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Predictive mathematical modeling for EC_{50} calculation of antioxidant activity and antibacterial ability of Thai bee products

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ABSTRACT

Antioxidant activities of bee products from Thailand (honey, bee pollen and propolis) *via* the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate)(ABTS) assays were determined. The prediction of the EC_{50} (the half maximal effective concentration) were studied using the logistic, sigmoidal, dose response, and asymmetric 5 parameters (5P) regression models. The antimicrobial ability was tested against *Staphylococcus aureus* (TISTR 517), *Bacillus cereus* (TISTR 687), and *Escherichia coli*(TISTR 1261). Propolis extract with higher total phenolic content (TPC) exhibited more effective antiradical action against the DPPH and ABTS, followed by bee pollen extract and honey. All four regression models could be used to estimate the EC_{50} of the bee products. However, the dose-respond and 5P provide the better EC_{50} prediction for the bee products than the others based on the comparability of their results to those of right-angled triangle method. Thai bee products had effective antimicrobial activities on each test microorganism. The antimicrobial potency of the bee products was ranged in the order: propolis> bee pollen >honey. Results revealed that antioxidant activity and antimicrobial ability of the bee products correlated with the TPC values.

INTRODUCTION

Anti-oxidative action is one of the physiological functions of many compounds found in foods (Nagai *et al.*, 2001). This action is assumed to protect living organisms from oxidation, resulting in the prevention of various diseases such as cancer and diabetes (Nagai *et al.*, 2001). The antimicrobial activity of chemical compounds, including antibacterial, antifungal and antiviral activity, is important against infections incited by microorganisms (Bogdanov, 2011). Plant polyphenols are potential natural alternatives to synthetic antioxidant and antimicrobial compounds (Siripatrawan *et al.*, 2013). Bee products, one of the essential sources of polyphenols, are well known in traditional medicine dating back to ancient times.

Man utilized bee products in many ways, and now a days their applications have expanded from healthy foods to medicinal products. Bee products such as honey, bee pollen, royal jelly and propolis from various geographical locations around the world have been found to possess antioxidant and antimicrobial activities (Buratti *et al.*, 2007; Choi *et al.*, 2006; Graikou *et al.*, 2011). Honey the nectar that the honey bees collect and process from many plants(Ferreira Isabel *et al.*, 2009)has been used in food as a sweetening agent (Nagai *et al.*, 2001) and food preservative since ancient times (Ferreira Isabel *et al.*, 2009; Nagai *et al.*, 2001). Honey normally consists of more than 150 substances, including complex mixture of sugars and small amount of polyphenolic compounds such as flavonoids and cinnamic acid derivatives (Buratti *et al.*, 2007; Ferreira Isabel *et al.*, 2009). Bee pollen is a fine, powder-like material produced by flowering plants and collected by worker honey bees formed into granules with added honey or nectar (Bogdanov, 2011).Bee pollen contains lipids, proteins, sugars, amino acids, vitamins, carotenoids and polyphenolics (Graikou *et al.*, 2011).

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Bee pollen is considered to be a nutrient-rich perfect food and is commercially promoted as a dietary supplement. Propolis is a sticky substance derived from plant resins collected by honeybees (Bogdanov, 2011).

Propolis contains more than 300 constituents such as polyphenols, sesquiterpene quinones, coumarins, steroids, amino acids, and inorganic compounds (Choi *et al*., 2006; Siripatrawan *et al.*, 2013).The composition of propolis varies depending on the season and on the botanical origin from which the plant resins have been collected (Bosio *et al.*, 2000). Propolisis now recognized to have a wide range of biological activities, such as antibacterial, anti-inflammatory, antioxidative, hepatoprotective, and tumoricidal activities (Bosio *et al.*, 2000; Miorin *et al.*, 2003).

Determination of the antioxidant power of the bee products involved the use of different methods such as the DPPH (2,2- diphenyl-1-picrylhydrazyl), ABTS [2,2´-Azino-bis(3-ethylbenzthiazoline-6-sulphonate)], TBARS (thiobarbituric acid reactive substances) and β-carotene bleaching assays (Buratti *et al.*, 2007; Ferreira Isabel *et al.*, 2009; Lachman *et al.*, 2010; Siripatrawan *et al.*, 2013). The DPPH and ABTS assays have been widely used to determine antioxidant activity of various plants and other materials since they are stable free radicals and the determination is simple. The half maximal effective concentration (EC_{50}), the concentration of antioxidant that causes a 50% decrease in the radical absorbance, is commonly expressed by measuring antioxidant results. In 1999, Alexader *et al*. (1999) presented a simple and accurate mathematical method for calculation of the EC_{50} called right-angle triangle. They suggested that the right-angle triangle method is simple, accurate, and non-computational technique for the calculation of the EC_{50} is needed. Now days, a number of methods and software have been developed which contain the functions for non-linear curve-fitting of the experimental data and estimation of the EC_{50} value, making this determination fast and particularly useful for laboratory test. Chen *et al*. (2013) studied the EC_{50} estimation of antioxidant standards (quercetin, catechin, ascorbic acid, caffeic acid, chlorogenic acid and acetylcysteine) with DPPH assay using various computer programs and mathematical models. All the statistical programs they used provided similar EC_{50} values, however, the asymmetric five-parameter equation in the GraphPad Prism software was found to point out a best fit for their experiment. Recently, the estimation of EC_{50} values for fungi with different methods using computer programs were also reported by Li *et al.*(2015).Results showed that among all the statistical programs they used, IBM SPSS, GraphPad Prism, DPS were appropriated for EC_{50} calculations of their samples.

To the best of our knowledge, there has been no research that publishes the EC_{50} prediction of antioxidant activity for honey, bee pollen and propolis using comparative different regression models.

Thus, the objectives of the present work are: (1) to evaluate the antioxidant activity of the extracts of propolis, bee pollen and honey from Thailand, and (2) to identify the best model for the prediction of EC_{50} from experimental data obtained *via* DPPH and ABTS assays. The *in vitro* antimicrobial activity was also investigated and it is reported.

EXPERIMENTAL

Materials

Longan honey and bee pollen were purchased from Chiang Mai Healthy Product (Chiang Mai, Thailand). Dried propolis extract selected from Chiang Mai province were purchased from T. Man Pharma Co., Ltd. (Bangkok, Thailand). Gallic acid, DPPH (2,2-Diphenyl-1-picrylhydrazyl), ABTS [2,2´-Azino-bis(3-ethylbenzthiazoline-6-sulphonate)], Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid),2.0 N Folin-Ciocalteu reagent and α-tocopherol were purchased from Sigma-Aldrich (MO, USA); sodium chloride, methanol and ethanol were purchased from QRëC (Auckland, New Zealand). L-ascorbic acid, sodium carbonate and potassium persulfate were purchased from Ajax Finechem Pty Ltd (New South Wales, Australia); NB (nutrient broth) and NA (nutrient agar) were purchased from HiMedia Laboratories Pvt. Ltd (Mumbai, India). All reagents were of analytical grade and used as received.

