A non-toxic approach to assess total antioxidant capacity (TAC) of exotic tropical fruits from Thailand

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Abstract A simple flow injection analysis (FIA) integrating with a metal-free approach for total antioxidant capacity (TAC) was developed. The non-toxic reaction was based on generating a vibrant blue radical from imipramine to avoid the potential interferents arising from the colorful fruit extracts. The blue radical can be rapidly scavenged by antioxidant compounds present in the sample. TAC values of Thai tropical fruit extracts were assessed by monitoring the quenching in absorbance of the test mixture following the addition of the antioxidant compounds/fruit extracts. The FIA co-operated in order to increase the sample throughput. The results demonstrated that *Antidesma thwaitesianum* Muell. Arg. has the highest capacity followed by *Terminalia chebula* Retz. and *Phyllanthus Emblica* Linn., respectively. An excellent correlation between the proposed method was found with the DPPH assay. The proposed method allowed the TAC determination of fruit extracts in a high-throughput and straightforward way in accordance with the principles of green analytical chemistry.

Keywords A non-toxic approach · Flow-based analysis · Total antioxidant capacity (TAC) · Imipramine · Tropical fruits

Introduction

Thailand is a kingdom abundant with various exotic tropical fruits, which can be found in almost every corner. Take advantage of this abundance; Thai fruits are inexpensive, flavorful and nutritious. Recent evidence suggests that tropical fruits pack with amounts of bioactive compounds and exhibit high antioxidant potential (Dembitsky et al. 2011). Mango (*Mangifera indica* L.), one of the most popular tropical Thai fruits is well known for its attractive color, sweet taste and high level of health-beneficial compounds such as phenolic compounds (Rumainum et al. 2018). Mangosteen (*Garcinia mangostana* L.), is plentiful with antioxidants especially Xanthone, which exhibits a high level of antioxidant activity (Gondokesumo et al. 2018). Durian contains relatively high polyphenols such as caffeic acid and quercetin (Arancibia et al. 2008). Apart from the commonly devoured fruits, another exotic indigenous Thai fruits with less known or known only specific area are also fascinating and contain various bioactive compounds (Kubola et al. 2011). Several studies have revealed that Thai wild fruits possess numerous health benefits, such as anti-inflammatory and anticancer activity (Bharucha and Pretty 2010; Chotchoungchatchai et al. 2012; Siriamornpun and Suttajit 2010). These hidden fruits have the potential to be developed into functional food. However, these wild crops have not received much attention and ingested only in some area such as in Northeast of Thailand due to a lack of information about their properties, especially antioxidant (Johnson 2002).

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Recently, there has been an increasing interest in a multiplicity presence of antioxidant compounds in fruit extracts because of the nutraceutical properties (Das et al. 2012). However, separating and quantifying individual antioxidant is complicated and time-consuming. Besides, the inaccurate result might also be due to the synergistic effects among the antioxidant components. Therefore, methods that can measure the total antioxidant capacity (TAC) is a more relevant tool for investigating the antioxidant capacity (Alam et al. 2013; Pisoschi and Negulescu 2012). TAC is an antioxidant estimated assay that integrated parameter of antioxidants existed in a sample (Wang et al. 1996). Therefore, it is necessary to establish and standardize methods which measure the TAC level directly from extracts containing a variety of antioxidant compounds. Since now many TAC assays were developed and modified to improve the accurate and convenient of the method such as ABTS, DPPH, FRAP method (Dawidowicz et al. 2015; Ghiselli et al. 2000; Kaprasob et al. 2018; Yan and Yu 2018).

Imipramine is a dibenzazepine derivative, generally used as an antidepressant. The oxidation of the compound with a metal such as Fe(III), Ti(IV), Ce(II) produces deep blue intense radical (Misiuk 2000). The former radical is stable and can be stabilized in strong acid (Borg 1965). From our previous investigation, Imipramine was used to estimate TAC in Thai medicinal plant extract (Panich 2018a, b) by using ammonium vanadate (NH_4VO_3) as an oxidizing agent. However, the vanadate agent is mutagenic and may cause damage to several organs such as kidneys, lungs, and liver (Elfant and Keen 1987; Wei et al. 1982). To solve this problem, in this assay imipramine was oxidized into a radical without using any metal. Therefore, there was no disposal as hazardous waste. The novel probe was scavenged by antioxidant compounds rapidly, and the color from the sample does not interfere because fruit presents in every color from red to purple however blue fruit is difficult to find.

