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Antioxidant capacity and nitrosation inhibition of cruciferous vegetable extracts

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Abstract

Cruciferous vegetables belong to the mustard family of plants such as Brussels sprouts, kale, broccoli, cabbage and cauliflower. They are well known for their cancer prevention properties which are due to high content of bioactive compounds, isothiocyanates (ITCs). This study was aimed to investigate nitrosation inhibition ability of the cruciferous vegetables commonly consumed with meat products namely, broccoli, cauliflower and cabbage. Aqueous extracts of fresh and steamed (2 and 4 min) vegetables were subjected to determination of antioxidant capacity (DPPH and FRAP assay) and chemical composition i.e. total phenolic and isothiocyanate (ITC) content. It was found that TPC, DPPH and FRAP values of raw vegetables were different in each vegetable and ranged from 17.12-38.91 mg GAE/100 ml, 44.09-63.31% and 1.36-6.81 mg TE/100 ml, respectively. Among three types of cruciferous vegetable, broccoli had the highest PEITC content being 0.21 mmol/100 g compared to cauliflower (0.15 mmol/100 g) and cabbage (0.06 mmol/100 g). Moreover, it was found that steaming process significantly enhanced antioxidant activity, TPC as well as PEITC content in a time-dependent manner up to 4 min ($p<0.05$). Nitrosation reaction was stimulated *in vitro* at pH 3.0 and spectrophotometric method was used to determine formation of nitrosodimethylamine (NDMA) in the presence and absence of cruciferous vegetable extracts. The highest NDMA inhibition was found in broccoli and steaming could promote the effect in all vegetables. Prevention of DNMA was believed to be a result of PEITC content in the vegetables because when anti-nitrosation ability of synthesized isothiocyanates was determined, PEITC acted as a potent inhibitor with 89.28% inhibition whereas that of ascorbic acid (positive control) was 88.57% and allyl isothiocyanate had the least effect (53.21%) at the same concentration. This study infers that the concomitant consumption of cruciferous vegetables may possess toxicity reduction of nitrosamine and these effects can be enhanced by a common heating process of the vegetables.

Introduction

Nitrite is added in a wide variety of foods such as cured meat products, smoked fish, bacon, dried malt and beer as a preservative, colouring or flavouring agent. However, high nitrite and amine-rich food promotes the formation of endogenous *N*-nitroso compound which is a potent carcinogen. Formation of *N*-nitroso compound is catalyzed when secondary or tertiary amines such as diethylamine, dimethylamine (DMA) or trimethylamine (TMA) interact with nitrite that presents in the food (Sun *et al*., 2007). Such interaction requires acidic conditions of the stomach (Sun *et al*., 2007). Hence, the possible trace amounts of *N*-nitroso compounds occurred in human food has led to an upsurge interest among food scientists. According to Yurchenko and Molder (2006), one of the most common nitrosamines found in the food is *N*-nitrosodimethylamine (NDMA). It is a strong carcinogen, hence limitation of NDMA formation in food is of important. Previous study demonstrated that the presence of NDMA is easily influenced by beneficial phytochemicals from fruits and vegetables such as ascorbic acid, α-tocopherol, sulphur-containing compounds and phenolic compounds (Sun *et al*., 2007). Therefore, consumption of nitrite containing food may endanger individuals with low serum levels of these antioxidants (Sun *et al*., 2007). One of the inhibition mechanisms of these compounds is competing with amines for nitrite and destroying or reducing the available nitrosating agents.

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Cruciferous vegetables, also known as Cruciferae or Cruciferaceae, belong to the botanical family of Brassicaceae (Klaus *et al*., 2011). The most consuming plants in Brassicaceae family include bok choy, Brussels sprouts, broccoli, cauliflower and cabbage (Albena and Rumen, 2012). Cruciferous vegetables contained abundant amount of sulphur-containing compounds, glucosinolates, that have been reported for their antioxidant and anti-carcinogenic properties which the later is considerable more effective (Cohen *et al*., 2000; Chu *et al*., 2002). Hydrolysis of glucosinolates by myrosinase enzyme occurs following chopping or mastication to produce isothiocyanates (ITCs). In addition, ITCs are more potent against cancer compared to their parent compounds (Keum *et al*., 2004).

