

Investigating the antioxidant and preventing DNA-damage properties of various honeys in Phitsanulok province

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Abstract

A honey has been well known as nutritional food products since it rich in nutrients and bioactive compound such as phenolic compounds possessing antioxidant activity. Many literatures have been reported that the difference sources of honey cause the significant differences in their antioxidant contents and their protective effects against DNA damage. Therefore, the aim of the present study was to evaluate bioactive components, antioxidant properties, and DNA damage protection of honey from different honey brands sold in the markets in Phitsanulok province, Thailand. The results revealed that eleven commercial honey products available in the markets were difference in physicochemical properties such as moisture, pH, ash, and sugar contents. Three out of tested eleven honey products had high amount of phenolic compounds, contributed to their antioxidant activities by DPPH assay and the mechanism is proven to be through free radical scavenging activities rather than ion-chelating activity by the protective DNA damage. These indicated that honey contains natural antioxidants and it could be used as an important ingredient of the functional food in particular functional beverage.

Keywords: Thai honey, antioxidant activity, DPPH assay, DNA-damage-inhibitory activity

1. Introduction

Honey is known to be rich in dietary nutritional value including amino acids, proteins (Alvarez-Suarez *et al.*, 2010) antioxidant compounds (flavonoids, phenolic acids, and carotenoid (Ahmad *et al.*, 2012; Tenore *et al.*, 2012), moreover it contains high content of phenolic compound (rho-hydroxybenzoic and rho-coumaric acids) that prevents DNA damage (Zhou *et al.*, 2012; Habib *et al.*, 2014). Previous literatures had been reported that the antioxidants identified in different honeys were effective in reducing the risk of degenerative disease development such as cardiovascular disease, diabetes, cancer, and aging-related disorders (Lachman *et al.*, 2010).

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The protective role against several diseases of honey has resulted in the widely increasing honey consumption; consequently there are several commercial honey brands globally available in the markets. However, the difference sources of honey cause the significant differences in terms of their antioxidant contents and activities as well as their protective effects against DNA damage. For example, eight buckwheat honeys, collected from China, obtained values of total phenolic content (TPC) ranging from 774 to 1,694 mg GAE/kg and prevented OH-induced DNA breaks by 21/kg and rho-coumaric acid (Zhou et al., 2012). Sixteen honeys, purchased from a local market the United Arab Emirates, obtained values of TPC (total phenolic compound) ranging from 30.81 to 132.60 mg GAE/100g of honey), and decreased DNA breaks (open circular form) by 2.15% to 26.52% (Habib *et al.*, 2014).

In Thailand, there are several commercial brands of honey products available in markets, and each brand come from the difference sources of bee species and plant species. There are no reports on the physical and chemical properties and components of nutrition among those brands available in the markets. The objectives of this research are to evaluate bioactive components, antioxidant activities, and DNA damage protection of honey from different honey brands sold in the markets in Phisanulok, Thailand. The information from this research reveals the nutritional value in terms of antioxidant activity and the DNA protective potential of some commercial honey products sold in the market at Phitsanulok and promotes honey as functional ingredients in household and food manufacture application.

2. Materials and Methods

2.1 Chemicals and reagents

All of the chemicals and reagents used were of analytical grade. DPPH (1,1-diphenyl-2-picrylhydrazyl), diamine dihydrochloride, sulphuric acid, sodium phosphate, ammonium molybdate, FeSO_4 , H_2O_2 , agarose, gallic acid, Folin–Ciocalteu's phenol reagent, HCl, were purchased from Sigma (Pacific Science co.ltd, Thailand, USA). The pGEM-T DNA was purchased from promega (Gibthai, Thailand).

2.2 Honey samples

Eleven commercial brands of honey products were randomly brought from supermarkets in Phitsanulok, Thailand (Table 1). Each sample (250 g) were kept in falcon tube, and stored at 4°C for further analysis and none of these samples exceeded the storage period beyond 6 months.

