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CHARACTERIZING THE ANTIOXIDANT AND ANTICANCER PROPERTIES OF SECONDARY METABOLITES FROM RED AND WHITE CABBAGES BRASSICA OLERACEA L. VAR. CAPITATA

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ABSTRACT

Vegetables belongs to *Brassicaceae*are rich in polyphenols, flavonoids, glucosinolates, vitamin C and their hydrolysis products which may have antioxidant and anticancer properties.This study was carried out to evaluate and differentiate the total phenolic content, total flavonoids, vitamin C and their antioxidant potential activities and cytotoxic effects between red and white cabbages after extraction with two different solvents (80% methanol and aqueous extracts). The total phenolic and total flavonoid contents varied from 29.13 to 11.44 mg/gdw and from 11.44 to 4.37 mg/gdw for red and white cabbage respectively that extracted with 80% methanol. Levels of total phenolic and flavonoids extracted with water were ranged from 21.38 to 10.36 mg/gdw and from 12.33 to 3.69 mg/gdw for red and white cabbages respectively. While, vitamin C ranged from 64.87 to 44.25 mg/100gfw. Red cabbage was the richest sources of dietary antioxidants, while their content was the lowest in white cabbage. Phenolic compounds present in the extracts showed antioxidant activities investigated using DPPH·radical scavenging activity, iron chelating and reducing power. The extract of red cabbage exhibited a stronger ability to scavenge DPPH· radicalas compared to white cabbage. Iron chelating and reducing power activities were also stronger in red cabbage. Results of the HPLC analysis indicated that, among phenolic compounds, luteolin and rutin were the main constituents in red cabbage extracted with 80% methanol. Red and white cabbages were tested for their anti-cancer activity using three cancer cell lines; hepatocellular cell line

(HePG2), colon cell line (HCT116), breast cell line (HCF7) and a normal cell line (human dermal fibroblast cell line). Each extract is evaluated cytotoxicity inhibition effects. Red cabbage aqueous extract showed cytotoxic inhibition effects of 46.40, 31.20 and 22.40 % for HePG2, HCT116 and MCF-7, respectively. Red cabbage seems to be a very good source of dietary antioxidants possessing high antioxidant and anticancer activities. Thus, it could be concluded that,Brassica vegetables contain bioactive substances such as phenolic and vitamin C with potential cancer preventive effects.

Key words: Red cabbage, White cabbage, Antioxidant, Anticancer.

INTRODUCTION

*Brassicaceae*vegetables are an abundant source of health-promoting substances, which reduce the risk of diseases. Apart from anticancerogenicglucosinolates, they possess antioxidants of both hydrophilic (vitamin C, polyphenols) and hydrophobic phases (carotenoids, vitamin E), which can neutralize active oxygen species and quench free radicals. Some studies have been conducted to quantify the phenolic compounds, carotenoids, vitamin C, and antioxidant potential [1], [2].It was found that, the majority of the antioxidant activity of Brassicaceae vegetables may be related to phenolic compounds such as flavonoids, isoflavone, flavones, anthocyanin, catechin and isocatechin[3].

The antioxidant properties were tested in many studies by using different approaches[4], [5]. The content of antioxidants depends on a lot of factors, especially on cultivars, stage of maturity and growing conditions [6]. The functional quality and antioxidant constituents of cabbage heads are strongly influenced by environmental factors and genetics. The ferric reducing antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC) and free radical scavenging activity ($DPPH^{\cdot}$) assays are the three most frequently used for assessing the antioxidant activities [7]. Different botanical compounds gained attention as therapeutic agents that relieve pain and inflammation [8]. The objective of this study was focused on characterizing the antioxidant and anticancer properties of secondary metabolites from red and white cabbages.

MATERIALS AND METHODS

Chemicals and cells

Ascorbic acid,Folin–Ciocalteau reagents,Gallic acid,Quercetin,DPPH: 2,2-diphenyl-1-picrylhydrazyl,Ferrozine:(3-(2-pyridyl) -5, 6-bis- (4-phenylsulfonicacid)-1, 2, 4-triazine, BHT: Butyl Hydroxy toluene and, were from (Sigma Chemical Co., St. Louis, MO, USA).

Preparation of plant extract

The leaves were extracted. Briefly, 10 g of the dried powder from red and white cabbages were soaked with 100 ml of 80% methanol and water and shaking at room temperature for 48 h. The extracts were filtered and the extraction was repeated twice. The resulting methanolic and aqueous extracts were used for the determination of total phenolic, flavonoid, antioxidant activity and cytotoxic effects on different human cancer cell line (HePG2, HCT116 and MCF-7).