In the last decades, researchers have only focused on the ability of ascorbic acid, α-tocopherol and phenolic compounds as nitrosation inhibitors in food. Lack of specific study carried out to disclose the mechanism of isothiocyanates on the inhibition of nitrosation. Therefore, in the current research, antioxidant capacity, chemical composition as well as nitrosation inhibition of cruciferous vegetables that are most commonly consumed with meat products i.e. broccoli, cauliflower and cabbage were investigated. The effect of thermal treatment on these properties was also evaluated.

Materials and Methods

Chemicals

Allyl isothiocyanate (AITC), benzyl isothiocyanate (BITC), phenethyl isothiocyanate (PEITC), trimethylamine (TMA), 2,4,6-diphenyl-s-triazine (TPTZ), benzene-1,2-dithiol, sodium citrate, sodium nitrite, ; nitrosodimethylamine (NDMA), dichloromethane and sodium sulphate were purchased from Sigma-Aldrich (St. Loius, MO). 1,1-diphenyl-2-picrylhydrazyl (DPPH) was purchased from Aldrich (Steinheim, Germany). Acetate buffer, and ferric chloride were obtained from Ajax Finechem (Seven Hills, Australia). Folin-Ciocalteu reagent, potassium phosphate and methanol were purchased from Merck.

Preparation of vegetable extracts

Broccoli (*Brassica oleracea var. italica*), cauliflower (*Brassica oleracea var. botrytis*) and white cabbage (*Brassica oleracea var. capitata)* were purchased during October to November 2015, from local supermarket in Chiang Rai, Thailand. The vegetables were washed thoroughly under running tap water to eliminate dusts and unwanted materials prior to chopping into a uniform size. The samples were steamed for 2 or 4 min while no steaming treatment was served as control (0 min). After that, the samples were blended using electric blender for 5 min. The collected extracts were filtered using Whatman filter paper No.1 and subjected to analysis directly or stored at -20°C for further investigation.

Total phenolic content

Total phenolic compound in cruciferous vegetable extracts were determined using Folin-Ciocalteu method as described by Yu and colleagues (2002). Briefly, 100 µl of each extracts was mixed with 0.5 ml of Folin-Ciocalteu reagent, and the mixture was vortexed for 10 seconds. After that, 7.9 ml of distilled water was added to the mixture and allowed to stand in the dark for 5 min prior to 1.5 ml of 20% sodium carbonate added. The absorbance measured using spectrophotometer (UV-Vis spectrophotometer, Thermo Scientific, Genesys 20) at 765 nm after incubation in the dark at room temperature for 2 hours. Standard curve of gallic acid was produced for calculation of TPC. The results expressed as mg of gallic acid equivalent (GAE)/100 ml extracts. The determination was carried out in triplicates.

DPPH free radical scavenging activity

Determination of DPPH of cruciferous vegetable extracts was based on previous method of Nuengchamnong and Ingkaninan (2010). 100 µl of each extract was mixed with 2.9 ml of 0.05 mM methanolic DPPH solution. The mixture was vortexed for 10 seconds and allowed to stand for 30 min at a dark place. Then, absorbance was measured using a spectrophotometer (UV-Vis spectrophotometer, Thermo Scientific, Genesys 20) at 517 nm. The experiment was carried out in triplicates. The percentage of the DPPH free radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

Where A_0 represents absorbance of DPPH and A_1 represents absorbance of DPPH and sample.