2.3. Physicochemical analysis

2.3.1. Moisture content

The moisture content was analyzed by using the assay as described in the Association of Official Analytical Chemists (AOAC) method number 925.45 (AOAC, 1990).

2.3.2. pH

The honey solution was prepared by 5 g of honey in 20 ml of distilled water., and was measured by a pH-meter (Metler Toledo, Model SevenEasy, Switzerland) according to AOAC method number 962.19 (AOAC1990).

2.3.3. Ash content

The honey sample (3 g) was placed in a crucible in a muffle furnace and heated at 640°C for 6 h, and the ash content (expressed as g%) was measured using method number 920.181 as described in AOAC (AOAC 1990).

2.4 Measurement of total phenolic contents (TPC)

TPC was measured by using the modified method (Socha *et al.*, 2011). Briefly, aliquot 400 µl of sample (200 mg/ml) was added to 1ml of Folin–Ciocalteu reagent (previously diluted to 10 folds with distilled water) and gently mixed by vortex, following by incubated at room temperature (25–28°C) for 5 min. Five milliliters of sodium carbonate (1 M) was added to the mixture and mixed gently by vortex. The mixture was adjusted to final volume 10 ml with distilled water, and incubated at room temperature for 1 h. The absorbance of mixture was read at 760 nm using a spectrophotometer (Optizen 3220 UV). The standard calibration (0.02–0.12 mg/ml) curve was plotted using gallic acid. The total phenolic content was expressed as mg gallic acid equivalents (GAE)/100 g honey sample.

2.5 Analysis of antioxidant activities by DPPH radical scavenging activity

Scavenging activity of honey on DPPH free radical inhibition was assessed by the slightly modified method of Wang *et al.* (2012). Briefly, the 200 µl honey solution (0.2g/ml) was added into methanolic solution (0.1 mM) 1 ml, and vigorously mixed by vortex, followed by incubation at room temperature for 30 min. The absorbance of the mixture was then measured by spectrophotometer (Optizen 3220UV) at 515 nm, and the absorbance without honey sample was used as the control. The percentage inhibition of DPPH radical was calculated according to the following equation:

$$\% \text{ inhibition} = [(A_{515 \text{ control}} - A_{515 \text{ test sample}}) / A_{515 \text{ control}}] \times 100$$

2.6 Examining the effect of honey on hydroxyl radical-mediated DNA break

The protective effect of honey on DNA strand break, induced by hydroxyl radical *in vitro*, was measured by the conversion of circular-pGEM-T DNA to open circular or linear form according to the slightly modified method described by Yeung *et al.* (2002). In brief, 1 μ l plasmid DNA (50 μ g) was incubated with 1 μ l FeSO₄ (1.0 mM), 1 μ l H₂O₂ (1%), and 2 μ l of different honey concentrations (0.2, 0.4, 0.8, or 1 g/ml), and the final volume was made up to 12 μ l with 50mM phosphate buffer (pH 7.0). Two reaction systems were involved in the production of either site-specific radicals absent EDTA or non-site-specific with the presence of EDTA. The mixture was incubated at 37°C for 30 min. This was immediately loaded on electrophoresis in a 0.8% agarose gel with TAE (Tris-acetate-EDTA) buffer. The supercoiled, open circular and linear DNA products were visualized with ethidium bromide (0.001%), and then photographed with gel documentation.

2.6. Statistical analysis

All the tests were performed in three to six biological replicates. The results were expressed as the mean values $\pm$ standard error. Data analysis was carried out using IBM SPSS statistics version 19. Statistically significant differences of mean values among the samples were evaluated by the Tukey's test at p value < 0.05 .

3. Results and Discussion

3.1 Physicochemical analysis of honeys

The physicochemical analysis of honey can indicate overall characteristics and quality of honey. In this study, eleven commercial honey samples collected from different brands sold in Phitsanulok province Thailand were used to assess the physicochemical parameters including moisture content, pH and ash. The moisture content of all examined samples had ranged from 14.22 ± 0.14 to $15.69 \pm 0.09\%$ (Table 3.1) which corresponded with the previously finding of Prica *et al.* (2015) reported that the moisture content in local honeys (collected from Vojvodina) was in the range from 14 to 18%. The result indicated that all honey samples were good quality because their moisture content were least than maximally permitted (20%) by relevant regulations regulated by EU Directive (EU Council 2002). The low moisture levels in all examined samples resulted in a longer shelf life (Fredes and Montenegro, 2006).

