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ORIGINAL RESEARCH

Biological Activities of Fractions from Methanolic Extract of *Picrasma quassioides*

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Abstract Antioxidant, antidiabetes, and anticancer activities of fractions (water, hexane, EtOAc, and BuOH) from methanolic (MeOH) extract of *Picrasma quassioides* were evaluated *in vitro*. Among the four fractions, BuOH fraction showed cytotoxic activity on the human stomach carcinoma cells (NCI-N87) without cytotoxic effect on the human normal kidney cells (293) by MTT assay. Considering these results, we adapted BuOH fraction of MeOH extract to the assessment of apoptosis by flow cytometry and mRNA expression levels of established apoptotic-related genes on NCI-N87 cells. The experiments showed that BuOH fraction could induce apoptosis on NCI-N87 cells significantly. Take together these results, *P. quassioides* showed that it could be used for potential pharmaceutical products.

Keywords Antioxidant · Antidiabetes · Anticancer · Fractions · MeOH extract · *Picrasma quassioides*

Reactive oxygen species (ROS) are ions or very small molecules that include oxygen ions, free radicals, and peroxides, both inorganic and organic. This cumulates into a situation known as oxidative stress. Strong oxidants like the various ROS can damage other molecules and the cell structures (Liu et al. 2002). The hydroxyl radical (OH) is the neutral form of the hydroxide ion and is a kind of free radical, belongs to ROS. The hydroxyl radical has a very short in vivo half-life of approximately 10^{-9} s and a high reactivity. This makes it a very dangerous compound to the organism (Ahmad et al. 2008). The hydroxyl radical cannot be eliminated by an enzymatic reaction, as this would require its diffusion to the enzyme's active site (Wood et al. 2008). As diffusion is slower than the half-life of the molecule, it will react with any oxidizable compound in its vicinity. It can damage virtually all types of macromolecules: carbohydrates, nucleic acids (mutations), lipids (lipid peroxidation), and amino acids (Beckman et al. 1990).

Diabetes mellitus, often referred to simply as diabetes, is a syndrome of disordered metabolism, due to a combination of hereditary and environmental causes, resulting in abnormally high blood sugar levels (hyperglycemia). Diabetes develops due to a diminished production of insulin in type 1 (Headig et al. 2008) or resistance to its effects in type 2 (Jain and Saraf 2008). One therapeutic approach for treating diabetes is to decrease the postprandial hyperglycemia. This is done by retarding the absorption of glucose through the inhibition of the carbohydrate-hydrolysing enzymes α-glucosidase in the digestive tract. Inhibitors of the enzyme delay carbohydrate digestion and prolong overall carbohydrate digestion time, causing a reduction in the rate of glucose absorption and consequently blunting the postprandial plasma glucose rise (Holman et al. 1999). In the human diet, starch is often the main source of exogenous glucose that appears in the blood circulation postprandially (Ells et al. 2005). It has been known that large differences can be found in the postprandial rise in blood glucose and insulin levels in response to different starch-rich foods containing the same amounts of starch (Wallace et al. 2008). α-amylase breaks starch down into sugar. Amylase is present in human saliva, where it begins the chemical process of digestion. *Picrasma quassioides* is distributed in China, Korea, India, and Japan. Its bark and stem contains a

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number of medicinal compounds and has been shown to be anthelmintic, antiamoebal, antiviral, bitter, hypotensive, and stomachic (Yoshikawa et al. 1995). It increases the flow of gastric juices. It is used in Korea in the treatment of digestive problems, especially chronic dyspepsia.

Therefore, the main aims of this study were to evaluate antioxidant and antidiabetes activities of four fractions (hexane, EtOAc, BuOH, and water) of *P. quassioides* MeOH extract and to determine the possible mechanisms of cell death elicited by BuOH fraction on stomach carcinoma cells.

Materials and Methods

Preparation of Fractions

P. quassioides stems were obtained in Chuncheon, Korea. Dried *P. quassioides* stems powder (100 g) was refluxed with methanol for 3 days at room temperature, and 12.324 g MeOH extract was obtained after evaporated. Then MeOH extract (10 g) was suspended in distilled water (2 L) and partitioned (Fig. 1) with 2 L of hexane, EtOAc, and BuOH in sequence to afford the fractions of hexane (1.494 g), EtOAc (0.941 g), BuOH (1.584 g), and water (5.972 g).