Total phenolic content (TPC)

The total phenolic content (TPC) of methanolic and water extracts of red and white cabbages werespectrophotometrically determined by FolinCiocalteu reagent assay using gallic acid for the preparation of calibration curve (20 – 120 mg/l) according to the method of [9].A suitable aliquot (1 ml) of each extract or standard solution was added to 25 ml volumetric flask, containing 9 ml of distilled water. One milliliter of FolinCiocalteu`s phenol reagent was added to the mixture and shaken. After 5 min. 10 ml of 7 % Na_2CO_3 solution were added to the mixture. The solution was diluted to 25 ml with distilled water and mixed. After incubation for 90 min. at room temperature, the absorbance was determined at 750 nm with spectrophotometer (Unicum UV 300) against prepared reagent as blank. A total phenolic content in samples wasexpressed as mg gallic acid equivalents (GAE)/g dry weight. All samples were analyzed in triplicates.

Total flavonoid content (TFC)

Total flavonoid content (TFC) of methanolic and water extracts of red and white cabbages werespectrophotometrically determined by the aluminum chloride method using quercetin as a standard [10].One ml of extract or standard solution (quercetin, 20–120 mg/l) was added to 10 ml volumetric flask, containing 4 ml of distilled water. To the flask 0.3 ml 5 % $NaNO_2$ was added and after 5 min 0.3 ml 10 % Al Cl_3 was added. At 6^{th} min, 2 ml 1M NaOH were added and the total volume was made up to 10 ml with distilled water. The solutions were mixed well and the absorbance was measured against prepared reagent blank at 510 nm by using spectrophotometer (Unicum UV 300). Total flavonoids in sample wase expressed as mg quercetin equivalents (QE)/ g fresh weight. Samples were analyzed in triplicates.

Determination of vitamin C

Ascorbic acid content in fresh samples of red and white cabbagewas measured colorimetrically according to[11].

Determination of free radical scavenging activity

Determination of free radical scavenging activity was performed spectrophotometrically as described by [12]. An aliquot of 0.5 ml of 0.1 mM DPPH·radical were added to a test tube with 1 ml of methanolic and aqueous extracts at different concentrations5, 10, 20, 40µg/ml. Methanol was used as blank.The reaction mixture was shaken vigorously at room temperature. , buthylatedhydroxyl toluene (BHT) was used as a positive control, and negative control contained the entire reaction reagent except the extracts. Then the absorbance was measured at 515 nm with spectrophotometer against blank (methanol pure).Lower absorbance of the reaction mixture indicated higher free radical scavenging activity.The capacity to scavenge the DPPH· radical was calculated using the following equation:

DPPH·scavenging effect (Inhibition %) = [(Ac – A_S / Ac) × 100].

Where Ac was the absorbance of the control reaction and $A_s$as the absorbance in the presence of Red and white extracts.

Determination of chelating effects on ferrous ions

Metal chelating effects on ferrous ions was carried out as described by [13]. One ml of methanolic and aqueous extracts, and or EDTA solution as a positive control at different concentrations (5, 10, 20, 40 µg/ml) were mixed with 0.1 ml of 2 mM $FeCl_2$- $4H_2O$ and 0.2 ml of 5 mMferrozine solution and 3.7 ml methanol or water were mixed in a test tube and reacted for 10 min, at room temperature .The absorbance was then measured at 562 nm. Mixture without extract was used as the control. A lower absorbance indicates a higher ferrous ion chelating capacity. The percentage of ferrous ion chelating ability was calculated using the following equation: Iron chelating activity (Inhibition %) = [(Ac – AS / Ac) × 100]

Where Ac was the absorbance of the control reaction and $A_s$as the absorbance in the presence of Red and white cabbage extracts.

Reducing power

The reducing power was assayed as described in [14]. Different concentrations(5, 10, 20, 40 µg/ml) of methanolic and aqueous extracts of red and white cabbages (1.0 ml) were mixed with 2.5 ml of phosphate buffer (50 mM, pH 7.0) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50^oC for 20 min. After, 2.5 ml of trichloroacetic acid (10 %) were added to the mixture, centrifuged at 3000 rpm for 10 min. Finally, 1.25 ml from the supernatant was mixed with 1.25 ml of distilled water and 0.25 ml $FeCl_3$ solution (0.1%,

w/v). The absorbance was measured at 700 nm. The assays were carried out in triplicate and the results were expressed as mean values ± standard deviations. Increased absorbance values indicate a higher reducing power. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against the extract concentration. BHT was used as standard.