Ferric reducing antioxidant power

Antioxidant activity of cruciferous vegetable extracts were determined using FRAP assay method as developed by Deetae and colleagues (2012). The stock solutions included 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6- tripyridyl-s-triazine (TPTZ) in 40 mM hydrochloric acid solution (HCl), and 20 mM ferric chloride solution. The working solution

was freshly prepared by mixing the acetate buffer, TPTZ solution, and ferric chloride solution in 10:1:1 ratio followed by incubation at 37°C for 8 min prior to analysis. To a 100 µl of cruciferous vegetable extracts, 3 ml FRAP reagent was added and left to stand for 30 min in the dark. The absorbance of the blue complex solution (ferrous tripyridyltriazine) formed was measured using spectrophotometer (UV-Vis spectrophotometer, Thermo Scientific, Genesys 20) at 593 nm. The linear standard calibration curve ranging from 0-100 mM Trolox was established. The final results were expressed in mg TE/100 ml of extracts. Determination was performed in triplicates.

Isothiocyanate content

A method developed from Zhang and Hamauzu (2004a) was used to determine the total isothiocyanate content in the cruciferous vegetable extracts with some modifications. Briefly, 900 µl of 100 mM potassium phosphate (pH 8.5), 900 µl methanol, 100 µl of sample extracts, and 100 µl of benzene-1,2-dithiol (80 mM prepared in methanol) were added in a 2 ml micro-centrifuge tube and gently mixed. The mixture was incubated in a tightly capped glass vial at 65°C for 2 hours and then was allowed to cool at room temperature. The reacting solution was measured using spectrophotometer (UV-Vis spectrophotometer, Thermo Scientific, Genesys 20) at 365 nm. Each extracts was done in triplicates. A calibration curve using PEITC as the standard (0-1000 mM) was established to obtain the total isothiocyanate content in the cruciferous vegetable extracts. Isothiocyanate content was expressed as mmol PEITC equivalents per 100 g.

Determination of nitrosodimethylamine: in vitro

Nitrosodimethylamine (NDMA) formation reaction was conducted by using *in vitro* study based on the method described by Tanaka and colleagues (1998). Trimethylamine (TMA) was incubated with sodium nitrite in the presence and absence of the inhibitory agent. 5 ml of TMA (200 mM) was incubated with 5 ml of sodium citrate (50 mM) and 5 ml of sodium nitrite (50 mM) in the presence of cruciferous vegetable extracts at 37°C for 12 hours. Nitrosation process was started by addition of 70% perchloric acid to the mixture in order to provide acidic condition at pH 3. The pH value of the reaction mixture was controlled throughout the reaction by addition of either perchloric acid or sodium hydroxide. The reaction was stopped after 24 hours by adding, sodium hydroxide, until the pH of the mixture was 13. Then, aliquots of sample were withdrawn to analyze NDMA content.

Quantification of NDMA was performed according to a method described by Lu and Chen (2007) with some modifications. Sodium chloride (1g) was mixed into 3 ml aliquot of sample, and the resultant mixture was extracted using dichloromethane. The dichloromethane solution was pre-dried with sodium sulphate and the combined total solution was made up to 10 ml. Quantification of NDMA formation in dichloromethane solution was performed by directly measuring the absorbance using spectrophotometer (UV-Vis spectrophotometer, Thermo Scientific, Genesys 20) at 350 nm. Each extracts were done in triplicates. The difference in NDMA concentrations between each extracts and control (absence of inhibitory agent) were expressed as percent inhibition:

$$\% \text{ NDMA inhibition} = \frac{[NDMA_C - NDMA_S]}{[NDMA_S]} \times 100 \quad (2)$$

Where $[NDMA_C]$ represents concentration of NDMA in control and $[NDMA_S]$ represents concentration of NDMA in sample.

Statistical analysis

The experiment data was statistically analyzed by Minitab Statistical Software (Version 15, 2008, Minitab Statistical Software, Minitab Inc.) for Microsoft Windows. One-way-analysis of variance (ANOVA) was used and the values were considered significantly different when $p<0.05$.

