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# EFFECT OF KOREAN WINE WASTE EXTRACTION ON NITRIC OXIDE AND CYTOKINE PRODUCTION IN LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION

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**ABSTRACT**

The effect of extract of Korean wine waste on the production of nitric oxide (NO) and cytokines in splenocytes and RAW 264.7 cells was investigated using lipopolysaccharide (LPS) as a stimulant. Treatment of RAW 264.7 cells with the 85% aqueous methanol (MeOH) fraction from Korean wine waste reduced LPS-induced NO production in a dose-dependent manner ($p$<0.05). Culture supernatants of splenocytes exposed to a co-treatment of 85% aq. MeOH fraction and LPS were harvested to determine the production levels of cytokines [IL-2, IL-4, IL-5, IL-6, IL-12/IL-23(p40) and IFN-γ]. As measured by ELISA, co-administration of 85% aq. MeOH fraction (10 μg/mL) with LPS significantly reduced the IL-2 expression after 48 h of treatment, as compared to treatment with only LPS ($p$<0.05), where the level of IL-6 expression was significantly lower after 24 h of the co-treatment ($p$<0.05). The IL-12/IL-23(p40) expression levels after 24 and 72 h of co-treatment were also significantly lower than that from LPS treatment only ($p$<0.05). However, the IL-4 and IFN-γ expression levels were significantly lower only after 72 h of co-treatment. These results indicate that the 85% aq. MeOH fraction from Korean wine waste decreased the production of NO as well as the expression of the pro-inflammatory cytokines IL-2, IL-6 and IL-12/IL-23(p40).

Keywords: Korean wine waste; nitric oxide; interleukin-2; interleukin-6; interleukin-IL-12/IL-23(p40).

## INTRODUCTION

Wine production entails the generation of huge amounts of by-products that consist mainly of organic wastes, wastewater, greenhouse gas emissions, and inorganic residues (Musse et al. 2007). By-products derived from the wine-making process also contain a high amount of secondary metabolites including phenolic acids, flavanols, proanthocyanidins, flavonols, anthocyanins, and stilbenes (Corrales et al. 2008). Several studies have reported high antioxidant activities of these by-products, based on the polyphenolic content, suggesting winery-derived grape pomace to be an interesting source for natural antioxidants with application in the pharmacological, cosmetic, and food industries (Boussetta et al. 2009; Rockenbach et al. 2011). Moreover, grape seed extracts and their constituents have

been proven to prevent inflammation, and have demonstrated protective effects on chemical-induced ulcerative colitis in rats (Mitjans et al. 2004; Cheah et al. 2013). Additionally, chronic inflammation causes an elevation of oxid ative stress in the affected tissues, which in turn, enhances the inflammatory response by activating redox-sensitive nuclear transcription factors (Alexander, 1995). Since grape pomace contains significant amounts of antioxidants (e. g. anthocyanins, catechin, epicatechin, quercetin, and a few phenolic acids), this material has been suggested as being beneficial for the prevention of oxidative stress and inflammatory conditions (Teixeria et al. 2014).

Korean domestic wines are mostly fermented using Campbell Early grapes (*Vitis labrusca* cultivar Campbell Early) since the variety constitutes more than 70% of the total grape production in Korea (Kim, 2005). However, little information as to the anti-inflammatory effect of Korean wine waste extracts has been reported. Our previous studies demonstrated that an 85% aqueous methanol (aq. MeOH) fraction from Korean wine waste, which contained higher levels of flavonoids and phenols, had both higher antioxidant (Baek and Lim, 2016) and anti-proliferative (Baek and Lim, 2017) effects than those observed with other fractions. Thus, in this study we have extended these previous results by investigating the effect of this 85% aq. MeOH fraction from Korean wine waste on nitric oxide (NO) and cytokine production in lipopolysaccharide (LPS)-induced inflamma-tion in macrophages and splenocytes, in order to evaluate the anti-inflammatory effect of wine waste.

## MATERIALS AND METHODS

### Materials and Cell Culture

6-week-old male C57BL6 mice (weighing approximately 25 g) were purchased from Daehan BioLink (Eumsong, Chungcheongbukdo, South Korea). Lipopolysaccharide (LPS), RPMI 1640, fetal bovine serum (FBS), phosphate buffer saline (PBS) and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank. The cells were maintained at 37$^{o}$C under 5% $CO_2$ in DMEM containing 10% FBS and 100 units/mL penicillin-streptomycin. Mouse enzyme-linked immunosorbant assay kits purchased from Biolegent (San Diego, CA, USA) were used to measure the expression of cytokines (IL-2, IL-4, IL-5, IL-6, IL-12/IL-23(p40), IFN-γ).