Cytotoxic effect on human cell line (HePG2 – MCF7 – HCT116)

Cell viability was assessed by the mitochondrial dependent reduction of yellow MTT (3-(4, 5-dimethylthiazol -2-yl)-2, 5-diphenyltetrazolium bromide) to purple formazan [15].

Procedure: All the following procedures were done in a sterile area using a Laminar flow cabinet bio safety class II level (Baker, SG403INT, Sanford, ME, USA). Cells were suspended in RPMI 1640 medium for HePG2- MCF7 and HCT116. The media are supplemented with 1% antibiotic-antimycotic mixture (10,000U/ml Potassium Penicillin, 10,000µg/ml Streptomycin Sulfate and 25µg/ml Amphotericin B), 1% L-glutamine and 10% fetal bovine serum and kept at 37 °C under 5% CO_2. Cells were batch cultured for 10 days, then seeded at concentration of 10x103 cells/well in fresh complete growth medium in 96-well microtiter plastic plates at 37 °C for 24 h under 5% CO_2 using a water jacketed Carbon dioxide incubator (Sheldon, TC2323, Cornelius, OR, USA). Media was aspirated, fresh medium (without serum) was added and cells were incubated either alone (negative control) or with different concentrations of sample to give a final concentration of (100 – 50 – 25– 12.5–6.25–3.125–0.78 and 1.56 µg/ml). After 48 h of incubation, medium was aspirated, 40µl MTT salt (2.5µg/ml) were added to each well and incubated for further four hours at 37°C under 5% CO_2. To stop the reaction and dissolving the formed crystals, 200µl of 10% Sodium dodecyl sulphate (SDS) in deionised water was added to each well and incubated overnight at 37ºC. A positive control which composed of 100µg/ml was used as a known cytotoxic natural agent who gives 100% lethality under the same conditions [16]. The absorbance was then measured using a micro plate multi-well reader (Bio-Rad Laboratories Inc., model 3350, Hercules, California, USA) at 595nm and a reference wavelength of 620nm. A statistical significance was tested between samples and negative control (cells with vehicle) using independent t-test by SPSS 11 program. DMSO is the vehicle used for dissolution of plant extracts and its final concentration on the cells was less than 0.2%. The percentage of change in viability was calculated according to the formula:

((Reading of extract / Reading of negative control) -1) x 100

A probit analysis was carried for IC_{50} and IC_{90} determination using SPSS 11 program.

Statistical Analysis

Data were statistically analyzed using Costat statistical package data according to [17].

RESULTS AND DESCUSSION

Total phenolic content, total flavonoids content and vitamin C

The quantitative contents of total phenolices, total flavonoids and vitamin C in the extracts of red and white cabbages are presented in (Table 1 and 2). The total phenolic and total flavonoid contents varied from 29.13 to 11.44 mg/gdw and from 11.44 to 4.37 mg/gdw for red and white cabbage respectively that extracted with methanol.Levels of total phenolic and flavonoids extracted with water were ranged from 21.38 to 10.36mg/gdw and from 12.33 to 3.69 mg/gdw for red and white cabbages respectively.While vitamin C ranged from 64.87 to 44.25 mg /100gfwof fresh red and white cabbage. Red cabbage methanolic extract was characterized by a higher content of total phenolices and total flavonoid.

Table 1. Total phenolic (TPC) and total flavonoids (TFC) in red and white cabbage

Extracts	Samples	TPC(mg*/g DW)	TFC(mg**/gDW)
Methanolic	Red cabbage	$29.13^{d} \pm 0.08$	$17.44^{d} \pm 0.11$
	White cabbage	$11.36^{b} \pm 0.02$	$4.37^{b} \pm 0.14$
Aqueous	Red cabbage	$21.38^{c} \pm 0.11$	$12.33^{c} \pm 0.11$
	White cabbage	$10.36^{a} \pm 0.04$	$3.69^{a} \pm 0.09$
LSD at 0.05		0.25	0.24

***** As gallic acid. ** As quercetin.

All values are the means of three replicates and are significantly different at $p \geq 0.05$

Table 2.Vitamine C in red and white cabbages

Samples	Vit C(mg/100gF W)
Red cabbage	$64.87^{b} \pm 0.14$
White cabbage	$44.25^{a} \pm 0.17$
LSD at 0.05	0.14

The results arecompatible with a study carried out by [18]who showed that the total phenolic content of the cabbage extract was 4.1 mg/g. Other authors reported that, in fresh white cabbage 0.4 mg total phenolices/g FM [19], 1.10-1.53mg total phenolices /g FM.[20],0.36mg total phenolices/g FM[21].

