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RESEARCH ARTICLE

Anticancer and Antioxidant Effects of Red Cabbage on Three Cancerous Cell Lines and Comparison with a Normal Cell Line (HFF-3)

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ABSTRACT

Red cabbage or scientifically *Brassica oleracea* is a rich source of anthocyanins exhibiting enormous antioxidant properties creating a perspective of its applications in healthcare sector. The aim of this study was the evaluation of antioxidant properties of red cabbage extract by DPPH radical scavenging assay, and exploration of its anticancer activity on the growth and viability of the desired human cancer cells by *in vitro* assay to detect cytotoxic activity. The results showed enhanced effects with increasing concentration exhibiting highest value of IC90+ at the concentration of 2500 µg/ml when it is compared with IC50 value of red cabbage extract at a concentration of 750 µg/ml. The polyphenol compounds were found 39.55 mg GAE/100 g of red cabbage extract and red cabbage extract increases the death rate of cancer cells and the cytotoxicity effect was dose dependent. It can be concluded that red cabbage extract should be used at lower concentrations than 6.4 mg/ml in order to prevent the normal human cell damage. Thus, it can be considered as a healthy foodstuff due to numerous phenolic compounds and powerful antioxidant and anticancer activity when it is used in moderate amount.

Keywords: Red cabbage, anticancer, antioxidant properties, polyphenol, cytotoxic activity

Phenolic compounds such as flavonoid acids (flavones, isoflavones, flavanones, anthocyanins, and catechins), ascorbic acid (vitamin C), tocopherols and tocotrienols (vitamin E), carotenoids (provitamin A) are also named phytochemical antioxidants, exist in abundance in some fruits and vegetables and protect plants against predators or environmental stress (Özgen *et al*., 2010; Brito *et al*., 2014; Guerrero *et al*. 2010). Anthocyanins and phenols are the responsible for red, purple, violet and blue pigments and for astringency and bitterness, respectively, which are able to operate and prevent as antioxidants against free radicals (Brito *et al*., 2014; Cliff *et al*., 2007). As the same way, they support the human health after swallowing by a wide range of antioxidant

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protection and therapeutic benefits including reduced risk of cardiovascular diseases, stroke, cancer and other degenerative disorders (Özgen *et al*., 2010; Guerrero *et al*. 2010) and several studies have focused on the correlation of food consumption including phytochemicals and the prevention of some diseases.

Brassicaceae vegetables are rich resources of ascorbic acid, α-tocopherol, β-carotene, lutein and anthocyanins such as, cyanidin-3-diglucoside-5-glucoside derivatives with various acylated groups connected to the diglucoside with high antioxidant property exhibiting cancer chemopreventive potency (Thounaojam *et al*., 2011; Koo *et al*., 2011). Among the *Brassica* species, Brussels sprouts, broccoli and red cabbage are as the best vegetables with the most efficient antiradical system. The antioxidant compounds can inhibit or control the foodstuff oxidation and Phenolic compounds are the major antioxidants in red cabbage (Ciska, Karamac, & Kosińska, 2005; Leja, Kamińska, & Kołton, 2010). They are natural antioxidants and may have many roles such as free radical scavenger and potential producer for pro-oxidants; and their chemical activity is related to the number of their hydroxyl groups. Anthocyanins are one of the nature pigment major groups in the fruits and flowers and possess antioxidant activity. Red cabbage (*Brassica oleracea* L.; family, Brassicaseae) has the high nutritional value as it is rich in minerals, vitamins, oligosaccharides and a number of bioactive substances, such as anthocyanins, flavonols, and glucosinolates, which possess a positive impact on human health (Wiczkowski, Szawara-Nowak, & Topolska, 2013). Red cabbage is also commonly used as a dietary supplement because it is rich in anthocyanins such as cyanidin-3-diglucoside-5-glucoside derivatives with the various connected acylated groups to the diglucoside, mostly sinapoyl esters (Sankhari, Thounaojam, Jadeja, Devkar, & Ramachandran, 2012; Scalzo, Genna, Branca, Chedin, & Chassaigne, 2008; Wu & Prior, 2005).