### Sample Extracts and Fractions

Korean red wine waste was collected in a wine farm named Cave Story (Yeoungcheon, Korea). Samples of Korean red wine waste were dried and sequentially extracted twice with acetone/methylene chloride (A+M) and methanol (MeOH) to obtain the maximum amount of extracts (Bae et al. 2014). Then the combined crude extracts were fractionated with *n*-hexane and 85% aqueous MeOH, and the aqueous layer was also further fractionated with *n*-butanol (*n*-BuOH) and water, resulting in the *n*-hexane, 85% aqueous MeOH, *n*-BuOH and distilled water fractions. The 85% aqueous MeOH fraction was concentrated to dryness at 40℃ using rotary vacuum evaporator (N-100, EYELA, Japan) and the residue was kept at 4$^{o}$C until analysis and assay. The 85% aqueous MeOH fraction was dissolved in DMSO.

### Measurement of Nitric Oxide Concentration

RAW 264.7 cells (5 x 10 cells/ mL/ well) suspended in a 96-well microplate. The cells were incubated at 37$^{o}$C under 5% $CO_2$ in

DMEM containing 10% FBS and 100 units/mL penicillin-streptomycin for 24 h. After changing with cell medium, samples were treated and incubated for 1 h. Then LPS (1 ppm) was treated to induce NO production and incubated for 48 h. NO concentration was measured by using Griess reagent as described (Green et al. 1982). Briefly, culture supernatants (50 μl) were mixed with 50 μl of the Griess reagent, and the NO concentration in the culture supernatant was measured 10 min after mixing at an absorbance of 570 nm. NO concentration was determined with reference to the standard curve using sodium nitrite.

**Spleen Cell Culture Supernatants**

Cell counts within spleen cell suspensions were adjusted to 2 x $10^6$ cells/mL using RPMI 1640 medium with 10% FBS, and the adjusted suspensions were distributed into 24-well tissue culture plates (Costar, Cambridge, MA, USA) at 1 mL/well. The cells were treated with sample and incubated at 37$^o$C in a humidified atmosphere of 5% carbon dioxide. Concomitant treatment with sample and LPS also was conducted. As a control, cells were treated with treated with 0.01% DMSO. Cells were incubated for 6, 24, 48, 72 h before collection. The suspensions then underwent initial centrifugation at 300 x g for 10 min and secondary centrifugation at 1000 x g for 30 min. The supernatants were stored at -70$^o$C until use in analysis of cytokine expression (Hwang et al. 2004). We measured the expression of IL-2, IL-4, IL-5, IL-6, IL-12/IL-23(p40) and IFN-γ in samples collected from 6, 24, 48, 72 h treatments.

**Measurement of Cytokines**

Capture antibodies for IL-2, IL-4, IL-5, IL-6, IL-12/IL-23(p40) and IFN-γ were diluted with coating buffer, loaded into 96-well plates at 100 μg/well, and incubated at 4$^o$C overnight. The wells were washed with washing buffer 4 times, and 200 μL of assay diluents was added to each well. The plates were then incubated at room temperature for 1 h. The wells then were washed with washing buffer 4 times again, and 100 μL of the sample was added to each well. The plates were then incubated at room temperature for 2 h. After washing 4 times, 100 μL of the detection antibody for the appropriate cytokine was added to each well, and the plates were incubated at room temperature for 1 h. The wells were washed with washing buffer 4 times, 100 μL of avidin-horseradish peroxides was added to each well, and plates were incubated at room temperature for 30 min. After washing 5 times with washing buffer, 100 μL of substrate fluid containing tetramethylbenzidine was added, and the plates were incubated at room temperature for 20 min. 100 μL of stop solution was added to stop reaction, and the optical density was measured at 450 nm using ELISA reader (Model 550 microplate reader, Bio-Rad, Richmond, VA, USA) (Kim et al. 2001).

**Statistical Analysis**

Data were presented as mean ± standard deviation (SD). To determine normal distribution, Kolmogorove-Smirnov test was done. Significance of differences observed between the control and experiment groups using Student's *t* test at $p<0.05$. Analyses were conducted using STATISTICA package.

