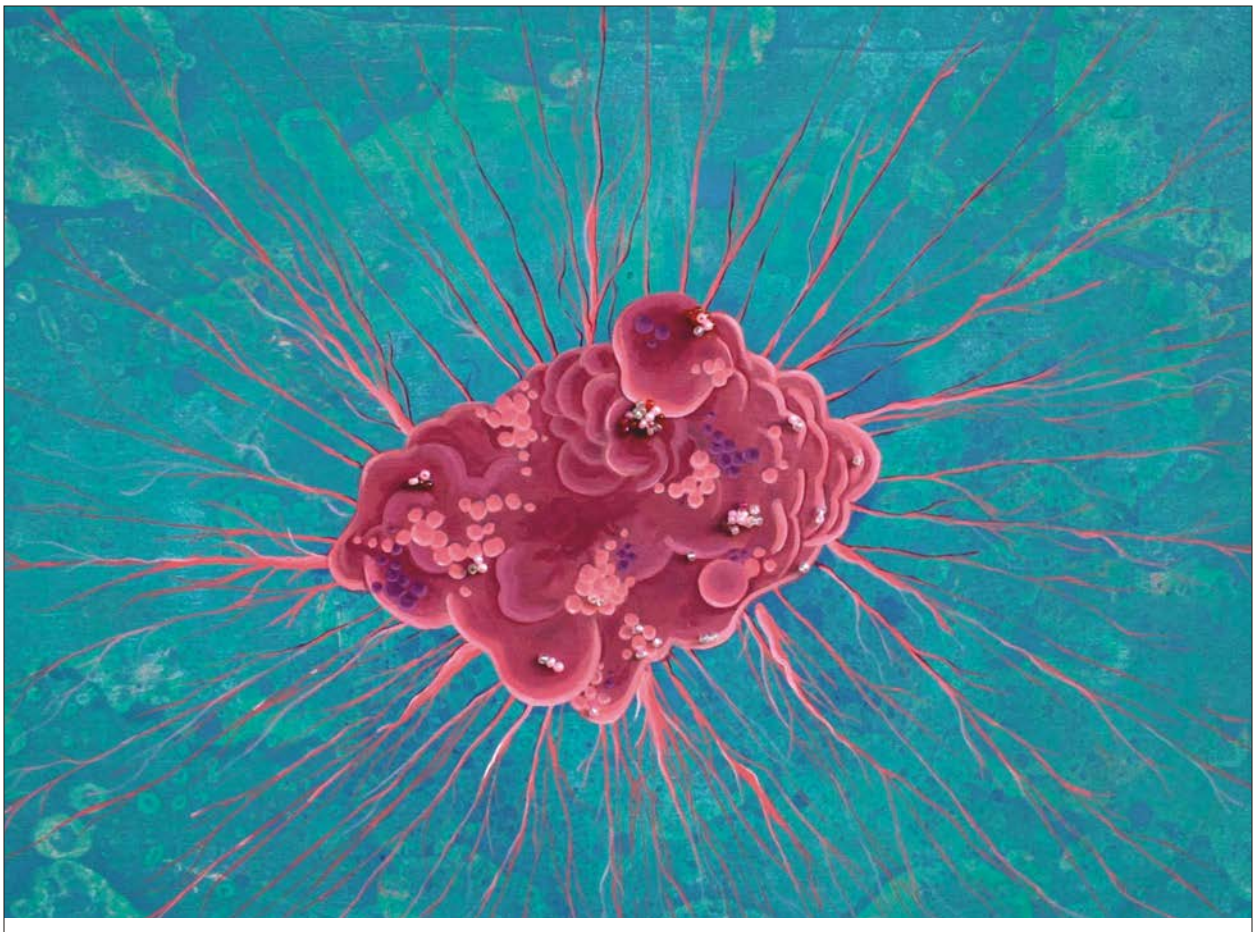

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Cytotoxic Effect of *Berberis vulgaris* Fruit Extract on the Proliferation of Human Liver Cancer Cell Line (HepG2) and its Antioxidant Properties