Various forms of the activated oxygen, called reactive oxygen species (ROS), and include such free radicals like superoxide ions and hydroxyl radicals, as well as non-free radical species such as hydrogen peroxide (Yildirim *et al*., 2000). ROS exerts oxidative stress toward the human and break antioxidant defense mechanism, then the free radicals attack cell macromolecules and lead to a number of physiological disorders (Uddin *et al*., 2011). Huang *et al*. (2005) have described the definition of antioxidant as it is "a substance that opposes oxidation or inhibits the promoted reactions by oxygen or peroxides that many of those are being used as preservatives in various products". A more biologically relevant definition of antioxidants is "synthetic or natural substances are added to products to prevent or delay their deterioration by the action of the air oxygen (Huang, Ou, & Prior, 2005).

In recent decades, the importance of vegetable dishes has increased in the diet. Epidemiological evidences indicate that high intake of plant products reduce the risk of many chronic diseases, such as atherosclerosis and cancer. The major antioxidants of vegetables are vitamins C and E, carotenoids and phenolic compounds, especially flavonoids (Podsędek, 2007).

However, red cabbage is used as a supplement, but there is sufficient literature on its pharmacological toxicity (Thounaojam *et al*., 2011). For this reason, Thounaojam *et al*. (2011) revealed that the no observable adverse effect level (NOAEL) red cabbage is 2000 mg/kg body weight and it is not mortal up to a dose of 5000 mg/kg body weight and its extract is nontoxic and can be consumed safely for the medicinal purposes.

The aim of this study was the evaluation of antioxidant properties of red cabbage extract by DPPH radical scavenging assay, and exploring its potential anticancer activity and its effects on the growth and viability of human cancer cells such as Caco-2, KYSE-30 and MCF-7.

The Caco-2 which is a human epithelial colorectal adenocarcinoma cell is derived from a colon carcinoma and adherent and confluent monolayer for

the laboratory researches. Therefore, these cells are caused the rapid tight junctions and are widely used in the pharmaceutical industry for the *in vitro* researches. KYSE-30 is also a human esophageal squamous carcinoma cell, adherent, epitheloid with long growing in monolayers which originate from well differentiated invasive esophageal squamous cell carcinoma resected. MCF-7 is a human adherent and epithelial breast cancer cell line which derived from metastatic site and is used for more abstracts reporting studies on the breast cancer. HFF-3 is a human, adherent and fibroblast normal cell from foreskin tissue.

Therefore, in this research, the cytotoxic effect of the red cabbage extract has been investigated on three cancerous cell lines such as Caco-2, KYSE-30 and MCF-7 and their results are compared to HFF-3 as a normal cell line. An RT-PCR assay was also described for the rapid detection of IBV which can be use directly on the tissues of infected chickens in the field.

MATERIALS AND METHODS

Plant material

Fresh red cabbage was purchased from a local market in Mashhad, northeast of Iran and after separating waste and decaying leaves, they were washed and chopped in small pieces and dried under room temperature and shade until the humidity was completely evaporated. After grounding to a fine powder using a hammer mill (Polymix, Switzerland), these eventually transferred to a dark glass and refrigerated for further uses.

Extraction methods

The powdered red cabbage was extracted for 24 h in a soxhlet apparatus with methanol having powder to solvent ratio of 1:10. The red cabbage hydroalcoholic extract was concentrated at a rotary evaporator (Heidolph, Germany) at 40˚ C to remove solvent. The Samples were located in a glass plate under the hood for 4 hours and then, they were placed in a vacuum oven for 72 hours. After that, the samples were scraped and depleted in the dark glass microtubes and conserved at 4˚C until further use. For preparation of final extract, 3 ml of methanol was added to 0.054 g of dry extract, completely dissolved, 13 (5.86, 11.72, 23.44, 46.87, 93.75, 187.5, 375, 750, 1500, 2000, 2500, 3000, 9000 µg/mL) and 6 (625, 1250, 2500, 5000, 10000, 20000 µg/mL) concentrations were prepared for DPPH and MTT methods, respectively (Sreejith, Mascarenhas, Praseeja, & Asha, 2012).