Preparation of bee pollen extracts

Bee pollen extract was prepared according to the procedure described by Morais *et al*. (2011). Bee pollen was soaked in methanol at pollen-to-methanol ratio of 1:2 (w/v). The mixture was left to macerate for 72 h at room temperature and shaken by hand for 5 min twice a day. The pollen extract was filtered through a Whatman filter paper No. 4 using a Buchner funnel. The methanol extract was evaporated in a vacuum evaporator (Thailand) and stored in an amber glass bottle at 4ºC for further analysis.

Determination of total phenol content

The total phenol content (TPC) of the bee product samples was determined using the Folin–Ciocalteau method as described by Ahn *et al*. (2004) with slight modifications. The sample (0.3 mL) was put in a test tube, and 3 mL of distilled water, 0.25 mL of 2.0 N Folin-Ciocalteu reagent and 2.5 mL of 7% (w/v) sodium carbonate were added. Each tube was covered with a cap and shaken with a vortex mixer (Dragon Lab, China). After 30 min of incubation in a dark place at 25˚C, the absorbance was measured at 760 nm with a spectrophotometer (Labomed, USA) and compared to a calibration curve of gallic acid. The results are presented as means of triplicate analyses and expressed in mg gallic acid equivalents/g of sample (mg GAE/g sample).

Determination of antioxidant activity

The DPPH (2,2- diphenyl-1-picrylhydrazyl) scavenging capacity of the bee products was monitored according to the method described by Brand-Williams *et al.* (1995). A different dilution of the samples (0.3 mL) was mixed with 0.06 mM DPPH-methanolic solution (2.7 mL). The mixture was placed in a dark room for 30 min. The absorbance at 516nm (A) was determined

with a spectrophotometer (Labomed, USA). This activity was given as % DPPH scavenging and calculated using equation 1:

$$\%\text{Inhibition DPPH}^{\bullet} = \frac{A_{control} - A_{sample}}{A_{control}} \text{x } 100 \quad (1)$$

where $A_{control}$ is absorption of DPPH solution, and A_{sample} is absorbance of the test sample. The half maximal effective concentration (EC_{50})is the amount of sample necessary to decrease the absorbance of DPPH by 50%. It was calculated by interpolation from the graph of inhibition percentage against sample concentration using a simple mathematical method based on the principle of right-angled triangle(Alexander *et al.*, 1999).Ascorbic acid and α-tocopherol were used as positive controls. All the analyses were carried out in triplicate.

Determination of Trolox equivalent antioxidant capacity (TEAC)

For the TEAC assay, the procedure followed the method described by Re *et al.*(1999). The TEAC assay is based on the scavenging of the 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) (ABTS) radical (ABTS•+). ABTS•+ was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate solution (1:1) (v/v) and storing it in the dark at room temperature for 16 h before use. The ABTS•+ solution (1 mL) was diluted to get an absorbance of 0.700±0.025 at 734 nm with methanol (40 mL). Bee product sample (0.3 mL) was added to the ABTS•+ solution (2.7 mL) and the absorbency was measured after 6 min. The %inhibition of the sample was calculated using the formula mentioned in the DPPH assay. The result was then compared with a standard curve made from the corresponding readings of Trolox (0–0.2 mM). Results were expressed as mg trolox equivalents/g dried sample (mg TE/g sample).

EC_{50} prediction using statistical models

Data analysis for the free radical scavenging activity of bee product samples was performed using the logistic, Boltzmann (sigmoidal), log (agonist) vs. normalized response−variable slope (dose−response), and asymmetric sigmoidal (five parameter, 5P) mathematical models indicated in equations 2 to 5, respectively, using JMP 10 (SAS Institute Inc., Cary, NC, USA) and SciDAVis (version 2, Boston, MA).

$$\text{Logistic } y = \frac{A_1 - A_2}{1 + (\frac{x}{x_0})^{\wedge} p} + A_2 \quad (2)$$

$$\text{Boltzman (sigmoidal) } y = \frac{A_1 - A_2}{1 + exp^{(\frac{x - x_0}{dx})}} + A_2 \quad (3)$$

$$\text{Dose–response } \quad y' = \frac{100}{1 + 10^{\wedge}[(x_0 - x) \cdot HillSlope]} \quad (4)$$

$$\text{Asymmetric sigmoidal } \quad y = A_1 + \frac{A_2 - A_1}{(1 + 10^{\wedge}[(Logx_b - x) \cdot HillSlope])^s} \quad (5)$$

where x is log of concentration, y is response, y' is normalized response (0 to 100%), A_1 is the baseline, $A_2$is the maximum response, x_0 is center or logEC_{50}, p is power, dx is time constant, *Hillslope* is the steepness of the curve which has no units and s is the symmetry parameter, which is unit less and x_b is concentration at the inflection point. For asymmetric sigmoidal model, the EC_{50} can be calculated from the x_b, *Hillslope* and s parameters by using equations 6 as followed:

$$LogEC_{50} = Logx_b - (\frac{1}{Hillslope} \times Log\left[\left(2 \times \frac{1}{s}\right) - 1\right]) \quad (6)$$

Antimicrobial ability

Preparation of inoculums

Gram-positive (*Staphylococcus aureus*TISTR517 and *Bacillus cereus* TISTR687) and Gram-negative (*Escherichia coli* TISTR1261) organisms were obtained from the Division of Biotechnology, Faculty of Agro-Industry, Chiang Mai University (Chiang Mai, Thailand). *S. aureus*, *B. cereus* and *E. coli* were cultured in NB at 30˚Cfor 24 h. The optical density (OD) of the bacteria was adjusted to the standard of McFarland No. 0.5(Hindler *et al.*, 1992)with 0.85 g sodium chloride/100 mL sterile solution to achieve a concentration of approximately 10^8 CFU/mL. The final concentration of the cell numbers was approximately 10^5-10^6 CFU/mL obtained by diluting 100 times with sterile sodium chloride solution.

Determination of minimum inhibitory concentrations (MIC) and minimal bactericidal concentration (MBC)

The MIC of the bee product samples was determined using a broth dilution assay according to the procedure described by Mazzola *et al.* (2009). One mL of NB medium was dispensed in each of the 12 numbered test tubes (16 mm x 150 mm), except for tube # 1. The tubes were autoclaved (IWAKI, Japan) at 121ºC. For tubes#1 and # 2, 1 mL of test sample was introduced; tube # 2 was stirred and 1 mL was withdrawn and transferred to tube #3. This serial dilution was repeated for all tubes up to tube # 11. Then 1 mL was removed from tube # 11.One mL of each test microorganism was added to each tube. All tubes were incubated at 30ºC for 24 h and the results were evaluated. The MIC of bacteria was defined as the lowest concentration at which no growth occurred. Tube # 12 is the positive control (NB + inoculation).