The range of the percentage ash content of all samples was narrow range from 0.14 to 0.17% (Table 1). The result suggested that all samples were good quality with high mineral contents and their values did not exceed the maximum permitted level of 0.60% specified by the Codex Standard, according to White and Landis (1980). Various ash

contents of samples might be from different harvesting processes of beekeepers during the foraging on the flora (Finola *et al.*, 2007). The values of ash contents in this study closed to some Indian honeys (0.03 to 0.43%) collected from different botanical regions (Saxena *et al.*, 2010). The pH of all eleven samples ranged from 4.43 to 6.56 (Table.1). About 82% (9 samples) of evaluated samples had values agreed with reporting from National Honey Board Food Technology (2006) which specified a pH range between 3.4 and 6.1.

Table 1 Physiochemical properties of the investigated 11 honeybee samples

Honey samples	Physiochemical properties		
	Moisture (%)	Ash (%)	pH
1	15.93±0.05 ^a	0.16±0.02 ^b	6.56±0.04 ^a
2	14.85±0.06 ^c	0.15±0.01 ^d	5.36±0.10 ^e
3	14.42±0.13 ^{de}	0.16±0.01 ^b	4.63±0.01 ^h
4	14.76±0.10 ^{cd}	0.15±0.02 ^c	5.53±0.02 ^d
5	14.98±0.06 ^{bc}	0.17±0.03 ^b	4.76±0.02 ^h
6	15.69±0.09 ^a	0.14±0.01 ^d	4.43±0.01 ⁱ
7	15.26±0.09 ^b	0.15±0.01 ^c	5.18±0.03 ^f
8	14.74±0.07 ^{cd}	0.17±0.03 ^a	6.14±0.01 ^b
9	14.22±0.14 ^e	0.15±0.04 ^d	5.17±0.04 ^f
10	14.56±0.16 ^{de}	0.17±0.05 ^{ab}	5.93±0.02 ^c
11	15.1±.058 ^{bc}	0.1627±0.01 ^b	4.90±.025 ^g

Note: Legend: values are expressed as means ± SE (with 6 biological replicates).

Different lower case letters (in same column) correspond to significant differences at $p < 0.05$, calculated by Tukey's test.

3.2 Sugar content in honeys

Reducing sugars, mostly glucose and fructose, were the main component in honey (Kucuk *et al.* 2007). Table 2 revealed that all eleven examined samples had glucose and fructose content less than the standard value (6 g/ 100 g) as specified by Codex Alimentarius Commission (CAC, 1998). About 55% of samples (6 samples) had high contents of both fructose and glucose ranged from 9.2 to 40.4 g/ 100g and 9.9 to 39.0 g/ 100 g respectively (Table 2). These values agreed with Al *et al.* (2009) found that the high content of combined glucose and fructose found in a lime honey samples from Romania.

Sucrose contents of all eleven samples were varied. Four samples (36%) had high sucrose content ranged between 14.1 and 32.4 mg/ 100 g (Table 2), indicating artificial fed with sugar to bees. Five samples (45%) did not have sucrose content and one sample contains very negligible amount of sucrose content (0.5 mg/ 100 g), indicating that the bees might collect nectar from floras (Babarinde *et al.*, 2011).