**RESULTS**

**Effect of 85% aq. MeOH Fraction from Korean Wine Waste on LPS-induced NO Production**

The effect of the 85% aq. MeOH fraction from Korean wine waste on LPS-induced

NO production was examined (Fig. 1). Whereas LPS induced a high level of NO production in RAW 264.7 cells, the addition of 85% aq. MeOH fraction reduced the LPS-induced NO production in a dose-dependent manner ($p$<0.05). Treatment with 2 mg/ mL of 85% aq. MeOH fraction resulted in 53% inhibition of the LPS-induced NO production.

**Effect of 85% aq. MeOH Fraction from Korean Wine Waste on Cytokine Expression**

Changes in the expression of cytokines [IL-2, IL-4, IL-5, IL-6, IL-12/IL-23(p40) and interferon-gamma (IFN-γ)] were investigated upon treatment of mouse splenocyte suspensions with LPS- only or with co-administration of LPS and different concentrations of the 85% aq. MeOH fraction from Korean wine waste. Table 1 shows the modulation of IL-2 expression induced by the 85% aq. MeOH fraction. Co-administration of 10 µg/mLof the fraction with LPS significantly reduced IL-2 expression after 48 h of treatment, compared with that from LPS treatment only ($p$<0.05). As shown in Table 2, the use of LPS as a stimulant increased IL-5 expression in a time-dependent manner. Co-administration of 3 µg/mL of 85% aq. MeOH fraction with LPS resulted in a significantly higher IL-5 expression after 48 h than that observed with LPS treatment alone ($p$<0.05). Table 3 shows the effects of the 85% aq. MeOH fraction on IL-6 expression. Co-administration of 10 µg/mL of 85% aq. MeOH fraction with LPS significantly reduced IL-6 expression after 24 h of treatment, relative to that from LPS treatment only ($p$<0.05), whereas a significant increase was observed at 6 and 72 h of co-treatment with 3 µg/mL of 85% aq. MeOH fraction and LPS ($p$<0.05). Co-administration of 10 µg/mL of 85% aq. MeOH fraction with LPS significantly decreased IL-12/IL-23(p40) expression after 24 and 72 h of treatment, as compared with treatment with LPS only ($p$<0.05) (Table 4). However, co-treatment with 3 µg/mL of the fraction and LPS significantly increased the IL-12/IL-23(p40) expression level after 6 h ($p$<0.05) relative to that from LPs treatment alone. Table 5 shows the expression of IL-4 induced by 85% aq. MeOH fraction from Korean wine wastes. Co-administration of 10 µg/mLof 85% aq. MeOH fraction with LPS resulted in significantly lower IL-4 expression than that observed with LPS alone after 72 h of treatment ($p$<0.05). As shown in Table 6, 10 µg/mL of the fraction with LPS significantly decreased the IFN-γ expression level after 72 h ($p$<0.05) relative to that from LPS treatment alone.

**Table 1. Effect of 85% aqueous MeOH fraction from wine waste on the production of lipopolysaccaride (LPS) induced interleukin-2 at different times in mouse spleen cells**

| Samples (µg/mL) | Concentrations (pg/mL) | | | |
|---|---|---|---|---|
| | 6 hr | 24 hr | 48 hr | 72 hr |
| LPS | 0.29±0.06 | 0.33±0.12 | 0.37±0.06 | 0.25±0.12 |
| LPS + 85% aq. MeOH 3 | 0.08±0.12 | 0.37±0.47 | 0.29±0.06 | 0.17±0.00 |
| LPS + 85% aq. MeOH 10 | 0.08±0.12 | 0.29±0.06 | 0.21±0.06* | 0.08±0.00 |

*The values were expressed as the mean ± SD and*
**significantly different between the control and each treatment at p<0.05*

**Table 2. Effect of 85% aqueous MeOH fraction from wine waste on the production of lipopolysaccaride (LPS) induced interleukin-5 at different times in mouse spleen cells**

| Samples (µg/mL) | Concentrations (pg/mL) | | | |
|---|---|---|---|---|
| | 6 hr | 24 hr | 48 hr | 72 hr |
| LPS | 0.10±0.14 | 0.27±0.10 | 0.31±0.05 | 0.31±0.05 |
| LPS + 85% aq. MeOH 3 | 0.17±0.05 | 0.41±0.00 | 0.48±0.00* | 0.61±0.10 |
| LPS + 85% aq. MeOH 10 | 0.20±0.10 | 0.44±0.05 | 0.31±0.05 | 0.20±0.00 |