Cell Lines and Culture Medium

NCI-N87 cells (human stomach carcinoma cell line) and 293 cells (human kidney normal cell line) were purchased from Korean cell line bank. NCI-N87 cells were cultured in RPMI 1640, supplemented with 10% fetal bovine serum (FBS, *v*/*v*) (Hyclone, Utah, USA), 100 U mL^{-1} penicillin–streptomycin solution (Hyclone, Utah, USA). Two hundred ninety-three cells were cultured in DEME, supplemented with 10% FBS (*v*/*v*), 100 U mL^{-1} penicillin–streptomycin solution.

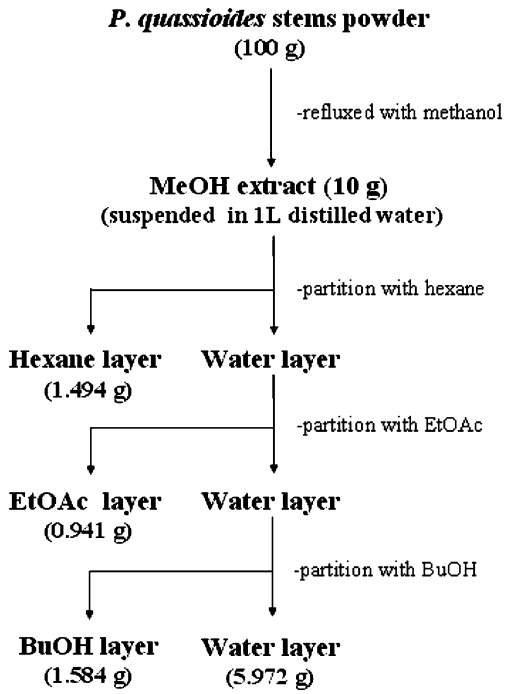

Fig. 1 Protocol of preparation fractions

Hydroxyl Radical Scavenging

Hydroxyl radical scavenging was carried out according to the method of Lee et al. (2007). The Fenton reaction mixture consisted of 200 μL of $FeSO_4 \cdot 7H_2O$ (10 mM), EDTA (10 mM), and 2-deoxyribose (10 mM). Two hundred microliters sample and 1 mL of 0.1 M phosphate buffer (pH 7.4) were added to make up a total volume of 1.8 mL. Thereafter, 200 μL of 10 mM H_2O_2 was added and the reaction mixture was incubated at 37°C for 4 h. After incubation, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added and the mixture was placed in boiling water for 10 min. It was centrifuged (5 min, 300 rpm) and the absorbance was measured at 532 nm. Each assay was performed in triplicate. The hydroxyl radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging activity } (\%) = (1 - (A_1 - A_2)/A_0) \times 100\%$$

where A_0 was the absorbance of the control (blank) and A_1 was the absorbance in the presence of sample, A_2 was the absorbance without 2-deoxyribose.

α-Amylase Inhibitory Activity

Twenty microliters of α-amylase (0.05 U μL^{-1}) was premixed with 20 μL of sample and 250 μL of 2% starch solution in 0.1 M sodium phosphate buffer (pH 6.9) was added as a substrate to start the reaction. The reaction was carried out at 37°C for 10 min and terminated by the addition of 200 μL of DNS reagent (1% 3,5-dinitrosalicylic acid and 12% sodium potassium tartrate in 0.4 M NaOH). The reaction mixture was heated for 15 min at 100°C and then diluted with 5 mL of distilled. α-amylase activity was determined by measuring absorbance at 540 nm. $IR=(1-(A_i-A_{iB})/(A_0-A_{0B}))\times 100$; A_i is the A_{540} of sample reactive solution, A_0 is the A_{540} of control reactive solution, A_{iB} is the blank of sample and A_{0B} is the blank of control.

α-Glucosidase Inhibitory Activity

One hundred microliters of 3 mM *p*-nitrophenyl α-D-glucopyranoside in 0.1 M sodium phosphate buffer (pH 6.9) was added as a substrate to the mixture of 50 μL of α-glucosidase (0.3 U mL^{-1}) and 50 μL of sample to start the reaction. The reaction was conducted at 37°C for

Table 1 The sequence of primers used in RT-PCR

Primer	Sequence (5′–3′)
c-myc Forward	GAACAAGAAGATGAGGAAGA
c-myc Reverse	AGTTTGTGTTTCAACTGTTC
p53 Forward	TGTGGAGTATTTGGATGACA
p53 Reverse	GAACATGAGTTTTTTATGGC
Caspase-3 forward	TCACAGCAAAAGGAGCAGTTT
Caspase-3 reverse	CGTCAAAGGAAAAGGACTCAA
β-Actin forward	TCACCCTGAAGTACCCCATC
β-Actin reverse	CCATCTCTTGCTGCAAGTCC

15 min and stopped by the addition of 750 μL of 0.1 M Na_2CO_3. α-glucosidase activity was assessed by measuring the release of *p*-nitrophenol from *p*-nitrophenyl α-D-glucopyranoside at 400 nm.

