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Antiproliferative Property of Wine Waste Extracts

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The study investigated the effect of wine waste extracts on antiproliferative property. Wine wastes were extracted using acetone/methylene chloride (A+M) and methanol (MeOH) and then fractionated using *n*-hexane, 85% aq. methanol (MeOH), butanol (BuOH) and distilled water. The cytotoxic activity of the wine wastes against AGS human gastric, HT-29 human colon and HT-1080 fibroblast cancer cell lines was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. All the extracts and fractions from the wine wastes decreased the growth of AGS cells, and the effect was concentration-dependent. The MeOH extract showed significantly higher inhibition against the growth of AGS cells compared with the A+M extract ($P<0.05$). The same trend was observed for the *n*-Hexane, 85% aq. MeOH, *n*-BuOH and water fractions. Among the fractions, the 85% aq. MeOH fraction showed the highest effect of 68% inhibition at the lowest concentration (0.025 mg mL^{-1}). In the HT-29 cancer cells, the pattern of growth inhibition by the crude extracts was a little different from that observed for the AGS cancer cells, with the A+M extract showing a higher effect ($P<0.05$). The *n*-BuOH and 85% aq. MeOH fractions were the most effective against the proliferation of HT-29 cancer cell lines ($P<0.05$). The pattern of growth inhibition in the HT-1080 cells was similar to that observed in the HT-29 cancer cells, with the A+M extract being the most effective. In addition, similar to the trend observed in the HT-29 cells, the 85% aq. MeOH fraction showed the highest inhibition of the growth of the HT-1080 cancer cells. Thus, the 85% aq. MeOH fraction from wine waste extracts would contain bioactive compounds such as polyphenols and flavonoids. There is a need for further research to separate and isolate these important compounds from the extracts.

Key Words: AGS gastric, antiproliferation, HT-29 colon, HT-1080 fibroblast, wine waste

Abbreviations: BuOH – butanol, DMEM – Dulbeco's modified Eagle's medium, DMSO – dimethylsulfoxide, FCS – fetal calf serum, MeOH – methanol, MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PWGPE – purified white grape pomace extract

INTRODUCTION

Grapes are among the major fruit crops and about 80% of the harvest is used by the winemaking industry. In Korea, the production of wine reached 26,000 tons in 2013 and thousands of tons of wine wastes are produced every year (MAFRA 2014). Winemaking is a seasonal activity, which leads to the generation of large quantities of wastes during a short period every year, especially in the high-production regions. Thus, it seems rational and economically profitable to extract the components of grape waste to be used as additives or dietary supplements in the food industry or as active compounds in the cosmetic and pharmaceutical sectors.

Grape waste is considered as a valuable by-product for oil extraction and for antioxidant and antibacterial preparations. Grape waste contains active compounds, such as dietary fiber (Yu and Ahmedna 2013), polyphenols (Deng et al. 2011), anthocyanins and flavonols (Downey et al. 2007), and resveratrol (Yang et al. 2009). In recent years, there has been a growing interest in the use of by-products rich in polyphenols obtained from the winemaking industry to produce extracts and novel products for the maintenance of human health (Kulkarni et al. 2011). Vergara-Salinas et al. (2015) found that grape pomace extracts exhibited potent protective activities on HL-60 cell growth and mitochondrial membrane potential, with an activity comparable to that of trolox. During winemaking, the polyphenols from grapes are transferred to the wine, but a high proportion of these compounds remain in the solid by-products obtained from the winemaking process.

There are several studies on Korean wine including metabolomic characterization (Son et al. 2008) and the role of yeasts in fermentation (Seo et al.

2007; Hong and Park 2013). However, there is limited information on extracts from Korean wine waste. Therefore the aim of our study was to investigate the antiproliferative effects of Korean wine waste extracts.

MATERIALS AND METHODS

Materials and Cell Culture

Red wine waste was collected in a wine farm named Cave Story (Yeoungcheon, Korea). Dulbeco's modified Eagle's medium (DMEM), fetal calf serum (FCS), phosphate buffer saline (PBS), dimethylsulfoxide (DMSO), penicillin-streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 2′-7′ dichlorofluorescein-diacetate (DCFH-DA) were obtained from Sigma-Aldrich (St. Louis, USA). AGS human gastric cancer, HT-29 human colon cancer, and HT-1080 human fibroblast cell lines were obtained from the Korea Cell Line Bank. The cells were maintained at 37 °C under 5% CO_2 in DMEM containing 10% FCS and 100 units per mL of penicillin-streptomycin.