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Abstract: Normal biochemical processes in human body may produce free radicals. These free radicals can, in turn, lead to oxidative stress related disease. This study examined the antioxidant activity of *Berberis vulgaris* fruit extract and its cytotoxic effect on human liver cancer cell line (HepG2).The antioxidant activity of *Berberis vulgaris* Fruit Extract (BFE) was assay by β-carotene bleaching and 1-diphenyl-2-picrylhydrazyl (DPPH) assay. The screening of cytotoxic effect was carried out by the microculture tetrazolium salt (MTT) assay on the human liver cancer cell line (HepG2). The BFE with concentration of 5-140 μg mL^{-1} was used. The control group cell was without any treatment. Intracellular Alkaline Phosphatase (ALP) activity is determined by p-nitrophenyl phosphate. The concentration of 5 μg mL^{-1} was chosen for this test. In β-carotene bleaching, ascorbic acid showed the mean total antioxidant activity of 96.16±5.09%, followed by BHT (66.71±2.52) and BFE (59.91±8.64). In DPPH, the EC_{50} of ascorbic acid was 0.252±0.000 mg mL^{-1}, BHT (0.612±0.009 mg mL^{-1}) and BFE (0.685±0.033 mg mL^{-1}). The IC_{50} of BFE was found 106.0±10.1 μg mL^{-1}. Beside reduction in cell proliferation the crude extract was capable of enhacing the intracellular protein content in cell cancer line by one fourth while intracellular alkaline phosphatase activity increased by 7 fold. The results showed that processed commercial *Berberis vulgaris* exhibited antioxidant properties, has the ability of reducing cell viability and may had the potential of enhancing the ALP activity probably through structural changes.

Key words: *Berberis vulgaris* fruit extract, antioxidant activity, cytotoxicity, Alkaline Phosphatase (ALP)

Introduction

Cancer is one of the major causes of death worldwide. It is estimated that 12.8% of the world population die due to cancer (World Health Orgnization, 2004). The number of new cases has been increasing every year. From the year 1990 to 2000 alone, there has been an increase of 22% in incidence and mortality (Parkin, 2001). In Malaysia, it is the fourth leading cause of death (Lim, 2002).

Primary liver cancer is one of the most common malignancies in the world. It is considered to be one of the major malignant diseases in the world today because of its high incidence in many of

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the most populous countries in the world, its fulminant course, poor response to conservative treatment, low resectability rate when symptomatic and grave prognosis. Among cancer deaths in 1998, it was among one of the commonest one diagnosed (Vital Statistics Malaysia, 1999). We should look into this area as it ranked second in creating the burden of disease in the world (World Health Organization, 2004).

Fruits and vegetables account for a small part of our daily caloric intake; however, their benefits to health surpass their caloric contribution. The contributory factors are due to the presence of vitamins and provitamins, such as ascorbic acid, tocopherols and carotenoids and in addition to that, they are also rich in a wide variety of phenolic substances (Loliger, 1991). The principle function of antioxidants is in delaying the oxidation of other molecules by inhibiting the initiation or propagation of oxidizing chain reactions by free radicals and they may reduce oxidative damage to the human body (Namiki, 1990). These molecules can inhibit the process of carcinogenesis to some extend (Kim *et al*., 1994).

Alkaline Phosphatase (ALP) is one of the enzymes tested in liver function test. Serum levels of ALP have been used in the clinical evaluation of numerous diseases, including malignancies. The aberrant expression of ALP genes in malignant tissues (Tsai *et al*., 2000) has led to suggestion that ALPs are oncofetal proteins and thus, could be involved in tumorigenesis. Thus, ALP has been established as a reporter for cancerous transformation (Millan, 1992; Bao *et al*., 2000).

Berberis vulgaris (barberry) grows in Asia and Europe. Medicinal properties for all parts of the plant have been reported to be able to treat various illnesses, including antimicrobial, antiemetic, antipyretic, antipruritic and cholagogue actions and it has been used in some cases like cholecystitis, cholelithiasis, jaundice, dysentry, leishmaniasis, malaria and gall stone (Lininger *et al*., 1999; Chi, 1994; Kim *et al*., 1998).

The purpose of this study examined the antioxidant properties of *Berberis vulgaris* fruit extract and its cytotoxic effect on human liver cancer cell line (HepG2).

Materials and Methods

Barberry (*Berberis vulgaris*) fruit was purchased from Iranian market at Kuala Lumpur in Malaysia. Linoleic acid, β-carotene, Tween 20, DPPH, L-ascorbic acid, BHT, cell culture medium (EMEM) and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St Louis, MO, USA). HepG2 cell line was obtained from Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. All other chemicals used were analytical grade.