MTT Assay

In this study, carcinoma cell growth inhibition activity was measured by using MTT assay (Athukorala et al. 2006). Tumor cells were seeded in a 96-well plate at the concentration of 2×10^5 cells mL^{-1}. After 4 h at 37°C, in a humidified atmosphere of 5% CO_2, all extracts were treated to the wells at a concentration of 100 μg mL^{-1}, 200 μg mL^{-1}, 300 μg mL^{-1}, and 500 μg mL^{-1}, respectively. The cells were then further incubated for an additional 72 h at 37°C. MTT stock solution (50 μL; 2 mg mL^{-1} in phosphate-buffered saline (PBS)) was then added to each well for a total reaction volume of 250 μL. After incubating for 4 h in a humidified atmosphere of 5% CO_2 at 37°C, the supernatants of each well were spilt out. The formazan crystals in each well were dissolved in 150 μL of DMSO. The amount of purple formazan was determined by measuring the absorbance at 540 nm after 15 to 20 min. For treated cells, viability was expressed as a percentage of control cells. All determinations were carried out in triplicate.

Assessment of Apoptosis by Annexin V

Apoptotic cell death of BuOH fraction solution treated with BuOH fraction was measured using fluorescein isothiocyanate (FITC)-conjugated Annexin V/PI assay (Biovision, Palo Alto, CA, USA) by flow cytometry (Becton–Dickinson, Franklin Lakes, NJ, USA) (Chiu et al. 2006). Briefly, 5×10^5 cells mL^{-1} were washed with ice-cold PBS, resuspended in 0.1 mL binding buffer, and stained with 10 μL of FITC-conjugated Annexin V (10 mg mL^{-1}) and 10 μl of PI (50 mg mL^{-1}). The cells were incubated for 15 min at room temperature in the dark, 400 μL of binding buffer was added, and analyzed by a FACScan flow cytometer Annexin V excitation 488 nm and emission 515 nm; PI excitation 488 nm and emission 580 nm.

Determination of the Expression Level of Apoptotic-Related Genes

The mRNA expression levels of established apoptotic-related genes, i.e., c-myc, p53, and caspase 3 were carried out using semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) as described (Kousteni et al. 1999). Briefly, the cells were cultured in T-25 flasks and starved in medium with 0.5% (*v*/*v*) FBS for 4 h before stimulation. Five hundred micrograms per milliliter of BuOH fraction needed to achieve 50% growth inhibition was used to stimulate the cells over the period of 24 h. Total cellular RNA was isolated from the untreated and treated cells using Trizol Reagent according to manufacturer's protocol. Subsequently, 1 μg RNA was reverse transcribed into cDNA and used as the template for PCR amplification. The template of PCR was denaturated for 5 min at 94°C, followed by amplification cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and terminated with an additional extension step for 7 min at 72°C. The PCR primers were designed on the human mRNA encoding the respective genes (Table 1). The PCR conditions including the quantity of RNA and cDNA samples used to amplify c-myc, caspase-3, p53, and β-actin genes were in the exponential phase of amplification indicating that the

Table 2 Hydroxyl radical scavenging activity of different fractions of *P. quassioides* MeOH extract

Sample	Concentration (μg mL^{-1})	Hydroxyl radical scavenging activity (%)[a]
Hexane fra.	100	1.65±0.45
	200	5.86±0.40
	500	9.04±1.07
EtOAc fra.	100	21.63±0.23
	200	35.80±0.99
	500	53.49±0.38
BuOH fra.	100	5.89±0.58
	200	28.31±2.09
	500	37.75±0.52
Water fra.	100	0.96±0.67
	200	4.72±0.20
	500	21.04±3.98
Positive control		
	100	85.61±0.69
α-Tocopherol[b]	200	92.31±0.81
	500	96.15±1.24