Sample Extracts and Fractions

Samples of wine waste were dried and sequentially extracted twice with acetone/methylene chloride (A+M) and methanol (MeOH) to obtain the maximum amount of extracts. First, samples were extracted with A+M to obtain fat-soluble extracts and then the residues were extracted with MeOH for the rest of the water-soluble 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 (BuOH) and water, resulting in the *n*-hexane, 85% aqueous MeOH, BuOH and distilled water fractions. The crude extracts and four types of fractions, with different polarities, were concentrated to dryness at 40 °C using a rotary vacuum evaporator (N-100, EYELA, Japan), and the residue was kept at 4 °C until analysis and assay. The extracts/fractions were dissolved in DMSO.

***In vitro* Cytotoxic Activity [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay]**

Cell viability was determined using the MTT assay (Stockert et al. 2012). MTT assay assesses the viability and the proliferation of cells. The cells were seeded in 96-well plates at a density of 5×10^3 cells/well. After incubation for 24 h, the cells were treated with different concentrations of samples. Then, after incubation for 48 h, the cells were incubated with 100 μL of MTT (1 mg mL^{-1}) for 4 h. Finally, the medium was removed and 100 μL of dimethylsulfoxide (DMSO) was added to solubilize the formed formazan crystals. The amount of formazan crystal was determined by measuring the absorbance at 540 nm using a microplate spectrophotometer (VICTOR3, Perkin Elmer, Waltham, USA). Cytotoxicity was measured in terms of inhibition of cell proliferation using the following formula:

$$\text{Cell viability} = \frac{\text{Abs. 540 nm Sample}}{\text{Abs. 540 nm Control}} \times 100$$

Statistical Analysis

Analytical data were subjected to one-way analysis of variance (one-way ANOVA) followed by Tukey's test for differences among treatment concentrations and extract fractions. Analyses were conducted using the statistical package for social science (SPSS) version 10.0 software package (SPSS Inc., Chicago, IL, USA). Significant ($P < 0.05$) differences were indicated by different superscript letters in the tables. Data are presented as mean ± standard deviation.

RESULTS AND DISCUSSION

The effects of the crude extracts and fractions from wine wastes on the proliferation of human cancer cell lines (AGS gastric, HT-29 colon, and HT-1080 fibroblast) were evaluated using the MTT assay, which measures the decrease in the mitochondrial activity of cells. Preliminary experiments showed that all the extracts and fractions from the wine wastes did not affect cell viability of NIH/3T3 normal cells at all concentrations analyzed. All the extracts and fractions from the wine wastes decreased the growth of the AGS cells, with the effect being concentration-dependent (Fig. 1). At concentrations of 0.25 and 0.5 mg mL^{-1}, the MeOH extract showed significantly higher inhibition against the growth of AGS cells compared with the A+M extract ($P < 0.05$). At the highest concentration (0.5 mg mL^{-1}), the MeOH extract showed 18% cell viability against the

AGS cells. The same trend was observed for the *n*-Hexane, 85% aq. MeOH, *n*-BuOH and distilled water fractions (Fig. 2). Among the different fractions, the 85% aq. MeOH fraction showed the lowest viability (32%) at the lower concentration of 0.025 mg mL^{-1} ($P < 0.05$).

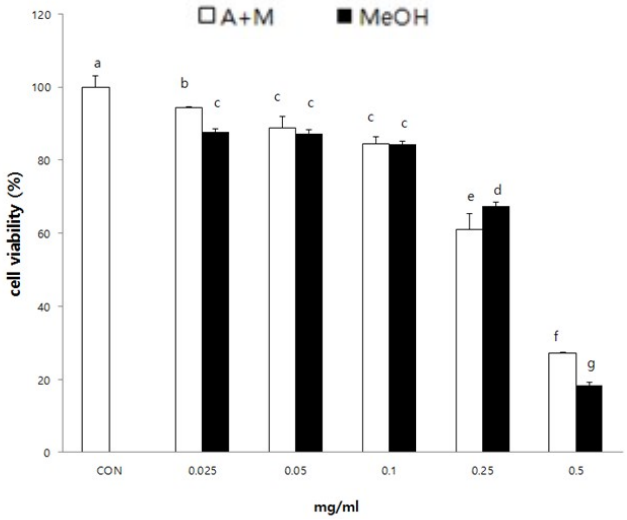


Fig. 1. Effect of acetone/methylene chloride (A+M) and methanol (MeOH) extracts from wine wastes on the cell viability of AGS human gastric adenocarcinoma cells. [a–g]Different superscripts indicate significant differences at $P < 0.05$.