Preparation of Plant Extract

Berberis vulgaris Fruit Extract (BFE) was prepared from method of Shamsa *et al*. (1999). *Berberis vulgaris* that was free from fungus, bacteria and any other plant diseases were selected randomly. The fruits were dried in an oven for 3 days at a constant temperature of 65°C. The fruits were cut into small pieces and were grounded into fine powder using a dry grinder. The grounded samples were sieved to get uniform particle size, then were kept in air-tight container and stored for further extraction. Water and ethanol were added in the ratio of 1:10 and stirred at 250 rpm in an orbital shaker for 1 h at room temperature. The extract was then separated from the residue by filtration through Whatman No.1 filter paper. The remaining residue was re-extracted twice and then the two extracts were combined. The residual solvent of ethanolic extract was removed under reduced pressure at 50°C using a rotary evaporator until thick syrup was collected. The thick syrup was evaporated completely using freeze drying system.

Antioxidant Activity

Antioxidant activity of BFE was measured according to the β-carotene bleaching method described by Gazzani *et al*. (1998). The effect of BFE on DPPH radical was achieved following the method by Yen and Hsieh (1998). BHT and ascorbic acid was used as standard comparison.

Cell Culture

Maintainance of the human liver cancer cell line (HepG2) was according to Ciccaglione *et al*. (2004).

Mtt Dye Reduction Assay

Stock solution of BFE was prepared in DMSO at a concentration of 1 mg mL^{-1}. For the experiments, substock were prepared from the stock solution and diluted to the final concentration of 5-140 μg mL^{-1} with the EMEM medium supplemented with 10% FBS. Semiconfluent cells were seeded in a 96-well plate at 1×10^4 cells/well with growth medium (100 μL) supplemented with different doses of BFE. After 72 h of incubation, cell viability was assessed by MTT, a method modified from Hirano *et al*. (1995).

Plasma Membrane Alkaline Phosphatase

The human liver cancer (HepG2 were cultured using EMEM medium supplemente. The cells, 2×10^5, were seeded in triplicate, into 6-well plate, incubated at 37°C in an incubator with 5% CO_2 atmosphere at 24 h. Then BFE (5 μg mL^{-1}) equvalent 0.01 mg of plant powder was added to the cells. and incubates for another 72 h. The cells viability was assessed by MTT method modified from Hirano *et al*. (1995).

Alkaline Phosphatase Isolation

The method of alkaline phosphatase isolation was modified from Chang *et al*. (1990). The cells were washed twice with PBS and harvested. The cells were washed again another two times with PBS, centrifuged at 3000 g for 5 min. Resuspension buffer (20 mM Tris/HCl, 1 mM $MgCl_2$, 150 mM NaCl pH 8.0) was added after that. The cells were solubilized by adding Triton X-100 was added to a final concentration of 1%. The cell free system was obtained by centrifugation at 12,000 g for 15 min.

Enzyme Assay

The enzyme activity was determined through enzymatic hydrolysis of 4-nitrophenyl phosphate (Chang *et al*., 1990). One unite of enzyme activity was made defined as the initial rate of 1 μmol substrate hydrolysis/min at 37°C. The specific activity was expressed in U mg^{-1} protein. The total protein concentration was determined by method of Lowry *et al*. (2001).

Statistical Analysis

All determinations except IC_{50} values were subjected to analysis of mean using one-way variance analysis (ANOVA). The Statistical Package for Social Science for windows version 11.5 was used to analyze the data.

Results

β-carotene Bleaching

The total antioxidant activity, which reflected the ability of the extract to inhibit the bleaching of β-carotene, was measured and compared with standards. The β-carotene bleaching rates

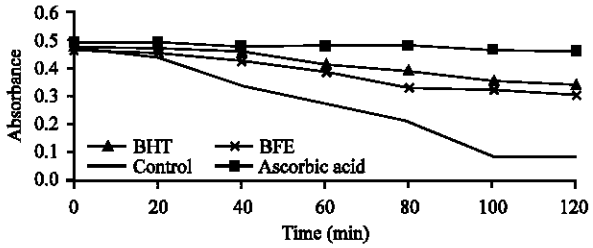

Fig. 1: β-carotene bleaching rates of *Berberis vulgaris* Fruit Extract (BFE) compared with ascorbic acid and Butylated Hydroxyl Toluene (BHT) at 1 mg mL^{-1} β-carotene chloroform