The MBC test determines the lowest concentration at which an antimicrobial agent will kill a particular microorganism. The MBC is defined using a series of steps, undertaken after the MIC test has been completed. The dilution representing the MIC and at least two more concentrated test sample dilutions was touched with a loop and streaked on a NA plate and incubated at 30 ºC for 24 h. The MBC was determined as the lowest concentration at which no growth appeared (Taemchuay *et al.*, 2009). The plates with streaking of each inoculation were used as the control.

Table 1: Total phenol content and antioxidant activity determined by DPPH and ABTS assays of bee product samples.

Sample	Total phenol content	DPPH assay	ABTS assay	
	(mg GAE/g sample)	EC50 (mg/mL)	EC50 (mg/mL)	TEAC (mg TE/g sample)
α-tocopherol (positive control)	-	0.055 ± 0.001 a	0.032 ± 0.002 a	768.4 ± 44.55 a
ascorbic acid (positive control)	-	0.023 ± 0.002 b	0.023 ± 0.002 b	1053 ± 101.2 b
honey	0.57 ± 0.01 a	276.7 ± 6.117 c	92.29 ± 5.638 c	0.263 ± 0.016 c
bee pollen	24.22 ± 0.34 b	1.499 ± 0.015 d	0.560 ± 0.039 d	43.35 ± 3.152 d
propolis	237.18 ± 6.76 c	0.150 ± 0.004 e	0.054 ± 0.008 e	456.7 ± 69.90 e

Note: Values in a column with the same letter are not significantly different ($P \leq 0.05$).

Statistical analysis

The data were analyzed by a one-way analysis of variance (ANOVA) and Tukey HSD's multiple range test ($p \leq 0.05$) using the SPSS software (Version 11, SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Total phenol content

Thai honey, bee pollen and propolis were analyzed by the proposed procedure and the results were expressed as mg GAE/g. Polyphenols were found in our bee products. The values of their total phenol content are shown in Table 1, and they are in the following decreasing order: propolis extract > pollen extract > honey. Buratti *et al*.(2007) also found a similar trend for bee products. The TPC of our propolis (237.18 mg GAE/g sample) agreed with values obtained by Ahn *et al.* (2004) in Korean propolis (85-283 mg GAE/g) and by Moreira *et al.*(2008)in Portuguese propolis (151-329 mg GAE/g). However, the TPC of our propolis was higher than the values reported by Siripatrawan *et al.*(2013) and Kumazawa *et al.*(2004)in Thai propolis,22.8-77.5 mg GAE/g and 31.2 mg GAE/g, respectively, and Choi *et al.* (2006) in Brazilian propolis (120 mg GAE/g). On the other hand, the TPC of Chinese propolis showed slightly higher values as ranged from 262 to 299 mg GAE/g (Kumazawa *et al.*, 2004). Meanwhile, the TPC of our bee pollen extracts (24.22 mg GAE/g) was in agreement with those of bee pollen reported from Portugal (25.3-28.8 mg GAE/g) and Spain (18.6-32.2 mg GAE/g) (Pascoal *et al.*, 2014).The TPC of our honey (0.57 mg GAE/g)was comparable to honey collected in Thailand (0.23-0.73 mg GAE/g) by Jantakee and Tragoolpua (2015) and in Portugal (0.23-0.73 mg GAE/g) by Ferreira *et al.* (2009). However, this value was lower when compared to honey from Brazil (1.05 mg GAE/g) (Sant'ana *et al.*, 2014).The variation of TPC from the bee products from various origins could be attributed to climate and environmental factors such as humidity, temperature and soil composition.

Antioxidant activity

Antioxidants from natural sources are attractive alternatives to synthetic antioxidants. Antioxidants can be used to prevent diseases and oxidation of food products (Morais *et al.*, 2011). According to the complex nature of natural antioxidants, Sakanaka and Ishihara (2008) suggested that the use of at least two methods is recommended to evaluate and compare the antioxidant capacity of a sample. In this research, we used the procedure based on the reduction of DPPH and ABTS, stable free radicals, to investigate the free radical-scavenging activity of the bee products. The DPPH and ABTS assay has been widely employed to determine the free radical scavenging ability of a variety of natural antioxidants (Chen *et al.*, 2013; Lachman *et al.*, 2010; Siripatrawan *et al.*, 2013).The underlying mechanisms of determining the activity using DPPH and ABTS can be represented as Reaction 1 and 2, respectively(Boligon *et al.*, 2014):

$$DPPH^{\bullet} + \text{antioxidant}\,(A-OH) \rightarrow DPPH-H + AO^{\bullet} \quad (1)$$

$$ABTS^{\bullet+} + A-OH \rightarrow ABTS + AO^{\bullet} + H^{+} \quad (2)$$

In the DPPH assay, the purple DPPH• is reduced by hydrogen–donating of antioxidant to the pale yellow DPPH–H. In the ABTS scavenging process, first, ABTS•+ is generated by reacting a strong oxidizing agent, potassium persulfate, with ABTS salt. The blue–green ABTS•+ is converted back to its colorless ABTS by hydrogen–donating of antioxidant.

The free radical scavenging activity of the methanolic fraction of the honey, pollen extract and propolis extract was measured at various sample concentrations by the DPPH assay and ABTS assay and expressed as the EC_{50}values in Table 1.Ascorbic acid and α-tocopherol, well known natural antioxidant, were used as standards. The EC_{50} values calculated from DPPH and ABTS assays for the bee products ranged between0.159–286.8 mg/mL and 0.059–93.19 mg/mL, respectively. The average antioxidant activities determined by the ABTS assay were two to three times lower compared to values determined by the DPPH assay (Lachman *et al.*, 2010).This may probably because DPPH may have limitation and show lower sensitivity to the bee products than ABTS.ABTS•+ is applicable to both hydrophilic and lipophilic antioxidants due to its solubility in both aqueous and organic solvents while DPPH• is useable to hydrophobic systems since it is dissolvable in organic media(Floegel *et al.*, 2011). Therefore, the ABTS method is reactive towards most antioxidants; whereas some compounds react very rapidly by the DPPH assay.

The lower EC_{50} value indicates a higher antioxidant activity for the product. The antioxidant activity values determined by these two different assays (Table 1) – revealed that among the bee products propolis had the stronger antioxidant power when compared to bee pollen and honey but lower than the standards. This outcome may be attributed to the large concentration of

phenolic compounds available in propolis. These results are in accordance with results provided by Buratti *et al.*(2007) and Nagai *et al*. (2001). The data collected by Buratti *et al*. (2007) *via* DPPH assay showed that within the Italian bee products, propolis (IC_{50} = 1.0–2.1 mg/mL) had the highest antioxidant capacity followed by royal jelly (IC_{50} = 1.4–2.3 mg/mL) and honey (IC_{50} = 5.0–15.5 mg/mL). Nagai *et al*. (2001) studied the anti-oxidative effects of some honeys, royal jelly, and propolis from Japan using a lipid peroxidation model. They discovered that the superoxide scavenging activities of the bee product decreased in the following order: propolis> royal jelly > honey.