DPPH radical scavenging assay

The DPPH radical scavenging activity was determined as described by Manosroi *et al* (2012). 50 µl of the sample (5.86, 11.72, 23.44, 46.87, 93.75, 187.5, 375, 750, 1500, 2000, 2500, 3000, 9000 µg/mL) and 50 µl of DPPH (Sigma-Aldrich, Germany) were put into each well of a 96-well microplate. They were mixed well and incubated at 25°C for 30 minutes. Absorbance was measured at 517 nm using a Microplate Reader System (BioTek Instruments, USA). BHA and ascorbic acid were used as the synthetic and natural standards, respectively. The assay was carried out in triplicate and the inhibition activity percentage was calculated as follow;

$$[(A_0 - A_1)/A_0] \times 100$$

Where A_0 was the absorbance of the control (containing all reagents except the extract), and A_1 was the absorbance of the extract or standard compounds.

The IC_{50} value was calculated by the plotted graph of the inhibition percentage against extract concentration (Manosroi *et al*., 2012).

Determination of total phenolic content

Total polyphenolic content (TPC) of plant extracts was estimated using Folin–Ciocalteau's phenol reagent (Sigma–Aldrich, , Germany) via the purposed method by Li *et al*. (2012). 25 µL gallic acid (Sigma–Aldrich, Germany) as a standard acid or vegetable extract were mixed with 125 µL Folin–

Ciocalteu reagent (10% v/v) and sodium carbonate (7.5% w/v) in 96-well microplates and allowed to react for 60 min at room temperature. The absorbance of the reaction mixture was read at 765 nm using a spectrophotometer (BioTek Instruments, USA). The total phenolic content (mg/g of plant extract) of extract has been expressed in gallic acid equivalents (GAE). Mean values were calculated by three measurement (H. Li *et al*., 2012).

Cell Culture

Four human cancerous cell lines used in this study which were arranged from the Pasteur Institute of Iran (PII), Tehran, Iran. KYSE-30 cells were cultured in RPMI-1640 medium (Gibco, USA) containing 100 units/ml penicillin and 100 mg/ml streptomycin and supplemented with 10% fetal bovine serum (FBS), and incubated at 37ºC in 5% CO_2. The Human Colon Adenocarcinoma cell line (HT-29), Human Breast Adenocarcinoma cell line (MCF-7), Colorectal Adenocarcinoma cell line (Caco-2) and Human Foreskin Fibroblast cell line (HFF3) as a normal cell line were cultured in DMEM supplemented with 10% FBS and antibiotics, as previously mentioned. The cells were incubated at 37ºC under 5% CO_2 for 24 and 48 h.

In vitro assay for cytotoxic activity

The viability of the cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Liu, Tang, Liang, You, & Yang, 2010). This method is a colorimetric assay and based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product. The cells were seeded in 96-well plates at a concentration of 1×10^4 cells/ml and treated with different concentrations of the extract (625, 1250, 2500, 5000, 10000, 20000 µg/mL). These cells were incubated in a humid atmosphere with 5% CO_2 for 24 and 48 h. After incubation, 20 µL of MTT was added to each well and incubated again at 37℃ for 4 hours. The medium was removed and the formed crystals were dissolved in DMSO. Eventually, the absorbance was measured at 570 nm using NanoDrop spectrophotometer (BioTek Instruments, USA).

Statistical Analysis

The standard error of the mean of the treatments was calculated by a single-factor analysis of variance. The data was analyzed using SPSS version 16.0. For all tests, differences were considered significant at $P < 0.05$. Duncan multiple range tests with a confidence interval of 95% were used to compare the means.

RESULTS AND DISCUSSION

Antioxidant activity of red cabbage extract

Brassicaceae vegetables naturally contain abundant antioxidant substances that could potentially reduce the risk of chronic and acute diseases. Oxidative stress may play an important role in the creation and continuation of the cancer process and this stress reduction may protect cells against carcinogens (Hwang & Bowen, 2007; Soengas Fernández, Sotelo Pérez, Velasco Pazos, & Cartea González, 2011). The Result of Scavenging effect of red cabbage extract on DPPH radical and comparison with ascorbic acid and BHT has been shown in Figure 1.

