Investigation of the Cytotoxicity, Anti-oxidant, and Anti-inflammatory Effects of *Ligusticum porteri* (Osha) on Human Peripheral Lymphocytes and Promyelocytic Leukemia Cells

by

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ABSTRACT

Background:

Ligusticum porteri is a traditional Native American herb. The roots of *L. porteri* have been used in treatments for many kinds of diseases as well as for boosting the immune system. Even though *L. porteri* has been widely used in traditional remedies, its acclaimed medicinal effects have been barely validated. This study is the first investigation into the medicinal effects of *L. porteri* on the cytotoxicity, anti-oxidative, and anti-inflammatory activity in human peripheral blood lymphocytes (PBLs) and promyelocytic leukemia (HL-60). This study also investigated the attenuating effects of *L. porteri* on the cytotoxicity of hydrogen peroxide induced oxidative damage in these cell cultures.

Methods:

Vacuum-dried ethanolic root extract of *L. porteri* was dissolved in dimethyl sulphoxide (DMSO) to prepare a stock solution. Appropriate volumes of stock solution of the root extract were added to cultured PBLs and HL-60 cells (1:10 v/v) so that the final concentrations of *L. porteri* root extract in each batch of cell culture were respectively 0 μg/ml (control), 50 μg/ml, 100 μg/ml, 200 μg/ml, and 400 μg/ml. Additionally, to investigate the attenuating effects of *L. porteri* in oxidative-damaged cell cultures, PBLs and HL-60 cells were challenged with 50 μM of hydrogen peroxide. The cell suspensions were incubated at 37^{o}C humidified with 5% CO_2. After each day during the incubation period, cell pellets and supernatants were harvested for the investigation of the cytotoxicity, anti-oxidation, and anti-inflammation induced by the cell cultures treated with *L. porteri* root extract.

Results:

Treatments with *L. porteri* at concentrations as high as 400 µg/ml could enhance the viability and proliferation of human PBLs and HL-60 cells. After 2 days incubation with 200 µg/ml and 400 µg/ml of root extract, the viability of PBLs was 2 and 2.5 fold higher than the control. The PBLs treated with *L. porteri* at 200 µg/ml and 400 µg/ml proliferated until day 3 while the untreated and those treated with lower concentrations (50 µg/ml and 100 µg/ml) of the root extract did not survive. After 7 days of incubation with 200 µg/ml and 400 µg/ml of *L. porteri* root extract, the proliferation of HL-60 cells was two-fold higher than the control ($P < 0.05$).

This study also found that the oxidative stress induced by hydrogen peroxide (H_2O_2) reduced the viability of PBLs and HL-60 cells. Data showed that the percentage viability of stress-induced PBLs and HL-60 cells was reduced by 54% and 42%, respectively ($P < 0.05$). The anti-proliferative effect of H_2O_2 was ameliorated by 400 µg/ml *L. porteri* treatment. This effective dose helped maintain the PBLs' viability at 1.5 times higher than the control after 2 days of incubation while this dose increased the proliferation of stressed HL-60 cells by 42% ($P < 0.05$). Treatments at lower concentrations did not have a significant proliferative effect which ultimately resulted in growth decline due to H_2O_2-exposure.

Lipid peroxidation was measured in terms of malondialdehyde (MDA) formed during the stress. Data showed that the root extracts reduced the MDA accumulation in stressed PBLs and HL-60 cells ($P < 0.05$). The addition of 400 µg/ml *L. porteri* significantly decreased the lipid peroxidation in stressed PBLs by 94% ($P < 0.05$). Treatment of stressed HL-60 cells with the root extract concentration equal or higher than 100 µg/ml reduced the lipid peroxidation by 12-13% ($P < 0.05$). Treatment with 400 µg/ml of the root extract resulted in 26.4% and 29.4%

increase of glutathione levels (GSH) in stressed PBLs and HL-60 cells respectively as compared to stressed cell cultures without the root extract ($P < 0.05$).

Studies on the superoxide dismutase (SOD) and catalase (CAT) activities in H_2O_2-challenged PBLs and HL-60 cells showed increased activities in response to oxidative stress. Positive modulatory effects of *L. porteri* to the activities of these enzymes in stressed cells were noted at concentrations as low as 100 μg/ml ($P < 0.05$). The activities of SOD and CAT increased significantly, by 17.5% and 55.2% respectively, when stressed PBLs were treated with 400 μg/ml *L. porteri* for 2 days. Treatment with root extract at 100 μg/ml significantly ($P < 0.05$) increased the activity of SOD in H_2O_2-challenged HL-60 cells. This study found that CAT activity in stressed HL-60 cells showed a 2- to 2.5-fold increase after treatment with more than 50 μg/ml *L. porteri*.

Treatment with 400 μg/ml *L. porteri* significantly ($P < 0.05$) increased IFN-γ and IL-2 in H_2O_2-challenged cells. Addition of the root extract did not cause a significant difference in IL-10 levels between stressed PBLs with and without 400 μg/ml *L. porteri* ($P > 0.05$). However, treatment with 400 μg/ml *L. porteri* diminished the effect of H_2O_2-induced decrease in IL-10 in stressed HL-60 cell cultures ($P < 0.05$).

Conclusion:

The use of ethanolic root extract of *L. porteri* at high concentration (400 μg/ml) enhanced the viability of human PBLs and HL-60 cells. Treatment with *L. porteri* may protect the cells against H_2O_2-induced oxidative stress by reducing lipid peroxidation and oxidation of GSH, as well as by elevating the activities of SOD and CAT. Treatment with the *L. porteri* root extract further enhanced the production of IFN-γ and IL-2. Along with the mild enhancement of secretion of IL-

10, cytokine stimulation by the addition of *L. porteri* suggested that the root extract may be a potential immune-modulating agent involving protective effects against oxidative damage.

Key words: *Ligusticum porteri*, herbal remedy, cytotoxicity, anti-oxidant, anti-inflammatory response, human peripheral blood lymphocytes, human promyelocytic leukemia cells.

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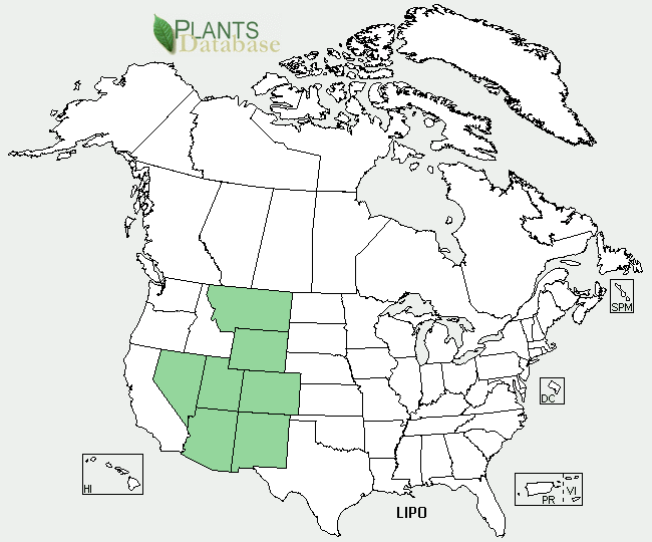
1. INTRODUCTION

1.1 Ethnobotany of *Ligusticum porteri*

Ligusticum porteri, commonly known as Osha, is a herbaceous perennial plant whose leaves and roots have been used since the 1600s by Hispanics and Native Americans as therapeutic treatments for a broad array of medicinal ailments such as influenza and respiratory problems [1]. The plant is classified in the carrot family, Apiaceae, which was previously known as Umbelliferae [2]. Today, products from the extract of *L. porteri* are widely traded and used for medicinal purposes. In commerce, *L. porteri* is most frequently known as Osha. Other common names of *L. porteri* include Porter's Licorice root, Mountain lovage, Colorado cough root, Porter's lovage, and Southern *Ligusticum* [3]. Historically, *L. porteri* was named "bear root" by the Native Americans when they observed bears digging up roots to eat or rubbing their fur with the *L. porteri* roots to repel insects and soothe bites. In Mexico, *L. porteri* is referred as chuchupaste, chuchupa, or chuchupate, which are folk remedies for many ailments, particularly respiratory infections [2].

Ligusticum porteri is native to the Rocky Mountains, which expand from Montana and Wyoming in the north through Colorado, Utah, Nevada, to Arizona and New Mexico (Figure 1). *Ligusticum porteri* has also been found in the states of Sonora and Chihuahua in Mexico [3, 4].

Figure 1: The distribution of *L. porteri* within the United States, including Arizona, Montana, New Mexico, Nevada, Utah and Wyoming.



The presence of *L. porteri* is highlighted in green–colored states. Adapted from United States Department of Agriculture [4].

Ligusticum porteri's habitat is often found in high elevation regions, ranging from 6,000 – 11,700 ft (1,829 – 3,567 m) above sea level [5]. It thrives in rich, moist soils in wooded habitats – nearby or in association with groves of aspen (*Populus spp.*), conifers, Douglas fir (*Pseudotsuga menziesii*), sagebrush (*Artemesia spp.*), spruce (*Picea spp.*) and oak (*Quercus spp.*). *Ligusticum porteri* is also found on slopes and upland meadows with drier, rocky soils [2,3].

Ligusticum porteri grows approximately 50-100 cm tall. During the winter, the rootstocks become thick, woody and very redolent because of resins produced by the plant [2,3]. The plant has profoundly engraved leaf segments which are 5-40 mm in width with larger leaves arising from the root that are 15-30 cm long [2,3]. *Ligusticum porteri* blossoms during late summer and their white flowers are 2-5 mm in diameter. Its fruits are characterized by a cherry-red color and 5-8 mm in length. The seeds of *L. porteri* are neither distributed by animals nor wind; instead, they are likely to remain close to their parental plants when they drop on the ground [3].

Figure 2: *Ligusticum porteri* plant and its root harvested in the Meadow site, near Cumbres Pass, Rio Grande National Forest in South Colorado.

Adapted from United States Department of Agriculture [4].

Adapted from Kindscher*et al.* [1].

The genus *Ligusticum* consists of 40-50 species [3]. Some Asian *Ligusticum* species are important in Chinese, Japanese, and Korean herbal medicine [3]. Ligusticum chuanxiong is known to have antioxidant, anti-inflammatory, and anti-bacterial effects [6]. Its medicinal benefits have been demonstrated by numerous pre-clinical and clinical studies [6]. The American *Ligusticum porteri* has been used medicinally by indigenous groups for centuries and subsequently implemented into the pharmacopeias of other cultures in both the Old and New

Worlds [3]. American *Ligusticum* species have been used as anticonvulsants, to stimulate appetite, and to treat anemia, hemorrhage, tuberculosis, stomach disorders, heart troubles, respiratory infections, earaches, sinus infection, congestion, and other ailments [3]. In Mexican folk medicine, *L. porteri* is highly prized for both medicinal and ceremonial purposes [7]. Hispanics use the roots of *L. porteri* to treat various respiratory ailments, including catarrh, colds, coughs, bronchial pneumonia, flu and other respiratory problems [3, 8]. Root preparations have been also used externally to treat aches and pains, digestive problems, scorpion stings, wounds and skin infections [3].

1.2 Literature reviews on the medicinal applications of *L. porteri*

Although *L. porteri* has been used to treat a wide range of ailments by generations of Native Americans, the knowledge pertaining to the pharmaceutical uses of *L. porteri* is scarce because of the shortage of scientific literature in this area. Research conducted over the years has provided similar information on the traditional medicinal uses of *L. porteri*, but little evidence of its efficacy and safety, leaving an area of potential study. As health care costs continue to rise, people are turning to natural sources of medicine such as herbs. *Ligusticum porteri* has gained a reputation for its medicinal effects [5].

Ligusticum porteri enhances the immune system, stimulates appetite, and also improves gastrointestinal discomforts such as indigestion and stomach upset associated with vomiting [9]. *Ligusticum porteri* has been used to treat acute influenza, acute bronchial pneumonia with dyspnea, and leukocytosis [10]. American *Ligusticum* species are effective as anticonvulsants, and treating anemia, hemorrhage, tuberculosis, stomach disorders, heart troubles, respiratory infections, earaches, sinus infection and congestion, and other ailments [11].

There has been a growing body of literature studying the cytotoxic effects of *L. porteri* root extract. For example, Beltran [12] evaluated the effects of various stock solutions of *L. porteri* root extract on *in vitro* cell lines using the 50% lethal dose as the criteria. Ethanolic root extract of *L. porteri* was not found to be toxic to human pro-monocytes (immature monocytes during their developmental stages) up to a concentration of 50-100 µg/ml. However, a low concentration of 5 µg/ml was toxic to *Mycobacterium tuberculosis*. The results indicated that the *L. porteri* extract had antimicrobial activities against *M. tuberculosis* at a sufficiently low concentration that did not affect the maturation of human monocytes *in vitro*. Ethanolic root extract of *L. porteri* has been reported to inhibit the growth of human breast cancer cell lines *in vitro* [13, 14]. Though the inhibitory mechanism remains unclear, a concentration of 32-60 µg/ml of ethanolic extract of *L. porteri* could kill 50% of human breast cancer cells [14]. These findings support some of the medicinal uses of *L. porteri* preparations among indigenous groups.

Compounds isolated from *L. porteri* have been shown to yield many medicinal activities. Butylidenephthalide and ligustilide are the most common constituents isolated from *Ligusticum* species [15]. Butylidenephthalide is effective as an anti-angina, anti-hypertensive, anti-oxidative, anti-platelet, anti-spasmodic, and vasodilator [15, 16]. Ligustilide has anti-microbial activities against gram positive, gram negative and yeast organisms [5]. 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* [17]. *Ligusticum spp.* are employed in folk medicine to boost the immune system [3, 7, 8, 15]. Despite its usage, there is little data about this effect. Recent phytochemical studies have been conducted to identify compounds and their corresponding bioactivity.