[a] Each value represents the mean±SD of three determinations

[b] α-Tocopherol was used as a positive control

Table 3 α-amylase and α-glucosidase inhibition activities of different fractions of *P. quassioides* MeOH extract

Sample	Concentrations (μg mL^{-1})	α-amylase inhibition ratio (%)[a]	α-glucosidase inhibition ratio (%)[a]
Hexane fra.	100	2.97±0.30	2.52±7.63
	200	5.93±0.60	7.55±6.80
	500	6.81±0.52	8.81±4.36
EtOAc fra.	100	4.01±0.60	3.14±3.93
	200	5.24±2.28	3.77±9.98
	500	9.25±0.30	6.29±3.93
BuOH fra.	100	5.41±0.60	1.26±3.93
	200	4.71±0.52	5.66±1.89
	500	6.98±0.80	6.92±4.75
Water fra.	100	5.06±1.60	0.63±3.93
	200	7.50±0.30	5.03±2.88
	500	7.68±0.30	7.55±3.77
Positive control			
Acarbose[b]	0.01	27.05±1.09	52.20±7.62

[a] Each value represents the mean±SD of three determinations

[b] Acarbose (0.01 μg mL^{-1}) was used as a positive control

conditions were optimized to be utilized for semi-quantitative studies (Tengku et al. 2000). The mRNA level of β-actin was used as an internal control for template levels. The PCR products were electrophoresed on a 1% (*w*/*v*) agarose gel and visualized with ethidium bromide staining.

Results

Antioxidant Activity

The hydroxyl radical is one of representative reactive oxygen species generated in the body. Hydroxyl radical scavenging activities of fractions were shown in Table 2. EtOAc fraction at 0.5 mg mL^{-1} showed that scavenging activity of 53.49% of the available free radicals was the highest activity among four fractions. α-tocopherol was used as a positive control, its hydroxyl radical scavenging activity was very high (85.61% at concentration of 100 μg mL^{-1}, 92.31% at concentration of 200 μg mL^{-1}, and 96.15% at concentration of 500 μg mL^{-1}).

α-Amylase and α-Glucosidase Inhibitory Activity

All fractions had α-amylase and α-glucosidase inhibitory activity at the concentration of 500 μg mL^{-1} (Table 3). EtOAc fraction inhibited α-amylase 9.25% and hexane fraction inhibited α-glucosidase 8.81% at the concentration of 500 μg mL^{-1}, respectively.

Cytotoxic Effect on Human Cells

The cytotoxic effect of fractions on NCI-N87 and 293 cells were determined. Cells were exposed to various concentrations of fractions (100 to 500 μg mL^{-1}) for 72 h. As showed in Fig. 2, hexane, EtOAc, and water fractions showed significant cytotoxic effect on both of NCI-N87 and 293 cells in dose-dependent manner. BuOH fraction also has high cytotoxicity on NCI-N87 cells (Fig. 2b), but has no cytotoxicity on human normal kidney cells (293; Fig. 2a). To study roles of fraction in apoptosis, BuOH fraction was used to set up apoptosis system on NCI-N87 cells. NCI-N87 cells were treated with concentration of fraction as 300 μg mL^{-1} and 500 μg mL^{-1} for 48 h (Wang et al. 2006). Quantitative analysis using Annexin V/PI assay further showed that the proportion of early stage apoptotic cells (Annexin V+/PI-) increased significantly from 20.48% to 37.55%, while the proportion of late stage apoptotic cells (Annexin V+/PI+) increased significantly from 2.70% to 29.51% (Fig. 3). In this assay, paclitaxel was used as a positive control, which induced early stage apoptosis