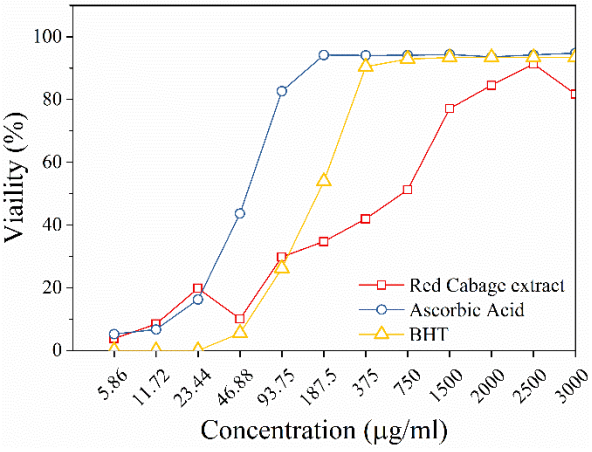

Figure 1. *Antioxidant activity of red cabbage extract, ascorbic acid and BHT assayed by DPPH test.*

Based on these results, the antioxidant effect increases with raising concentration and this makes a significant difference for the red cabbage extract in a concentration of 2500 µg/ml, so that the highest percentage of inhibition calculated in this concentration. The highest percentage of inhibition was observed for ascorbic acid and BHT at concentrations of 375 and 750 µg/ml, respectively. The IC50 value is defined as the amount of the required extract for scavenging 50% of DPPH radicals and it was 700, 150, 170 µg/ml for red cabbage extract, ascorbic acid and BHT, respectively.

Several highly conjugated anthocyanins have been identified in red cabbage with potential antioxidant activities. These anthocyanins are highly conjugated with sugars and acylated groups, and thus, their structures are very complicated (Farag & Motaal, 2010; Wu & Prior, 2005). The results showed that similar to the previous studies, the red cabbage has a high antioxidant activity (Cartea, Francisco, Soengas, & Velasco, 2010; Kataya & Hamza, 2008; H. Li *et al*., 2012; Podsędek, 2007; Singh *et al*., 2006). In the study carried out by Li *et al*. (2012) twelve highly pigmented (red or purple) vegetables (carrots, cabbage, cauliflower, potatoes, onions, asparagus and eggplant) were investigated for antioxidant activities by DPPH, and some other assays. The two cruciferous vegetables (purple cauliflower and red cabbage) and two kinds of the purple carrots showed the highest antiradical activity in the radical scavenging method (H. Li *et al*., 2012).

Similar results have been reported in many studies about cruciferous vegetables, for example, Farag and Motaal (2010) evaluated anticancer and antioxidant activity of several crucifer vegetables such as broccoli, brussels sprout, green cabbage, red cabbage, Chinese kale and turnip. They found out that red cabbage and Chinese kale only showed the high inhibition activity 73% and 54%, respectively. Other cruciferous extracts displayed the moderate to weak capacity in scavenging DPPH radicals.

Determination of Total Polyphenol Content (TPC)

Phenolic compounds are a large group of phytochemicals can be found widely in plants. According to their structure, they can be divided into several simple groups such as phenols, phenolic acids, hydroxycinnamic acid derivatives and flavonoids. Phenolic compounds have attracted many attentions in the recent year due to the high protection potential against cancer and heart diseases because of their powerful antioxidant properties and their widespread presence in foods derived from plants. The beneficial effects of brassica vegetables on human health is immense because of the presence of a complex mixture of phytochemicals and their antioxidant activity (Cartea *et al*., 2010) .

For determination of the polyphenolic compounds amount in red cabbage extract, the absorbance of these compounds was read at 765nm in triplicate by spectrophotometer and their averages were calculated for later considerations. Based on the three repetition averages of the gallic acid absorbency, a standard curve and the best fit line was plotted obtained that its result was the following equation with 97.7 percent correlation coefficient ($R^2 = 0.977$).

$$\boldsymbol{Y = 0.0362\,X + 0.3199}$$

Based on this equation and the average of triplicate results, the content of polyphenol compounds in red cabbage extract was estimated as 39.55 mg GAE/100 g of red cabbage extract.

In a study that was carried out for the characterization of antioxidant activity and phenolic compounds of traditional Chinese medicinal plants, total phenolic content of methanolic extracts was from 0.22 to 50.3 g of gallic acid equivalent/100 g DW, respectively. The correlation between antioxidant activity and total phenolic content showed that phenolic compounds are the dominant antioxidant compounds in the studied plants and herbs (Cai, Luo, Sun, & Corke, 2004).

