

Cytotoxic Effects of Trans-resveratrol and Red Grape Products on Liver Ultrastructures of Mice, and Apoptosis Induction in Human Cancer Cells

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Abstract

Trans-resveratrol and red grape products have been known to be antioxidants and anticarcinogens. The present study investigated the total phenolic compound (TPC) contents of red grape products; wine, juice and pomace. The effects of grape products and trans-resveratrol on ultrastructure of mouse liver tissue, cytotoxicity, and apoptotic induction on human cancer cell lines were investigated. The TPC content of ethanolic grape pomace extract ($4,407.33 \pm 13.65$ mg/L) was significantly higher than those of red wine ($3,613.00 \pm 15.13$ mg/L) and grape juice ($1,102.67 \pm 21.96$ mg/ml). Exposure of ICR mice to grape products and trans-resveratrol daily for six months reduced ultrastructural pathologic of hepatocytes, included minimal glycogen, fat accumulation, and organelle abnormality, compared to their corresponding vehicle controls. Trans-resveratrol and ethanolic grape pomace extract exhibited cytotoxic effects on pancreatic Panc 2.03 and cholangiocarcinoma SNU 1079 cells in a dose dependent manner assessed by MTS assay. The cytotoxic activity was mediated via apoptosis as demonstrated by DAPI staining and decreased pro-caspase 3 and Bcl-2 protein expressions. These data suggest a possible mechanism of cytotoxicity in both cancer cell lines, at least in part, through the regulation of apoptosis-related proteins and/or cell cycle dysregulation.

Key words: Trans-resveratrol/ Ultrastructure/ Apoptosis/ Cancer

Introduction

Several polyphenol compounds extracted from plants possess an antioxidant activity, and the research on polyphenols occurring in plants has attracted considerable interest due to the numerous and health-beneficial effects, such as antimutagenic, anticarcinogenic, antiatherogenic, etc. Recently, a wide variety of polyphenolic compounds and non-flavonoids have been found mainly in vegetables and fruits especially in grapes and their derivatives (Frankel et al., 1993). One of the main phytochemicals which is found in red grapes (*Vitis vinifera*) is trans-resveratrol. The beneficial effects of trans-resveratrol consumption include suppression of lipid peroxidation and eicosanoid synthesis, inhibition of platelet aggregation, anti-inflammatory, and vasorelaxant activities. Trans-resveratrol also has anticancer activities by affecting cell signaling pathway, modulating transcription factors, gene induction, regulation of enzyme activities and protein interactions in several *in vivo* and *in vitro* study. Specifically, only a few studies are available on the anti-proliferative effects of resveratrol on human pancreatic cancer cells (Athar et al., 2007; Althar et al., 2009).

Cholangiocarcinoma is the highest incident primary liver cancer in the Northeast of Thailand (Vatanasapt et al., 1993) and is still a major health problem of people in this area.

Pancreatic cancer is one of the most aggressive malignancies, with a persistently high mortality and poor prognosis. This cancer has the highest mortality rate and the lowest 5-year survival (*i.e.*, less than 5%) (Strimpakos et al., 2008). This malignant tumor is highly fatal and poor prognosis because there is no method for early detection and lack of effective treatments. Failure to Surgical resection of pancreatic cancer is available only in 15-20 % of all patients, while medical approaches, such as chemotherapy or radiation, have no cure. Induce apoptosis is a major factor limiting the efficacy of common treatment for cancer: surgical treatment, chemotherapy and radiotherapy (Dive, 1997). The resistance of pancreatic cancer and cholangiocarcinoma to chemotherapeutic agents is one of the serious problems in clinical situations. Therefore, suppression of apoptosis may be a feature of tumor promotion by chemical carcinogens. Indeed, many chemopreventive agents may act through the induction of apoptosis as a mechanism of anticarcinogenic action. Though there is enormous amount of data supporting trans-resveratrol's and certain grape products such as wine and juice possess anticancer effects *in vitro* and *in vivo*, there is not much data on the chemopreventive and therapeutic effects of grape pomace especially those that prepared from Zinfandel red grapes.