Antioxidant activity

DPPH· radical scavenging activity

All the cabbages extracts at the tested concentrations were capable of directly reaction with and quenching DPPH· radicalsTable (3). The red cabbage aqueous extract were found to have the highest DPPH· scavenging activity ranged from (62.51 to 89.04µg/ml), from (31.58 to 59.42 µg/ml) in white cabbage.

Table 3.DPPH· Scavenging activity of red and white cabbage

Extracts	Samples	Inhibition %			
		5 µg/ml	10µg/ml	20µg/ml	40µg/ml
Methanolic	Red cabbage	70.52^{d} ± 0.31	81.07^{c} ± 0.31	88.83^{d} ± 0.31	93.71^{d} ± 0.21
	White cabbage	35.50^{b} ± 0.33	42.75^{a} ± 0.26	57.80^{b} ± 0.36	70.24^{b} ± 0.26
Aqueous	Red cabbage	62.51^{c} ± 0.16	76.84^{b} ± 0.36	81.17^{c} ± 0.16	89.04^{c} ± 0.31
	White cabbage	31.58^{a} ± 0.31	42.82^{a} ± 0.31	51.03^{a} ± 0.41	59.42^{a} ± 0.31
BHT as Standard		71.55^{e} ± 0.27	81.99^{d} ± 0.79	89.86^{e} ± 0.36	94.40^{e} ± 0.36
LSD at 0.05		0.47	0.86	0.68	0.60

In comparison with white cabbage cultivar, antioxidant value was lower than that cabbage value used in other study [22]. The antioxidant activity of the plant can be attributed to flavonoids and polyphenolic compounds found in it [23]. Total phenol and flavonoid contents were significantly different between the studied cabbage cultivars ($p \leq 0.05$).However, the antioxidant capacity also depends on several other factors including genetics, environmental conditions, production techniques used, date of harvest and post-harvest storage conditions [24].

Fe-chelating activity of red and white cabbage

Red and white cabbages have significant ferrous ion chelating activity but in all cases it was significant lower than EDTA. The highest Fe chelating was found in red cabbage aqueous extract it ranged from 19.52 to 77.42 µg/ml and from 13.05 to 73.55 µg/ml in white cabbage (Table 4).

Table 4. Fe-chelating activity of red and white cabbage

Extracts	Samples	Inhibition %			
		5 µg/ml	10µg/ml	20µg/ml	40µg/ml
Methanolic	Red cabbage	$35.90^{c} \pm 0.49$	$54.70^{d} \pm 0.55$	$82.95^{d} \pm 0.28$	$90.03^{d} \pm 0.28$
	White cabbage	$19.84^{b} \pm 0.49$	$30.18^{a} \pm 0.28$	$58.70^{c} \pm 0.49$	$79.04^{c} \pm 0.41$
Aqueous	Red cabbage	$19.52^{b} \pm 0.41$	$38.64^{c} \pm 0.47$	$56.91^{b} \pm 0.21$	$77.42^{b} \pm 0.47$
	White cabbage	$13.05^{a} \pm 0.56$	$34.10^{b} \pm 0.41$	$55.33^{a} \pm 0.27$	$73.55^{a} \pm 0.27$
EDTA as Standard		$49.48^{d} \pm 0.41$	$68.65^{e} \pm 0.55$	$83.67^{e} \pm 0.49$	$93.43^{e} \pm 0.28$
LSD at 0.05		0.97	0.96	0.66	0.68

Antioxidant activity in *Brassica* species is correlated with vitamin C and total phenolic content [25]. In the previous investigations [26], ascorbic acid content in red and white cabbages was not differentiated as phenolic substances estimated in the present study, hence, high antiradical activity determined in red cabbage in comparison with the samples of white cabbage cultivars was probably due to phenolics, especially those of the phenylpropanoids and anthocyanins groups. According to [27], polyphenols possess the ideal chemical structure for free radical scavenging and seem to be more effective antioxidants than vitamins E and C. The phenolic acids, antiradical activity are positively correlated with the number of hydroxyl groups bonded to the aromatic ring [28]. In the present study, radical scavenging activity is measured by two methods: $DPPH^{\cdot}$, Fe-chelating and power reducing reagents. In the case of $DPPH^{\cdot}$, antiradical activity was higher as related to theFe –chelating and power reducing method. A similar effect was described by [2]for Radical scavenging activity (RSA) estimated in white cabbage assays. The various results obtained by these three chemical methods might have been due to the reaction of different phenolics with free radicals of different chemical structures. Several studies have reported on the relationships between phenolic content and antioxidant activity[29],[30],[31]. Some authors found a correlation between the phenolic content and the antioxidant activity[32],[33],[28].