Figure 3 shows the inhibitory effect of the crude extracts from the wine wastes on the growth of HT-29 cancer cells. The pattern of cell viability of these cells by the crude extracts was a little different from that observed in the AGS cancer cells, with the A+M extract showing a significantly higher effect than the MeOH extract ($P < 0.05$). Among the different fractions, the *n*-BuOH and 85% aq. MeOH fractions were most effective against the proliferation of HT-29 cancer cell lines ($P < 0.05$) (Fig. 4). Figure 5 shows the inhibitory effect of the crude extracts obtained from the wine wastes on the growth of HT-1080 cancer cells. Again, all the extracts and the fractions from wine wastes decreased the growth of HT-1080 cancer cells, and the effect increased with increasing concentrations. The pattern of cell viability was similar to that observed in the HT-29 cancer cells, with the A+M extract being the most effective ($P < 0.05$). The A+M extract showed the growth of the HT-1080 cancer cells by 14% at a concentration of 0.5 mg mL^{-1}. As shown in Figure 6, the 85% aq. MeOH fraction showed the highest inhibitory effect on the

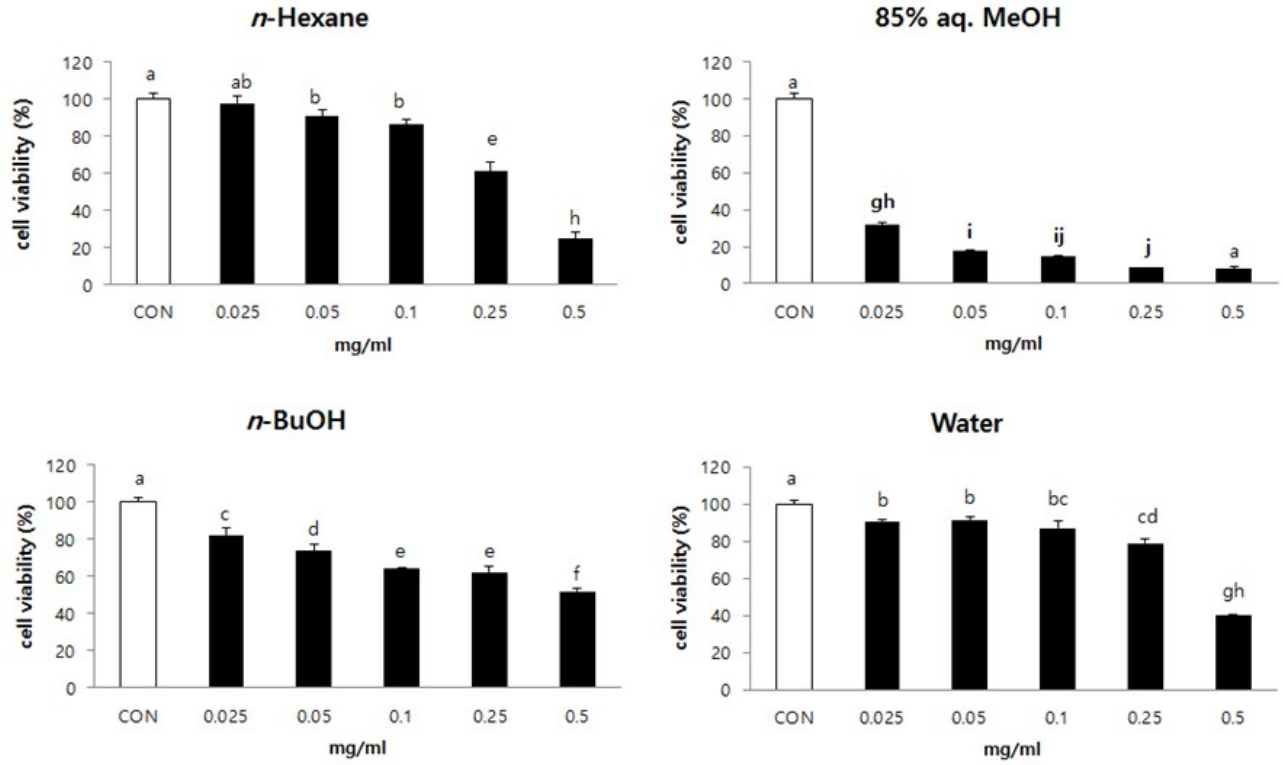


Fig. 2. Effect of fractions from wine waste extracts on the cell viability of AGS human gastric adenocarcinoma cells. [a–h]Different superscripts indicate significant differences at $P < 0.05$.

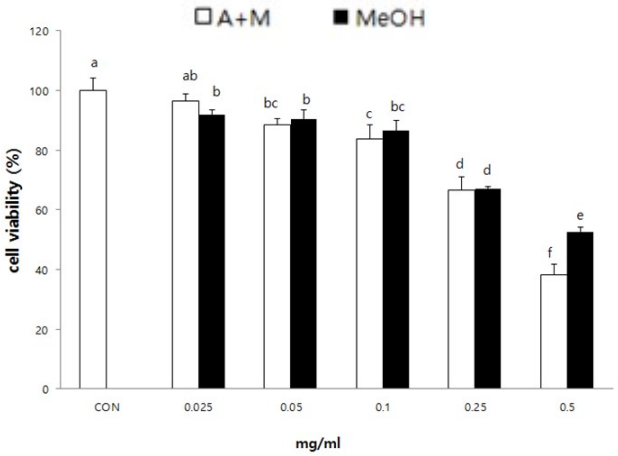
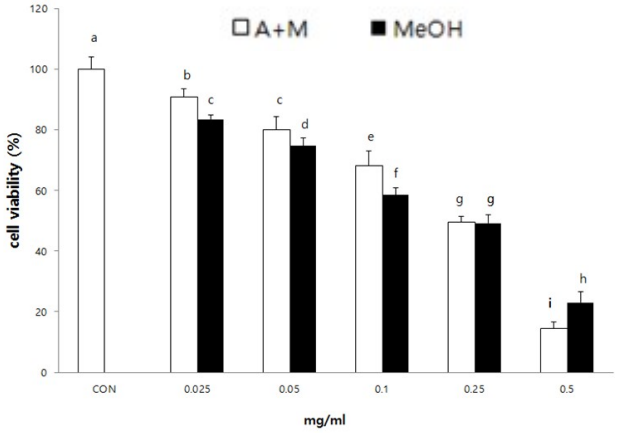


Fig. 3. Effect of acetone/methylene chloride (A+M) and methanol (MeOH) extracts from wine wastes on the cell viability of HT-29 human colon cancer cells. [a–f]Different superscripts indicate significant differences at $P < 0.05$.



Fig, 5. Effect of acetone/methylene chloride (A+M) and methanol (MeOH) extracts from wine wastes on the cell viability of HT-1080 human fibroblast cells. [a–h]Different superscripts indicate significant differences at $P < 0.05$.

