200, and 400 µg/mL, respectively). The vehicle (0.1% DMSO) was used as the control. The plate was incubated for 3 and 7 days. Cytotoxicity was determined using CCK-8 solution. Ten microliters of CCK-8 solution was added to each well of the plate and incubated for 4 h at 37°C. The absorbance of formazan was measured at 450 nm (Technical manual Cell Counting Kit-8, Maryland, USA) as previously described.[17]

Lipid peroxidation, reduced glutathione levels and antioxidant enzymes

After treatment of HL-60 cells with 50, 100, 200, 400, and 0 µg/mL (control) of L. porteri root extract, the cell pellets were harvested for the assays of lipid peroxidation and GSH levels,[21,25] and SOD and CAT activities[21,26] as previously described.[21]

Determination of interferon-gamma, interleukin-2, and interleukin-10

Cultures of HL-60 were induced to differentiate by addition of 1.0 µg/mL all-trans retinoic acid (Sigma-Aldrich, Minnesota, USA) and incubated for 4 days.[24] The differentiated HL-60 cells (10^6 cells/mL) were exposed to 50 µM H₂O₂, followed by treatment with 400 µg/mL L. porteri extract or the control. After 2 days of incubation, the supernatants were removed for analyses of cytokines. Cytokine levels of IFN-γ, IL-2, and IL-10 in the culture supernatants were determined using commercial enzyme-linked immunosorbent assays (ELISA) obtained from Thermo Scientific (Instructions for human IFN-γ, IL-2, and IL-10 ELISA kits, Illinois, USA).

Statistical analysis

Data are presented as mean ± standard error of the mean. Data were obtained from three separate experiments and each experiment included the control and four other concentrations of L. porteri extracts performed in triplicate.[21] The results among different concentrations were evaluated by one-way ANOVA (P < 0.05). Duncan’s multiple range test at significance level P < 0.05 was used to test for significant difference among the means.

RESULTS

Figure 1 shows that treatment with L. porteri root extract at doses higher than 100 µg/mL enhanced the viability of HL-60 cells after 7 days of incubation. Growth of HL-60 cells in the control reached a peak on day 3, followed by a decrease in their viability on day 7 of incubation. The viability of HL-60 cells treated with 100 µg/mL of L. porteri extract increased by 31% as compared to the control after 7 days of incubation. The HL-60 cells treated with 200 µg/mL and 400 µg/mL of L. porteri

![Figure 1](image)

**Table 1: Effects of Ligusticum porteri on lipid peroxidation in H₂O₂-induced stress human promyelocytic leukemia cells after 2 days**

| L. porteri concentration (µg/mL) | MDA (µmol/mg protein) | Inhibition (%) by L. porteri treatment |
|----------------------------------|------------------------|--------------------------------------|
| 0                                | 684.9 ± 20.03          |                                      |
| 50                               | 665.53 ± 18.52         | 2.8                                  |
| 100                              | 601.77 ± 21.47         | 12.1                                 |
| 200                              | 596.41 ± 20.26         | 12.9                                 |
| 400                              | 601.07 ± 33.39         | 12.2                                 |

Values that have different letter superscripts are significantly different (P < 0.05). MDA: Malondialdehyde; L. porteri: Ligusticum porteri
extract showed a 2-fold increase in viability after 7 days of incubation. Figure 2 shows that H$_2$O$_2$ exerted a cytotoxic effect to HL-60 cells after 7 days of incubation. The viability of HL-60 cells was declined by 42% after 7 days of incubation ($P < 0.05$).

Figure 3 shows that treatment with 400 µg/mL L. porteri root extract significantly ameliorated the adverse effects of H$_2$O$_2$ in HL-60 cells. Only treatment with 400 µg/mL L. porteri root extract was effective in boosting the HL-60 cell survival; it increased by 30% after 7 days of incubation compared to the control. Other lower concentrations of the root extract (≤200 µg/mL) did not considerably relieve the deleterious effect of H$_2$O$_2$ after a period of incubation. On day 0, viability of HL-60 cells was not detected in the groups treated with different concentrations of L. porteri root extract. However, HL-60 cell viability was seen in treated groups on day 3 and 7.

Table 1 shows that HL-60 cells treated with the extract doses of 100, 200, and 400 µg/mL reduced lipid peroxidation by 12%–13%.

Table 2 shows that treatments with 200 µg/mL and 400 µg/mL of L. porteri root extract elevated GSH levels by approximately 29%–30% while other treatments with lower concentration showed 7%–8% elevations.

Table 3 shows that treatment with L. porteri at the concentration as low as 100 µg/mL enhanced the activities of SOD ($P < 0.05$). The antioxidant effect of L. porteri on the enzyme activity was greater with increasing concentrations of root extract. The modulatory effect of L. porteri on SOD activity was seen when the root extract was above 100 µg/mL.

Table 4 shows that CAT activity in stressed HL-60 cells increased by 2.5-fold after treatment with L. porteri root extract at a dose above 200 µg/mL.

