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ORIGINAL ARTICLE

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

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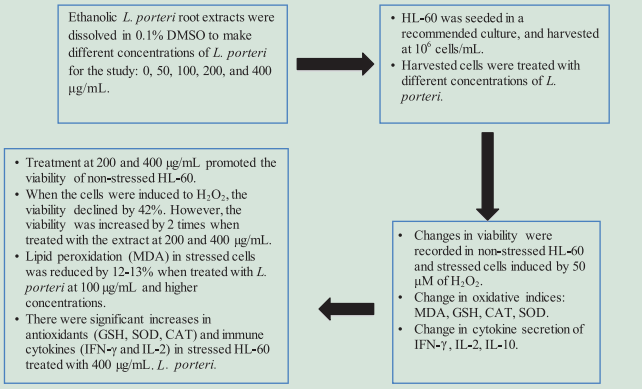
ABSTRACT

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_2O_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_2O_2-induced-stressed HL-60 were measured after 2 days. **Results:** The viability of HL-60 challenged with H_2O_2 declined by 42% compared to unstressed 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_2O_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.



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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.[3-5]

Phytochemical studies conducted identified butylidenephthalide and ligustilide as the most common constituents isolated from *Ligusticum*.[6] 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 yeast organisms. The essential oil (100 μg/mL) prepared from the roots increased the antimicrobial activity of antibiotic norfloxacin against the norfloxacin-resistant strain of *Staphylococcus aureus*.[8] *Ligusticum* spp. is employed in traditional medicine to boost the immune system.[4-6,9] Z-ligustilide was reported to possess anti-inflammatory activity.[6] 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.[14] 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, antioxidative and immune-modulatory effects of *L. porteri* root extract on human peripheral blood lymphocytes.[17] 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.[18] 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 0.20 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 Cellgro, 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.[17,19]

Preparation of cultured human promyelocytic leukemia cells

HL-60 cells (ATCC, Virginia, USA) at the seeding concentration of 10^5 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% CO_2. 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% CO_2. 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,[21-24] and SOD and CAT activities[21,25] as previously described.[17]

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.[26] The differentiated HL-60 cells (10^6 cells/mL) were exposed to 50 μM H_2O_2, 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.[17] 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*

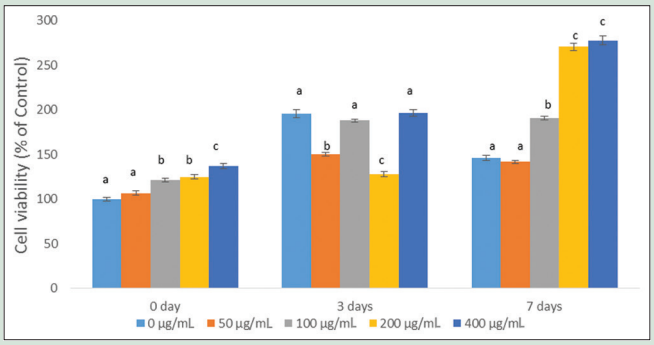

Figure 1: Change in the viability of human promyelocytic leukemia cells cells treated with different concentrations of *Ligusticum porteri* during 7 days of incubation. Figures that share different letters are significantly different ($P < 0.05$)

Table 1: Effects of *Ligusticum porteri* on lipid peroxidation in H_2O_2-induced-stress human promyelocytic leukemia cells after 2 days

L. porteri concentration (μg/mL)	MDA (μmol/mg protein)	Inhibition (%) by *L. porteri* treatment
0	684.91±20.03[a]	
50	665.53±18.52[a,b]	2.8
100	601.77±21.47[b]	12.1
200	596.41±20.26[b]	12.9
400	601.07±33.59[b]	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_2O_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_2O_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_2O_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 4 shows that there was no detection of IFN-γ in untreated HL-60 (no additives) and the group treated with only 50 μM H_2O_2. However, a significant enhancement of IFN-γ production was detected in the group of root extract treatment.

Table 2: Effects of *Ligusticum porteri* root extract on glutathione content in H_2O_2-induced-stress human promyelocytic leukemia cells after 2 days

L. porteri concentration (μg/mL)	GSH (μM/mg protein)	Elevation (%) by *L. porteri* treatment
0	41.97±1.81[a]	
50	45.26±1.46[a]	7.8
100	45.6±1.11[a]	8.7
200	54.17±2.04[b]	29.1
400	54.48±2.15[b]	29.8

Values that have different letter superscripts are significantly different ($P<0.05$). GSH: Reduced glutathione; *L. Porteri*: *Ligusticum porteri*

Table 3: Effects of *Ligusticum porteri* root extract on antioxidant enzyme superoxide dismutase activity in H_2O_2-induced-stress human promyelocytic leukemia cells after 2 days

L. porteri concentration (μg/mL)	SOD (mU/mg protein/min)	Increase of SOD activity by *L. porteri* treatment (%)
0	40.78±6.39[a]	
50	39.94±3.92[a]	
100	57.58±7.48[b]	41.2
200	75.07±7.49[c]	84.1
400	77.76±8.03[c]	90.7