Reducing Power of red and white cabbage

Reducing power antioxidant activity was expressed as EC_{50} (effective concentration at which the absorbance is 0.5 at 700 nm).Reducing power of methanolic and aqueous extracts of red and white cabbage in comparison with BHT as standard showed in (Fig 1 and Table 5).

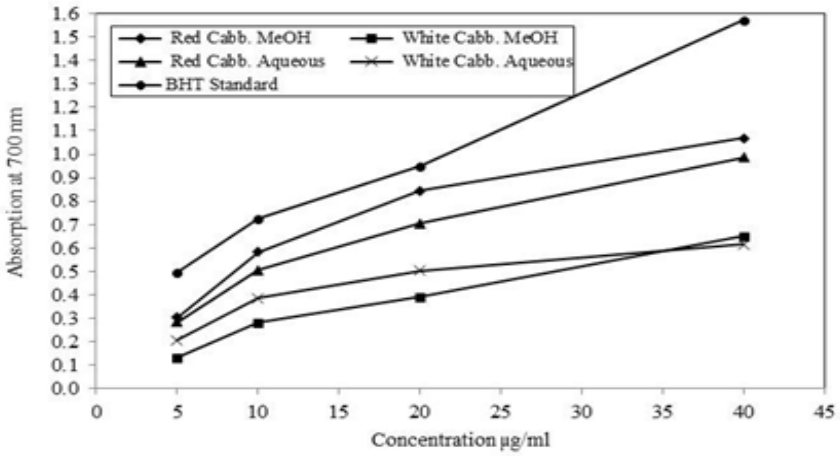

Fig.1. Reducing power of 80% methanolic and aqueous extracts of red and whiteCabbage

Table 5.Reducing power activityof**red and white cabbage**

Extracts	Samples	EC_{50}µg/ml
Methanolic	Red cabbage	8.23^{b} ± 0.02
	White cabbage	9.87^{e} ± 0.13
Aqueous	Red cabbage	20.47^{c} ± 0.03
	White cabbage	28.49^{d} ± 0.14
BHT as Standard		4.09^{a}± 0.10
LSD at 0.05		0.21

It was observed that methanolic extract of red and white cabbage had higher reducing power (EC_{50} = 8.23 – 9.87 µg/ml) than aqueous extract (EC_{50} = 20.47 – 28.49µg/ml), respectively. The reducing power activity may be due to the presence of reductonesas they electron donors and are capable of converting them into a more stable product and terminating the free radical reaction.The reducing power activity of the extracts examined in this study was similar with the results of [34]and [35] who reported that reducing power of broccoli extracts was used as a method for antioxidant activity investigation. The extract of white cabbage possessed also the stronger reducing power [36].

Identification of phenolics compounds by HPLC

The HPLCprofile of crude methanolic extracts of Red and white cultivars extracted with methanol and water are illustrated in Table (6).

Table 6. Phenolic compounds of red and white cabbage cultivars (mg/100gD.W)

Phenolic compounds mg/ 100g DW	Methanolic		Aqueous	
	Red Cabbage	White Cabbage	Red Cabbage	White Cabbage
Pyrogallol	-	16.22	5.12	-
Gallic acid	-	3.07	3.67	3.10
Chlorogenic acid	1.59	0.43	2.29	0.79
Vanillic	-	0.38	0.32	0.77
Catechines	-	15.92	22.34	22.53
Caffeic acid	-	0.70	0.39	8.12
Rutin	58.36	5.76	13.48	4.49
P-Coumaric acid	-	-	10.72	1.47
Ferulic	7.82	1.45	1.30	5.19
Benzoic	14.40	1.80	2.15	6.34
Acacetin	8.06	3.32	1.78	4.55
Myricetine	7.61	-	-	0.82
Coumarin	5.36	-	-	0.79
Luteolin	119.65	10.82	38.56	2.95
Quercetin	36.33	7.98	-	-
Cinnamic acid	0.74	0.38	1.17	0.31
Genistein	2.57	-	2.22	0.27
Kaempferol	1.88	2.05	0.33	3.23

Methanolic extract enhanced phenolic compounds in both cultivars of cabbage as compared to water aquase extract. Methanolic extract of red cabbagecultivars showed higher content in most phenolic compounds especially Luteolin (119.65 mg/ 100gDW), Rutin (58.36 mg/100g DW), Qurectein (36.33 mg/100g DW),and Benzoic (14.40 mg/100g DW).