Interestingly, the extracts of the bee products, which exhibited higher activity, were those that contain a high phenol level. Propolis was obviously most active among all the bee product samples. The antioxidant activity seemed to be related to the total phenol content of the extract. Similar phenomena have been reported for propolis from Korea (Choi *et al.*, 2006), Italian bee products (Buratti *et al.*, 2007), Czech honey (Lachman *et al.*, 2010), Brazilian honey (Sant'ana *et al.*, 2014), Portuguese bee pollen (Morais *et al.*, 2011), Greek bee pollen (Graikou *et al.*, 2011), and Thai propolis (Siripatrawan *et al.*, 2013). Flavonoids and phenolic components played an important role in the free radical scavenging capacity of the extract (Graikou *et al.*, 2011). Likewise, the different origins of the extracts may provide different types and contents of the phenolic compounds in propolis. Rutin, quercetin and naringenin were found to be the main phenolic compounds in propolis collected from Nan province, Thailand (Siripatrawan *et al.*, 2013). Kumazawa *et al*. (2004), who study the antioxidant activity of propolis of various geographic origins, found that propolis contained antioxidative compounds such as kaempferol and phenethylcaffeate showing the strong antioxidant activity. Phenols exhibits an excellent property of reducing spontaneous autoxidation of organic molecules (Ingold, 1961) through a general class of mechanism called chain-breaking. Chain breaking antioxidants operate by neutralizing peroxide radicals to stop chain propagation of the radicals. To inhibit the oxidation, an H atom from the phenols is transferred to the oxidative chain carrying peroxyl radicals (ROO•)as exemplified in Reaction 3(Foti, 2007):

$$\text{Phenol} - \text{OH} + \text{ROO}^{\bullet} \rightarrow \text{Phenol} - \text{O}^{\bullet} + \text{ROOH} \qquad (3)$$

Phenoxyl radical (Phenol-O•), generated as a product, is normally nonreactive to oxygen (O2) and substrates (RH) (Reaction 4 and 5). This reduces the rate of the oxidation reaction(Ingold, 1961). The Phenol-O• is then degenerated *via* the bimolecular self-reaction or the reaction by another ROO• radical (Reaction 6 and 7).

$$\text{Phenol} - \text{O}^{\bullet} + \text{O}_2 \xrightarrow{\text{very slow}} \text{Phenol}(=\text{O})\text{OO}^{\bullet} \qquad (4)$$

$$\text{Phenol} - \text{O}^{\bullet} + \text{RH} \xrightarrow{\text{very slow}} \text{Phenol} - \text{OH} + \text{R}^{\bullet} \qquad (5)$$

$$\text{Phenol} - \text{O}^{\bullet} + \text{Phenol} - \text{O}^{\bullet} \xrightarrow{\text{fast}} \text{Phenol} - \text{OO} - \text{Phenol} \qquad (6)$$

$$\text{Phenol} - \text{O}^{\bullet} + \text{ROO}^{\bullet} \xrightarrow{\text{fast}} \text{non} - \text{radical products} \qquad (7)$$

Figure 1 shows the correlation between the total phenol and antioxidant activity with $1/EC_{50}$ values measured from DPPH assay of the tested bee products. Correlations of some natural products from previous studies (Barreira *et al.*, 2008; Harzallah *et al.*, 2016; PhomkaivonAreekul, 2009) are also showed (Figure 1). The antioxidant activity of the products correlated with the total phenolic contents. Ferreira *et al.*(2009)have tested honey form Northeast Portugal and found that the higher antioxidant contents and the lower EC_{50} values for antioxidant activity were obtained in the darker honey which contained higher total phenolics. In the study of Moreira *et al.*(2008), propolis from northeast and center of Portugal was analyzed. Lower values of EC_{50} on DPPH scavenging assay were obtained for northeast of Portugal, which could be related with the higher total phenols content. However, the strong relation between the phenolic compounds and antioxidant activity was not found for the bee pollen studied by Pascoal *et al.* (2014) and Morais *et al.* (2011), and they did not give any reason.Previous studies have presented the effect of phenolic compounds on antioxidant activity of other natural products. He *et al.*(2015) studied antioxidant activity of *Pyruspashia* flowers in China, and they found that antioxidant effect of *P.pashia* flowers was related with phenolics content. Barreira *et al.* (2008) determined an antioxidant activity and polyphenols content of the extracts from various part of chestnut such as flowers, leaves and fruits. They found that chestnut flowers and leaves presented very good antioxidant activity while chestnut fruits revealed the highest EC_{50} values. Their obtained results are in agreement with the phenol contents determined for each sample. In this work, we also show a correlation of the EC_{50} value with the total phenolic contents.

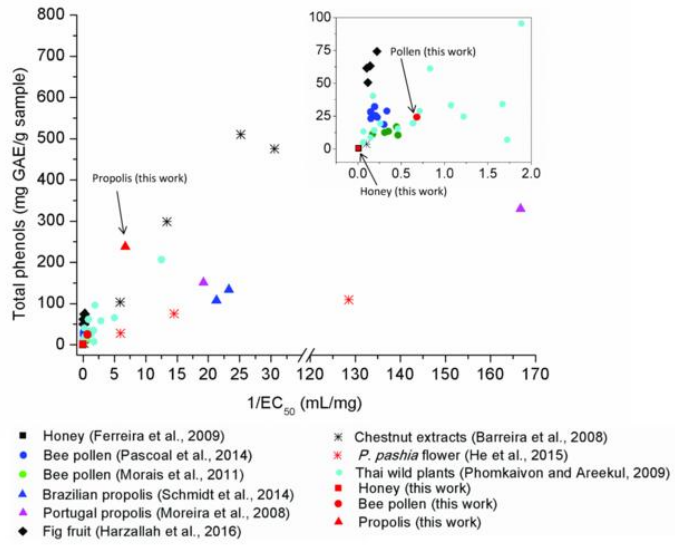

Fig. 1: Total phenolic (mg GAE/g) vs. $1/EC_{50}$ (mL/mg) of bee products and some natural products. EC^{50} was obtained from DPPH assay.

EC_{50}prediction using statistical models

EC_{50} is an important parameter to evaluate the antioxidant activity of materials and it could be used to compare the antioxidant capacity of various materials. The EC_{50} could be

determined by interpolating data from an appropriate curve or by a non-linear regression of the data by using different models (Chen *et al.*, 2013). Various models can be used to determine EC_{50}. In this work, we fit the experimental data to the logistic, Boltzmann (sigmoidal), log (agonist) vs. normalized response–variable slope (dose–response), and asymmetric sigmoidal (five parameters, 5P) to predict EC_{50}.