Materials and methods

Materials

Chemicals and reagents

All reagents used in this work were analytical grade and was used without further purification. Folin-ciocalteu reagent (FCR), diphenylpicrylhydrazyl ($\text{C}_{18}\text{H}_{12}\text{N}_5\text{O}_6$), L-ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6$), gallic acid ($\text{C}_7\text{H}_6\text{O}_5$), trolox ($\text{C}_{14}\text{H}_{18}\text{O}_4$), quercetin ($\text{C}_{15}\text{H}_{10}\text{O}_7$), uric acid ($\text{C}_5\text{H}_4\text{N}_4\text{O}_3$), caffeic acid ($\text{C}_9\text{H}_8\text{O}_4$), catechin ($\text{C}_{15}\text{H}_{14}\text{O}_6$), rutin ($\text{C}_{27}\text{H}_{30}\text{O}_{16}$), hesperidin ($\text{C}_{28}\text{H}_{34}\text{O}_{15}$), and imipramine

hydrochloride ($\text{C}_{19}\text{H}_{24}\text{N}_2\text{-HCl}$) were purchased from Sigma-Aldrich. Ultrapure water (resistivity $> 18 \text{ M}\Omega \text{ cm}$, TKA-GenPure, TKA) and absolute methanol (Fisher Scientific, Leicestershire, UK) were used as fruit extraction solvents.

Methods

Preparation of fruit extracts

Fruits for antioxidant analyses were collected from the North-East region of Thailand. Only the edible parts were washed with water and chopped into a small slice ($1 \times 1 \times 1 \text{ cm}$). Then all samples were dried in a freeze dry (super modulyo freeze dry, Thermo) for 24 h. For extraction step, 3.000 g of dried fruit was extracted with 25 mL of methanol at 4°C for three days. The solvent was removed by a rotary evaporator (210/R-215, Buchi). Then the extracts were ready for the further process.

Determination of total phenolic compounds

The total phenolic content of 22 fruit extracts was quantified according to the Folin-Ciocalteu reagent (FCR) method modified from ISO 14502-1 (ISO 14502-1. 2005). Briefly, an aliquot of 1 mL of a series concentration of Gallic acid or fruit extracts was mixed with 5 mL of FCR reagent (20% v/v) and was allowed to react for 5 min at room temperature. Then 4 mL of saturated Na_2CO_3 solution was added and allowed to stand for 60 min before the absorbance of the reaction mixture was monitored using the spectrophotometer (UV-1700, Shimadzu). A calibration curve of the Gallic acid solution at 10–60 mg/L was created. The total phenolic compounds in the samples were expressed as mg Gallic acid equivalent per g dry sample. All determinations were performed in triplicate.

Antioxidant capacity by DPPH radical scavenging assay

The DPPH method used in this work was modified from (Kim et al. 2002). Briefly, an aliquot of 50 μL of Ascorbic acid or sample were vortexed with 2.92 mL of 0.050 mM DPPH in methanolic solution (prepared by dilute 80% methanol in water). Then the solution was incubated at room temperature for 30 min. The absorbance of the solution was then measured at 517 nm. The antioxidant capacity of the extract samples, thus, was reported as equivalent to mg Ascorbic acid equivalent per g dry of the sample. All determinations were performed in triplicate.

Free radical scavenging activity by FIA-imipramine method

This study is the first time that imipramine was used as a novel probe for antioxidant study without any heavy-metal for green analysis propose. The FIA manifold was modified from (Panich 2018a, b). Briefly, the carrier channel consists of a two-channel manifold which is composed of a peristaltic pump equipped with a 30 cm mixing coil where the imipramine was oxidized to produces free-radicals by H_2SO_4 as shown in Fig. 1a. The sample solution (100 μL) of antioxidants/fruit extracts was injected via a six-port valve sample injector into a deep visible blue imipramine free radical stream. The absorbance of the product formed was measured at 620 nm using the spectrophotometric method.

Results and discussions

The principle of imipramine-FIA measurement

The oxidizing mechanism of imipramine is fascinating as the active nitrogen atom exhibits many interesting

analytical properties, especially a blue chromophore free radical. Herein, the fundamental of TAC evaluation is based on the generating of the vibrant blue imipramine free radical, as shown in Fig. 1b. The formation of the cation radical is the first step then the rapid coupling itself leads to form an intensively blue colored product. Once antioxidant compounds present in the sample scavenges the dimeric compound, its blue color disappears depending on the antioxidant activity of the sample.