Z-ligustilide was the most well-known bio-effective substance with anti-inflammatory activity [15]. Ma *et al.* [18] noted that 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 (VEGF-α), and IL-17 in endotoxin-infected mice within 24 hours. The suppression of these pro-inflammatory mediators has been found to reduce the severity of the inflammatory reaction. Tsun [19] observed that Z-ligustilide and Senkyunolide A contributed to 50% of the organic constituents in *Ligusticum* species. Liu *et al*. [20] found that Z-ligustilide and Senkyunolide A suppressed the production of TNF-α during inflammation. The results showed that only 50 μM of these compounds could inhibit the TNF-α production. The mechanism was due to the down-regulation of TNF-α mRNA transcription in lymphocytes [20]. These studies not only support the traditional use of *L. porteri* for anti-inflammatory effects, but also points out the individual substances responsible for this anti-inflammation. It is clear that the anti-inflammatory effects of *Ligusticum spp.* are due to the ability to reduce the production of pro-inflammatory mediators in infected cells.

1.3 Reactive oxygen species and their sources of production

In a normal situation, there is a balance in the generation of oxygen-free radicals and the antioxidant defense mechanisms used to deactivate free radical toxicity [21-23]. Reactive oxygen species (ROS) or oxidants are characterized as the reduced metabolites of oxygen, generated intracellularly during normal aerobic metabolisms [24]. Reactive oxygen species include superoxide anion ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), and other non-radical molecules such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and so on (Figure 3) [24, 25].

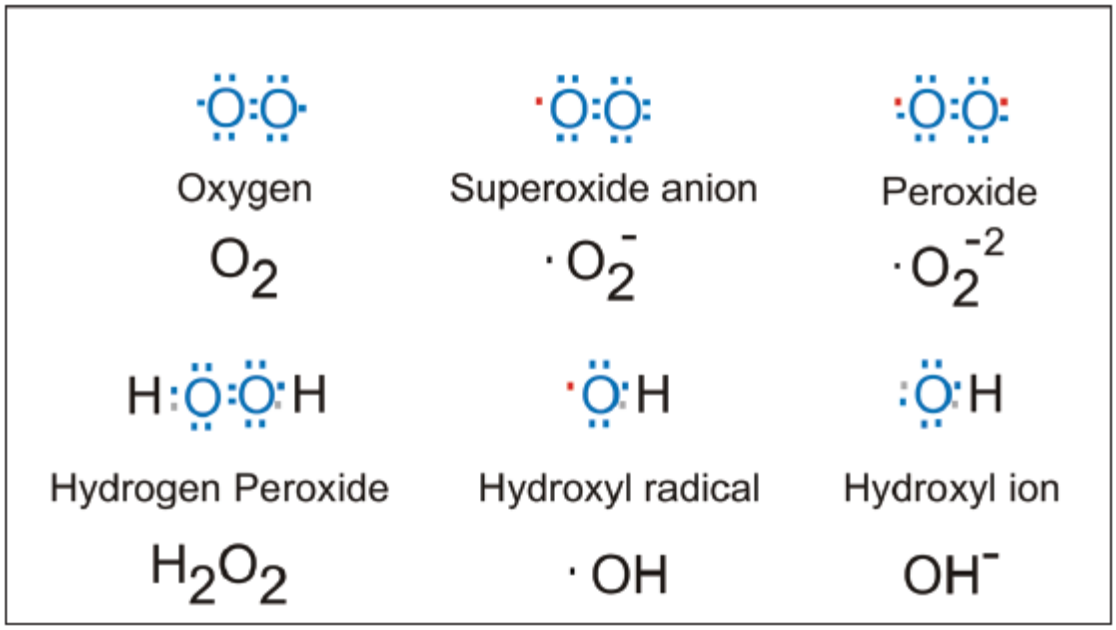


Figure 3: Chemical structure of common reactive oxygen species. The red (·) indicates the unpaired electron. Adapted from [25].

Reactive oxygen species are generated through a stepwise reduction of molecular oxygen (O_2) due to high-energy exposure or electron-transfer reactions [26]. The severity of ROS depends on their concentration, duration of exposure, and type of cells. Low doses of ROS are mitogenic and stimulate cell proliferation. Intermediate doses cause growth arrest either temporarily or permanently, such as replicative senescence while high doses of ROS result in cell apoptosis or necrosis (Figure 4) [24]. Chkhikvishvili *et al.* [26] reported that the introduction of H_2O_2 at 25 μM or 50 μM to Jurkat T cells resulted in oxidative stress, increased the production of superoxide and peroxyl radicals, restricted cell proliferation by altering the cell-cycle phase distribution, and promoted cell apoptosis [27].

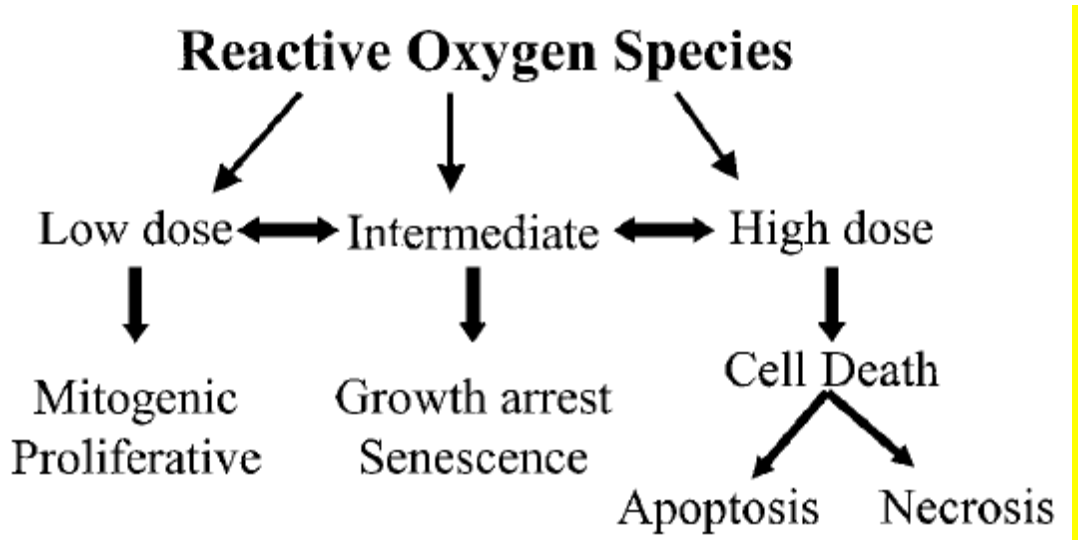


Figure 4: ROS concentration and their cellular effects. Adapted from [24].

In aerobic organisms, ROS are generated via intracellular processes including mitochondrial respiration, oxidation of fatty acids in peroxisomes, and phagocytosis by the immune system [28]. Mitochondrial respiration is a primary source of ROS in mammalian cells [29]. The mitochondria organelle is the main site of oxygen metabolism, which contributes to the consumption of 85-90% oxygen absorbed by the cells [30, 31]. Although mitochondrial respiration is an efficient metabolism, it was evident that 1-3% of the consumed oxygen was not completely metabolized [32, 33]. It was also reported that 1-2% of the electrons leaks out of the mitochondrial electron transport chain and combines with molecular oxygen to form ROS [28]. The partial processing of oxygen in mitochondria results in toxic side-products such as superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$) [34]. Superoxide radicals are mostly produced by complexes I (NADH dehydrogenase) and III (ubisemiquinone) (Figure 5) [32, 33, 35]. The mitochondrial enzyme manganese superoxide dismutase (MnSOD) catalyzes superoxide to H_2O_2. Hydrogen peroxide is still a potentially toxic compound, therefore, the scavenging system requires the enzyme glutathione peroxidase (GSPx) and its co-enzyme

reduced glutathione (GSH) to convert H_2O_2 to water, completing the detoxification of ROS [34]. However, H_2O_2 can react with reduced metals to produce hydroxyl radicals (·OH), which possess a highly oxidant property (Figure 5) [36].

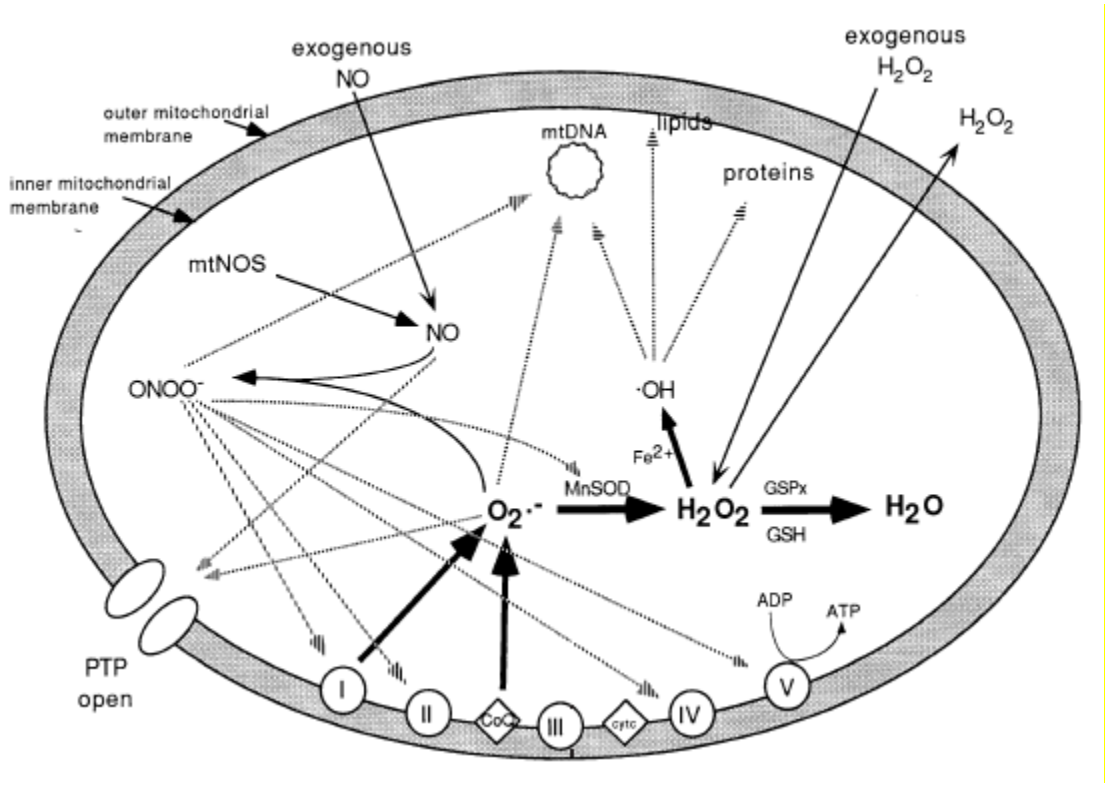


Figure 5: Diagram of ROS in mitochondria. Solid arrows with solid arrowheads indicate the generation of molecules. Solid arrows with pointed arrow heads show the diffusion of molecules. Hatched arrows point out the damaging effects of ROS [Adapted from 34].

Among mitochondria-derived ROS, superoxide is the first highly reactive radical generated in the inner membrane of mitochondria. Due to its high oxygen reactivity, it does not diffuse easily throughout the cell. Thus, superoxide is believed to target the mitochondrial DNA (Figure 5) [37]. Hydrogen peroxide is the second damaging ROS produced by the detoxification effect of enzyme MnSOD. Compared to other ROS, hydrogen peroxide lacks free electrons and is small in size, thus it easily diffuses far from the site of production to mediate toxic effects. For instance,

H_2O_2 can react with free Fe^{2+} in the Fenton reaction ($Fe^{2+} + H_2O_2 <-> Fe^{3+} + OH^- + \cdot OH$) to produce hydroxyl radical (·OH). This free radical has a very short half-life and is extremely reactive, rendering its ability to react with any neighbor molecules (Figure 3). Kirkinezos *et al.* [34] reported that there are no cellular scavengers to directly neutralize hydroxyl radical, and suggested that the production of hydroxyl radicals can be prevented through enzymatic action on H_2O_2 and most importantly by lowering the abundance of free transition metals such as Fe^{2+} and Cu^{2+} [34].

The β-oxidation of fatty acids for ATP production occurs in both mitochondria and peroxisome [38]. Although the energy is generated via the catalysis of different enzymes in different organelles, their functions are complementary [39-42]. Mitochondria is the main site for the beta-oxidation of short- (< C_8), medium- (C_8-C_{12}), and long- (C_{14}-C_{20}) chain fatty acids while the very long chain fatty acids (> C_{20}) are exclusively beta-oxidized in peroxisomes because mitochondria lack very-long-chain acyl-CoA synthetases [42-44]. Due to the abundance of long chain fatty acids (C_{14} - C_{20}) in dietary fat, these fatty acids become a dominant source for lipid metabolism; and thus mitochondria is the main cellular compartment to beta-oxidize fatty acids for energy production [39-42, 45]. However, the peroxisome also participates in beta-oxidation of these substrates. Evidence showed that the metabolic activities in peroxisomes and mitochondria cooperate at different levels to maintain various metabolic and signaling pathways [46-48]. Similar to mitochondria, peroxisomes are a relevant source of different types of reactive species [49]. It was hypothesized that the low levels of ROS in peroxisomes signal the proliferation and survival of cells [49]. However, the excessive generation of ROS in peroxisomes increases the mitochondrial redox state and triggers mitochondrial fragmentation; subsequently leading to apoptosis in cells [50]. The down-regulation of catalase activity in peroxisomes contributes to the

oxidative stress in mitochondria which consequently leads to mitochondrial dysfunction [51-53]. The enhanced catalase activity in peroxisomes restores the mitochondrial redox balance and function [51, 53, 54]. Oxidative stress is strongly associated with inflammation and there are evidences that the peroxisome plays a potential role in the inflammatory process [55]. Peroxisomes regulate the availability of important inflammatory mediators such as H_2O_2, $NO^{\cdot}$, prostaglandin, and leukotriene [56, 57]. It was found that when levels of very long chain fatty acids and lipid derivatives are excessively accumulated, the inflammatory responses and demyelination are enhanced [50].