Another study was carried out by Leja *et al*. 2010 describing its total phenol compounds of red cabbage as 31-43 mg $100g^{-1}$ FW and was compared with two white cabbage cultivars in that study. Also Singh *et al*. in 2006 obtained the total phenol content between 12.58 to 34.41 mg per 100 g^{-1} FW for 14 types of white cabbage (Leja *et al*., 2010). Syska *et al*. (2005) determined the total phenolic content of sauerkraut and white cabbage 8.25 and 5/72 mg per g, respectively.(Ciska *et al*., 2005).

Cytotoxicity of red cabbage on three cancerous cell line and comparing to a normal cell

Direct correlation between antioxidant power and anticancer activity in various herbs and vegetables has been reported in several different studies. Li *et al*. (2007) investigated the aqueous extracts antioxidant and anticancer effects of several herbs that are commonly used in traditional Chinese medicine formulae against cancer. By comparing their free radical scavenging capacity and growth inhibition percentages on A549 and MCF-7 cells, they realized a positive linear relationship between antioxidant properties and anticancer effect of aqueous extracts of studied plants (W.-Y. Li, Chan, Guo, & Yu, 2007). Another study has been carried out to evaluate anticancer properties of garlic extract anticancer effects of different type of processed garlic extracts on WEHI-164 tumor cells in inbred BALB/c mice. The results indicated a linear correlation between the anticancer activity of the kinds of garlic with the different levels of allicin, flavonoids, phenolic compounds and antioxidant activity (Shirzad, Taji, & Rafieian-Kopaei, 2011).

In this research, the cytotoxic effect of the red cabbage extract was investigated on three cancerous cell lines such as Caco-2, KYSE-30 and MCF-7, and their responses against this extract were compared to HFF-3 as a normal cell line during 24 and 48 hr (Figs 2, 3, 4 and 5).

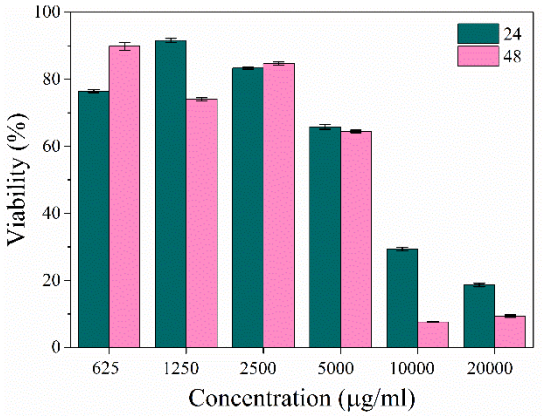

Figure 2. *The effect of hydroalcoholic extract of red cabbage on the viability Percentage of the Caco-2 cell line (P<0.05).*

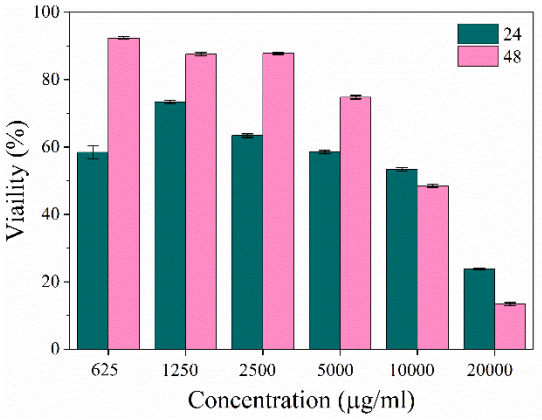

Figure 3. *The effect of hydroalcoholic extract of red cabbage on the Percentage viability of the KYSE-30 cells (P<0.05).*

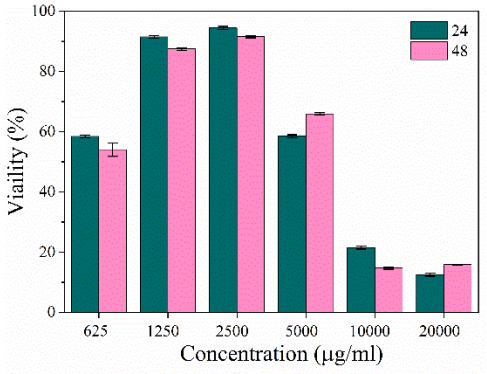

Figure 4. *The effect of hydroalcoholic extract of red cabbage on the Percentage viability of the MCF-7 cells (P<0.05).*