Table 2 Sugar contents of the investigated 11 honeybee samples

Honey samples	Sugar content		
	Fructose (g/100g)	Glucose (g/100g)	Sucrose (mg/100g)
1	20.8±0.2 ^e	25.2±0.3 ^f	0.0
2	9.2±0.1 ^h	9.9±0.1 ^j	32.4±0.3 ^a
3	37.6±0.2 ^{cd}	30.4±0.2 ^d	0.00
4	12.6±0.1 ^g	14.4±0.1 ⁱ	18.2±0.2 ^b
5	37.3±0.1 ^d	30.8±0.1 ^d	6.2±0.1 ^e
6	38.7±0.2 ^b	32.0±0.1 ^c	0.0
7	38.1±0.1 ^c	34.1±0.2 ^b	0.0
8	16.2±0.2 ^f	20.6±0.2 ^g	8.9±0.2 ^d
9	40.4±0.2 ^a	39.0±0.2 ^a	0.5±0.0 ^f
10	13.0±0.1 ^g	15.0±0.1 ^h	14.1±0.1 ^c
11	37.4±0.2 ^d	29.5±0.2 ^e	0.0

Note: Legend: values are expressed as means ± SE (with 6 biological replicates).

Different lower case letters (in same column) correspond to significant differences at $p < 0.05$, calculated by Tukey's test.

3.3 Assay of total phenolic content (TCP) and DPPH-free radical-scavenging in honeys

Total phenol content (TPC) can be widely used as important indicator for determining antioxidant activity for food products because its redox properties act as reducing agents such as hydrogen donors (Negri *et al.*, 2011). In this study, the TPC and antioxidant activity (AA) of eleven different honey samples were investigated by the Folin-Ciocalteu method and DPPH scavenging assay, respectively. The results found that out of eleven samples, only two samples had the highest both TPC and AA values with range of 9.78 to 9.83 mg GAE/100 g and 4.72 to 5.53%, respectively (Table 3). These TPC and AA values suggest that both samples have potential to exhibit bioactivity on the protective DNA damage by free radical.

Table 3 Phenolic compound of the investigated 11 honeybee samples

Honey samples	Total phenolic content (mg GAE/100g)	Antioxidant activity (% inhibition)
1	4.41±0.06 ^e	0.00
2	3.78±0.04 ^f	0.00
3	8.30±0.12 ^b	4.72±0.07 ^b
4	2.62±0.12 ^h	0.00
5	6.96±0.073 ^d	3.91±0.04 ^c
6	9.78±0.079 ^a	5.53±0.08 ^a
7	6.88±0.16 ^d	3.68±0.09 ^c
8	3.11±0.06 ^g	0.00
9	7.87±0.05 ^c	3.78±0.06 ^c
10	3.07±0.12 ^g	0
11	9.83±0.08 ^a	4.97±0.05 ^b

Note: Legend: values are expressed as means ± SE (with 6 biological replicates).

Different lower case letters (in same column) correspond to significant differences at $p < 0.05$, calculated by calculated by Tukey's test.

3.4. Assay the effects of honey sample on hydroxyl radical-mediated DNA strand break

Since honey contains phenolic compounds and exerts antioxidant activity which has potential to scavenge biological free radical such as hydroxyl radical, leading to the prevention of DNA damage, we further investigated whether it could protect the damage of DNA from free radicals. There are two tested hypothesizes that honey can prevent hydroxyl radical-mediated DNA strand break *in vitro* through present or absent chelating EDTA. Three honey samples (3, 6 and 11) with high total phenolic contents were selected in this test. The plasmid DNA (supercoiled DNA) was incubated in FeSO_4 and H_2O_2 with present chelating EDTA according to the slightly modified method as described by Yeung *et al.* (2002). With the attack of hydroxyl radical generated from the Fenton reaction, supercoiled plasmid DNA was broken into open circular and linear form. The DNA products were visualized with ethidium bromide, and then photographed with gel documentation.

For the present EDTA assay (non-site specific) (Figure 1), the result showed that all three honey sample can prevent hydroxyl-radical-mediated-DNA-strand break, by giving both supercoiled-DNA (with size approximately 3kb) and linear-DNA bands (with size greater than 3kb) (Figure 1 sample lane 1–12). In the control (Figure 1 lane C), there was no DNA plasmid recover because the DNA plasmid was degraded by H_2O_2 and hydroxyl radicals,

resulting in increasing DNA break (Aruoma, 2003). This indicated that these three honey samples had an antioxidant effect which can prevent directly scavenging hydroxyl radical-mediated DNA strand break as agreement with the report of the protective effects of buckwheat honey on DNA damage induced by hydroxyl radicals (Zhou *et al.*, 2012).