In addition, to the best of the author's knowledge, no studies of cytotoxic activities of trans-resveratrol and grape products against human cholangiocarcinoma SNU 1079 and pancreatic Panc 2.03 cells are conducted. Since both cholangiocarcinoma and pancreatic cancers are very poor prognosis, resistant to the available chemotherapeutic agents and hence represent the serious problems in clinical treatment, the present study aimed to explore the therapeutic potential of trans-resveratrol and certain grape products against two human cholangiocarcinoma and pancreatic cancer cell lines, SNU 1079 and pancreatic Panc 2.03 cells, respectively. In initial phase of the study, the total phenolic contents of the products of red grapes grown at Suranaree University of Technology (SUT) farm and trans-resveratrol absorption in mice were determined. Then the chemopreventive effect of the products of red grapes on ultrastructural changes of liver tissue in mice was investigated. The last part of the study was to assess the cytotoxic and antiproliferative effects of trans-resveratrol and grape products on SNU 1079 and Panc 2.03 cells. The alteration of signaling protein factors in apoptotic pathway as the molecular mechanism of cytotoxicity on the cancer cell lines was also explored.

Materials and Methods

Total phenolic content (TPC) was determined by a modified Folin Ciocalteu's method (Swain and Hills, 1959; Matthaus, 2002). Effects of grape products and trans-resveratrol on ultrastructural pathologic of liver tissues: ICR mice were daily exposed in the morning through oral gavage for six months. Every two months liver tissues were taken in order to study of ultrastructural examination under the standard techniques for Transmission electron microscope (TEM) (Bozzola and Russell, 1999). *In vitro* cytotoxicity studies, the normal human fibroblast, Cholangiocarcinoma; SNU 1079 and Pancreatic cancer; Panc 2.03 cells were cultured in completed RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS). All cell lines were maintained at 37°C in 5% CO₂ humidified incubator. The MTS assay was used in this study to indirectly determine cytotoxic effects of ethanolic grape pomace extract and trans-resveratrol by measuring at 490 nm using ELISA plate reader (Wallac Model 1420 Multilabel counter, michigan, USA). IC₅₀ value was expressed as concentration of extract in microgram per milliliter that caused a 50% growth inhibition comparing with controls. Apoptosis assay was determined by the detection of nuclear morphology using DAPI staining as

described by Hotz et al (1992) with slight modification. Apoptotic protein was detected and visualized by using Ecl chemiluminescence system as described in the manufacturing instruction (Pierce Biotechnology, Inc., Rockford, USA).

Results

The determination of TPC content

The determination of TPC content from grape products was measured by Folin-Ciocalteu's phenol reagent as modified from the method of Matthaus (2002). The amount of TPC in ethanolic grape pomace extract (4,407.33±13.65 mg/L) was higher than red

wine (3,613.00±15.13 mg/L) and juice (1,102.67±21.96 mg/L) (Figure 1). They were significant differences ($p \leq 0.01$).

Effect on Ultrastructural pathologic changes study

Electronmicrograph of mice hepatocytes treated with test compounds: vehicle control, grape products and trans-resveratrol for up to 6 month. The result was shown in table 1. The prominent pathology were lipid droplets and glycogen accumulation, organelle abnormality, and focal cytoplasmic degeneration. Mitochondria abnormalities were observed, including variation in size and shape. The endoplasmic reticulum was disarranged.

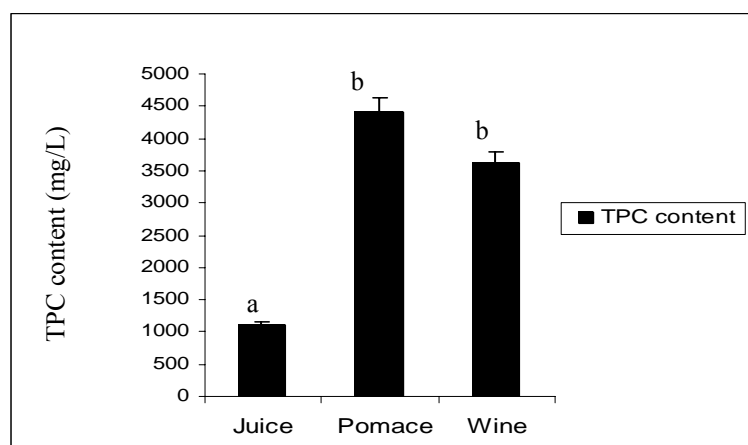


Figure 1 Comparison of TPC contents of Zinfandel grape products: juice, ethanolic grape pomace extract and wine expressed as mg/L GAE. Each sample was measured in triplicates. All values are mean±S.D. Values with differing alphabets are significantly different from each other at $p < 0.01$.

Table 1 Ultrastructural pathologic characteristic of hepatocytes from mice treated with grape products and trans-resveratrol during different period of time post administration.