Figure 2 shows the effect of different concentration of honey, bee pollen and propolis in free radical scavenging tests: (a) DPPH assay and (b) ABTS assay. The data was fitted with sigmoidal model, as an example. The results showed that the relationship between radical inhibition and logarithm of the bee products concentration is not a straight line, but a sigmoidal or S–shape. Four mathematical model including logistic, sigmoidal, dose-response and 5P were selected to fit the curve and estimated EC_{50} of the bee products. The results indicated that these four mathematic models could be used to fit our antioxidant data sets and provide the EC_{50} values. The EC_{50} values of the same sample among the four models did not show large difference (Table 2). This might be because of the log-logistic based equation they used. For the DPPH-assay, no statistical differences were found between the EC_{50} of each model and the right-angled triangle method (simple method) for honey ($P>0.05$). For bee pollen, the dose-response and 5P models show no significant different between their EC_{50} to that of the simple method ($P>0.05$) while the logistic and sigmoidal models show opposite results ($P\leq0.05$). Significant differences were found between the EC_{50}values of propolis estimated by the four models to the simple method ($P\leq0.05$). However, the EC_{50} value obtained from the 5P model was the closest to the simple method. The ABTS-assay estimated EC_{50} of the bee products is also represented in Table 2.The results of honey for the ABTS assay are similar to those for the DPPH assay ($P>0.05$). For bee pollen, there are no statistic significant between the EC_{50} from dose-respond and the 5P model to that of the simple method ($P>0.05$). For propolis, only dose-response model shows no significant difference between its $EC_{50}$to that of simple method ($P>0.05$). These results indicated that it might be better to use dose-response and 5P models for prediction of the EC_{50} of the bee products *via* DPPH assay and to use dose-response *via* ABTS assay. The reason that the 5P model was more appropriate than the logistic and sigmoidal models in estimation of EC_{50} for DPPH assay might be an impact of number of its parameters in the log–logistic model. Dose-response was more appropriate to estimate the EC_{50} than the other models for ABTS assays might be because the normalized–responses were used in an equation. Dose-response, a log–logistic model, has four-parameter as logistic and sigmoidal models but the response is normalized to run from 0% to 100%. This model assumes that the data have been normalized thus forces the curve to run between 0–100%. Then the EC_{50} is reflected as a response equal to 50%. Nonlinear modeling with data normalization and constrains has been found to produce more sigmoidal curves than nonlinear modeling without data manipulation (Wenner *et al.*, 2011). Wenner *et al.* (2011) have reported that normalizing and constraining parameters increased statistical power and minimized the need to exclude data because of poor curve fitting. Although the overall interpretation of the two modeling; with and without normalization methods of curve fitting was similar.

Table 2: Estimated EC_{50} (mg/mL) of honey, bee pollen and propolis obtained by the different models: above, using DPPH assay; below: using ABTS assay.

DPPH assay					
sample	right-angled triangle	logistic	sigmoidal	dose–response	5P
α-tocopherol	0.055 ± 0.001 $^{a, A}$	0.067 ± 0.004 $^{a, B}$	0.068 ± 0.004 $^{a, B}$	0.051 ± 0.001 $^{a, A}$	0.060 ± 0.002 $^{a, C}$
ascorbic acid	0.023 ± 0.002 $^{b, AB}$	0.025 ± 0.001 $^{b, B}$	0.026 ± 0.001 $^{b, B}$	0.021 ± 0.002 $^{b, A}$	0.024 ± 0.001 $^{b, B}$
honey	276.7 ± 6.117 $^{c, A}$	361.4 ± 96.86 $^{c, A}$	317.4 ± 78.52 $^{c, A}$	267.3 ± 15.99 $^{c, A}$	283.8 ± 75.90 $^{c, A}$
bee pollen	1.499 ± 0.015 $^{d, AB}$	2.376 ± 0.047 $^{d, C}$	1.920 ± 0.031 $^{d, D}$	1.396 ± 0.021 $^{d, A}$	1.625 ± 0.224 $^{d, B}$
propolis	0.150 ± 0.004 $^{e, A}$	0.171 ± 0.004 $^{e, B}$	0.180 ± 0.003 $^{e, C}$	0.136 ± 0.005 $^{e, D}$	0.161 ± 0.004 $^{e, E}$
ABTS assay					
sample	right-angled triangle	logistic	sigmoidal	dose–response	5P
α-tocopherol	0.032 ± 0.002 $^{a, ABC}$	0.036 ± 0.002 $^{a, BC}$	0.037 ± 0.001 $^{a, C}$	0.032 ± 0.003 $^{a, AB}$	0.028 ± 0.004 $^{a, A}$
ascorbic acid	0.023 ± 0.002 $^{b, A}$	0.023 ± 0.004 $^{b, A}$	0.023 ± 0.004 $^{b, A}$	0.022 ± 0.002 $^{b, A}$	0.022 ± 0.004 $^{a, A}$
honey	92.29 ± 5.638 $^{c, A}$	93.41 ± 2.407 $^{c, A}$	92.20 ± 2.174 $^{c, A}$	95.02 ± 9.776 $^{c, A}$	81.61 ± 10.34 $^{b, A}$
bee pollen	0.560 ± 0.039 $^{d, A}$	0.744 ± 0.117 $^{d, B}$	0.744 ± 0.117 $^{d, B}$	0.566 ± 0.024 $^{d, A}$	0.698 ± 0.088 $^{c, AB}$
propolis	0.054 ± 0.008 $^{e, A}$	0.068 ± 0.006 $^{e, B}$	0.073 ± 0.002 $^{e, B}$	0.054 ± 0.009 $^{e, A}$	0.073 ± 0.004 $^{d, B}$

Note: Values followed by the same lowercase letter within the same column and for the same uppercase letter within the same row are not statistically significantly different (*p>0.05*).