There are two tested hypothesizes that honey can prevent hydroxyl radical-mediated DNA strand break *in vitro* through present (non-site specific) or absent chelating EDTA (site-specific) as the result shown in Figure 1 and 2 respectively. We suspect whether antioxidant activity of honey exhibit through chelating properties. Consequently, the site-specific system was performed and the result as shown in Figure 2.

In the site-specific system, EDTA is omitted. Iron ion can bind to DNA and produce hydroxyl radicals at the non-site-specific site (Aruoma *et al.*, 1989). So iron chelating compounds can reduce the extent of DNA damage even if they are not effective hydroxyl radical scavengers. The data showed that the recovery of band intensity of supercoiled in Figure 2 was less than those in Figure 1. This also suggested the honeys which protect more effectively in the non-site-specific system than in the site-specific system are due to its stronger hydroxyl radical scavenging activity than iron chelators in the present assay.

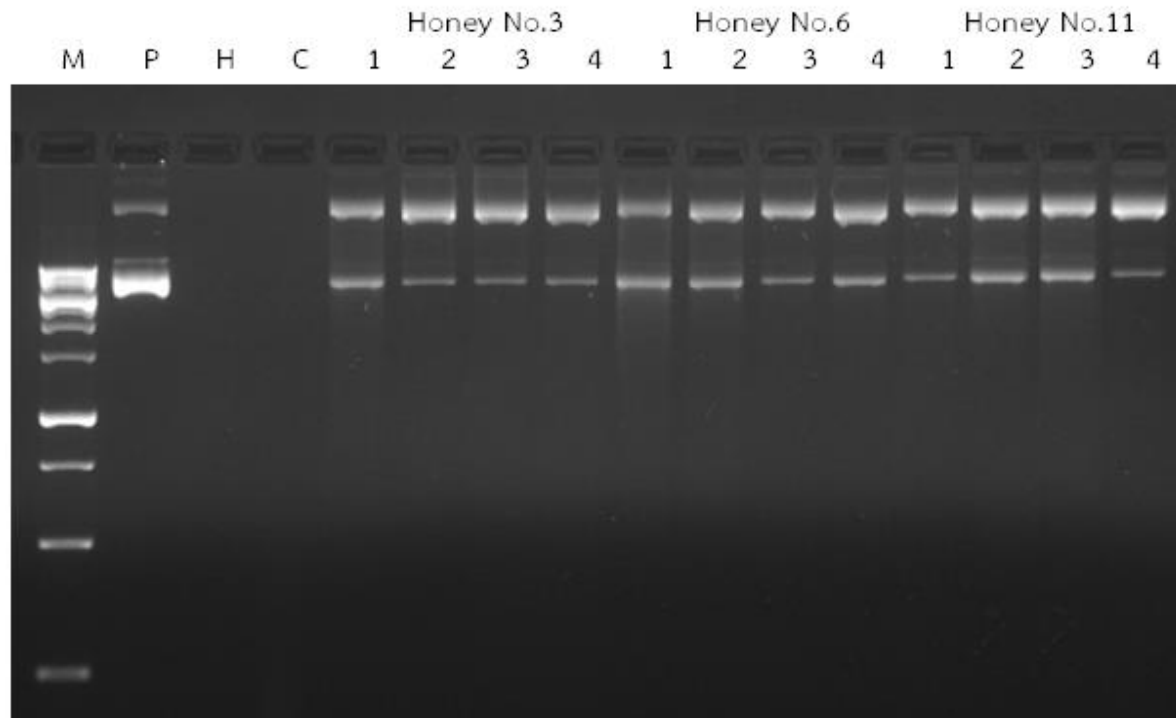


Figure 1 Determination of protective effects of honey bee on hydroxyl radical-mediated plasmid-DNA strand breaks with site-specific condition (present EDTA/ **non site specific**) on gel electrophoresis; Legend: Lane M represents 1 kb DNA ladder, Lane P represents only plasmid (pGEM-*Bph14*) (100 μ g), : Lane H represents only honey (0.2 g/ml), Lane C represents the mixture of pGEM-*Bph14* DNA (100 μ g) + 2% H_2O_2 (1 μ l) + 1 mM FeSO_4 (1 μ l), Lane 1-4 represents FeSO_4 and H_2O_2 in presence of honey sample (No.3, 6, and 11) with concentration 0.2, 0.4, 0.8, and 1.0 g/ml respectively.