Results and Discussion

Total phenolic content

Secondary metabolites known as phenolic compounds present in plants in high concentrations. Total phenolic content (TPC) in aqueous extracts of cruciferous vegetables was determined using Folin-Ciocalteu phenol reagent. Table 1 shows that TPC values were different among vegetables. As can be seen, broccoli had the highest TPC compared to cauliflower and cabbage which the values were 38.97, 33.99 and 17.12 mg GAE/ 100 ml, respectively. It has been reported that Brassica crops are rich in polyphenol. For this reason, they are of nutritional interest. However, the polyphenol composition can be quite different among species and even among crops from the same species. Similar to the current study, antioxidant activity between different vegetables, red cabbage and Brussels sprouts, were 5 to 2.2-fold higher than that for white and savoy cabbages and this might be due to the presence of different

types and levels of phenolic compounds (Cartea *et al*., 2011). In addition, increasing steaming time led to an increase of TPC values ($p<0.05$). It has been shown that cooking method such as steaming could improve or enhance the availability of bioactive compounds in the vegetables (Zhang and Hamauzu, 2004a ; Miglio *et al*., 2008). The current results were in accordance with previous studies where TPC in steamed cauliflower was increased compared to the raw sample (Wachtel-Galor *et al*., 2008; Mazzeo *et al*., 2011). Phenolic compounds present in vegetables can be found in both soluble forms or bound with cell-wall complexes. When the vegetables are exposed to lengthy cooking times and high temperatures, cell walls of the vegetables are disrupted. Subsequently, dietary fibre-bound phenolic compounds are released and free phenolic contents are increased in steamed vegetables (Fresco *et al*., 2010). These results infer that even though cruciferous vegetables are well known for both disease prevention and curing, the extension of these effects also depends on plant species.

Table 1. Total phenolic content of cruciferous vegetables at different steaming time

Steaming time (min)	Total Phenolic Content (mg GAE/ 100ml)		
	Broccoli	Cauliflower	Cabbage
0	38.97 ± 0.85^{bA}	33.99 ± 1.05^{bB}	17.12 ± 0.29^{bC}
2	46.61 ± 4.04^{abA}	34.19 ± 0.64^{bB}	21.11 ± 1.10^{abC}
4	49.17 ± 5.50^{aA}	54.93 ± 1.43^{aA}	22.38 ± 3.99^{aB}

Each value is expressed as mean±SD (n=3).

Means with lower case letters in the same column and means with upper case letters in the same row are significantly different ($p<0.05$) where GAE refers to gallic acid equivalent.

Antioxidant capacity

DPPH radical scavenging activity in broccoli, cauliflower, and cabbage were determined by measuring the disappearance of DPPH radical at 517 nm as shown in Table 2. It can be seen that broccoli had the highest DPPH scavenging activity (50.76%) whereas slightly lower values were observed in cauliflower and cabbage being 44.09 and 43.31%, respectively. Moreover, the DPPH values were elevated in a time dependent manner in all samples when the vegetables were steamed ($p<0.05$) and at 4 min steaming the DPPH values were not different among the plants (73.98-75.46%) ($p>0.05$). Similarly, it has been demonstrated that DPPH values of methanolic extracts of cooked and uncooked cauliflower were 78.18% and 35.13% before respectively (Turkmen *et al*., 2005). Furthermore, a superior DPPH values from previous study compared to our results is thought to be a result of a higher extraction power of methanol compared to the normal crude extracts employed in the current study. In addition, the higher DPPH values of broccoli and cauliflower were in agreement with their high TPC content which is able to scavenge reactive oxygen species due to their electron donating properties (Tupe *et al*., 2013). It is well established that phenolic compounds have positive correlation with high antioxidant activity in fruits and vegetables. However, in the current study high DPPH value was observed in cabbage even though TPC value was low. This is thought to be due to the different types of polyphenols present in cabbage which offer higher efficacy of antioxidant activity compared to broccoli and cauliflower. Antioxidant capacity of individual phenolic compound depends on many factors such as the degree of methoxylation and the number and location of the hydroxyl groups present (Podsędek, 2007). Likewise, it has been demonstrated previously that individual phenolic compounds in *Terminalia chebula* showed different strength of radical scavenging activity in the order of casuarinin > 1,6-di-O-galloyl-β-D-glucose > chebulinic acid > chebulanin, with the IC50 values of 2.82, 9.03, 18.81 and 19.63 mg/mL, respectively (Cheng *et al*., 2003). On the other hand, it is not surprising that the FRAP values in the cabbage were in agreement with TPC values. The discrepancy of FRAP and DPPH values is according to the difference in principle of determination, while DPPH assay determines ability of antioxidant to scavenge free radical, reducing capacity is evaluated in the case of FRAP assay (Prior *et al*., 2005).