*The values were expressed as the mean ± SD and *significantly different between the control and each treatment at p<0.05*

**Table 3. Effect of 85% aqueous MeOH fraction from wine waste on the production of lipopolysaccaride (LPS) induced interleukin-6 at different times in mouse spleen cells**

| Samples (µg/mL) | Concentrations (pg/mL) | | | |
|---|---|---|---|---|
| | 6 hr | 24 hr | 48 hr | 72 hr |
| LPS | 0.99±0.20 | 1.56±0.20 | 2.08±0.27 | 1.75±0.33 |
| LPS + 85% aq. MeOH 3 | 1.56±0.20* | 1.75±0.07 | 2.41±0.07 | 3.02±0.13* |
| LPS + 85% aq. MeOH 10 | 0.99±0.07 | 1.09±0.20* | 1.18±0.07 | 1.13±0.00 |

*The values were expressed as the mean ± SD and *significantly different between the control and each treatment at p<0.05*

**Table 4. Effect of 85% aqueous MeOH fraction from wine waste on the production of lipopolysaccaride (LPS) induced interleukine-12/ interleukine-23(p40) at different times in mouse spleen cells**

| Samples (µg/mL) | Concentrations (pg/mL) | | | |
|---|---|---|---|---|
| | 6 hr | 24 hr | 48 hr | 72 hr |
| LPS | 1.16±0.07 | 1.81±0.07 | 2.09±0.20 | 2.70±0.26 |
| LPS + 85% aq. MeOH 3 | 1.72±0.07* | 2.60±0.79 | 2.28±0.33 | 2.28±0.07 |
| LPS + 85% aq. MeOH 10 | 1.67±0.13* | 1.63±0.07* | 2.23±0.00 | 1.58±0.13* |

*The values were expressed as the mean ± SD and *significantly different between the control and each treatment at p<0.05*

**Table 5. Effect of 85% aqueous MeOH fraction from wine waste on the production of lipopolysaccaride (LPS) induced interleukin-4 at different times in mouse spleen cells**

| Samples (µg/mL) | Concentrations (pg/mL) | | | |
|---|---|---|---|---|
| | 6 hr | 24 hr | 48 hr | 72 hr |
| LPS | 0.34±0.36 | 0.56±0.18 | 0.94±0.36 | 0.77±0.12 |
| LPS + 85% aq. MeOH 3 | 0.26±0.12 | 0.73±0.42 | 0.56±0.18 | 0.47±0.06 |
| LPS + 85% aq. MeOH 10 | 0.34±0.24 | 0.56±0.18 | 0.51±0.12 | 0.43±0.12* |

*The values were expressed as the mean ± SD and *significantly different between the control and each treatment at p<0.05*

**Table 6. Effect of 85% aqueous MeOH fraction from wine waste on the production of lipopolysaccaride (LPS) induced interferone-γ at different times in mouse spleen cells**

| Samples (µg/mL) | Concentrations (pg/ mL) | | | |
|---|---|---|---|---|
| | 6 hr | 24 hr | 48 hr | 72 hr |
| LPS | 29.27±13.80 | 51.22±3.45 | 70.73±24.15 | 51.22±10.35 |
| LPS + 85% aq. MeOH 3 | 24.39±6.90 | 65.85±44.84 | 39.02±20.70 | 26.83±17.25 |
| LPS + 85% aq. MeOH 10 | 48.78±0.00 | 58.54±6.90 | 51.22±17.25 | 27.97±6.90* |

*The values were expressed as the mean ± SD and *significantly different between the control and each treatment at p<0.05*