Figure 2: The effect of 50 µM H$_2$O$_2$ on human promyelocytic leukemia cells after 7 days. Figures that share different letters are significantly different ($P < 0.05$)

Figure 3: Change in the viability of H$_2$O$_2$-induced-stress human promyelocytic leukemia cells after treatment with Ligusticum porteri root extract for 7 days. Figures that share different letters are significantly different ($P < 0.05$)

Figure 4: Change in the levels of interferon-gamma-induced by human promyelocytic leukemia cells after treatment with 400 µg/mL Ligusticum porteri root extract for 2 days. ***This value is significantly different from other group treatments ($P < 0.05$)

Figure 5: Change in the levels of interleukin-2 induced by human promyelocytic leukemia cells after treatment with 400 µg/mL Ligusticum porteri root extract for 2 days. Figures that share different letters are significantly different ($P < 0.05$)
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16.09±2.49
2
89.7

Effects of
12.79±1.45
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148.4
16.62±2.56
- induced-stress human promyelocytic leukemia cells

[28]
Change in the levels of interleukin-10 induced by human peripheral blood lymphocytes treated by oxidative stress.

Lipid peroxidation is an important indicator of cellular damage caused by oxidative stress. It resulted in significant increase in MDA content and depletion of GSH levels, which may be indicative of increased oxidative damage in these cells. However, we noted increase in GSH levels, SOD and CAT activities in stressed HL-60 supplemented with 400 µg/mL L. porteri root extract which is indicative of the protection of acute promyelocytic leukemia cells against oxidative stress.

To evaluate the immune-modulatory effects of the extract in HL-60 cells, we measured IL-2, IFN-γ and IL-10. We noted increase production of inflammatory mediators (IL-2 and IFN-γ) in stressed HL-60 cells treated with 400 µg/mL L. porteri root extract. We also noted significant up-regulation of IL-2 after a 2-day incubation with 400 µg/mL L. porteri root extract. Although the amount of IFN-γ was low compared to other interleukins (IL-2 and IL-10) in stressed cell cultures treated with 400 µg/mL L. porteri root extract, there was a significant increase in IFN-γ production when compared to the control. These findings suggest that the root extract of L. porteri has positive effect on oxidative stress and inflammation in acute promyelocytic leukemia cells.

CONCLUSION

Findings from this study do not support the use of L. porteri root extract as an anti-proliferation and differentiation therapy for acute promyelocytic leukemia cells. However, the results also showed that L. porteri root extract possess an antioxidation function and regulation on immune factors in HL-60 cells.

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Conflicts of interest
There are no conflicts of interest.

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Table 4: Effects of Ligusticum porteri root extract on antioxidant enzyme catalase activity in H2O2-induced-stress human promyelocytic leukemia cells after 2 days

| L. porteri concentration (µg/mL) | CAT (U/mg protein/min) | Increase of CAT activity by L. porteri treatment (%) |
|---------------------------------|------------------------|--------------------------------------------------|
| 0                               | 6.69±0.58              |                                                    |
| 50                              | 12.79±1.45             | 91.2                                              |
| 100                             | 12.69±1.23             | 89.7                                              |
| 200                             | 16.09±1.49             | 140.5                                             |
| 400                             | 16.62±2.56             | 148.4                                             |

Values that have different letter superscripts are significantly different (P<0.05). L. porteri: Ligusticum porteri; CAT: Catalase

Figure 5 shows that there was an increasing trend in IL-2 secretion when the cells were stress induced (50 µM H2O2) or treated with L. porteri root extract.

Figure 6 shows that hydrogen peroxide suppressed the production of IL-10. Treatment with 400 µg/mL of L. porteri root extract showed an increase of 63% in IL-10 levels, but this amount was still lower that the control, indicating that the root extract suppressed the inhibitory effect of H2O2.

DISCUSSION

The observed decline in HL-60 cells viability may be due to the inability of the medium to sustain the growth of the cells after 7 days of incubation. The enhancing survivability of HL-60 cells after treatment with L. porteri root extract may not be advantageous due to the malignant nature of the cells. Findings from this study do not support the application of L. porteri root extract as an antileukemic therapy. It is possible that the viability of HL-60 cells on day 0 was below the threshold of detection by the cell counting assay kit used. It is hypothesized that the addition of root extract to stressed HL-60 cells results in the suppression of the cell viability on day 0. It was previously reported that induction of 50 µM H2O2 for 4 h resulted in DNA fragmentation and triggered cell death in HL-60 cells.[27] Findings from this study showed that the treatment with root extract of L. porteri may protect HL-60 cells from oxidative stress caused by H2O2 similar to the effect observed in human peripheral blood lymphocytes.[17]

Lipid peroxidation is an important indicator of cellular damage caused by oxidative stress.[26] We have earlier reported the inhibition of lipid peroxidation, increased GSH, and increase in the activities of superoxide dismutase and catalase in human peripheral blood lymphocytes treated with L. porteri root extract.[13] In this study, the exposure of HL-60 cells to 50 µM H2O2 resulted in a significant increase in MDA content and depletion of GSH levels, which may be indicative of increased oxidative damage in these cells. However, we noted increase in GSH levels, SOD and CAT activities in stressed HL-60 supplemented with 400 µg/mL L. porteri root extract which is indicative of the protection of acute promyelocytic leukemia cells against oxidative stress.

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