Whereas, Methanolic extract of white cabbage cultivar showed lower content in phenolic compounds especially in Pyrogallic acid (16.22mg/100gdw), Catchiness acid (15.92mg/100gDW), and Luteolin (10.82 mg/100gDW).

The least phenolic constituent was Acacetin, which was detected in red and white cabbage aqueous water extract.Caffeic acid and Kaempferol in red cabbage. Whereas, Ggenistein (0.27mg/100gDW) and Cinnamic 0.31mg/100 gDW).The results obtained showed significant quantitative variation in the polyphenolic profile as well as variation in the concentration of each individual compound. In this concern, [37] mentioned that phenolic compounds ranged between plants according to their genus species, varieties, cultivars, and types of fertilizer.

Cytotoxicity effect of red cabbage and white cabbage

Cancer is a global health problem with high morbidity and mortality and poses both economic and psychological challenges [38],[39].It is known that different cell lines might exhibit different sensitivities towards an antiproliferative compound, so the use of more than one cell line is therefore considered necessary in the detection of antiproliferative compounds.

Red and white cabbages were tested for their anti-cancer activity using three cancer cell lines hepatocellular cell line (HePG2), colon cell line (HCT116) and breast cell line (HCF7), beside normal cell line (human dermal fibroblast cell line). The cytotoxic activity data are presented in Table (7).

Table 7. Cytotoxicity effect of red and white cabbage

Extracts	Samples	Remarks % at 100 ppm		
		HePG 2	HCT116	HCF7
Methanolic	Red cabbage	35.8	26.7	19.8
	White cabbage	27.4	28.3	13.7
Aqueous	Red cabbage	46.4	31.2	22.4
	White cabbage	32.6	22.7	16.5

HePG2 :Human hepatocellular carcinoma cell line.

MCF7 : Human Caucasian breast adenocarcinoma.

HCT116:Human Colon Cancer Cells

Red cabbage aqueous extract exhibited a pronounced cytotoxic effect (46.40, 31.20 and 252.40 % for HePG2, HCT116 and MCF-7 respectively cell survival at 500 µg mL^{-1})46.40, 31.20 and 252.40%, for HePG2, HCT116 and MCF-7 respectivelyas compared to that of white cabbage (32.605, 22.70, 16.50%).Cabbages were found to possess very potent inhibitory activities against all tested cell lines. These properties are in agreement with the known anticancer properties of cruciferous vegetables observed in both epidemiological and laboratory studies[40],[41],[42],[43].

Cruciferous vegetables contain several chemical compounds act as antioxidants or as inhibitors and/or inducers of phase I and phase II enzymes [44]. Phytonutrients in crucifers' vegetables work at a much deeper level and actually signal genes to increase production of enzymes involved in the detoxification.anti-carcinogenic actions of cruciferous vegetables are

attributed to their content of glucosinolate (GLS)[45]. Cruciferous vegetables contain sulforaphane, which has anticancer properties[46]. The anticancer effects of cruciferous vegetables may attribute to organic sulfur compounds (diallyl disulfide) and isothiocyanates, which had the ability to modulate expression/activity of antioxidative and phase 2 drug metabolizing enzymes and scavenging free radicals[41].Also it was reported that administration of red cabbage extract to rats resulted in chemoprevention of liver and colon cancers induced by heterocyclic amine[43]. The same authors showed that breakdown products of glucosinolate such as isothiocyanates in red are responsible for the chemopreventive properties of cruciferous vegetables.

CONCLUSION

In conclusion, here in our study we demonstrated that the effects of white and red cabbages were found to have significant antioxidant and anticancer activity. These chemo preventive effects are likely related to the formation of organosulfur compounds following mechanical disruption of these vegetables, namely isothiocyanates from cruciferous.This may be a therapeutically insight in the treatment of cancers using *Brassicaceae* family.

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