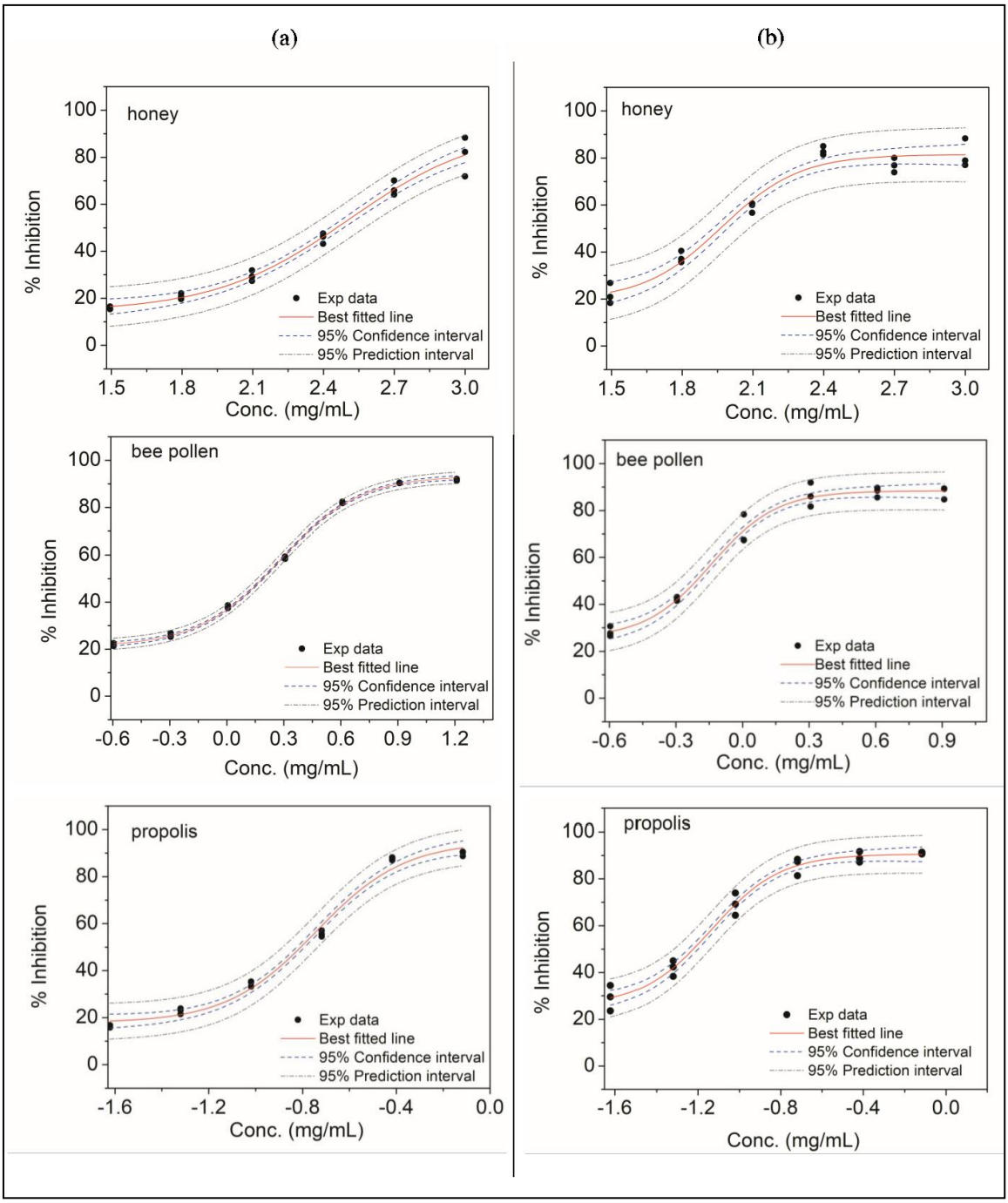

Fig. 2: Effect of different concentration of honey, bee pollen and propolis in free radical scavenging tests: (a) DPPH assay and (b) ABTS assay. Data are fitted with sigmoidal model.

Antimicrobial ability

S. aureus is a gram-positive bacterium, which is an important cause of gastroenteritis resulting from the consumption of contaminated food (Loir *et al.*, 2003). *B. cereus* is a gram-positive bacterium, common soil saprophyte and is easily spread to many types of foods (Granum Lund, 1997). *E. coli* is a gram-negative bacterium that can be found in contaminated water or food, especially raw vegetables and raw meat products (Siripatrawan *et al.*, 2013). It has been identified as a particularly dangerous pathogen due to its resistance to many commonly used antibiotics (Rahman *et al.*, 2010). These three bacteria are commonly recognized to cause food poisoning or food spoilage.

The MIC values of Thai honey, bee pollen and propolis against *S. aureus*, *B. cereus* and *E. coli* are listed in Table 3.A turbidity assay was used to identify the growth of microorganisms compared to the positive control. Each bee product tested caused inhibition of bacterial growth. The MIC values of honey were of

340 mg/mL for *S. aureus* and *B. cereus* while it was680 mg/mL For *E. coli*. Concentrations of 153.71–614.83 mg/mL of pollen extract inhibit growth against these three microorganisms. The MIC values of the propolis were 1.88, 0.94 and 3.75 mg/mL against *S. aureus*, *B. cereus* and *E.coli*, respectively. The MBC values of the three types of bee products against the microorganisms grown in NA plate are listed in Table 3. The digital photographs of the MBC determination for honey, bee pollen and propolis against *S. aureus*, *B. cereus* and *E. coli* are shown in Figures3, 4 and5 respectively. NA plates streaked from positive control tubes show the appearance of colonies of *S. aureus* (Figure 3d), *B. cereus* (Figure 4d) and *E.coli* (Figure 5d). The MBC assays showed growth, no growth and inhibition of growth following bacterial streaks (Figure 3-5). Honey was lethal to *B. cereus* and *E. coli* at the same concentration (680 mg/mL), but did not kill *S. aureus.* A concentration of 614.83 mg/mL of the pollen extract demonstrated effectiveness in killing *S. aureus* while only 307.42 mg/mL was sufficient to kill *B. cereus* and *E. coli*. The MBC of the propolis extract was 15.01 mg/mL for all the bacteria *S. aureus*, *B. cereus* and *E. coli.* It was found that the propolis extract solution had the lowest MIC and MBC values for these bacteria.

Table 3: MIC values (mg/mL) and MBC values (mg/mL) of Thai bee products against food borne microorganisms. *Abbreviations:*N.D. – without efficacy.

Microorganism	Honey		Pollen		Propolis	
	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus 517	340.0	N.D.	153.7	614.8	1.9	15.0
B. cereus 687	340.0	680.0	153.7	307.4	0.9	15.0
E. coli 1261	680.0	680.0	153.7	307.4	3.8	15.0