Values that have different letter superscripts are significantly different ($P<0.05$). SOD: Superoxide dismutase; *L. Porteri*: *Ligusticum porteri*

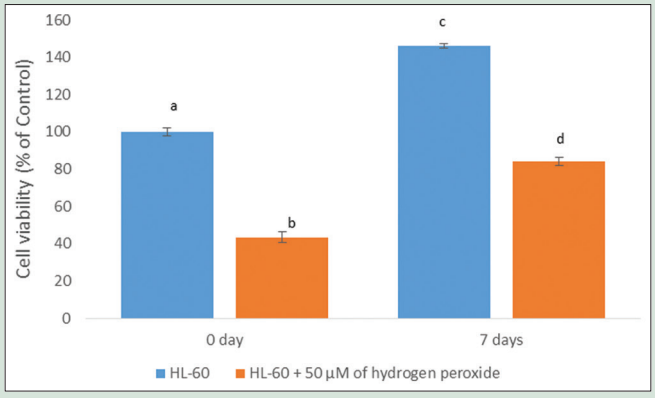

Figure 2: The effect of 50 μM H_2O_2 on human promyelocytic leukemia cells after 7 days. Figures that share different letters are significantly different ($P < 0.05$)

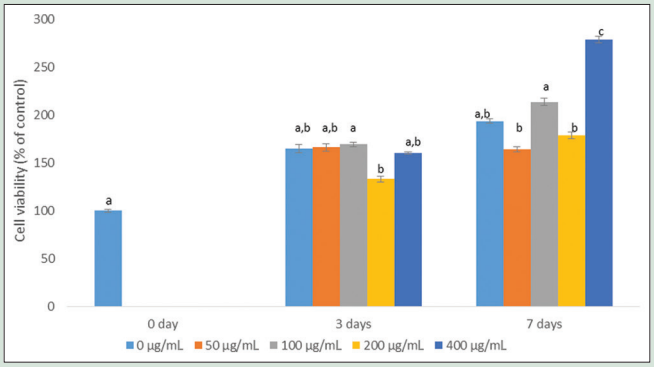

Figure 3: Change in the viability of H_2O_2-induced-stress human promyelocytic leukemia cells after treatment with *Ligusticum porteri* root extract for 7 days. Figures that share different letters are significantly different ($P < 0.05$)

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 ($P < 0.05$)

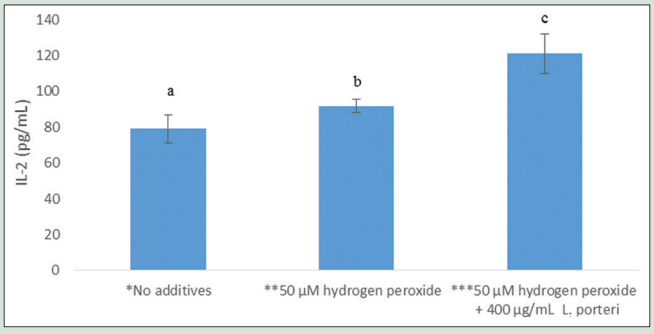

Figure 5: Change in the levels of interleukin-2 induced by human promyelocytic leukemia cells after treatment with 400 μg/mL *Ligusticum porteri root extract* for 2 days. Figures that share different letters are significantly different ($P < 0.05$)

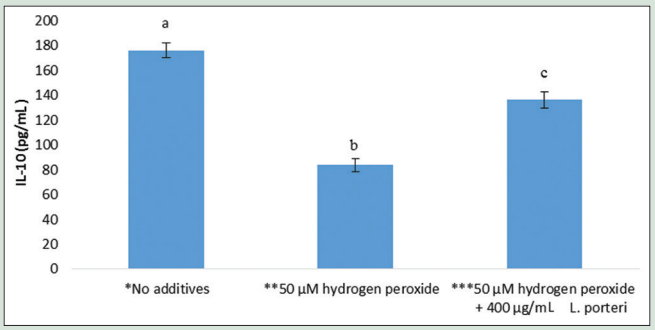

Figure 6: Change in the levels of interleukin-10 induced by human promyelocytic leukemia cells after treatment with 400 µg/mL *Ligusticum porteri root extract* for 2 days. Figures that share different letters are significantly different ($P < 0.05$)

Table 4: Effects of *Ligusticum porteri* root extract on antioxidant enzyme catalase activity in H_2O_2-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[a]	
50	12.79±1.45[b]	91.2
100	12.69±1.23[b]	89.7
200	16.09±2.49[c]	140.5
400	16.62±2.56[c]	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 H_2O_2) 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 H_2O_2.

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 H_2O_2 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 H_2O_2 similar to the effect observed in human peripheral blood lymphocytes.[17]

Lipid peroxidation is an important indicator of cellular damage caused by oxidative stress.[28] 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.[17] In this study, the exposure of HL-60 cells to 50 µM H_2O_2 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.

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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Nil.

Conflicts of interest

There are no conflicts of interest.

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