Phagocytosis is the first line of the innate immune system in which phagocytic cells kill and digest pathogens that penetrate the epithelial barriers [58]. In phagocytosis, phagocytic cells, mostly including macrophages and neutrophils, consume oxygen molecules to produce ROS [28, 59]. This metabolic process is referred as a "respiratory burst", during which ROS are produced by non-mitochondrial respiration [60]. The induction of ROS is to kill the invading microorganisms [58]. In phagosomes, the enzyme NADPH oxidase (NOX) is responsible for producing ROS. Oxygen molecules are converted to $\cdot O_2^-$ by NOX and then these highly reactive free radicals are further converted to H_2O_2 by additional enzymes [59]. At the same time, the enzyme myeloperoxidase (MPO) is released into the phagosomes and catalyzes the reaction of chloride ion (Cl^-) and H_2O_2 to form hypochlorous acid (HOCl), which is a major oxidant weapon of neutrophils [61]. Winterbourn *et al.* [60] measured the concentrations of superoxide and H_2O_2 during phagocytosis by modelling the phagocytic activity of neutrophils *in vitro* and stated that the steady-state concentration of superoxide and H_2O_2 during phagocytosis is 25 μM and 2 μM respectively [61]. It was also stated that HOCl is generated at a rate of 134 mM/min [61]. These HOCl molecules can induce the peroxidation of polyunsaturated lipids and react with sulfur

containing amino acids. They also attack the amine groups in amino acids resulting in the formation of chloramines, which possess oxidizing activity. These reaction products may extend the ability to kill microbes in time and space [60].

Reactive oxygen species are generated when cells are exposed to environmental stresses [28]. The exposure to ROS is constant and inevitable. The transient fluctuation in ROS serves as an important regulator. When ROS are present at high and/or prolonged levels, they can severely damage DNA, protein, and lipids [24].

1.4 Oxidative damage

The imbalance between oxidants and antioxidants is defined as oxidative stress [62]. Oxidative stress contributes to cellular injuries and subsequently leads to several degenerative diseases in humans, such as coronary heart disease, muscle degeneration, aging, and cancer [63]. The harmful effects of ROS result from their ability to get an electron from the adjacent stable molecule, leading to the formation of a new free radical that is able to ionize other molecules [64]. This cascade results in the disruption of cell components [62, 65]. The excessive levels of ROS cause lipid peroxidation, protein oxidation, DNA damage, enzyme inhibition, and the activation of programmed cell death pathway (Figure 6) [66].

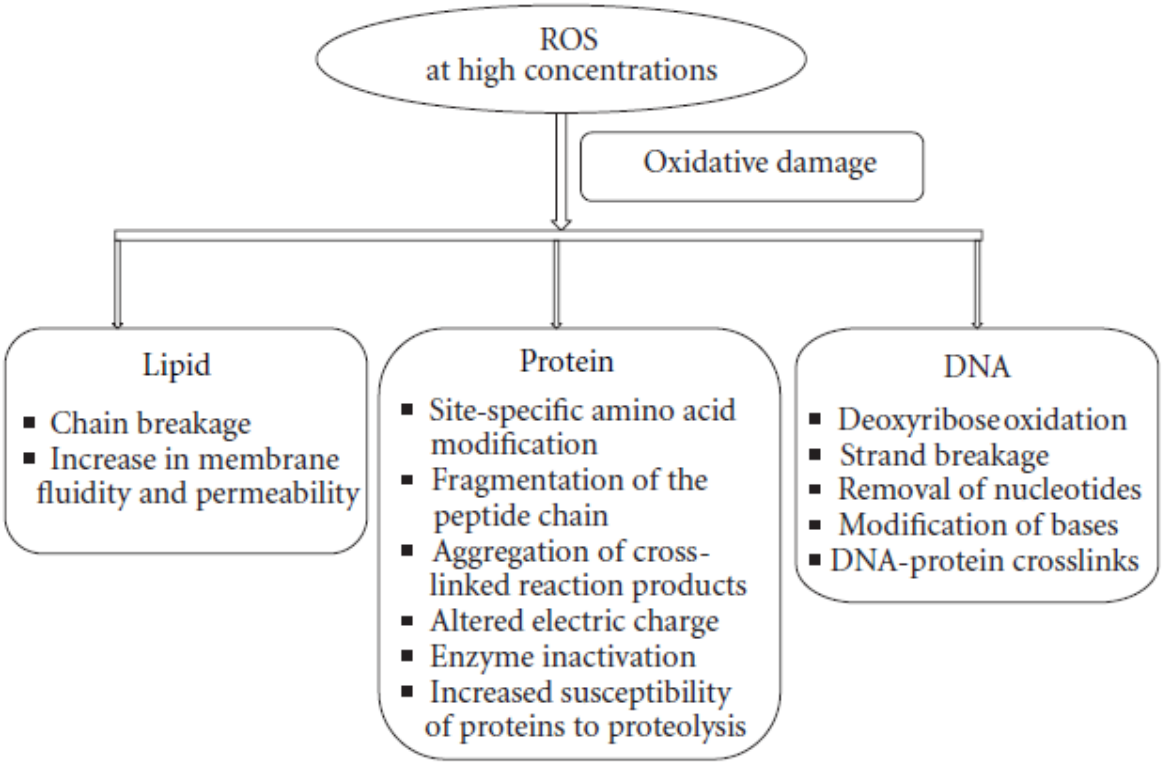


Figure 6: The oxidative damage on lipid, protein, and DNA induced by Reactive oxygen species (ROS). Adapted from [66].

El-Aal [64] reported that cellular membranes are vulnerable to oxidative attack due to the presence of unsaturated fatty acids. These unsaturated fatty acids serve as excellent substrates for lipid peroxidation because of the presence of bis-allylic methylene groups. The carbon-hydrogen bonds on these methylene units have low bond-dissociation energies, hence the hydrogen atoms are easily removed from the unsaturated fatty acids [65]. The susceptibility of a particular unsaturated fatty acid toward peroxidation is dependent on the number of unsaturated sites in the lipid chain [65]. The destruction of lipids due to peroxidation results in the formation of a wide range of primary products such as conjugated dienes, lipid hydroperoxides, and secondary products like malondialdehyde (MDA) and F2-isoprostane [64]. Among the products of lipid

peroxidation, MDA is most commonly measured through its reaction with thiobarbituric acid. The reaction generates thiobarbituric reactive species and the concentration is used as an index of lipid peroxidation [62]. This method is acceptably recognized as a general biomarker of lipid peroxidation [67]. The lipid peroxidation itself interferes with fluidity and permeability of cellular membranes [66]. Reactive byproducts from lipid peroxidation can further react with proteins and nucleic acids, which contributes to protein and DNA damage [68, 69].

Yamauchi *et al.* [68] stated that protein oxidation can occur in two ways as a consequence of cellular oxidative stress. First, ROS interfere with proteins via nitrosylation, carbonylation, formation of disulphide bonds, and glutathionylation. Second, protein molecules themselves can react with byproducts from lipid peroxidation [68]. Protein oxidation causes modified amino acids, fragmented peptide chains, aggregated cross-linked reaction products, and altered electric charge. Thiol- and sulfur-rich amino acids are extremely vulnerable to the attack of ROS [66]. Gardner *et al.* [70] reported that the oxidation of iron-sulfur centers by superoxide radicals is irreversible, resulting in enzyme inactivation.

Nucleic acids are also the target of ROS during oxidative stress. Evan *et al.* [71] indicated that ROS attack nucleic acids through sites of sugar and base moieties. It was also noticed that each type of ROS have different targets in DNA damage. Hydroxyl radicals ($^{\cdot}OH$) target purine and pyrimidine bases, as well as the C4$^{'}$position in deoxyribose backbone [72]. While singlet oxygens ($^{1}O_2$) only attack guanine, H_2O_2 and superoxide anion ($^{\cdot}O_2^{-}$) do not attack any kinds of bases. The consequences of DNA damage are the oxidation of deoxyribose, strand breakage, removal of nucleotides, and various modifications in bases of nucleotides [66]. These genetic changes may contribute to the inactivation or malfunctions of the translated proteins.

1.5 Oxidative defense system

To avoid oxidative stress due to the excessive levels of ROS, cells have their own anti-oxidative mechanisms to keep the ROS levels below the stress threshold. The detoxification of excessive ROS is achieved by the cooperation between non-enzymatic anti-oxidant molecules and enzymatic anti-oxidant scavengers. Halliwell [73] stated that there is a cascading reaction to remove free radicals by anti-oxidant enzymes involving superoxide dismutase (SOD), catalase (CAT), and peroxidases. The enzyme SOD converts free radical superoxide anions ($O_2^{\cdot -}$) into H_2O_2 molecules which are further catalyzed to become water and oxygen. In human cells, enzyme glutathione peroxidases (GSH-Px) are also responsible for converting H_2O_2 into non-toxic molecules. The H_2O_2-removal mechanism of GSH-Px is through the oxidation of the GSH. This reaction results in the formation of oxidized glutathione (GSSG). Reversely, enzyme glutathione reductase is responsible for the reduction of GSSG to GSH [66]. Thus, the ratio GSH/GSSG is an indicator of intracellular oxidative status. In normal redox status, the high ratio GSH/GSSG is in favor, which means more production of GSH is generated [74].

Although the anti-oxidative enzymes are localized in different cellular compartments, they respond simultaneously to help cells combat oxidative stress [66]. Superoxide dismutase is an important enzyme in the defense mechanism of aerobic organisms [75]. It is present in all eukaryotic cells and its function is to convert superoxide anions into H_2O_2 which is less reactive for cellular damage as compared to the free radicals. There are three isoforms of SOD in eukaryotic cells. The isoform Copper-/Zinc-SOD is localized in cytoplasm, nucleus, and plasma while Manganese-SOD is primarily present in the mitochondria [66].

In animals, catalase is mainly found in peroxisomes. It is known to catalyze H_2O_2 generated in this organelle during the β-oxidation of fatty acids, and other enzymatic reactions to remove free

radicals [76]. Glutathione peroxidase (GSH-Px) scavenges H_2O_2 through the oxidation of glutathione. While peroxisomes are the major sites for CAT, GSH-Px is mainly present in the cytoplasm or mitochondrial matrix. Besides the ability to scavenge H_2O_2, GSH-Px can also reduce other peroxides, such as fatty acid hydroperoxides [66].

Besides anti-oxidative enzymes, non-enzymatic components also play an important role in the oxidative defense system. Non-enzymatic antioxidants are glutathione, vitamin C, vitamin E, carotenoids, and other phenolic compounds [66]. Many of these antioxidant molecules can be supplemented through daily diets such as β-carotene, vitamin E, and vitamin C. Because β-carotene and vitamin E are lipid-soluble, their anti-oxidant properties are efficient in lipid environments such as cellular membranes. Vitamin C, on the other hand, is water-soluble so its anti-oxidant activity takes place in the aqueous phase of cytoplasm [77]. Among non-enzymatic anti-oxidant components, glutathione displays an essential role in the defense system against oxidative stress. Reduced glutathione (GSH) is present in a variety of organelles including cytosol, chloroplasts, endoplasmic reticulum, vacuoles, and mitochondria [78]. First, GSH acts as a direct free radical scavenger because glutathione itself can react directly with superoxide anions ($\cdot O_2^-$), hydroxyl ($\cdot OH$), and H_2O_2. Second, GSH can prevent oxidation to macromolecules such as lipids, proteins, and nucleic acids due to its ability to neutralize electrophiles or ROS [66]. Reduced glutathione was found to participate in the regeneration of antioxidant ascorbic acid [79, 80]. Due to GSH's crucial role in the oxidative defense system, glutathione content is used as a stress indicator [66].

1.6 Inflammation

Inflammation is an immediate defense of the host to eliminate harmful stimuli such as pathogens, toxins, and local injuries as well as to activate the healing process [81-83]. Inflammation is induced by the activation of various inflammatory cells such as fibroblasts, endothelial cells, tissue macrophages, and mast cells as well as the recruitment of monocytes, lymphocytes, and neutrophils [84]. The aggregation of these inflammatory cells at the site of inflammation is induced by a number of soluble cytokines [84]. Cytokines that promote the inflammatory response are called “pro-inflammatory cytokines”, while “anti-inflammatory cytokines” act to down-regulate the inflammatory response by suppressing the activity of pro-inflammatory cytokines [85]. The function of the immune system is to balance the activity of pro-inflammatory and anti-inflammatory mediators [86]. Inflammation can be described as a double-edged sword because it eliminates invading pathogens and initiates the healing process, but uncontrolled inflammation can lead to injuries of cells and tissues [87]. For instance, the activation of pro-inflammatory mediators induces a variety of oxidant-generating enzymes such as NADPH oxidase, xanthine oxidase, and myeloperoxidase in order to produce oxidants to eliminate the infectious agents [86]. The over-expression of the inflammatory response could produce excessive ROS, resulting in damage to DNA and cellular membranes [86]. Most inflammatory diseases are treated with the conventional anti-inflammatory drugs, such as steroidal anti-inflammatory drugs (SAIDs) and nonsteroidal anti-inflammatory drugs (NSAIDs) [22]. However, the long-term administration of these drugs may produce adverse side effects, such as gastrointestinal ulcers, hemorrhage, renal damage, immunodeficiency and humoral disturbances [22, 88]. The development of anti-inflammatory drugs has recently focused on discovering plant-derived extracts that are potent and safe [89].

Previous studies as reviewed by Reuter *et al.* [90] revealed thatoxidative stress, chronic inflammation, and cancers are closely linked. Under a sustained environmental stress, ROS are produced over a long time that can lead to chronic inflammation, which in turn mediates most chronic diseases including different types of cancers (table 1) and other diseases (table 2) [90]. However, pharmaceutical treatments for diseases are commonly marked by side effects, leading to the search for natural substances with less adverse side effects. Hence, this study investigated the effects of *L. porteri* root extract on the oxidative and inflammatory indices that are often associated with the development of many diseases.