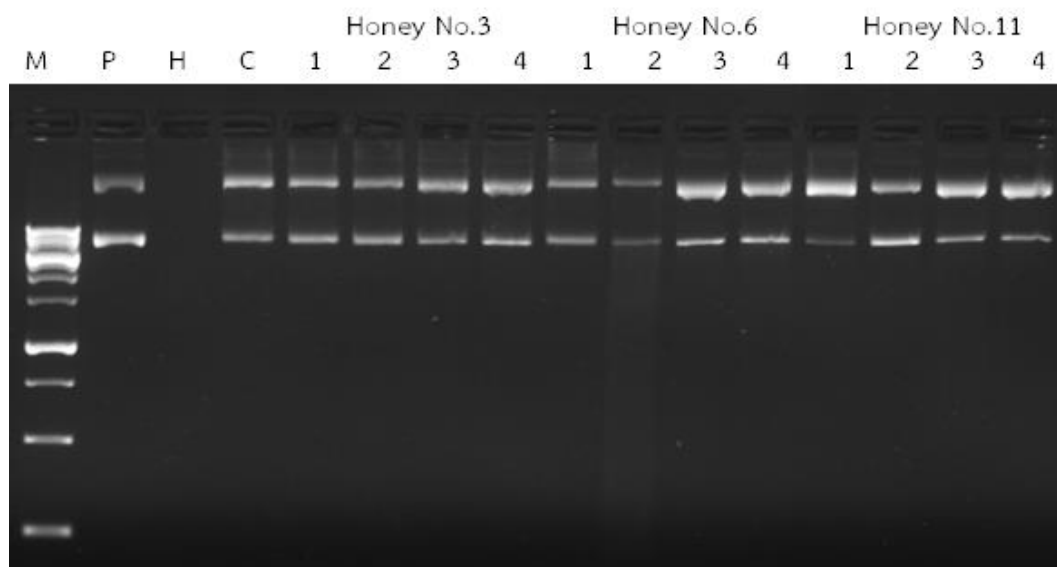


Figure 2 Determination of protective effects of honey bee on hydroxyl radical-mediated plasmid-DNA strand breaks with site-specific condition (absent EDTA/site specific) on gel electrophoresis; Legend: Lane M represents 1 kb DNA ladder, Lane P represents only plasmid (pGEM-*Bph14*) (100 μ g), : Lane H represents only honey (0.2 g/ml), Lane C represents the mixture of pGEM-*Bph14* DNA (100 μ g) + 2% H_2O_2 (1 μ l) + 1 mM EDTA- FeSO_4 (1 μ l), Lane 1-4 represents EDTA- FeSO_4 and H_2O_2 in presence of honey sample (No.3, 6, and 11) with concentration 0.2, 0.4, 0.8, and 1.0 g/ml respectively.

4. Conclusion

The results obtained in this study showed that some of eleven commercial honeys available in the market were difference in physicochemical properties such as moisture, pH and ash content, under permitted by regulation standards. Moreover, they contained different amount of phenolic compound, contributed to the antioxidant activities. We found here that some commercial honeys possessed the strong reducing power, DPPH and hydroxyl radical scavenging activities rather than ion-chelating activity on protective DNA damage. These indicated that some of them were proven as natural antioxidants and as an important ingredient of the functional food in particular functional beverage.

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