Ferric reducing antioxidant power (FRAP) is a quick and reliable method related to the content of antioxidant in food (Benzie and Strain, 1996). Table 2 exhibits that cauliflower had the highest FRAP values (6.81 mg TE/100 ml) whereas that in broccoli was slightly lower (4.13 mg TE/100 ml) and cabbage was found to have the lowest FRAP value of 1.36 mg TE/100 g. When heating was applied before extraction, FRAP values were increased and the antioxidant activity were also increased in the following order of broccoli (11.32 mg TE/100 ml) > cauliflower (9.66 mg TE/100 ml) > cabbage (4.76 mg TE/100 ml).

The different antioxidant capacity in each vegetable could be due to the presence of different phytochemicals. Moreover, heat treatment increased the extraction ability of these compounds (Saikia and Mahanta, 2013). Besides that, higher antioxidant activity could also be contributed to the polymerization of polyphenols in the cruciferous vegetables during heat treatment (Nicoli *et al*., 1999).

Table 2. DPPH radical scavenging activity and ferric reducing antioxidant power of cruciferous vegetables at different steaming time

Antioxidant activity	Steaming time (min)	Broccoli	Cauliflower	Cabbage
DPPH (%)	0	50.76±2.94^{bA}	44.09±3.73^{bB}	43.31±1.49^{bB}
	2	71.91±0.58^{aA}	45.38±4.71^{bB}	73.46±2.49^{aA}
	4	74.01±3.03^{aA}	73.98±1.92^{aA}	75.46±1.01^{aA}
FRAP (mg TE/100ml)	0	4.13±0.66^{bB}	6.81±1.38^{bA}	1.36±0.08^{bC}
	2	9.84±0.26^{aA}	6.98±0.45^{bB}	4.67±0.77^{aC}
	4	11.32±0.65^{aA}	9.66±0.49^{aB}	4.76±0.44^{aC}

Each value is expressed as mean±SD (n=3).

Means with lower case letters in the same column and means with upper case letters in the same row are significantly different ($p<0.05$) where TE refers to trolox equivalent.

Isothiocyanate content

Isothiocyanates are stored in cruciferous vegetables as glucosinolates. However, during preparation of these vegetables such as cutting, myrosinase is segregated from the damaged plant cell walls and hydrolyses glucosinolates into isothiocyanates (Li *et al*., 2013) which their association with cancer prevention has been well explained (Ioannides and Konsue, 2015). In relation to nitrosation inhibition study, isothiocyanate content of cruciferous vegetables employed was determined using cyclocondensation reaction of carbon atom in N=C=S group of isothiocyanate with 1,2-benzenedithiol (Li *et al*., 2013). From this reaction, 1,3-benzenedithiol-2-thione is formed and then spectrophotometric measured at 365 nm. According to Choi (2004), 1,2-benzenedithiol was used because it reacts with all isothiocyanate and it is very stable during reaction conditions.

Table 3 demonstrates that broccoli contained significantly ($p<0.05$) highest PEITC content (0.21 mmol/100 g) whereas that of cauliflower and cabbage were 0.15 and 0.06 mmol/100 g, respectively. The ranges of PEITC content from current study were slightly higher than total ITCs content reported by Li *et al*. (2013). The authors found that ITCs content in broccoli, cauliflower and cabbage were 2.60-18.10, 0.70-2.70 and 0.50-77.90 µmol/100 g, respectively. Similarly, a study conducted by Totusek *et al*. (2011), found that both broccoli and cauliflower contained high total ITCs content compared to cabbage. The different results among studies and the different amount of ITCs content in cruciferous vegetables are possibly due to various factors. Isothiocyanates content can be varied even with across the same family and species. In addition, the most significant influences are variety and age of plant (Watson, 2008).