Table 1: List of cancers associated with ROS. Adapted from [90]

Breast cancer	Multiple Myeloma
Bladder cancer	Leukemia
Brain tumor	Lymphoma
Cervical cancer	Oral cancer
Gastric (stomach) cancer	Ovarian cancer
Liver cancer	Pancreatic cancer
Lung cancer	Sarcoma
Melanoma	Prostate cancer

Table 2: List of diseases associated with ROS. Adapted from [89]

Acute Respiratory Distress Syndrome	Cardiovascular disease	Obesity
Aging	Diabetes	Parkinson
Alzheimer	Inflammation	Pulmonary fibrosis
Atheroscerosis	Inflammatory joint disease	Rhematoid arthritis
Cancer	Neurological disease	Vascular disease

The HL-60 and lymphocytes were chosen as models to investigate the effects of *L. porteri* root extract. The HL-60 were established in 1977 from the peripheral blood of a patient with acute myeloid leukemia [91]. 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* [92]. Granulocytes, mostly neutrophils that make up 50-70% of the population of white blood cells, are important cells in the innate immune system which is considered as the front lines of attack to clear threatening agents from the body [58]. Also, lymphocytes are fundamental cells in the adaptive immune response [58]. They are key producers of antibodies and cell-mediated immunity which are the ultimate products needed to effectively protect the host from pathogens or foreign substances [58]. It is clear that there are interactions between the innate immune and adaptive immune systems in which they work in harmony to exert an effective protection against invaders [58]. Hence, in this study, both HL-60 and lymphocytes were used as models for the *in vitro* investigation of the anti-inflammatory and anti-oxidative effects of *L. porteri* extract.

1.7 Objectives

Objective 1: To investigate the cytotoxicity of *L. porteri* root extract used at the concentration of 50 μg/ml, 100 μg/ml, 200 μg/ml, and 400 μg/ml after every 24 hours on human lymphocytes and promyelocytic leukemia cells (until 3 days and 7 days, respectively) with and without oxidative stress induction by 50 μM H_2O_2. The results provided knowledge on the cytotoxicity of *L. porter* as well as the herb's ability to protect stress-induced cells against the anti-proliferative effect caused by H_2O_2. Quantification of cell viability post-treatment was done at 24 hour intervals. Lethal dose 50 (LD_{50}) was used as the standard measurement of toxicity. This indicator represented the amount of root extract (μg/ml) that is sufficient to kill 50 percent of human cell cultures [12].

Objective 2: To investigate the antioxidant effects of *L. porteri* root extract in H_2O_2-induced-stress lymphocytes and promyelocytic leukemia cells. Superoxide dismutase and catalase were the target anti-oxidative enzymes whose activity levels will reflect the degree of oxidative stress. The level of oxidative damage (represented by the degree of lipid peroxidation) as well as the level of protein oxidation (represented by the glutathione contents) on H_2O_2-challenged cells after treatment with the root extract were determined. The purpose was to test whether the root extract of *L. porteri* protects stressed cells against oxidative damage.

Objective 3: To measure cytokines secreted from H_2O_2-challenged lymphocytes and promyelocytic leukemia cells pre- and post-treatment with root extract of *L. porteri*. The IL-2, IL-10 and IFN-γ were target cytokines to test the hypothesis that *L. porteri* stimulates cells to secret cytokines for anti-inflammatory effects. These cytokine levels were measured by Enzyme-Linked Immunosorbent Assay (ELISA) [Thermo Scientific, Illinois, USA].

2. MATERIALS & METHODS

2.1 Herbal harvest and sample preparations

This study was conducted in Drs. Sparks' and Omoruyi's lab at Texas A&M University-Corpus Christi, Natural Resources Center building, room number 3416. This room provided sufficient tools for all the assays that were performed. *Ligusticum porteri* root was collected from Crested Butte, Colorado and used for the preparation of the extract.

Preparation of L. porteri extract: The extract was prepared by following Beltran's method with modifications [12]. About 30 grams of dry *L. porteri* root were 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^{o}C for further use. The dried sample was weighed and then dissolved in dimethyl sulphoxide (DMSO) [Corning Cellgro, VA, USA] at a concentration of 4400 µg/ml (stock solution). Final working concentrations of the root extract at 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, and 0 µg/ml (the control containing only DMSO) were prepared by diluting the stock solution in DMSO right before the experiments. The range of concentrations of *L. porteri* root extract prepared in this study was based on a previous study that used concentrations ranging from 12 to 400 µg/ml of *L. porteri* crude extracts to study the cytotoxicity of the plant extract on the culture media of U-937 cell line [12].

Preparation of cultured lymphocytes: Peripheral Blood Mononuclear Cells (PBMCs) [Sanguine Biosciences, California, USA] were cultured at the concentration of 10^5 cells/ml in RPMI 1460 medium containing 2 mM of glutamine, supplemented with 1% (v/v) nonessential amino acids, 1% (v/v) sodium pyruvate, 50 U/ml penicillin, 50 mg/ml streptomycin, and 20% (v/v) Fetal Bovine Serum [Corning Cellgro, VA, USA]. Ten µg/ml of lipopolysaccharide [obtained from

Escherichia coli serotype O55:B5, Sigma Aldrich, Missouri, USA] was added to PBMCs culture to activate lymphocyte differentiation. The cell suspension was incubated at 37ºC in humidified atmosphere containing 5% CO_2. When cell growth reached 10^6 cells/ml, cells were harvested according to the manufacturer's instructions [Sanguine Biosciences, California, USA].

Preparation of cultured HL-60 cells: HL-60 cells [ATCC, Virginia, USA] at the seeding concentration of 10^5 cells/ml was suspended in Iscove's Modified Dulbecco's Medium (IMDM) [ATCC, Virginia, USA] supplemented with 20% (v/v) Fetal Bovine Serum [ATCC, Virginia, USA]. The HL-60 cells were incubated at 37ºC in humidified atmosphere containing 5% CO_2. When the cell concentration reached 10^6 cells/ml, the cell culture was diluted for sub-culturing according to the manufacturer's instruction [ATCC, Virginia, USA].

Determination of the cell concentration: The number of viable cells was assessed by 0.4% Trypan Blue solution [Corning Cellgro, VA, USA]. A volume of 0.5 ml 0.4% Trypan Blue solution was transferred to a test tube, followed by adding 0.2 ml of cell suspension and 0.3 ml Hanks' Balanced Salt Solution (HBSS) [ATCC, Virginia, USA]. The dilution factor used in this study was 5. Then the suspension was mixed thoroughly. With the cover-slip in place, 10 µl mixture was pipetted to a chamber of the hemocytometer. The number of cells in the one-millimeter-center square and four one-millimeter-corner squares were counted. Non-viable cells stained blue while viable cells remained transparent [93].

Each large square of the hemocytometer, with cover-slip in place, represents a total volume of 0.1 mm^3 or 10^{-4} cm^3. Since 1 cm^3 is equivalent to 1 ml, the subsequent cell concentration per milliliter, the total number of cells, was determined using the following formula [93]:

$$Cells/ml = Average\ cells\ counted/square \times Dilution\ factor \times 10^4$$

2.2 Objective 1: The cytotoxicity of *L. porteri* root extract on PBLs and HL-60 cells with and without H_2O_2-exposure

Cell Counting Kit-8 (CCK-8) is a sensitive colorimetric assay used to determine the number of viable cells post-treatment with various substances. CCK-8 assays utilize the activity of enzyme dehydrogenases produced by living cells [94]. This enzyme-based method helps distinguish between healthy cells and cells that are alive but losing their functions, which makes it superior to Trypan Blue cell staining method. Water-soluble tetrazolium salt (WST-8), known as 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, is utilized in CCK-8 solution. An orange color product (formazan) is formed when WST-8 is reduced by the dehydrogenases produced by living cells. The amount of formazan generated by the dehydrogenases in living cells is directly proportional to the number of metabolically active cells [91]. In this study, human PBLs and HL-60 cells treated with different concentrations of *L. porteri* extract (50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml and the control) were harvested every day to evaluate the cytotoxicity.

2.2.1 The cytotoxicity of *L. porteri* on human PBLs and HL-60 cells

One hundred µl of cell suspension (10^6 cells/ml) was seeded in each well in a 96-well plate. The plate was pre-incubated for 24 hours in a humidified incubator at 37^{o}C with 5% CO_2. Subsequently, 10 µl of different concentrations of *L. porteri* (50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, and the control) was added to test for cytotoxicity (ratio of root extract and cell suspension is 1:10 v/v), followed by incubation for 3 days for PBLs and 7 days for HL-60 cells. Then, 10 µl of CCK-8 solution was added to each well of the plate and incubated for 4 hours at 37^{o}C. The absorbance of formazan was measured at 450 nm [94].

$$\text{Cell viability (\%)} = \frac{A_{substance} - A_{blank}}{A_{0\ substance} - A_{blank}} \times 100$$

$A_{substance}$: absorbance of a well with cell culture, CCK-8 solution and root extract.

$A_{0\ substance}$: absorbance of a well with cell culture, CCK-8 solution.

A_{blank}: absorbance of a well with medium (no cells) and CCK-8 solution.

2.2.2 The effect of *L. porteri* on H_2O_2-challenged PBLs and HL-60 cells

An appropriate volume of 0.1 M H_2O_2 was added to fresh cell culture medium so that the final concentration of H_2O_2 was 50 μM [27]. The cells were suspended in the prepared medium containing 50 μM H_2O_2 and adjusted to the concentration of 10^6 cells/ml by using Trypan Blue method [93], followed by the addition of the root extract.

2.3 Objective 2: Modulatory effects of *L. porteri* on lipid peroxidation, GSH levels, anti-oxidant enzyme SOD and CAT in H_2O_2-challenged PBLs and HL-60 cells

After treatment with 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml, and 0 μg/ml (control) of *L. porteri* root extract, the cell pellets were harvested for the assays of lipid peroxidation, GSH levels, and superoxide dismutase and catalase activities.

2.3.1 Lipid peroxidation

The pellets (10^6 cells/ml) were lysed in ice-cold physiological saline by sonication, followed by centrifugation at 28000 x g for 5 minutes at 4ºC. The cellular supernatants were used immediately for the measurement of the levels of MDA [95]. The formation of MDA, an end product of fatty acid peroxidation, were measured at 532 nm by using a thiobarbituric acid (TBA) reactive substance as described by Genet *et al.* [96]. Briefly, the final reaction mixture of 3 ml contained the following: 1.5 ml of 10 mM potassium phosphate buffer (pH 7.4), 0.5 ml of

the cellular supernatant, 0.5 ml of 30% trichloroacetic acid and 0.5 ml of thiobarbituric acid (TBA) (0.53%). The mixture was heated for one hour at 80°C, cooled and centrifuged for 5 minutes at 2700 x g. The absorbance of the clear supernatant was measured at 532 nm against a blank. Total protein in the cellular supernatant was determined by using Stanbio kit [97].

2.3.2 Glutathione levels

The reduced GSH levels were measured by following Ellman's method [98]. The harvested pellets were lysed in hypotonic solution for 45 minutes at 37^{o}C and then processed for the assay [99]. One hundred µl of the lysate was mixed with 10% trichloroacetic acid (TCA) and centrifuged at 2000 x g for 15 minutes. One milliliter of supernatant was treated with 0.5 ml of Ellman's reagent [19.8 mg of 5,5'-dithiobisnitro benzoic acid (DTNB) in 100 ml of 0.2 M phosphate buffer (pH 8.0)]. The absorbance was read at 412 nm.

2.3.3 Superoxide dismutase

The cell pellets (10^6 cells/ml) were lysed by sonication in buffer (cold 20 mM HEPES buffer (pH 7.2) containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose) [100]. After sonication, the lysate was centrifuged at 1500 x g for 5 minutes at 4^{o}C. The cellular supernatant was used for the measurement of SOD activity. One ml of reaction mixture was prepared by adding 500 µl of 0.1 M sodium phosphate buffer, 32 µl of 3.3 mM ethylenediaminetetraacetic acid (EDTA), 60 µl of 8.1 mM pyrogallol, and followed by adding cellular supernatant containing 7-10 µg protein. The change in absorbance at 420 nm of the mixture was monitored for 2 minutes at 25^{o}C against the blank that contains all ingredients except the supernatant. One unit of enzyme is defined as the amount of enzyme that causes half maximal inhibition of pyrogallol autoxidation [96].

2.3.4 Catalase

The cell pellets (10^6 cells/ml) were lysed by sonication in buffer containing 50 mM potassium phosphate (pH 7.0), 1 mM EDTA. The lysate was centrifuged at 10,000 x g for 15 minutes at 4^{o}C. The activity of catalase was measured in the supernatant [101]. Reaction mixture was prepared by adding 500 µl of 0.1 M sodium phosphate buffer pH 7.0 (50 mM), 100 µl of H_2O_2 (10 mM), and 100 µl cellular supernatant treated with 30 µl of 1% Triton X-100. The decrease in absorbance was measured at 240 nm for 5 minutes at 25^{o}C against a blank containing all the ingredients without the supernatant [96].

2.4 Objective 3: Modulatory effects of *L. porteri* on the production of IL-2, IL-10, and IFN-ɤ in H_2O_2-challenged PBLs and HL-60 cells

To investigate the immune responses induced by neutrophils, which are main components of the innate immune system, the HL-60 cells was induced to differentiate to neutrophil-like cells by addition 1 µg/ml All Trans-Retinoic Acid [Sigma-Aldrich, Minnesota, USA] and incubated for 4 days [102]. Then, lymphocytes and differentiated HL-60 cell cultures (10^6 cells/ml) were exposed to 50 µM H_2O_2 (see section 2.2.2). It was followed by the treatments with 400 µg/ml or 0 µg/ml (control) of *L. porteri* extract. After 2 days of incubation, the supernatants were removed for analyses of cytokines. Cytokine levels of IL-2, IL-10, and IFN-γ in the culture supernatants were determined by using commercial enzyme linked immunosorbent assays (ELISA) obtained from Thermo Scientific (Illinois, USA) [103-105].