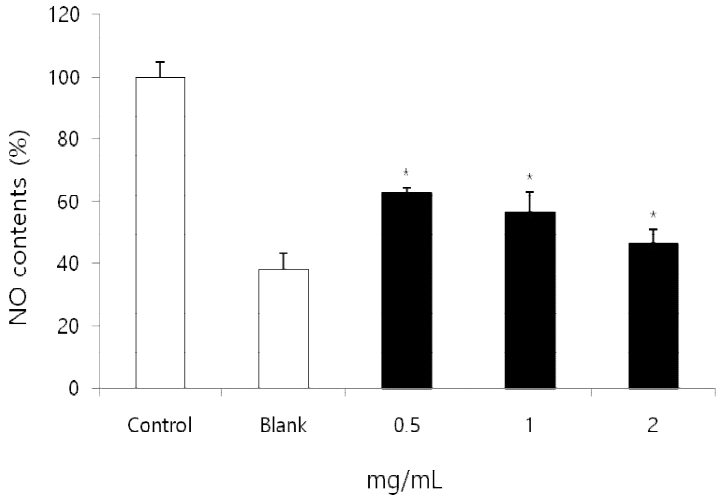


**Fig. 1. Effect of 85% aqueous MeOH fraction from wine waste on the production of nitric oxide (NO) in RAW 267.4 cells**

**significantly different between the control and each treatment at p<0.05*

## DISCUSSION

Wine by-product represents approximately 20% of the weight of the grapes that are processed, and it is already known that a large range of products can be recovered from these by-products, such as phenolic compounds, grape seed oil and dietary fiber. These by-products are known to have important roles in protection against lipid oxidation and degenerative diseases like cancer and atherosclerosis, and in inflammation processes. In the present study, we have provided evidence that the 85% aq. MeOH fraction from Korean wine waste decreases the production of NO as well as the expression of pro-inflammatory cytokines in splenocytles and macrophages. NO is an important regulatory and effector molecule with a variety of biological functions (Magazine, 1995; MacMicking et al. 1997; Bredt, 1999). Its overproduction has been associated with oxidative stress (Sies and Mehlhorn, 1986) and with the pathophysiology of various diseases such as arthritis, diabetes, stroke, septic shock, autoimmune disease, and chronic inflammation (Monaca et al. 1991; Arteel et al. 1999). Thus, the determination of foods and beverages with the potential to decrease NO has become increasingly important. Rebelo et al. (2014) demonstrated that phenolic extracts of Duuro wine exerted a dose-dependent decrease of NO production in RAW 264.7 macrophages. Kamap et al. (2000) observed the antioxidants of wine behaved as modulators of NO production. Qureshi et al. (2012) found that resveratrol and pterostilbene, which are present in grapes, blueberries, and red wine, inhibited the production of NO and also significantly suppressed the expression of tumor necrosis factor-alpha (TNF-α), IL-1β, IL-6, and inducible nitric oxide synthase genes in LPS-stimulated RAW 264.7 cells.

Cytokines regulate the growth and differentiation of different lymphocyte subsets and they activate and regulate cells

that participate in the inflammatory response. IL-12/IL-23(p40) has been implicated as key mediators of immune-mediated inflammatory diseases and multiple sclerosis (Wang et al. 2004). Since grape pomace contains significant amounts of anthocyanins, catechin, epicatechin, quercetin, and a few phenolic acids, this material has been suggested to be beneficial for the prevention of oxidative stress and inflammatory conditions (Teixeria et al. 2014). Recently, there have been accumulating reports on the anti-inflammatory effect of grape wastes including seeds, although little information on wine wastes is available. Nunes et al. (2013) reported that a red wine extract suppressed cytokine-induced IκB degradation and IL-8 production in a dose-dependent manner. Bognar et al. (2013) demonstrated that malvidin, the most abundant polyphenol ingredient of red wine, induced a series of cellular and signaling events that protected LPS-stimulated cells against inflammation-mediated chronic conditions. Cho et al. (2009) reported that grape seed proanthocyanidin extract treatment significantly reduced the numbers of TNF-α- or IL-17- producing cells in the synovial tissue, with the spontaneous production of TNF- α and IL-17 by splenocytes compared with that in control mice. Mukherjee et al. (2012) found that treatment with grape skin or grape flesh significantly increased the IL-4 level, but reduced TNF-α, IFN-γ, transforming growth factor and vascular endothelial growth factor-A compared to the ethanol-treated group. Hogan et al. (2010) found that dietary supplementation with 250 mg grape pomace/kg per day for 12 weeks showed significant anti-inflammatory effects in obese mice. Our results indicated that the 85% aq. MeOH fraction from Korean wine waste decreased the expression of the pro-inflammatory cytokines IL-2, IL-6, and IL-12/IL-23(p40). Our previous study had shown that the 85% aq. MeOH fraction contained higher contents of total flavonoids and phenols than that in other fractions, suggesting that the anti-inflammatory ability of Korean wine waste might be related to these phenolic compounds (Baek and Lim, 2016). Thus the recycling of winery co-products or side streams constitutes an opportunity for providing valuable materials to the pharmaceutical, cosmetic nutraceutical, and food industries, contributing to reductions in both the costs and environmental impact linked to the disposal of these by-products in the production areas. These results are is in accordance with published effects of phenolic compounds, reinforcing the beneficial health effects of wine wastes.