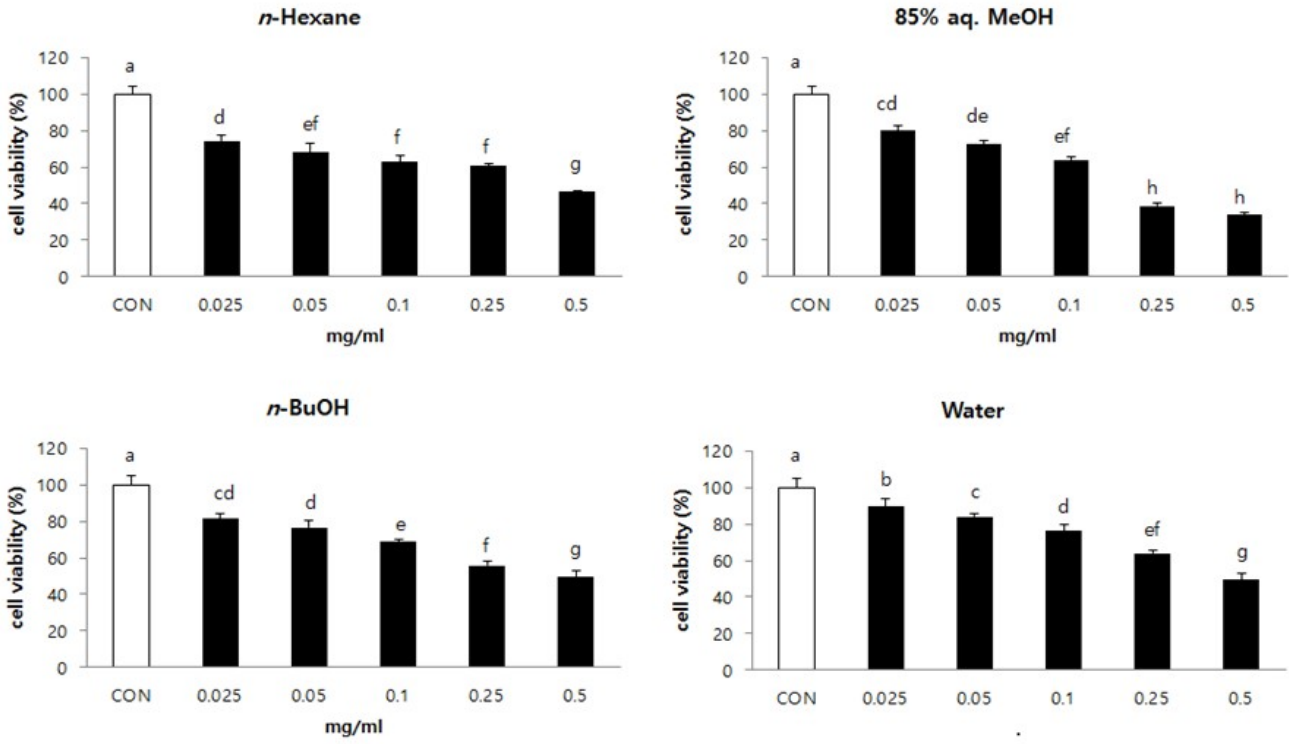


Fig. 4. Effect of fractions from wine waste extracts on the cell viability of HT-29 human colon cancer cells. [a–h]Different superscripts indicate significant differences at $P < 0.05$.

growth of the HT-1080 cancer cells ($P < 0.05$), which is similar to what was observed in the HT-29 cancer cells.

After fermentation during wine production, the original polyphenolic content remains in the by-products (Vergara-Salinas et al. 2015). Polyphenols have great value as micronutrients in the human body and can exert preventive action against cancer, cardiovascular diseases, and neurodegenerative diseases (Flamini et al. 2013). In our preliminary study, the amounts of total flavonoids and phenols in the 85% aq. MeOH fraction were 105.1 and 3.6 mg g^{-1}, respectively. Jara-Palacios et al. (2015) suggested that the most abundant flavonol in purified white grape