Treatment	Ultrastructural pathologic characteristic of hepatocytes					
	2 months		4 months		6 months	
	1	2	1	2	1	2
Normal control	-	-	-	-	-	-
10 % DMSO	++	++	+++	+++	+++	+++
12 % Ethanol	++	++	+++	+++	++++	++++
Grape juice	-	-	-	-	-	-
Ethanolic grape pomace extract	+	+	++	++	++	++
Wine	+	+	++	++	++	+++
Trans-resveratrol	+	+	++	++	++	++

++++ = most change, +++ = mild change

++ = less change, - = normal

***In vitro* Cytotoxicity studies**

Cytotoxic effect of trans-resveratrol and ethanolic grape pomace on SNU 1079 and Panc 2.03 cell lines was measured by MTS cell proliferation assay. Pronounced cytotoxic activities of trans-resveratrol and ethanolic grape pomace extract were observed on SNU 1079 and Panc 2.03 cells with IC₅₀ values of 18.10±0.11 and 5.09±0.04 µg/mL and 716.42±0.05 and 274.46±0.82 µg/mL, respectively. They were markedly decreased cell viability of both cell lines in a dose-dependent manner.

Apoptosis assay

Following DAPI staining, it was found that apoptotic cells could be clearly seen under fluorescent microscope. These apoptotic cells exhibited characteristic of nuclear morphological changes, including chromatin condensation, segmentation of nuclear chromatin of irregular size in treated cell. This was clear contrast to the spherical and regular form of control nuclei (Figure 2). Overall, the apoptotic Panc 2.03 and SNU 1079 cells were increased in dose dependent manner after trans-resveratrol or ethanolic grape pomace extract treatment.

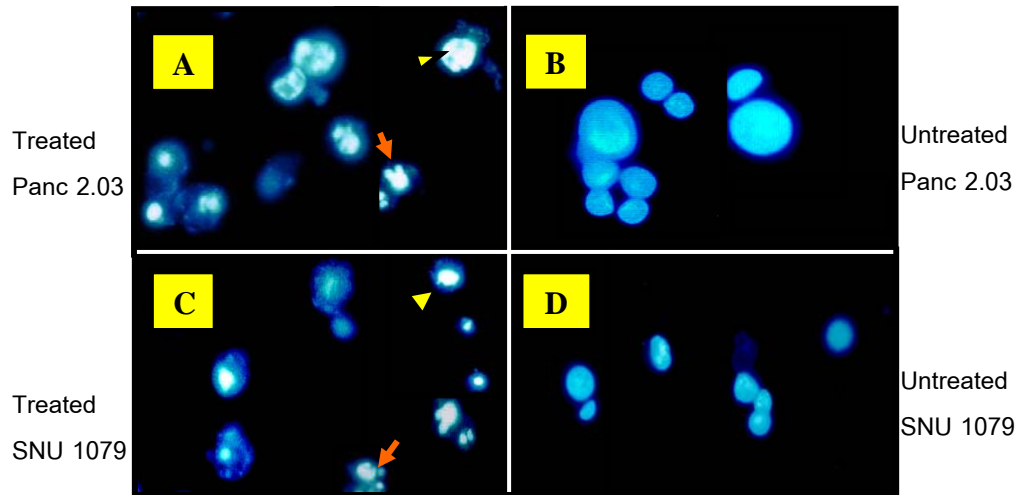


Figure 2 Effects of trans-resveratrol on nuclear morphology of Panc 2.03 and SNU 1079 cells for 48 h and 72 h. Cells showed chromatin condensation (arrow head) and nuclear fragmentation (arrow) under fluorescence microscope (40X). A= treated Panc 2.03 cells, B= untreated Panc 2.03 cells , C= treated SNU 1079 cells, D= untreated SNU 1079 cells.

Effect trans-resveratrol and ethanolic grape pomace extract on the levels of Bcl-2 and caspase-3 proteins

Trans-resveratrol and ethanolic grape pomace extract induced a dose dependent

reduction of Bcl-2 and increasing caspase-3 level in both cell lines, the alterations which may partly explain the induction of apoptosis observed in this study as shown in Figure 3.