2.5 Statistical Analysis

Data are presented as means ± standard error of the mean (n = 3). Statistical significance of differences was performed using unpaired Student's t test at the significance level of $P < 0.05$ for comparative analysis between two different groups (i.e. the control versus treated groups). 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.

3. RESULTS AND DISCUSSIONS

3.1 The cytotoxicity of *L. porteri* root extract on PBLs and HL-60 cells

The cytotoxicity of *L. porteri* at the concentrations of 50 μg/ml, 100 μg/ml, 200 μg/ml, and 400 μg/ml was investigated in PBLs and HL-60 cells. The goal was to determine whether the *L. porteri* root extract at these concentrations is deleterious for the viability of PBLs and HL-60 cells. The PBLs and HL-60 cells were treated with different concentrations of *L. porteri* root extract and collected data was compared to the control (treatment without the root extract) (Figure 7 and Figure 8). Overall, the addition of *L. porteri* at high concentrations (200 μg/ml and 400 μg/ml) enhanced the cell survival (Figure 7). The LD_{50} was not used in this study because the application of *L. porteri* at the concentrations as high as 400 μg/ml increased cell viability during the incubation period. Treatment of PBLs with 200 μg/ml and 400 μg/ml *L. porteri* resulted in 2 and 2.5 fold increases in cell viability compared to the control after 2 days of incubation (Figure 7). However, the observed decrease in PBLs viability in the control group (0 μg/ml) on day 1 versus increased cell viability on day 2 is unclear. The non-detection of PBLs viability in the control group after three days of incubation could be due to the shortage of growth factor in the media. It has been reported that long-term proliferation and survival of *in*

vitro lymphocytes is dependent on IL-2 growth factor and that the repeated addition of IL-2 into the media is essential to maintain cell proliferation [106, 107]. Keong *et al.* [108] used different lymphocyte separation techniques to optimize the *in vitro* viability of lymphocyte for 3 days [108]. Human peripheral lymphocytes did not survive in the groups treated with lower concentrations of *L. porteri* (≤ 100 µg/ml) on day 3 of incubation. This would suggest that PBLs viability was not sustainable beyond day 2 of the treatment with lower concentrations of the root extracts. However, the viability of PBLs was maintained beyond 3 days of incubation when treated with 200 µg/ml and 400 µg/ml *L. porteri* extract (Figure 7).

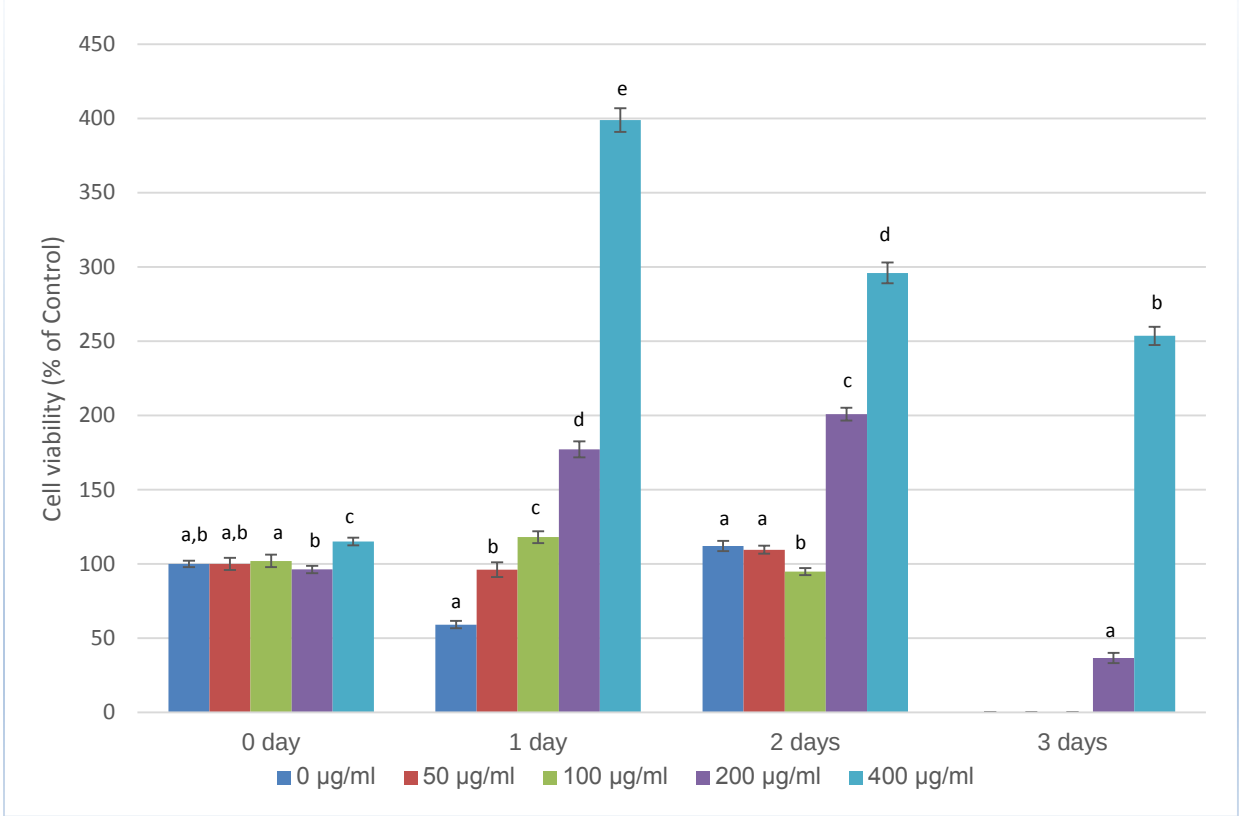


Figure 7 : Change in the viability of PBLs treated with different concentrations of *L. porteri* during 3 days of incubation. Figures that share different letters are significantly different (P <

0.05). Day zero (0 day) represents the change in the viability of PBLs immediately after treatment with different concentrations of *L. porteri.*

The growth of HL-60 cells (control) reached a peak on day 3, followed by a decrease in their viability on day 7 of incubation (Figure 8). The observed decline in HL-60 cell viability may be due to the inability of the medium to sustain the growth of the cells after 7 days of incubation. However, treatment of HL-60 cells with *L. porteri* root extract at doses higher than 100 µg/ml enhanced the viability of HL-60 cells after 7 days of incubation (Figure 8). 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. Overall, further studies are needed to isolate and identify the active principles in the root extract that are able to sustain HL-60 cell proliferation after 7 days of incubation. Promyelocytic leukemia cells treated with 200 µg/ml and 400 µg/ml of *L. porteri* extract showed 2-fold increase in viability after 7 days of incubation.

Data indicated that *L. porteri* promoted the viability of lymphocytes and HL-60 cells (Figure 7 and Figure 8). The enhancement of the viability of normal PBLs by *L. porteri* extract may be beneficial to boost the immune system. However, the observed enhancing viability effect of *L. porteri* on HL-60 cells 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 extracts as an anti-leukemic therapy.

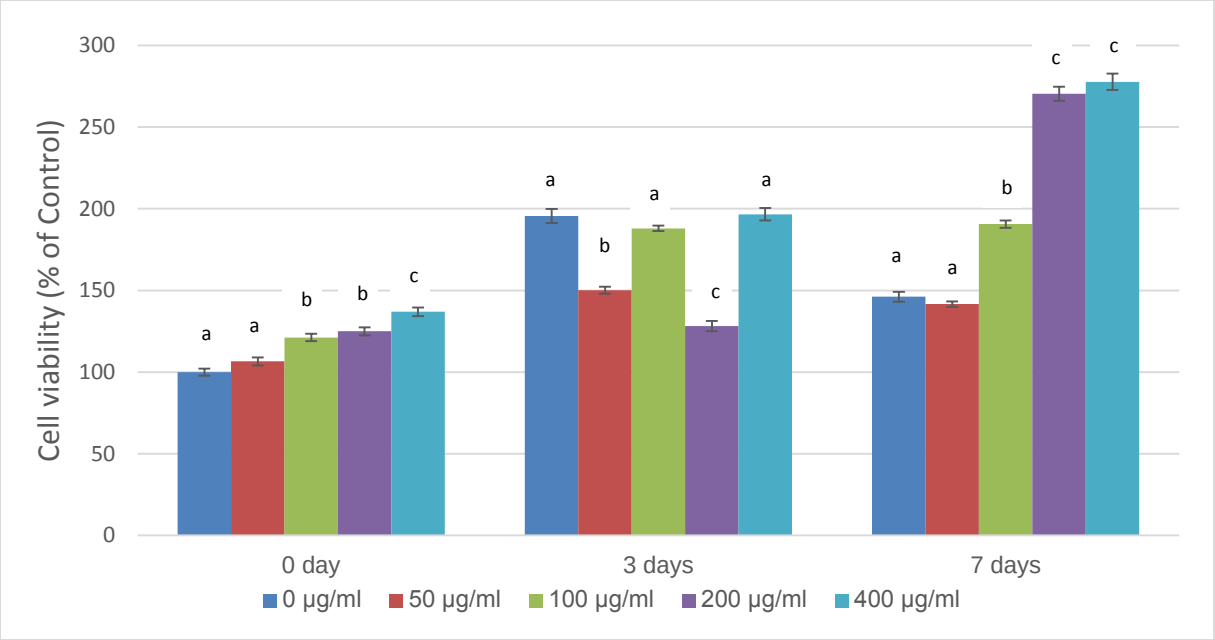


Figure 8: Change in the viability of HL-60 cells treated with different concentrations of *L. porteri* during 7 days of incubation. Figures that share different letters are significantly different ($P < 0.05$). Day zero (0 day) represents the change in the viability of HL-60 immediately after treatment with different concentrations of *L. porteri.*

3.2 Effects of *L. porteri* on the viability of H_2O_2-induced-stress in PBLs and HL-60 cells

Oxidative stress induced by H_2O_2 reduced the viability of lymphocytes and HL-60 cells (Figure 9 and Figure 10). In Figure 9, due to the adverse effect of H_2O_2, the survival of PBLs was reduced after 2 hours of incubation in the presence of 50 µM H_2O_2 (day 0). Hydrogen peroxide is a highly toxic agent that is capable of exerting a strong adverse effect on cell proliferation. Saiko *et al*. [109] reported that cells treated with 50 µM H_2O_2 activated apoptotic caspase-3 and caspase-9 with subsequent cell death [109]. In this study, the survival of H_2O_2-induced-stress PBLs declined by 54% after 2 days of incubation when compared to PBLs that were not treated with 50 µM H_2O_2 ($P < 0.05$) (Figure 9).

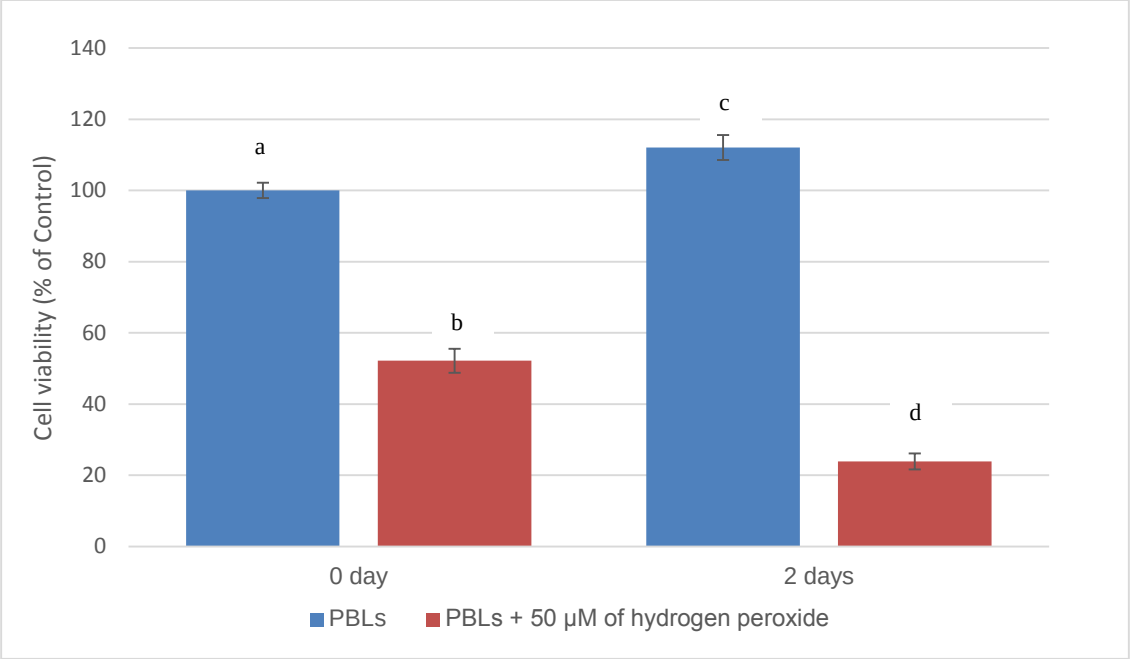


Figure 9: The effect of 50 µM H_2O_2 on PBLs after 2 days. Figures that have different letters are significantly different ($P < 0.05$). Day zero (0 day) represents the effect immediately after treatment of PBLs with 50 µM H_2O_2.

The addition of 50 µM H_2O_2 to HL-60 cell culture significantly reduced the viability of the cells on day 0 (the percentage of viability was measured immediately after treatment with 50 µM H_2O_2). In the presence of exogenous H_2O_2, the viability of H_2O_2-induced stress HL-60 cells declined by 42% after 7 days of incubation as compared to unstressed HL-60 cells ($P < 0.05$) (Figure 10).