## CONCLUSION

Wine by-product represents approximately 20% of the weight of the grapes that are processed, and it is already known that a large range of products can be recovered from these by-products, such as phenolic compounds, grape seed oil and dietary fiber. These by-products are known to have important roles in protection against lipid oxidation and degenerative diseases like cancer and atherosclerosis, and in inflammation processes. In the present study, we have provided evidence that the 85% aq. MeOH fraction from Korean wine waste decreases the production of NO as well as the expression of pro-inflammatory cytokines in splenocytles and macrophages. These results are is in accordance with published effects of phenolic compounds, reinforcing the beneficial health effects of wine wastes.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

## References

Alexander, R. W. (1995). Theodore cooper memorial lecture. Hypertension and the pathogenesis of atherosclerosis: Oxidative stress and the mediation of arterial inflammatory response: A new perspective. Hypertension. 25:155-161.

Arteel, G. E., Briviba, K. and H. Sies. (1999). Protection against peroxynitrite. FEBS Lett. 445:226-230.

Bae, J. H., Jang, J. R. and Lim S. Y. (2014). Production of glutathione and reactive oxygen species in cell line HT-1080 treated with chub mackerel (*Scomber japonicus*) extracts. Philipp. Agric. Sci. 97:180-184.

Baek, J. Y. and Lim S. Y. (2016). Flavonoid and phenol contents and antioxidant effect of wine by-product extracts. J. Life Sci. 26:948-954.

Baek, J. Y. and Lim, S. Y. (2017). Flavonoid and Phenol contents and antioxidant effect of wine by-product extracts. Philipp. Agric. Sci. 100:156-162.

Bognar, E., Sarszegi, Z., Szabo, A., Debreceni, B., Kalman, N., Tucsek, Z., Sumegi, B. and Gallyas, F. (2013). Antioxidant and anti-inflammatory effects in Raw 264.7 macrophages of Malvidin, a major red wine polyphenol. PLoS ONE. 8:e65355.
DOI: 10.1371/journal.pone.0065355

Boussetta, N., Lebovka, N., Vorobiev, E., Adenier, H., Bedel-Cloutour, C. and Lanoiselle, J. L. (2009). Electrically assisted extraction of soluble matter from chardonnay grape skins for polyphenol recovery. J. Agric. Food Chem. 57:1491-1492.

Bredt, D. S. (1999). Endogenous nitric oxide synthesis: Biological functions and pathophysiology. Free Radic. Res. 31:577-596.

Cheah, K. Y., Bastian, S. E., Acott, T. M., Abimosleh, S. M., Lymm, K. A. and Howarth, G. S. (2013). Grape seed extract reduces the severity of selected disease markers in the proximal colon of dextran sulphate sodium-induced colitis in rats. Dig. Dis. Sci. 58:970-977.

Cho, M. L., Heo, Y. J., Park, M. K., Oh, H. J., Park, J. S., Woo, Y. J., Ju, J. W., Park, S. W., Kim, H. Y. and Min, J. K. (2009). Grape seed proanthocyanidin extract (GSPE) attenuated collagen-induced arthritis. Immunol. Letters. 124:102-110.

Corrales, M., Toepfl, S., Butz, P., Knorr, D. and Tausher, B. (2008). Extraction of anthocyanins from grape by-products assisted by ultrasonics, high hydrostatic pressure or pulsed electric fields: A comparison. Innov. Food Sci. Technol. 9:85-91.

Green, L. C., Wagner, D. D. A., Glowgowski, J., Skepper P. L., Wishnok, J. S. and Tannenbaum, S. R. (1982). Analysis of nitrate, nitrite and $^{15}N$ nitrate in biological fluids. Anal. Biochem. 126:131-138.