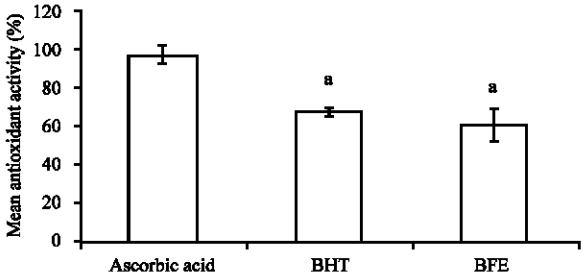

Fig. 2: Mean total antioxidant activity of *Berberis vulgaris* Fruit Extract (BFE), ascorbic acid and Butylated Hydroxyl Toluene (BHT) measured by β-carotene bleaching assay. The same letter (a) indicate values not significantly different from each other with $p>0.05$. Results are means of three determinations

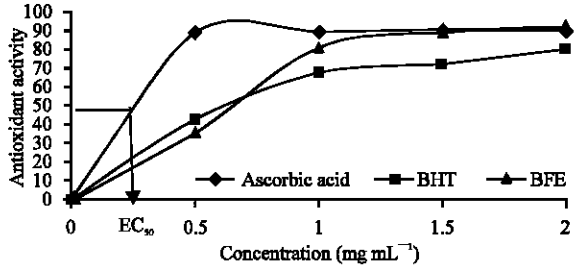

Fig. 3: Scavenging activity of ascorbic acid, Butylated Hydroxyl Toluene (BHT) and *Berberis vulgaris* Fruit Extract (BFE) on DPPH• radical

(Fig. 1). It showed a decrease in absorbance of β-carotene in the presence of different samples due to the oxidation of β-carotene and linoleic acid. The high absorbance values indicated that BFE possessed antioxidant activity.

The mean total antioxidant activity of ascorbic acid is the highest (96.16±5.09%), followed by BHT (66.71±2.52%) and BFE (59.91±8.64%) (Fig. 2). There is no significant difference observed between BFE and BHT.

Dpph Free Radical Scavenging

The dose response curve for the free radical scavenging activity of studied sample extract and standards at different concentrations are presented (Fig. 3). The scavenging activity of all the samples on the DPPH radical was found to be strongly dependent on the concentration.

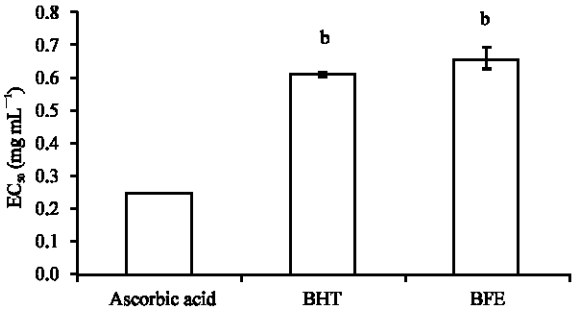

Fig. 4: Free radical scavenging activities (EC_{50}) of *Berberis vulgaris* Fruit Extract (BFE), ascorbic acid and Butylated Hydroxyl Toluene (BHT). The DPPH• radical concentration was 0.250 mM in all reaction mixtures. The same letter (b) indicates values not significantly different from each other with $p>0.05$. Results are means of three determinations. The EC_{50} value is defined as the amount of extract necessary to decrease the initial DPPH• radical concentration by 50%