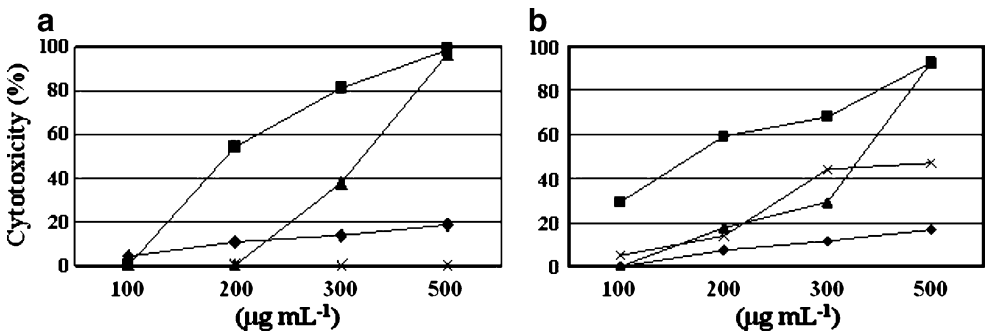

Fig. 2 Cytotoxicity test of fractions from *P. quassioides* MeOH extract on the human kidney normal cell line (293) (**a**) and the human stomach cancer cell line (NCI-N87) (**b**) by MTT assay. The concentrations of each fraction are 100 μg mL^{-1}, 200 μg mL^{-1}, 300 μg mL^{-1}, and 500 μg mL^{-1}, respectively. (*filled diamond* water fra., *filled square* hexane fra., *filled triangle* EtOAc fra., *multiplication symbol* BuOH fra.)

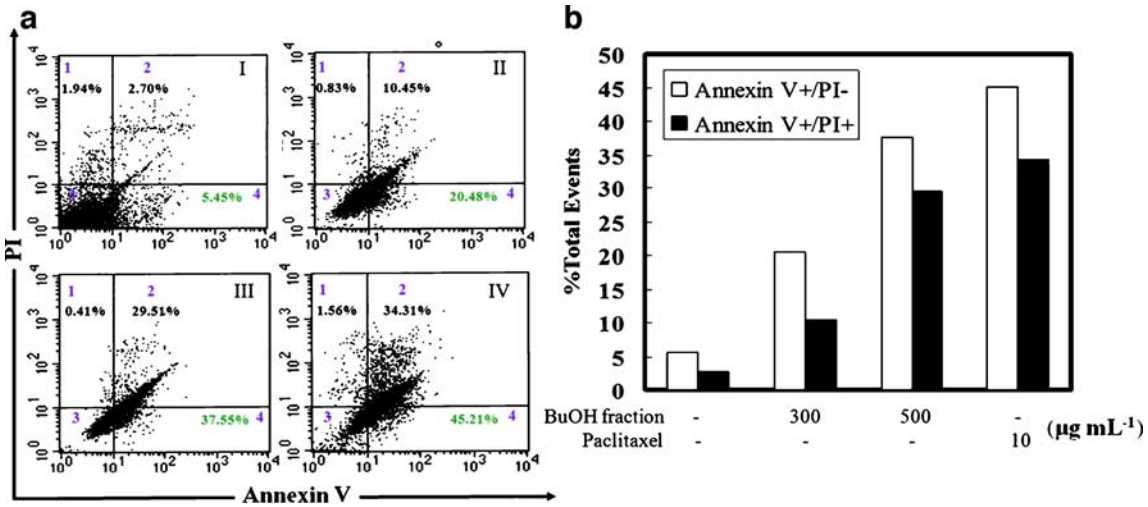

Fig. 3 Assessment of apoptosis by Annexin V on the human stomach cancer cell line (NCI-N87). **a** The cells were pre-treated with BuOH fraction (panels *II* and *III*) and paclitaxel (panel *IV*) or without BuOH fraction and paclitaxel (panel *I*) 48 h. The necrotic cells lost cell membrane integrity that permits PI entry. Viable cells exhibit Annexin V-/PI- (symbol *3* in the plot); early apoptotic cells exhibit Annexin V+/PI- (symbol *4* in the plot); late apoptotic cells or necrotic cells exhibit Annexin V+/PI+ (symbol *2* in the plot). **b** Percentage of cell death based on the assessment of apoptosis by Annexin V in (**a**). Paclitaxel was used as a positive control

45.21% at concentration of 10 μg mL^{-1} on NCI-N87 cells. These results suggested that BuOH fraction can induce apoptosis on NCI-N87 cells.

Expression Level of Apoptotic-Related Genes

In order to determine the expression level of apoptosis-related genes induced in the BuOH fraction treated NCI-N87 cells, the mRNA levels of c-myc, p53, and caspase 3 (Table 1) were evaluated by RT-PCR (Ju et al. 2004; Qi and Xu 2006). The steady state mRNA levels of caspase 3 had no change, c-myc decreased when the cells were treated with the fraction at 3 h and 12 h on NCI-N87 cells, and p53 were decreased very significantly (Fig. 4). Thus, the results indicated that apoptosis mechanism of the BuOH fraction killed NCI-N87 carcinoma cells was not through caspase 3, c-myc, and p53.