Although previous study claimed that heat treatment reduced isothiocyanate content in these vegetables (McNaughton and Marks, 2003), it was found in the present study that steaming at 2 and 4 min led to an increase of PEITC content (Table 3). The study carried out by Wachtel-Galor (2008) demonstrated that steaming of cruciferous vegetables for 10 min resulted in lower amount of antioxidant activity and low amount of isothiocyanate. Disagreements of the result are believed to be due to shorter steaming period employed in the current study, hence myrosinase enzyme was not completely destroyed. Moreover, the disrupted cell wall of the plants due to steaming promotes the release of myrosinase and breakdown of glucosinolates into isothiocyanates, thus isothiocyanate content in all samples increased.

Table 3. PEITC content of cruciferous vegetables at different steaming time

Steaming time (min)	PEITC content (mmol/100 g)		
	Broccoli	Cauliflower	Cabbage
0	0.21±0.02^{cA}	0.15±0.05^{bA}	0.06±0.03^{bB}
2	0.44±0.14^{bA}	0.33±0.03^{aA}	0.06±0.01^{bB}
4	1.01±0.02^{aA}	0.40±0.12^{aB}	0.15±0.07^{aC}

Each value is expressed as mean±SD (n=3).

Means with lower case letters in the same column and means with upper case letters in the same row are significantly different ($p<0.05$) where PEITC refers to phenethyl isothiocyanate.

Inhibition of NDMA formation; in vitro

Reaction of secondary amines has been well established. According to the previous study by Shapley (1976), the presence of nitrite in acidic environment of stomach can enhance nitrosation reaction which leads to the formation of *N*-nitrosamines. Table 4 shows that NDMA was only detected following *in vitro* digestion as expected. However, prior to exposure to gastrointestinal tract simulation system, broccoli was the most potent NDMA inhibitor with % inhibition of 76.77 compared to cauliflower (52.88%) and cabbage (26.04%). Maximum inhibition of NDMA was anticipated in broccoli extract due to its high TPC and PEITC content

among the other vegetables. The antioxidant ability of TPC has been found to allow them to scavenge both active oxygen species and electrophiles and therefore, to inhibit nitrosation (Zhang and Hamauzu, 2004b). Considering the effect of ITCs on NDMA inhibition, the major ITC that responsible to this is thought to be contributed to PEITC content. Even though PEITC content was not abundant in these vegetables it was the most potent inhibitor of NDMA formation compared to AITC and BITC. Inhibition of NDMA (%) by synthesized isothiocyanates (AITC, BITC and PEITC) and ascorbic acid are illustrated in Table 5. PEITC and ascorbic acid showed a strong inhibition of NDMA formation whereas weak inhibition was observed when AITC and BITC were employed. It was clear that inhibition of NDMA formation by these isothiocyanates and ascorbic acid were concentration dependent. Moreover, PEITC and ascorbic acid were able to inhibit nitrosation reaction even at low concentration. It is believed that ITCs are a competitive inhibitor of amines where they might readily react with nitrite better than the amines.

Heat treatment of vegetables also influenced NDMA inhibition. As the steaming time increased, the inhibition of NDMA formation for all samples also increased (Table 4). This might be the result of higher antioxidant activity, TPC and isothiocyanate content after steaming treatment led to superior scavenging ability and, therefore, less nitrosating agents available for nitrosation.

Conclusion

Cruciferous vegetable extracts could inhibit NDMA formation when *in vitro* study was carried out. The highest inhibition of NDMA was observed in broccoli followed with cauliflower, whereas cabbage had the lowest effect. The mechanisms of action to these findings are believed to be PEITC content and the ability to scavenge the free radicals by ITCs and other antioxidants such as phenolic compounds. Moreover, steaming up to 4 min increased antioxidant activity, phenolic content and isothiocyanate content hence, maximizing the inhibition of nitrosation reaction that could occur in human body.

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