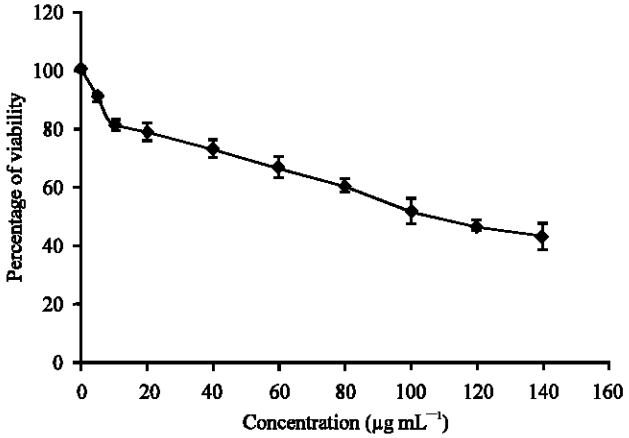

Fig. 5: The effect of *Berberis vulgaris* Fruit Extract (BFE) on HepG2 cell lines after 72 h incubation. Cell were plated with 1×10^4 cells per well in 96-well culture plates. All tests were conducted in triplicate and the means were used. MTT assay was applied to assess cytotoxic effect. The IC_{50} value of BFE on HepG2 was 106.0±10.1 µg mL^{-1}. IC_{50} is defined by the amount of extract necessary to inhibit the cell viability by 50%

Fig. 4 shows the comparison of the mean concentration of 50% free radical scavenging activity (EC_{50}) of sample and standards against 250 µM DPPH radical. The EC_{50} of ascorbic acid is the strongest (0.252±0.000 mg mL^{-1}), followed by BHT (0.612±0.009 mg mL^{-1}) and BFE (0.658±0.033 mg mL^{-1}). There is no significant difference observed between the EC_{50} of BFE and BHT. The EC_{50} value is defined as the amount of extract necessary to decrease the initial DPPH• radical concentration by 50%.

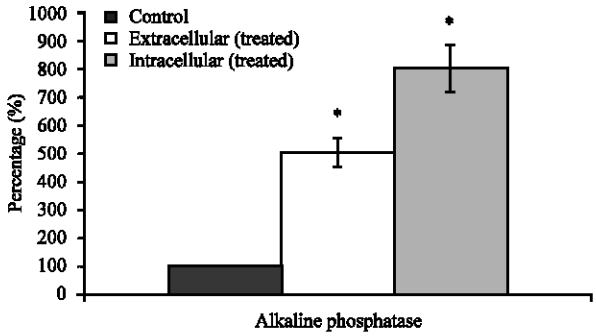

Fig. 6: The effect of *Berberis vulgaris* fruit extract (5 μg mL^{-1}) on extracellular and intracellular alkaline phosphatase content with respect to the untreated (control), which is set at 100%, respectively. Asterisks (*) indicate the significant difference between treated group compared with the control group, $p<0.05$. Results are means of three determinations

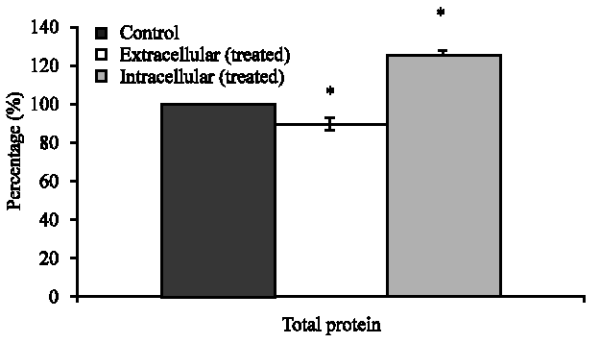

Fig. 7: The effect of *Berberis vulgaris* fruit extract (5 μg mL^{-1}) on extracellular and intracellular total protein content with respect to the untreated (control), which is set at 100%, respectively. Asterisks (*) indicate the significant difference between treated group compared with the control group, $p<0.05$. Results are means of three determinations

Cytotoxic Study

Figure 5 shows the viability of HepG2 cells after 72 h of incubation with BFE. Cell were plated with 1×10^4 cells per well in 96-well culture plates. MTT assay was applied to assess cytotoxic effect. There is a dose dependent reduction on the viability of the cells. The IC_{50} of the BFE on HepG2 cells was established to be around 106.0±10.1 μg mL^{-1}. It was evident, as represented in the Fig. 5 the cell number were reduced by the plant extract in HepG2 cells.

Alkaline Phosphatase

The ALP activities in the medium of the cell line (extracellular) increased by 4 fold while the intracellular level increased by 7 fold after 72 h of incubation with 5 μg mL^{-1} BFE as shown in Fig. 6. Protein variation of the cells with the plant extract decreased the extracellular total protein by 10.25% while the intracellular total protein was increased by 25.34% in comparison to control cells (Fig. 7).