Discussions

Oxidant damage caused by free radicals can theoretically contribute to chronic diseases, such as cancer, cardiovascular diseases, and age-related macular degeneration, and to aging (Lin et al. 2004). Significant hydroxyl radical (ROS) inhibition activities of four fractions extracted from MeOH extract *P. quassioides* were observed in Table 2.

The best way to control postprandial plasma glucose levels was with medication combined with dietary restrictions and exercise programs for diabetes (Yki-Jarvinen 1997). Inhibiting α-amylase and α-glucosidase are new alternative therapeutic approaches of treating non-insulin diabetes mellitus, which are the key enzymes involved in starch breakdown and intestinal absorption. The digestion and uptake of carbohydrate decreases by the inhibition of these enzymes significantly, thereby decreasing the post-prandial blood glucose level in the non-insulin-dependent diabetes mellitus patients (Puls et al. 1977). Inhibitors of intestinal α-glucosidase have used in the treatment of non-insulin-dependent diabetes mellitus and represented at the huge proportion of antidiabetic drug market (Inzucchi 2002). Table 3 showed four fractions extracted from MeOH extract of *P. quassioides* have a certain inhibition on α-glucosidase and α-amylase assay, meaning that α-glucosidases and α-amylase inhibitors can be isolated from fractions.

The ability to induce tumor cell apoptosis is an important property of a candidate anticancer drug, which discriminates between anticancer drugs and toxic compounds (Tan et al. 2005). The MTT assay is thought to be produced by

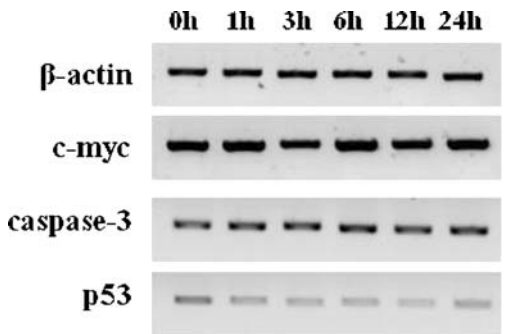

Fig. 4 Time dependency effects of the p53, caspase 3, and c-myc mRNA levels in human stomach cancer cell line, NCI-N87, incubated in the absence or presence of BuOH fraction (500 μg mL^{-1}) from *P. quassioides* MeOH extract. β-actin was used as an internal control for integrity

the mitochondrial enzyme succinate dehydrogenase (Lee et al. 2008a, b) and can be dissolved and quantified by measuring the absorbance of the resultant solution. The absorbance of the solution is related to the number of live cells. A multiwell spectrophotometer assay can be semi-automated to process a large number of samples and provide a rapid object measurement of cell number (Kogure et al. 2003). Our study showed that BuOH fraction extracted from MeOH extract of *P. quassioides* exerted a significant proliferation inhibitory activity against NCI-N87 cells in a dose-dependent manner and had no cytotoxic effect on 293 cells (Fig. 2). Much effort has been directed towards the searching for compounds that influence apoptosis and understanding their mechanisms of action (Hu and Kavanagh 2003). The apoptosis evoked by BuOH fraction was confirmed by the observation of phosphatidylserine translocation (Fig. 3).

Caspase 3 belongs to caspase family, which directly responsible for many of the molecular and structural changes in the cells undergoing apoptosis (Thornberry and Lazebnik 1998). C-myc, a member of the Myc-family of transcription factors, is a regulator of cell cycle progression and can also cause cells to undergo apoptosis (Peckham and Cleveland 1994; Wagner et al. 1994). Gene of p53, known as protein 53 or tumor protein 53, is a transcription factor which regulates the cell cycle and thus functions as a tumor suppressor that involved in preventing cancer. In this study, the mRNA expression levels of three apoptotic-related genes, p53, c-myc, and caspase 3, in NCI-N87 cells treated with the BuOH fraction were investigated. It was found that the apoptosis elicited by the BuOH fraction on NCI-N87 cells was not mediated via caspase 3, c-myc, and p53 (Fig. 4). It should be other mechanism induced apoptosis of the BuOH fraction on NCI-N87 carcinoma cells.

In conclusion, the results suggest that fractions of *P. quassioides* MeOH extract may contain bioactive compounds that have antioxidant activity, α-glucosidase and α-amylase inhibition activities and kill NCI-N87 stomach carcinoma cell by apoptosis. So, *P. quassioides* could be considered as a functional food ingredient and pharmaceutical. However, the identification of individual compounds and apoptosis mechanism are needed to understand their mechanisms of action.

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