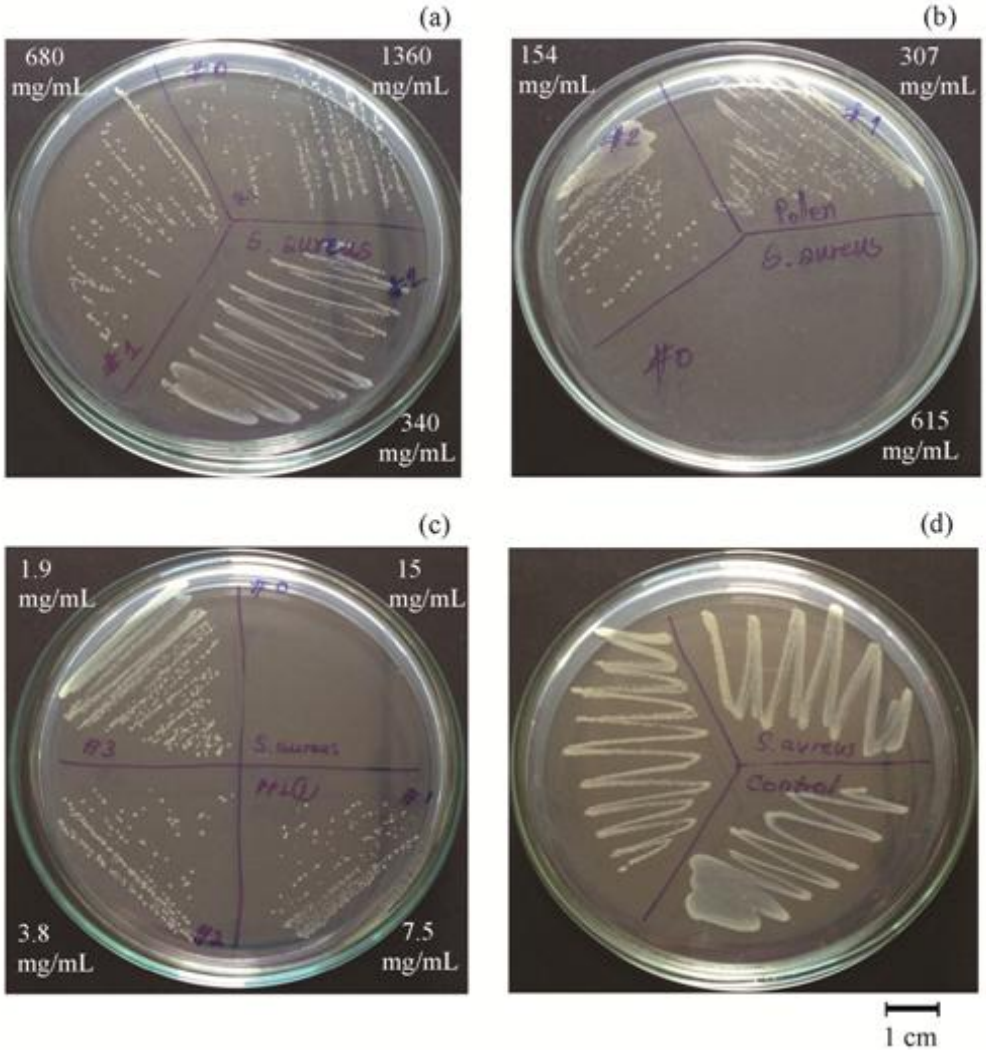

Fig. 3: Digital photographs of the MBC of (a) honey. (b) bee pollen and (c) propolis against *S. aureus* and (d) control.

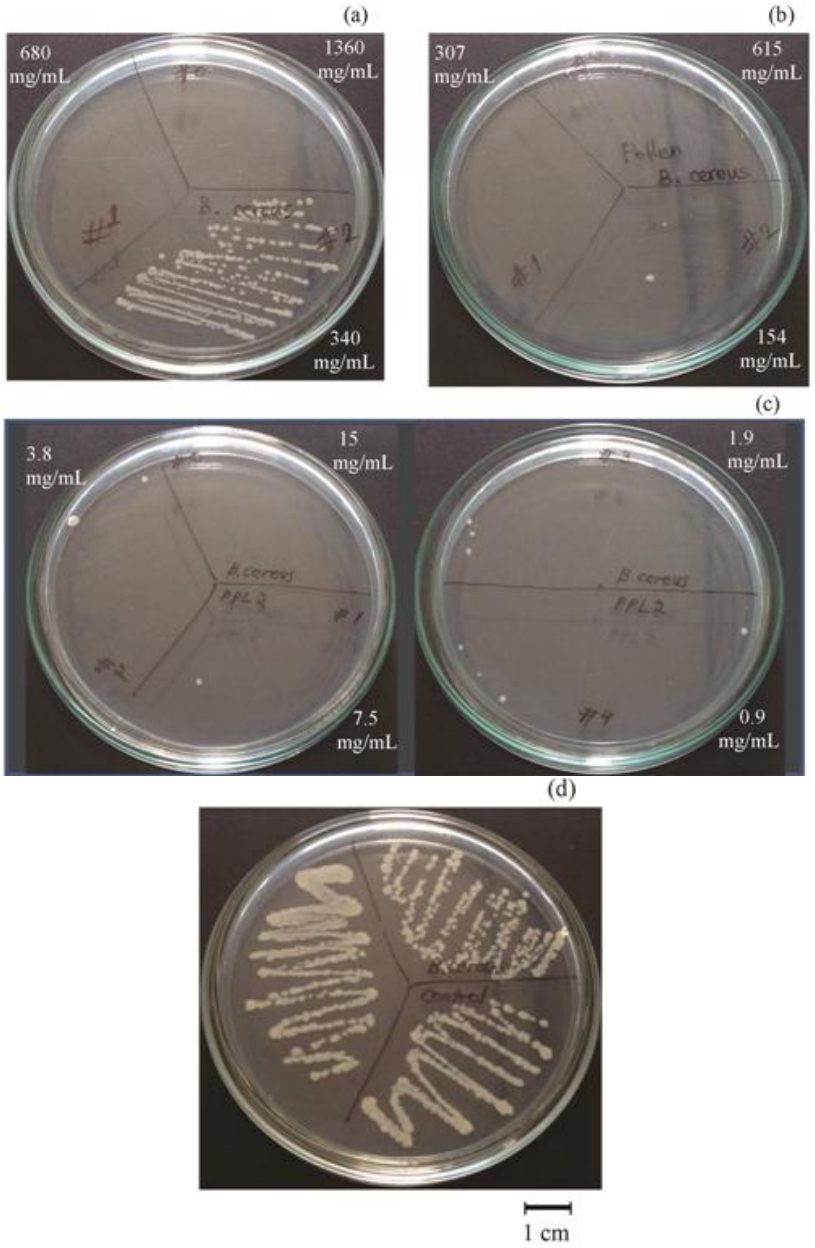

Fig. 4: Digital photographs of the MBC of (a) honey. (b) bee pollen and (c) propolis against *B. cereus*. and (d) control.

The MIC and MBC techniques are simple and easily used to investigate the inhibitory doses of antibiotics or disinfectants for particular bacteria (Rahman *et al.*, 2010). In this work, honey, pollen and propolis demonstrated antibacterial activity against *S. aureus*, *B. cereus* and *E. coli via* the MIC and MBC techniques. However, honey did not show an MBC against *S. aureus*. The antimicrobial activity of honey is due to its osmotic effect, natural acidity, hydrogen peroxide, phenolic acids, flavonoids and lysozyme (Boukraâ *et al.*, 2013). The antimicrobial activity of pollen is attributed to its phenolic compounds (Boukraâ *et al.*, 2013), and the antimicrobial activity of propolis is caused by the phenolic compounds such as flavonoids (Boukraâ *et al.*, 2013). Different antimicrobial agents possess different mechanisms.