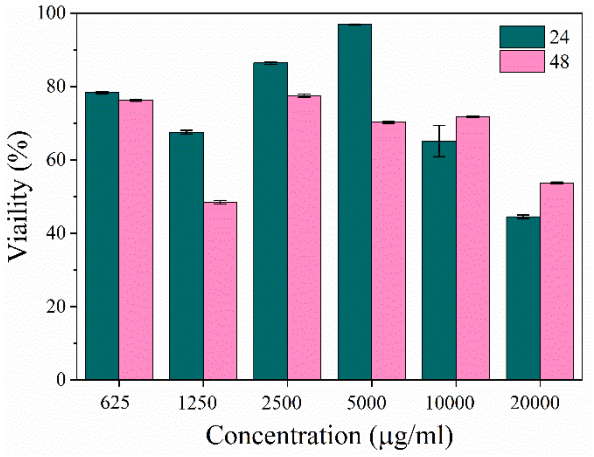

Figure 5. *The effect of hydroalcoholic extract of red cabbage on the Percentage viability of the HFF-3 cells (P<0.05).*

As it has been shown in figures 2, 3, 4 and 5, all of desired cell lines have shown primary resistance against the low concentrations of red cabbage extract, but the cytotoxicity effect rose with increasing of the extract concentration. According to the results, there was a significant difference of red cabbage extract cytotoxicity effect between HFF-3 cells as a normal cell line while in comparison with the cancerous cells. On the other hand, red cabbage extract affects normal cells with minimal side effects.

The obtained results of this research showed that the cytotoxic effects of red cabbage extract on three cancerous cell lines were significantly different (P<0.05) from normal cell line. The cytotoxic effect of red cabbage extract on MCF-7 (CC50 = 5.8 mg/ml) was lower than on KYSE-3 (CC50 = 11.3 mg/ml) and Caco-2 (CC50 = 6.75 mg/ml) and even HFF-3 (CC50=6.4 mg/ml). Whereas the cytotoxicity effect was the dose dependent, so red cabbage extract should be used at concentrations of lower than 6.4 mg/ml to prevent the damage of normal human cells. It is obvious about the good selectivity and safety of red cabbage extract is only optimal for MCF-7 and Caco-2 cells treatment as an agent of anticancer, of course, it is not evidence that this extract is able to perform a selective cytotoxicity effect and it is necessary to plan another supplement research for the selective inducing apoptosis verification.

CONCLUSION

As mentioned previously, anthocyanins are one of the phenolic compouns in red cabbage. Thus, it is possible that the results of this research are related to anthocyanins as a subclass of the flavonoids. However, in this study the total polyphenol content and antioxidant and cytotoxic effects were evaluated and it was revealed that the hydroalcoholic extract of red cabbage possess a radical scavenging activity and also act as dose-dependent.

Based on the results, red cabbage extract causes the high death rate of Caco-2, KYSE-30 and MCF-7 cancerous cells as compared with HFF-3 as a normal cell and it can be considered as a healthy food due to its numerous phenolic compounds and powerful antioxidant activity.

It was also observed that red cabbage extract possesses a good potential of controlling cancerous cells growth especially for MCF-7 on a low concentration equal 5.8 mg/ml (Gafaar *et al*., 2014). The viability percentage of all cells are diminished with the increasing of the red cabbage extract concentration after 24 and 48 hr, but HFF3 which is normal cell line survives and resists more than others against the high concentrations of extract.

The other research of cytotoxicity effect of red cabbage extract exhibited 73% viability at 500 µg/mL against human A-549 cells and its antioxidant activity was detected at a dose of 10 mg/mL (Farag and Motaal, 2010), of course, it was found more toxic for cancer cells than normal cell line. Also, it has been proven that anthocyanins reduce the cell growth of human colon cancer cell lines HT 29 and HCT 116 (Kang *et al*., 2003).

Therefore, the red cabbage extract showed in agreement with previous reports on phenolic compounds and their antioxidant effects and ability to prevent the cancer cell growth. This extract shows the anticancer and antioxidant properties that these

effects are likely related to the existence of anthocyanins (Kang *et al*., 2003). For this reason, it can be applied in daily food consumption and also as a natural and suitable alternative for synthetic food colorants which used in the food industry.

However, red cabbage has a potential of cancer chemoprevention and helps in cancer prevention, but it is important to discover their chemical structures and biochemical pathway which determine their anticancer and antioxidant properties in future studies.

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