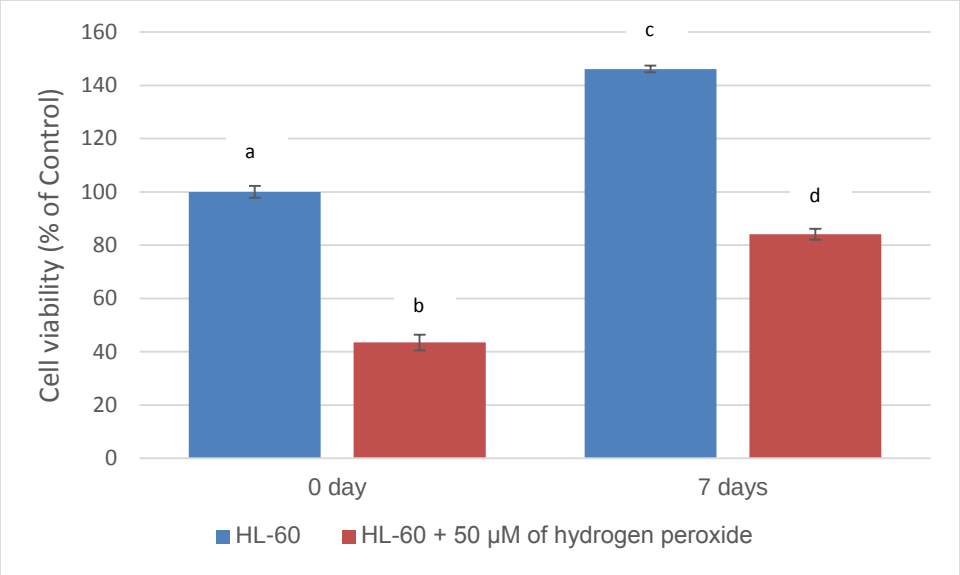


Figure 10: The effect of 50 µM H_2O_2 on HL-60 cells after 7 days. Figures that have different letters are significantly different ($P < 0.05$). Day zero (0 day) represents the effect immediately after treatment of HL-60 cells with 50 µM H_2O_2.

Treatment with 400 µg/ml *L. porteri* significantly ameliorated the adverse effects of H_2O_2 in lymphocytes and HL-60 cells (Figures 11 and 12). Treatment of PBLs with 400 µg/ml of *L. porteri* extract was most effective in boosting the cell viability when compared to other treatment groups (Figure 11). Treatment with 50 µg/ml of the extract did not ameliorate the effect of H_2O_2. Similar to the control, the cell viability in this group was reduced as much as 47% after 2 days of incubation (Figure 11).

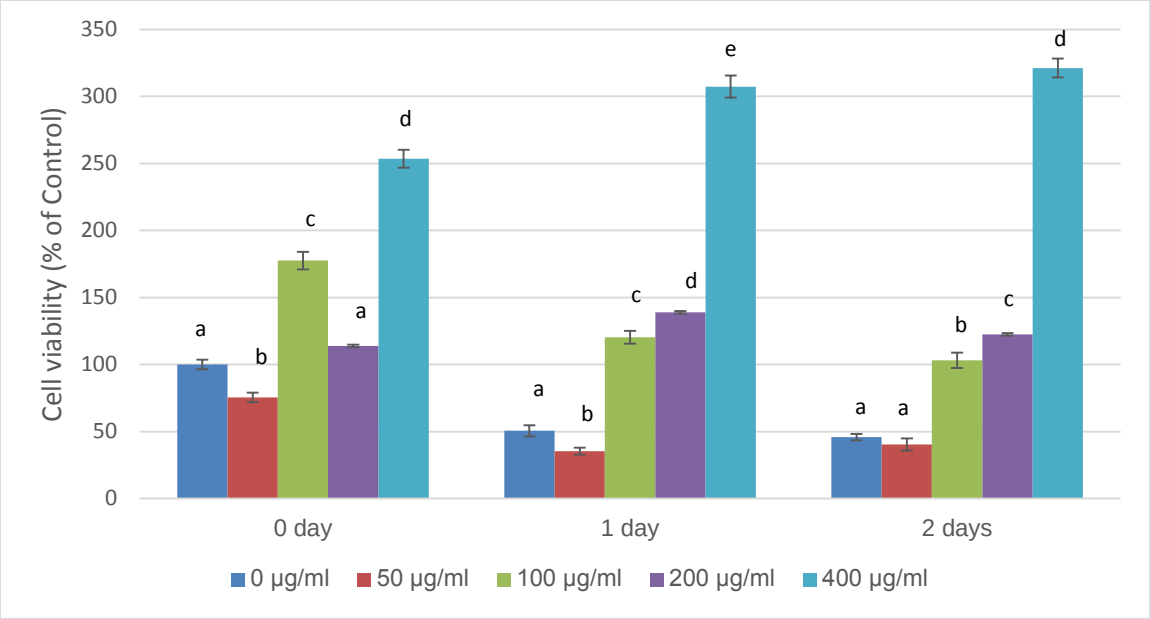


Figure 11: Change in the viability of H_2O_2-induced-stress PBLs after treatment with *L. porteri*. Figures that have different letters are significantly different ($P < 0.05$). Day zero (0 day) represents change in viability of H_2O_2-induced-stress PBLs immediately after treatment with *L. porteri*.

Only treatment with 400 µg/ml *L. porteri* was effective in boosting the HL-60 cell survival, it increased by 30% after 7 days of incubation compared to the control (Figure 12). 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* extract (Figure 12). However, the viability of HL-60 cells was seen in treated groups on day 3 and 7. It is possible that the viability of HL-60 cells on day zero 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 zero (Figure 12).

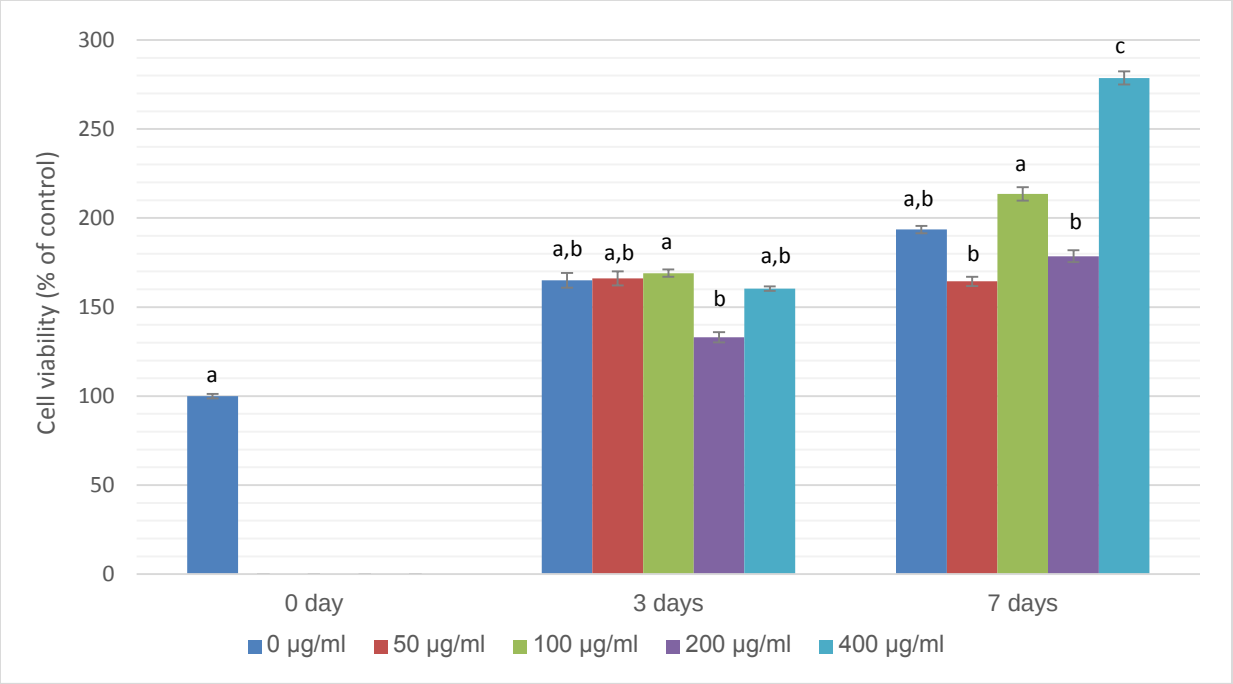


Figure 12: Change in the viability of H_2O_2-induced-stress HL-60 cells after treatment with *L. porteri*. Figures that have different letters are significantly different ($P < 0.05$). Day zero (0 day) represents the change in the viability of H_2O_2-induced-stress HL-60 cells immediately after treatment with *L. porteri*.

A low dose of intracellular H_2O_2 signals the activation of lymphocytes and fights against invading pathogens [110]. Excessive induction of H_2O_2 caused oxidative stress and impaired cell activity. A common consequence of oxidative stress is cell apoptosis which is programmed cell death. It was previously reported that 50 µM of H_2O_2 caused cellular apoptosis while the use of H_2O_2 at 500 µM induced necrosis in human T-lymphoma Jurkat cells [108]. The treatment with 50 µM of H_2O_2 for 4 hours resulted in DNA fragmentation and triggered cell death in HL-60 cells [111]. Findings from this study demonstrated for the first time that the treatments with root extracts of *L. porteri* may protect PBLs and HL-60 cells from oxidative stress caused by H_2O_2.

3.3 Effect of *L. porteri* on lipid peroxidation

Lipid peroxidation is an important indicator of cellular damage caused by oxidative stress [69]. To determine lipid peroxidation, MDA levels were assessed in H_2O_2-induced-stress PBLs and HL-60 cells after being treated for 2 days with different concentrations of *L. porteri* (50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml, and control) (Tables 3 and 4). The two-day incubation of human lymphocytes with 50 μM H_2O_2 caused a significant formation of MDA (Table 3). This high level of MDA was significantly inhibited by the supplementation of *L. porteri* extract. The inhibitory effect of *L. porteri* on lipid peroxidation was greater with increasing *L. porteri* concentrations. Treatment with 400 μg/ml *L. porteri* significantly decreased the lipid peroxidation by 94%, when compared to oxidative-stressed PBLs untreated with the root extract ($P < 0.05$). Fifty μM of H_2O_2 caused an elevation of MDA in HL-60 cells after 2 days of incubation (Table 4). The HL-60 cells treated with 100 μg/ml, 200 μg/ml, and 400 μg/ml reduced the lipid peroxidation by 12-13% in comparison with the control ($P < 0.05$).

Previous studies proposed that a mechanism to attenuate the deleterious effect of H_2O_2 is through the inhibitory effects on the levels of MDA [112]. This study revealed that the exposure of PBLs and HL-60 cells to 50 μM H_2O_2 led to a significant increase in MDA content. The improvement of H_2O_2-induced-stress cell cultures after *L. porteri* treatment suggests a protective influence of *L. porteri* against oxidative stress.

Table 3: Effects of *L. porteri* on lipid peroxidation in H_2O_2-induced-stress PBLs after 2 days.

L. porteri concentration (µg/ml)	MDA (µmol/mg protein)	Inhibition (%) by *L. porteri* treatment
0	421.69 ± 23.36^{a}	
50	337.28 ± 11.63^{b}	20.0
100	326.32 ± 14.84^{b}	22.6
200	296.73 ± 20.56^{b}	29.6
400	23.83 ± 1.91^{c}	94.3

Values that have different letter superscripts are significantly different ($P < 0.05$)

Table 4: Effects of *L. porteri* on lipid peroxidation in H_2O_2-induced-stress HL-60 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	$665.53 \pm 18.52^{a,b}$	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 ($P < 0.05$)

3.4 Effects of *L. porteri* on glutathione levels

Reduced glutathione, also known as GSH, is an important cellular antioxidant, involved in the protection against free radicals and other cytotoxic compounds. The cellular GSH content is an important determinant to regulate the redox status in cells. When the level of GSH is elevated, the oxidative stress is reduced and the level of lipid peroxidation, represented by MDA content, is low. In contrast, low levels of GSH are an indicator for the rising amount of MDA, which consequently leads to impaired cellular oxidative status [113].

This study showed that 50 μM of H_2O_2 decreased GSH levels in PBLs and HL-60 cells as compared to other treatments with the root extract (Table 5 and Table 6). When oxidative stress was induced in PBLs, the oxidation of GSH was ameliorated by treatments with increasing concentrations of *L. porteri* (Table 5). Oxidation of GSH in stressed PBLs was significantly inhibited when the cells were treated with 400 μg/ml *L. porteri*. This effect was marked by an elevation of 26.4% in GSH levels as compared to the control ($P < 0.05$). Root extract concentrations lower than 200 μg/ml reduced the GSH levels but their ameliorating effects were not as remarkable as that induced by the addition of 400 μg/ml *L. porteri* (Table 5). In stressed HL-60 cells, treatments with 200 μg/ml and 400 μg/ml inhibited the GSH decrease by 29-30% while other treatments with lower concentration showed 7-8% inhibition as compared to the control (Table 6) ($P < 0.05$).

The findings from this study indicate that the GSH levels in H_2O_2-induced-stress PBLs and HL-60 cells after treatment with *L. porteri* were significantly higher than those observed in the stressed cell cultures without *L. porteri* treatment. Previous studies suggested that oxidative stress and the deficiency of thiol compounds may be the primary cause for the development of

immune deficiencies [114-116]. Moreover, the shortage of intracellular GSH may interrupt T-cell function [117]. Depletion of GSH levels was seen in cells exposed to 50 μM H_2O_2 alone (Tables 5 and 6), which indicated increased oxidative damage in these cells. However, the supplementation with *L. porteri* at high doses significantly increased the GSH content as compared to the control. These results suggest that the *L. porteri* root extracts may be a potential antioxidant that possesses a protective effect against oxidation of GSH when the cells are exposed to oxidative stress.