Discussion

According to Jayaprakasha *et al*. (2001) the bleaching mechanism of β-carotene is a free radical mediated phenomenon resulting from the formation of hydroperoxides from linoleic acid. The presence of antioxidants in the samples (ascorbic acid, BHT and BFE) protects the extend of β-carotene bleaching by neutralizing the linoleate-free radical and other free radicals formed in the system.

The antioxidant activity of the extract were further assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay (Blois, 1952). Apart from β-carotene bleaching, DPPH free radical scavenging activity assay was carry out in order to provide additional data on antioxidant potential of the ability of the *Berberis vulgaris* Fruit Extract (BFE) to act as free radical scavengers or hydrogen donors. β-carotene bleaching assay is a direct measurement of antioxidant activity while DPPH free radical scavenging assay, which is an indirect measurement of antioxidant activity. Both methods compliment each other well in providing a more precise and reliable data (Roginski and Lisi, 2004). This is shown when both methods gave the same result where the antioxidant level of ascorbic acid is significantly higher than BFE, but no significant difference was between BFE and BHT (Fig. 2 and 4). The antioxidant level of BFE was of considerable amount since the extract was a crude extract while the ascorbic acid and BHT standards are pure compounds.

The anticancer activity of BFE was investigated using MTT assay on HepG2, a human liver cancer cell line. The MTT assay relies on the ability of viable cells to reduce a tetrazolium dye to formazan. The yellow tetrazolium salt was cleaved by the cellular microsomal enzymes, succinate-dehydrogenase, converting the MTT to insoluble purple formazan crystals. Therefore, the amount of formazan produced is directly proportional to the number of viable cells (Mossman, 1983; Wu *et al*., 1993) assuming that dead cells or their products do not reduce tetrazolium. Decreased viability indicates that the BFE contain cytotoxicity ability.

For the MTT assay, only liver cancer and no other cell line is used since it is our interest to study on liver cancer. The result show cytotoxic level of BFE on HEPG2 cells which is 106.0±10.1 μg mL^{-1}. The cytotoxic ability might be due to the antioxidant content of the extract. Antioxidants have been shown to be able to prevent carcinogenesis in some extend (Kim *et al*., 1994; Hiros *et al*., 1994).

Total intracellular protein content in the treated group was increased by one fourth (Fig. 7). Based on this observation, we expect almost the same enhancement in all protein, including ALP. In reality, that is not the case as the ALP activity is increased by 7 fold (Fig. 6). This can only be explained by the reason that total protein content is increased not so much by general increment but mostly due to enhancement of ALP enzyme. Similar results have been reported by (Sadeghi and Yazdanparast, 2003; Sorimachi and Yasumura, 1986) with K562, CCRF-CEM, HL-60 and LNCaP-FGC-10 cell lines. Based on these, they suggest a regulatory role of extract on the intracellular ALP activity.

They also suggest that the enhancement of molecular activity and intracellular accumulation of ALP in the cells may be due to at least two factors. Firstly, the enhanced ALP is based on the increased intracellular protein content of the treated cell line. Although the increase in the intracellular protein content is not very high, there still exists the possibility. Secondly, there might be structural modification of the enzyme, probably through noncovalent aggregation to form isozymes. These structural modifications can be a better explanation because the intracellular protein variation from control is low but the intracellular ALP activity is enhanced significantly (Fig. 6).

The extracellular ALP level in the treated group was increased 4 fold (Fig. 7). The first possible reason might be due to damage to the cell, causing it to be leaky or dead. This is not very likely since

the total extracellular protein was decreased by 10.25% compared to control and not increased as expected (Fig. 7). Sorimachi and Yasumura (1986) have stated before that there can be an increase in extracellular ALP level under various conditions due to the modulation of ALP molecule accompanied by changes in the enzyme activity without new protein synthesis. Since total extracellular protein level is decreasing instead of increasing or maintaining at the same level, we think the reason is because the extract might also affect other protein regulations.

Conclusions

In conclusion, the processed commercial *Berberis vulgaris* exhibited considerable amount of antioxidant properties. The cytotoxic effect might be due to antioxidant properties. The extract also had the ability to enhance the ALP activity probably through structural changes. However, further studies are needed to identify the active components of the extract, its cytotoxic effect and effect on ALP activity. Further research is in progress in our laboratory, to explore the mode of action of the purified component on the molecular structure of the ALP of the treated cell lines and elucidate the chemical structure of the purified component of *Berberis vulgaris*.

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