Hogan, S., Canning, C., Sun, S., Sun, X. and Zhou, K. (2010). Effects of grape pomace antioxidant extract on oxidative stress and inflammation in diet induced obese mice. *J. Agric. Food Chem.,* 58: 11250-11256

Hwang, S. A., Dasgupta, A. and Actor, J. K. (2004). Cytokine production by non-adherent mouse splenocyte cultures to Echinacea extracts. J. Clin. Chim. Acta. 343:161-166.

Kampa, M., Hatzogiou, A., Notas, G., Damianaki, A., Bakogeorgou, E., Gemetzi, C., Kouroumalis, E., Martin, P. M. and Castanas E. (2000). Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. Nutr. Cancer. 37: 223-233.

Kim, S.K. (2005). The present state of grape cultivation in Korea. In: Symposium on development of yeongdond grape cluster regional innovation. Yeongdong Grape Cluster Organization, Yeongdong. 4-10.

Kim, K. H., Kim, S. H. and Park, K. Y. (2001). Effects of kimchi extracts on production of nitric oxide by activated macrophages, transforming growth factor-beta 1 of tumor cells and interleukin-6 in splenocytes. J. Food Sci. 6:126-132.

MacMicking, J., Xie, Q. W. and Nathan, C. (1997). Nitric oxide and macrophage function. Ann. Rev. Immunol. 15:323-350.

Magazine, H. J. (1995). Detection of endothelial cell derived nitric oxide: Current trends and future directions. Adv. Neuroimmunol. 5:479-490.

Mitjans, M., Del Campo, J., Abajo, C., Martinez, V., Selga, A., Lozano, C., Torres, J. L. and Vinardell M. P. (2004). Immunomodulatory activity of a new family of antioxidants obtained from grape polyphenols. J. Agric. Food Chem. 52:7297-7299.

Monaca, S., Palmer, R. M. and Higgs, E. A. (1991). Nitric oxide: Physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43:109-142.

Mukherjee, S., Das, S. K. and Vasudevan, D. M. (2012). Dietary grapes (*Virus vinifera*) feeding attenuates ethanol-induced oxidative stress in blood and modulates immune functions in mice. Indian J. Biochem. Biophys. 49:379-385.

Musse, N., Lprenzen, L. and Aldrich, C. 2007. Cellar waste minization in the wine industry: A system approach. J. Clean Prod. 15:417-431.

Nunes, C., Ferreira, E., Freitas, V., Almeida, L., Barbosa R. M., and Laranjinha, J. (2013).

Intestinal anti-inflammatory activity of red wine extract: Unveiling the mechanisms in colonic epithelial cells. Food Funct. 4:373-383.

Qureshi, A. F., Guan, X. Q., Reis, J. C., Papasian, C. J., Jabre, S., Norrison, D. C. and Qureshi, N. (2012). Inhibition of nitric oxide and inflammatory cytokines in LPS-stimulated murine macrophages by resveratrol, a potent protezsome inhibitor. Lipids in Health Disease. 11:76. Available:http://www.lipidworld.com/contents/11/1/76

Rebelo, M. J., Sousa, C., Valentao, P., Rego, R. and Andrade, P. B. (2014). Phenolic profile of Douro wines and evaluation of their NO scavenging capacity in LPS-stimulated RAW 264.7 macrophages. Food Chem. 163:16-22.

Rockenbach, I. I., Rodrigues, E., Gonzaga, L. V., Caliari, V., Genovese, M. I., Souza Schmidt Goncalves A. E. and Fett, R. (2011). Phenolic compounds content and antioxidant activity in pomace from selected red grapes (*Vitis vinifera* L. and *Vitis labrusca* L.) widely production in Brazil. Food Chem. 127:174-179.

Sies, H. and Mehlhorn, R. (1986). Mutagenicity of nitroxide-free radicals. Arch. Biochem. Biophys. 25: 393-396.

Teixeira, A., Baenas, N., Dominguez-Perles, R., Barros, A., Rosa, E., Moreno, D. A. and Garcia-Viguera, C. (2014). Natural bioactive compounds from winery by-products as health promoters: A review. Int. J. Mol. Sci. 15:15638-15678.

Wang, S. Z., Bao, Y. X., Rosenberger, C. L., Tesfaigzi, Y., Stark, J. M., Harrod, K. S. (2004). IL-12p40 and IL-18 modulate inflammatory and immune response to respiratory syncytial virus infections. J. Immunol. 173:4040-4049.