Table 5: Effects of *L. porteri* on GSH content in H_2O_2-induced-stress PBLs after 2 days

L. porteri concentration (μg/ml)	GSH (μM/mg protein)	Elevation (%) by *L. porteri* treatment
0	47.8 ± 1.02^a	
50	48.48 ± 1.56^a	1.4
100	48.57 ± 2.03^a	1.6
200	50.17 ± 2.45^a	5.0
400	60.46 ± 2.84^b	26.4

Values that have different letter superscripts are significantly different ($P < 0.05$).

Table 6: Effects of *L. porteri* on GSH content in H_2O_2-induced-stress HL-60 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 ($P < 0.05$)

3.5 Effects of *L. porteri* on the activity of anti-oxidant enzymes CAT and SOD

The effects of *L. porteri* on CAT and SOD activities in PBLs and HL-60 cells after inducing oxidative stress with 50 µM H_2O_2 are shown in Tables 7-10. Fifty µM H_2O_2 caused a significant decrease in CAT and SOD activities as compared to other cell cultures treated with *L. porteri*. Treatment with the root extract significantly increased CAT and SOD activities in PBLs ($P < 0.05$) (Tables 7 and 8). The activities of CAT and SOD were increased by 55.2% and 17.5% respectively when stressed PBLs were incubated with 400 µg/ml *L. porteri* for 2 days. After HL-60 cells were exposed to oxidative damage, treatment with *L. porteri* at the concentration as low as 50 µg/ml and 100 µg/ml respectively enhanced the activities of CAT (Table 9) and SOD (Table 10) ($P < 0.05$). The anti-oxidant effect of *L. porteri* on CAT and SOD activities was greater with increasing concentrations of *L. porteri*. Results showed that CAT activity in stressed

HL-60 cells increased by 2.5-fold after treatment with *L. porteri* at a dose above 200 µg/ml (Table 9). The modulatory effect of *L. porteri* on SOD activity was seen when the root extract was above 100 µg/ml (Table 10).

CAT and SOD activity were increased in oxidative-stressed PBLs and HL-60 cells treated with the herbal extract as compared to the untreated cell cultures (Tables 7-10). In the cellular defense system against oxidative stress, these two enzymes are responsible for catalyzing the reactions to convert toxic reactive oxygen species into non-toxic compounds. The role of SOD is to convert superoxide radicals to H_2O_2 which is further degraded by catalase into water and oxygen. Thus, the activities of SOD and CAT are known to play a key role in modulating the cellular redox status [118]. The results of the present study indicate that *L. porteri* may be effective in preventing oxidative damage through increasing the activities of anti-oxidant enzymes SOD and CAT.

Table 7: Effects of *L. porteri* on anti-oxidant enzyme CAT activity (U/mg protein/min) in H_2O_2-induced-stress PBLs after 2 days.

L. porteri concentration (µg/ml)	CAT (U/mg protein/min)	(%) Increase of CAT activity by *L. porteri* treatment
0	9.33 ± 0.36^{a}	
50	11.14 ± 2.34^{b}	19.4
100	11.02 ± 1.59^{b}	18.1
200	11.48 ± 1.23^{b}	23.0
400	14.48 ± 1.41^{c}	55.2

Values that have different letter superscripts are significantly different ($P < 0.05$)

Table 8: Effects of *L. porteri* on anti-oxidant enzyme SOD activity (mU/mg protein/min) in H_2O_2-induced-stress PBLs after 2 days.

L. porteri concentration (µg/ml)	SOD (mU/mg protein/min)	(%) Increase of SOD activity by *L. porteri* treatment
0	38.05 ± 1.05^a	
50	38.05 ± 1.64^a	0
100	44.39 ± 2.09^b	16.7
200	44.39 ± 1.92^b	16.7
400	44.71 ± 1.83^b	17.5

Values that have different letter superscripts are significantly different ($P < 0.05$)

Table 9: Effects of *L. porteri* on anti-oxidant enzyme CAT activity (U/mg protein/min) in H_2O_2-induced-stress HL-60 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 ($P < 0.05$)

Table 10: Effects of *L. porteri* on anti-oxidant enzyme SOD activity (mU/mg protein/min) in H_2O_2-induced-stress HL-60 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 ($P < 0.05$)

3.6 Effects of *L. porteri* on the expression of cytokines

To determine whether the enhanced viability of PBLs and HL-60 cells were associated with cytokine production, cytokines including IL-2, IL-10, and IFN-γ were quantitated by ELISA. The total soluble cytokines were measured in the cell culture supernatants after incubation for 2 days, with or without *L. porteri* root extract treatment. Because 400 µg/ml *L. porteri* resulted in significant increases in cell viability and antioxidant levels, this concentration of *L. porteri* was used to further investigate the production of cytokines. There was no detection of cytokines in untreated cell cultures (no additives) and the group of cell culture treated with only 50 µM of H_2O_2 (Figures 13 and 14). Similarly, there was no detection of cytokines in untreated cell cultures – no additives (Figure 15). Treatment with 400 µg/ml of *L. porteri* extract induced a significant increase in IFN-γ and IL-2 levels in H_2O_2-induced-stress PBLs and HL-60 cells (Figures 13-16) ($P < 0.05$). Hydrogen peroxide (50 µM) reduced IL-10 levels in PBLs. However,

treatment with 400 µg/ml root extract did not significantly alter IL-10 levels in these stressed cells ($P > 0.05$) (Figure 17). Hydrogen peroxide (50 µM) also suppressed the production of IL-10 in stressed HL-60 cells (Figure 18). Treatment with 400 µg/ml of *L. porteri* extract showed an increase of 63% in IL-10 levels but still lower that the control (no additives), indicating that the root extract suppressed the inhibitory effect of H_2O_2 (Figure 18).

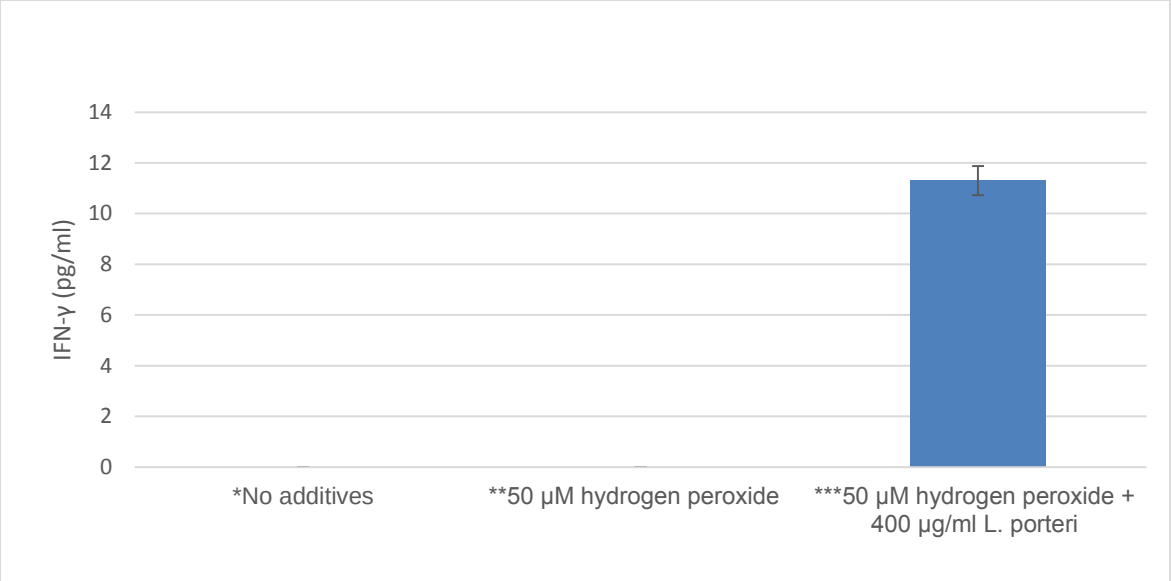


Figure 13: Change in the levels of IFN-γ induced by PBLs after treatment with 400 µg/ml *L. porteri*for 2 days. This valueis significantly different from other group treatments ($P < 0.05$).*No additives represents a cell culture that was not treated with 50 µM H_2O_2 and 400 µg/ml *L. porteri*. **50 µM H_2O_2 represents a cell culture that was treated with only 50 µM H_2O_2. ***50 µM hydrogen peroxide + 400 µg/ml *L. porteri* represents a cell culture that was treated with both 50 µM H_2O_2 and 400 µg/ml *L. porteri* extract.

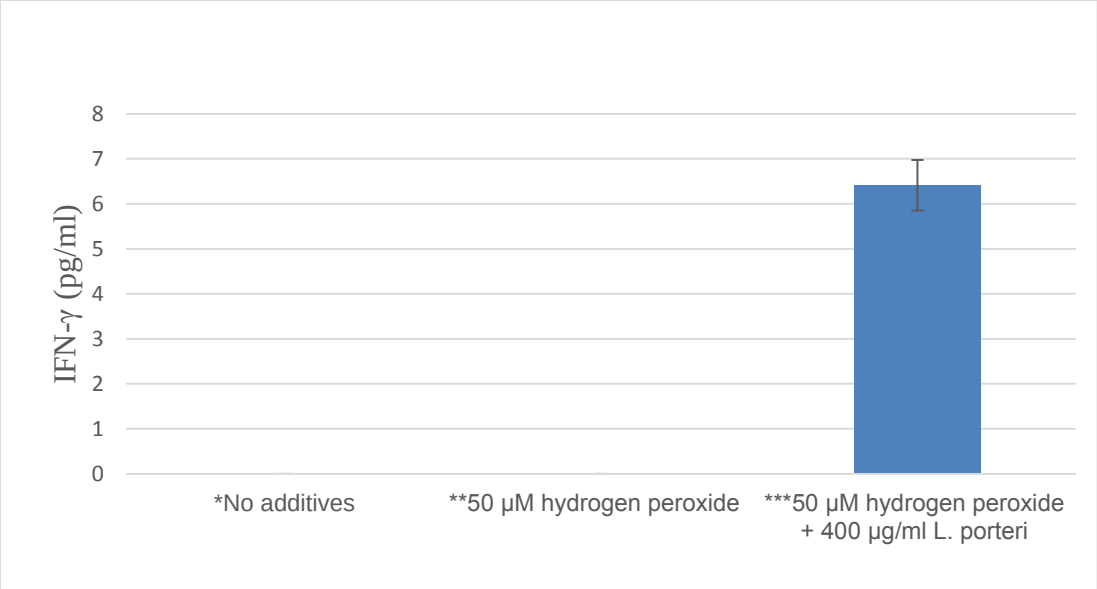


Figure 14: Change in the levels of IFN-γ induced by HL-60 cells after treatment with 400 µg/ml *L. porteri* for 2 days. This value is significantly different from other group treatments ($P < 0.05$). *No additives represents a cell culture that was not treated with 50 µM H_2O_2 and 400 µg/ml *L. porteri*. **50 µM H_2O_2 represents a cell culture that was treated with only 50 µM H_2O_2. ***50 µM hydrogen peroxide + 400 µg/ml *L. porteri* represents a cell culture that was treated with both 50 µM H_2O_2 and 400 µg/ml *L. porteri* extract.

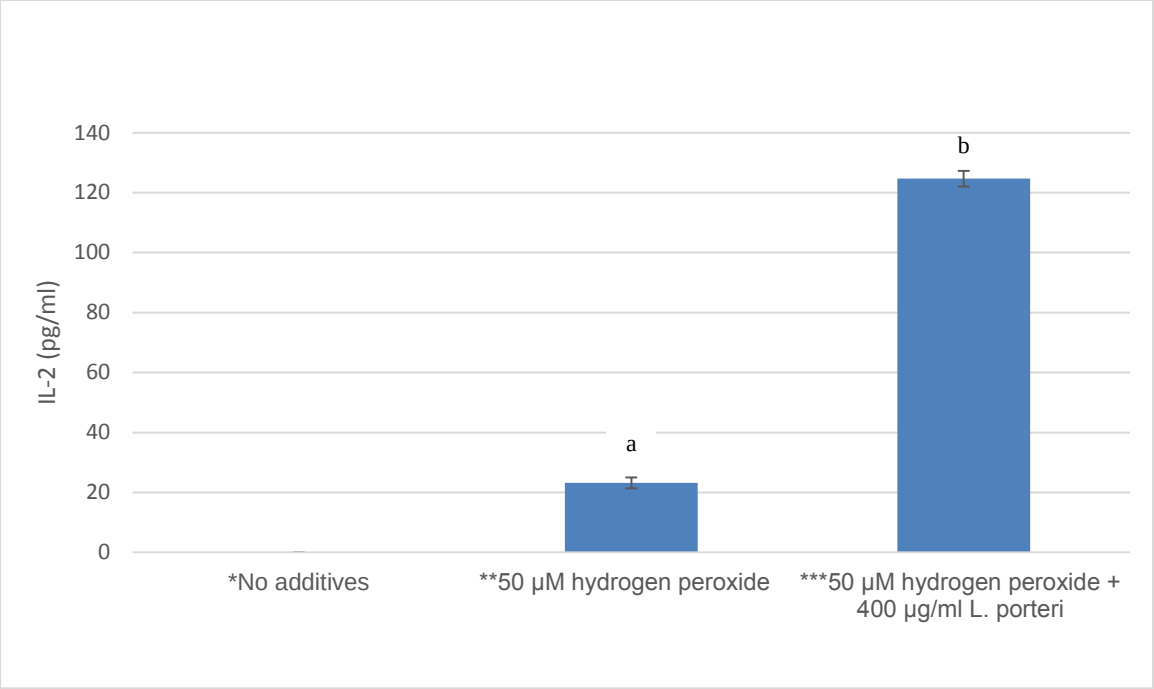


Figure 15: Change in the levels of IL-2 induced by PBLs after treatment with 400 µg/ml *L. porteri* for 2 days. Values that have different letters are significantly different from other group treatments ($P < 0.05$). *No additives represents a cell culture that was not treated with 50 µM H_2O_2 and 400 µg/ml *L. porteri*. **50 µM H_2O_2 represents a cell culture that was treated with only 50 µM H_2O_2. ***50 µM hydrogen peroxide + 400 µg/ml *L. porteri* represents a cell culture that was treated with both 50 µM H_2O_2 and 400 µg/ml *L. porteri* extract.