The mechanism of antimicrobial resistance of honey, bee pollen and propolis is involved degrading the cytoplasm membrane of the bacteria (BellikBoukraâ, 2012). This leads to a loss of potassium ions and the damage effected provoking cell autolysis (BellikBoukraâ, 2012). Quercetin, a flavonoid found in both honey and propolis, can increase membrane permeability of the bacterial and dissipates bacterial potency (Mirzoeva *et al.*, 1997). This makes the bacteria lose their motility, membrane transport and capacity to synthesis adenosine triphosphate (ATP). The present study revealed that these bee products seemed to inhibit the gram-positive bacteria more than gram-negative. This is in an agreement with Brazilian propolis studied by Schmidt *et al*. (2014). In general, plant extracts normally have a higher activity against

gram-positive bacteria than gram-negative bacteria (Rahman *et al.*, 2010). Gram-negative bacteria are more resistant than the gram-positive bacteria because they have more complex chemical structures(Morais *et al.*, 2011). This bacterial group has a polysaccharide as one of the components in the cell wall, which is involved in the antigenicity, toxicity and pathogenicity of the microorganisms. Furthermore, the gram-negative bacteria possess a higher lipid amount than that observed in gram-positive bacteria (Morais *et al.*, 2011).This lipid is a component of an endotoxin, which has responsible toxicity in the cell wall of gram-negative bacteria.

Graikou *et al.* (2011) reported that the MIC values of a Greek pollen-methanol extract were 0.74 and >10 mg/mL against *S. aureus* and *E. coli*, respectively. Morais *et al.* (2011) found that the honeybee-collected pollen from Portuguese Natural Parks provided the MIC of 0.17% (w/v) for *B. cereus*, 0.21% (w/v) for *S. aureus*, and <5% (w/v) for *E. coli*. Choi *et al*. (2006) verified that the propolis from Korea had much more powerful antimicrobial activity than another from Brazil. Rahman *et al.* (2010) investigated propolis and honey from Canada against *S. aureus* and *E. coli.* Propolis and honey concentrations at rates of 2.74–5.48 and 375.0 mg/mL could inhibit *S. aureus*. For *E. coli*, negative growth was found in propolis at a concentration of only 5.48 mg/mL, but honey was no effective. These MIC and MBC values are different than the MIC and the MBC found to be active in this work. These variations of antimicrobial property of honey, bee pollen and propolis may be because of the different plants in the sources where the bee lives. In addition, the results showed that the antimicrobial activity of bee products in this study related to total phenol content of the extracts as shown in Table 1. This is because the phenolic compounds are the main sources of antimicrobial action of honey, bee pollen and propolis. The other works in bee products by Choi *et al.* (2006) and Morais *et al.* (2011) were also in agreement. Miorin *et al.* (2003) suggested that the effectiveness of honey or propolis depends on differences in chemical composition, bee species and geographic region. Another factors such as the nature of the phenolic fraction might be involved (Morais *et al.*, 2011), and they should be further study.

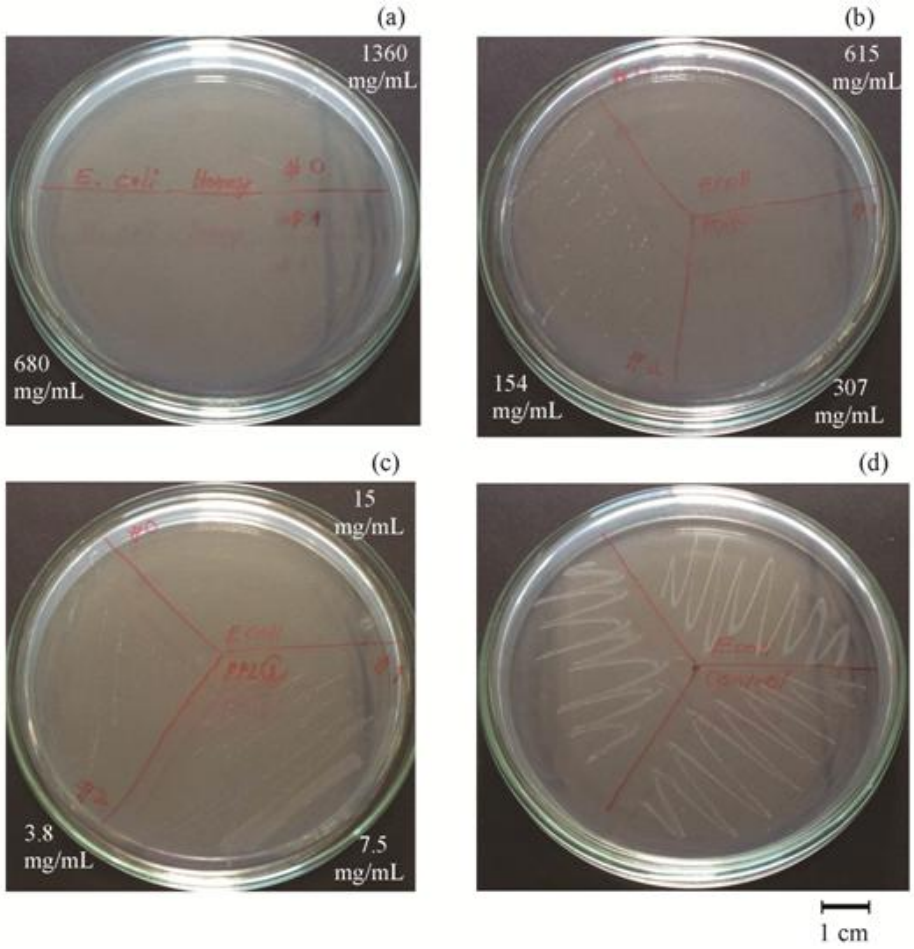

Fig. 5: Digital photographs of the MBC of (a) honey. (b) bee pollen and (c) propolis against E. coli, and (d) control.

CONCLUSIONS

This work emphasized the value of honey, bee pollen and propolis from Thailand as essential sources of natural antioxidant and antimicrobial. Among these bee products, propolis possessed the most powerful anti-free radical and antibacterial activities following by bee pollen and honey, respectively. Phenolic compounds play an important role to their strong effectiveness. In order to evaluate the prediction of the EC_{50}, four mathematic equations logistic, sigmoidal, dose-response and 5P models could be applied for calculations of EC_{50} values. Among these four models, dose-response and 5P gave the nearest results to a right-angled triangle method as a reference. Thus we recommended dose-response and 5P model as the effective methods for the curve fitting and prediction the EC_{50} *via* DPPH and ABTS assays. Future work, we are interested to study the incorporating of the bee products in the packaging materials in order to extend shelf life of the prospective product.

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