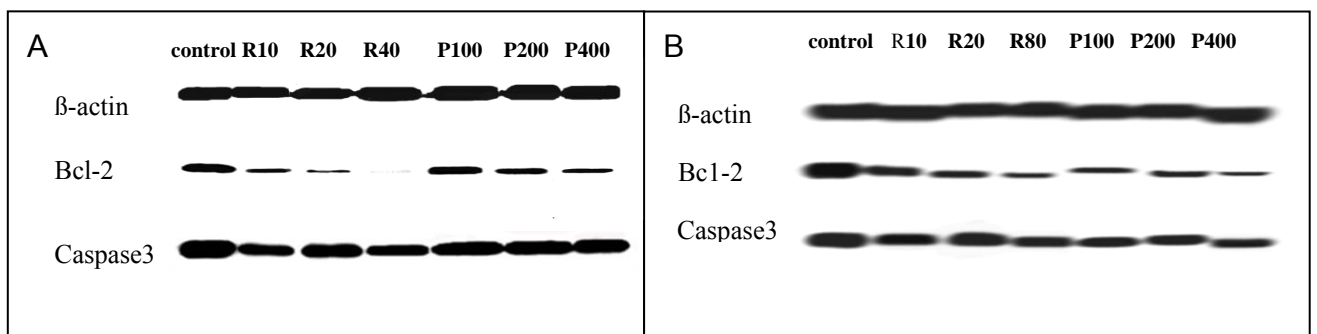


Figure 3 Bcl-2, caspase 3 and β -actin protein expressions in Panc 2.03 cells (A) and SNU 1079 cells (B). Cells were treated with a series of concentrations of trans-resveratrol and ethanolic grape pomace extract for 48 h, the levels of Bcl-2, caspase 3 and β -actin expression by western blotting analysis.

Discussion

Determination of TPC content in red grape products, the results were similar to those presented by Teissedre and Landrault (1996) who reported the variability in the levels of TPC ranged from 1,847 – 2,600 mg/L of red wine. Jang et al. (1997) reported about 50-100 µg of trans-resveratrol per gram in grape skin. This finding was similar to the result of Jeandet et al. (1991) and Okuda et al. (1977) who reported that trans-resveratrol, one of the important polyphenol, was found only in grape skin. Cytotoxic effects of trans-resveratrol and ethanolic grape pomace, the data exerted a greater cytotoxic effect on Panc 2.03 and SNU 1079 cells than normal human fibroblast. Joe et al. (2002) reported that there is limited information on the toxicity of resveratrol in experimental animals, and there are, apparently, no clinical toxicity data on the use of pure resveratrol in human. Clement et al. (1998) demonstrated that resveratrol is minimally toxic to human peripheral blood cells. The different susceptibility of these cell lines are likely due to the different in genetic background. The results in this study agree with several previous reports which showed the growth inhibitory activity of resveratrol in various human cancer cell lines including epidermoid carcinoma A 431 cells

(Ahmad et al., 2001), human SW480 colorectal tumor cells (Delmas et al., 2002), melanoma cells (Niles et al., 2003), Seg-1 esophageal adenocarcinoma cells, MCF7 breast carcinoma cells, HL60 promyelocytic leukemia cells (Joe et al., 2002). These results supported our hypothesis that trans-resveratrol or ethanolic grape pomace extract could inhibit cancer cell growth or enhance the molecular mechanism of the chemopreventive effects on cancer. In addition, morphological characterization of treated cells revealed that the mode of action of cell death induced by resveratrol or ethanolic grape pomace extract was mediated through apoptosis. Thus, chromatin condensation and nuclear fragmentation of treated cells were clearly evident. A decrease in expression of the anti-apoptotic protein Bcl-2 was observed in both treated cells in a dose dependent manner. Trans-resveratrol and ethanolic grape pomace extract could also up-regulate the expression of pro-caspase 3 in a dose dependent manner. These data suggest a possible underlying molecular mechanism whereby trans-resveratrol and ethanolic grape pomace extract could induce the apoptosis signaling pathway in Panc 2.03 and SNU 1079 cells by the regulation of apoptosis related proteins. Grape product and

trans-resveratrol induced ultrastructural pathologic characteristic changes of liver cells, it appeared that there might be a cytoprotective changes. The effects observed in our study were similar to previously reported (Zhou et al., 2002).

Summary

This study demonstrates that the use of red grape products and trans-resveratrol possess anticancer and antiproliferative activities. The characterization of grape products is depended on the concentrations of total phenolic compound (TPC) contents and activities. The study also suggests that trans-resveratrol and ethanolic grape pomace extract exhibit the potent cytotoxic and apoptotic activities towards cancer cells. This study demonstrated that trans-resveratrol and ethanolic grape pomace extract exhibited cytotoxic effect on Panc 2.03 and SNU 1079 cell lines in a dose dependent manner. In addition, both compounds could induced apoptosis in Panc 2.03 and SNU 1079 cell lines. Therefore, trans-resveratrol and ethanolic grape pomace extract possess antiproliferative properties towards cancer cells and could be promising anticarcinogens. Further explanation in the development of both compounds as chemopreventive agents should be highly warranty.

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