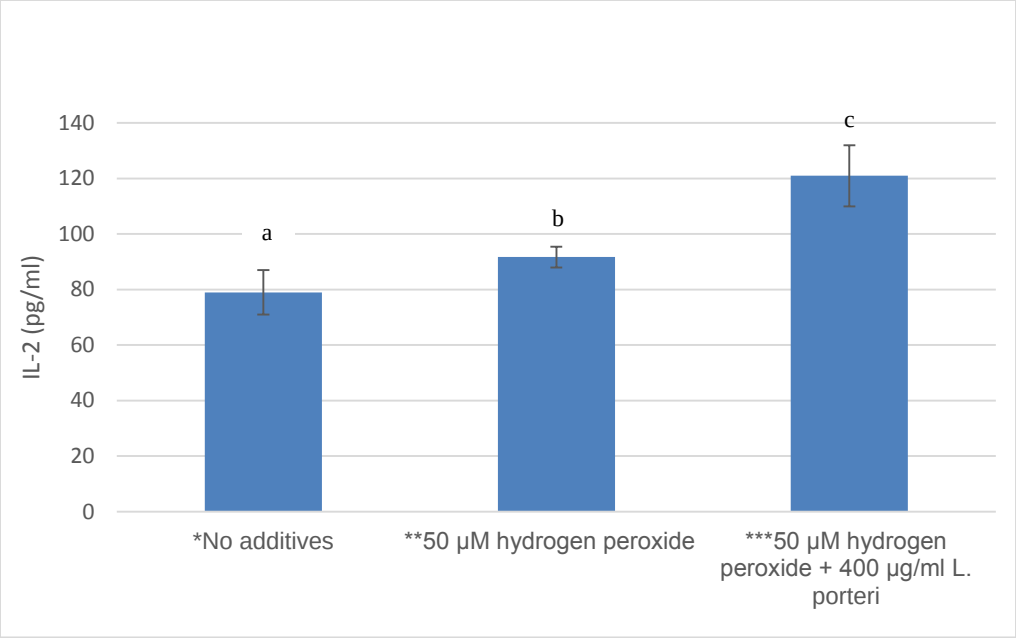


Figure 16: Change in the levels of IL-2 induced by HL-60 cells after treatment with 400 µg/ml *L. porteri* for 2 days. Figures that have different letters are significantly different ($P < 0.05$). *No additives represents a cell culture that was not treated with 50 µM H_2O_2 and 400 µg/ml *L. porteri*. **50 µM H_2O_2 represents a cell culture that was treated with only 50 µM H_2O_2. ***50 µM hydrogen peroxide + 400 µg/ml *L. porteri* represents a cell culture that was treated with both 50 µM H_2O_2 and 400 µg/ml *L. porteri* extract.

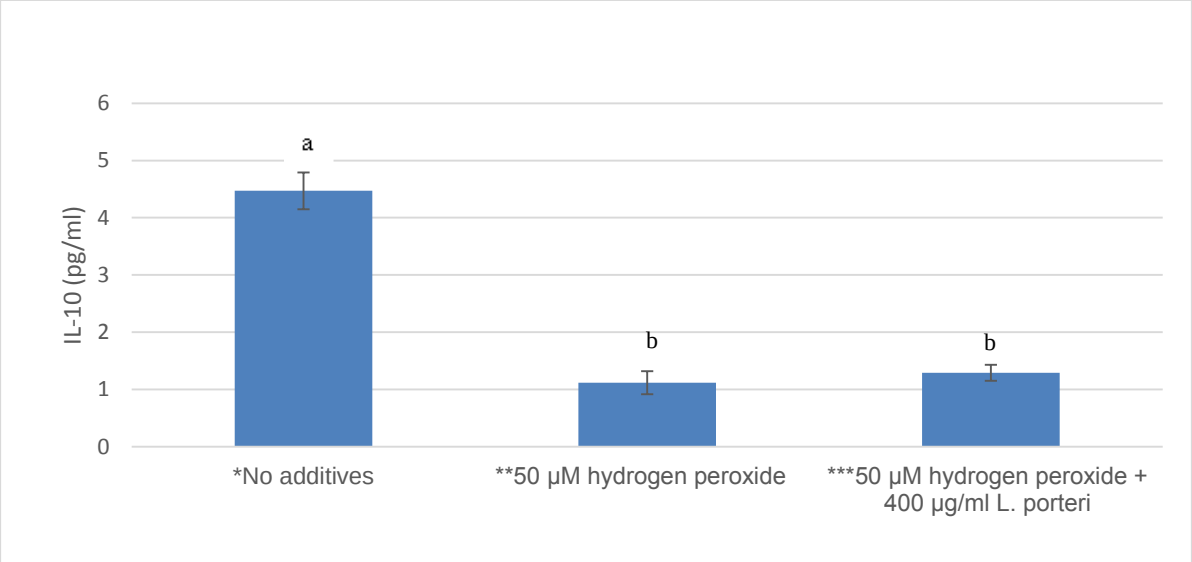


Figure 17: Change in the levels of IL-10 induced by PBLs after treatment with 400 µg/ml *L. porteri*for 2 days. Figures that have different letters are significantly different ($P < 0.05$).*No additives represents a cell culture that was not treated with 50 µM H_2O_2 and 400 µg/ml *L. porteri*. **50 µM H_2O_2 represents a cell culture that was treated with only 50 µM H_2O_2. ***50 µM hydrogen peroxide + 400 µg/ml *L. porteri* represents a cell culture that was treated with both 50 µM H_2O_2 and 400 µg/ml *L. porteri* extract.

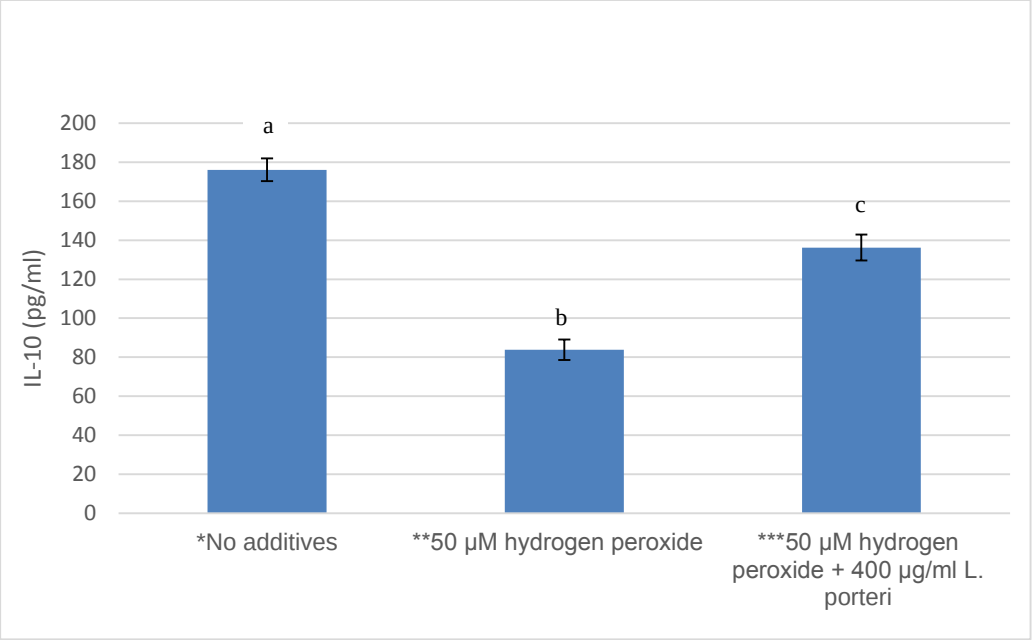


Figure 18: Change in the levels of IL-10 induced by HL-60 cells after treatment with 400 µg/ml *L. porteri*for 2 days. Figures that have different letters are significantly different ($P < 0.05$). *No additives represents a cell culture that was not treated with 50 µM H_2O_2 and 400 µg/ml *L. porteri*. **50 µM H_2O_2 represents a cell culture that was treated with only 50 µM H_2O_2. ***50 µM hydrogen peroxide + 400 µg/ml *L. porteri* represents a cell culture that was treated with both 50 µM H_2O_2 and 400 µg/ml *L. porteri* extract.

The root extract of *L. porteri* has been commonly used in traditional Native American medicine to treat inflammation and respiratory infectious diseases [3]. Data from this study showed that the ethanolic root extract of *L. porteri* enhanced cell viability in PBLs and HL-60 cells. The underlying mechanism might be through the herb's ability to stimulate the expression of IL-2, IL-10, and IFN-γ in these cells. It has been proposed that most cytokines are expressed transiently and could be produced or prohibited by other cytokines. It is the "cytokine network" that determines which cytokines regulate the others. The IL-2 and IFN-γ are pro-inflammatory cytokines [74]. In this study, their production was increased after PBLs and HL-60 cells were

challenged with H_2O_2, indicating that the inflammatory response was stimulated in these cells due to stress damage. Interleukin-10 is an anti-inflammatory cytokine, mainly secreted by Th2 lymphocytes. The induction of IL-10 is believed to inhibitor suppress the activity of other pro-inflammatory cytokines and down-regulate eosinophil activity [117]. It was shown that due to the treatment of 50 μM H_2O_2, the cellular balance between pro-inflammation and anti-inflammation was impaired. The imbalance was marked by low amounts of induced IL-10 and high amounts of IL-2 and IFN-γ secreted after exposure to the stress. The root extract *L. porteri* used at 400 μg/ml rendered a mild anti-inflammatory response toward the H_2O_2-induced stress.

The secretion of IFN-γ, IL-2, and IL-10 plays an important role in the activation and proliferation of immune cells. It has been known that IL-2 is a key cytokine in stimulating T-cell proliferation, cytokine production, and functions of B cells, macrophages, and Natural Killer cells [119]. The IFN-γ is known as a major pro-inflammatory cytokine [120] and it is exclusively produced by activated lymphocytes and Natural Killer cells in the adaptive immune response [121]. Upon secretion, IFN-γ could up-regulate a number of lymphoid cell functions; as well as it could exert strong regulatory influences on the proliferation, differentiation, and effect or responses of B cell and T cell subsets [122]. The production of IL-2 and IFN-γ in stressed PBLs and HL-60 cells were increased by the treatment with 400 μg/ml *L. porteri* as compared to the control ($P < 0.05$). This study showed that IL-2 was significantly up-regulated after a 2-day incubation with 400 μg/ml *L. porteri* 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* (11.3 pg/ml and 6.41 pg/ml in Figure 13 and Figure 14, respectively), there was a significant increase in IFN-γ production when compared to the control ($P < 0.05$). Zhou *et al.* reported that IL-10 generates T-cell tolerance [123]. This cytokine (IL-10) is released in order to balance the

dramatic increase in pro-inflammatory cytokines in stressful situations, and therefore it could control the intensity and duration of the inflammatory response [124].

4. CONCLUSIONS

This study is the first to report the medicinal effects of *L. porteri* on peripheral blood lymphocytes and HL-60 cells. The herb has been used in traditional medicine for years but its acclaimed effects remain unknown. Today, due to the rising cost of pharmaceuticals, it is essential to investigate herbal remedies which are affordable and efficient in treating diseases. This study identified three properties of *L. porteri*: the proliferative effect, the antioxidant effect and the immune-modulatory effect on human lymphocytes and HL-60 cells.

Data suggests that the applications of ethanolic root extract of *L. porteri* at concentrations as high as 400 μg/ml increased the viability of human lymphocytes and HL-60 cells. Addition of *L. porteri* at 400 μg/ml increased the viability of lymphocytes and HL-60 cells by 1.5 and 2.5 times. The above enhancement of the viability of normal PBLs by *L. porteri* root extract may be beneficial to boost the immune system. However, the observed proliferative effect of *L. porteri* extract on HL-60 cells may not be advantageous due to the malignant nature of the cells. The stress-induction by 50 μM H_2O_2 significantly inhibited the growth of cell cultures. However, the addition of 400 μg/ml *L. porteri* reduced the effect of H_2O_2.

Exposure to 50 μM H_2O_2 also resulted in the elevation of MDA, depletion of GSH levels, and decreased activities of SOD and CAT. These changes were indicative of increased oxidative damage in these cells. However, the addition of the root extract at concentrations as low as 100 μg/ml significantly increased GSH levels, reduced MDA formation, and increased activities of SOD and CAT. These changes suggest that *L. porteri* root extracts may be protective against

H_2O_2-induced cytotoxicity in PBLs and HL-60 cells through reducing the lipid peroxidation, and increasing both the non- enzymatic and enzymatic antioxidant indices.

Another noteworthy finding of this study was the stimulation of cytokines in H_2O_2-challenged PBLs and HL60 after these stressed cells were treated with 400 μg/ml of *L. porteri* root extract. The activity and expression of IFN-γ and IL-2 were up-regulated significantly as compared to stressed cells that were not treated with the root extract. Data from this study showed that *L. porteri* may be a potential immune-modulating agent that may accrue some health benefits.

Recommendations for further study:

1) Study the cytotoxicity of *L. porteri* at concentrations higher than 400 µg/ml.
2) Study the cell viability at shorter time intervals.
3) Determination of the active compounds present in the root extract
4) Evaluations of oxidative damage by different concentrations of H_2O_2 to validate the intensity of oxidative damage to the cell culture.
5) Study the presence of ROS in the cell cultures after being exposed to stress stimulator to further identify how the cells metabolically respond in stressful condition.
6) Determination of other cytokines to better understand the cytokine network induced by these immune cells and the effects of the extract.
7) Determine whether the extract chemically interacts with GSH in cells or the H_2O_2 added to cell cultures.

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