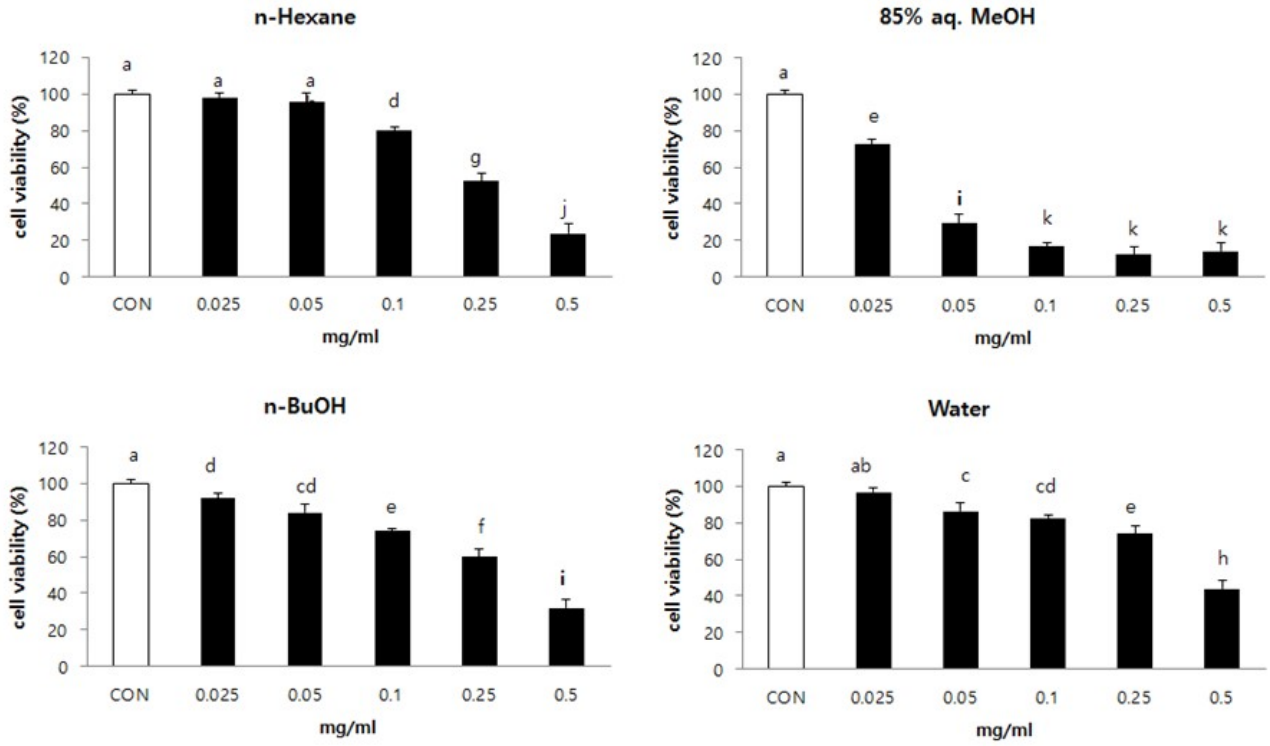


Fig. 6. Effect of fractions from wine waste extracts on the cell viability of HT-1080 human fibroblast cells. [a–h]Different superscripts indicate significant differences at $P < 0.05$.

pomace extract (PWGPE) was procyanidin B1, followed by catechin, procyanidin B2-3-*O*-gallate, and procyanidin tetramer 1. They also found that PWGPE induced dose-dependent inhibition of cancer cell proliferation following 24, 48, and 72 h of exposure, suggesting that PWGPE obtained from the winemaking process is capable of inhibiting adenocarcinoma cell proliferation by a combination of its antiproliferative activity and via a direct initiation of cell death. Notably, polyphenol-rich foods such as olive oil, red wine and tomato extract were shown to inhibit cancer cell growth over a similar period (Corona et al. 2007; Gomez-Alonso et al. 2012; Saunders 2009). It is estimated that for each 6 L of wine, 1 kg of grape pomace is produced, which is mainly used for animal feed and for compost elaboration (Mendes et al. 2013). In this context, the phenolics present in grape pomace represent bioactive substances with many applications also related to healthy benefits such as scavenging activity against free radicals, anti-inflammatory properties (Terra et al. 2007) and potential for use in anti-proliferation and cancer therapy (Nandakumar et al. 2008). Grape pomace extracts display widespread uses in pharmaceutical and cosmetic industries: they have also been recently found to be useful in a new class of "phytosanitary bioproducts" that can help control the incidence of crop diseases. Tournour et al. (2015) suggested that the ethanol/water extracts of red grape pomace exhibited satisfactory antioxidant properties with higher levels of total polyphenols. Based on our results, the 85% aq. MeOH fraction was the most effective in inhibiting the proliferation of cancer cells. Thus, the 85% aq. MeOH fraction from wine waste extracts would contain bioactive compounds such as polyphenols and flavonoids.

This study is a preliminary and first attempt to prove the uses and benefits of Korean wine wastes since there have been few studies on their biological effects. Further research needs to be undertaken to separate these important compounds from wine waste extracts.

CONCLUSION

Wine wastes show preventive biological effects in cancer cell lines, which could be related to the properties of the individual phenolic constituents.

Our data show that wine waste extracts are highly efficient as antiproliferative agents against cancer cells and are therefore worthy of further study for their possible use as food additives or chemopreventive agents. The research is just the first step in understanding the biological activities of Korean wine waste. The complete identification and characterization of the profile of bioactive compounds from wine wastes need to be conducted. The sustainable development of methodologies for wine waste disposal/use will be a useful strategy for wineries with the aim of reducing environmental contamination.

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