Optimization studies

Influence of oxidants

The oxidation reaction of imipramine is strongly influenced by the concentration of oxidant (Misiuk 2000). In this study, the blue chromophore produced from imipramine was used as an indicator for antioxidant activity so as maximizing the absorbance is expected. The effect of the concentration of H_2SO_4 as the oxidants was optimized as shown in Fig. S1a. At the concentration 1.0, 1.5, 2.0, 3.0, 4.0 and 5.0 M, the noticeable deep and intense blue color was recorded. Due to the linear absorbance range of most spectrometers is between 0.1 and 1 thus the concentration

Fig. 1 **a** The schematic of an imipramine-FIA system used for the TAC assay. The blue radical produced from imipramine is rapidly scavenged by antioxidant compounds in the sample caused the decreasing in the absorption spectrum which related to the antioxidant capacity. The antioxidant capacity was expressed as % radical scavenging. By comparing the scavenging activity with Ascorbic acid, TAC in the sample was then reported as mg ascorbic equivalent/g dried sample. (%Radical scavenging is $[(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$, where A_{control} is absorbance in the absence of the antioxidant compounds, and A_{sample} is absorbance in the presence of antioxidant compounds) **b** The oxidation of the imipramine. Starting from imipramine compound (I) to imipramine cation radical (II) and the final blue dimeric product (III) modified and reprinted with permission from (Panich 2018a, b)

of H_2SO_4 at 1.5 M was sufficient and was chosen as an optimum condition.

The effect of the injection volume

The effect of the injection volume was examined by varying it from 100 and 300 μL . It was observed that the injection at 300 μL provides a higher change in the absorbance as shown in Fig. S1b. However, sample injection more than this volume is not recommended because the amount of sample extractions is always limited and may induce the transient negative signal to be a broad peak.

The influence of the flow rate

The flow rate of the reagent stream is the most critical parameter as it influences the signal, reagent consumption and analysis time. Increasing the flow rate can improve sample throughput and can reduce the analysis time. On the other hand, it can also reduce the sensitivity of the method. According to the result in Fig. S1c, the optimum flow rate is 1.5 mL/min due to the highest reproducibility, short analysis time. The optimized condition can be found in Table S1.

The reaction of imipramine with pure antioxidant compounds

The reaction of the imipramine probe with several pure standards was reported previously (Panich 2018a, b). The results showed that caffeic acid was the most sensitive toward the novel probe follow by trolox and ascorbic acid whereas the excellent linearity was found in trolox, ascorbic acid, gallic acid, and caffeic acid, respectively. Previously, several antioxidant standards were used as a candidate to verified the assay. The most commonly used is trolox. However, trolox is not a natural antioxidant. As a result, ascorbic acid is a better candidate because it often presents in fruits and vegetables. Figure 2 demonstrated the flow injection profile of imipramine probe reacted with ascorbic acid at different concentrations. Calibration curve was constructed with r^2 value equal to 0.9924 proves linearity of the proposed method.

TAC from imipramine method, the total phenolic content and DPPH antioxidant activity of fruit extracts

The antioxidant capacities of the fruit extracts were different in relation to the assay used; thus, the same sample often ranked differently depending on the assay. The results from the developed method was demonstrated in Fig. 3. The TAC level ranked in the top-three was found at

Fig. 2 The flow injection profile of imipramine probe which was scavenged by ascorbic acid at 0.0, 5.0, 10.0, 25.0 and 40.0 ppm, respectively. The higher the concentration of ascorbic acid, the more decrease in absorbance at 620 nm

3.01 ± 0.02 , for *Antidesma thwaiteianum* Muell. Arg., 1.68 ± 0.02 for *Terminalia chebula* Retz. and 1.36 ± 0.01 as mg ascorbic acid/g sample for *Phyllanthus Emblica* Linn. extracts, respectively. The results from the FCR method showed remarkably high total phenolic content in the extract of *Morus alba* L. extract and *Phyllanthus Emblica* Linn. at 9.90 ± 0.02 and $8.414.91 \pm 0.01$ as mg gallic acid/g dry, respectively. The methanolic extract of *Antidesma thwaiteianum* Muell. Arg. showed the highest DPPH antioxidant activity at 9.45 ± 0.01 as mg ascorbic acid/g dry sample follow by *Terminalia chebula* Retz and *Phyllanthus Emblica* Linn. at 3.92 ± 0.01 and 4.91 ± 0.01 as mg ascorbic acid/g dry sample, respectively. (More information about TAC results can be found in Fig. S2.)

The correlation analysis test was applied to observe the strength of association between the three methods, FCR, DPPH, and the developed method as shown in Fig. S3. A strong correlation was found between the developed imipramine method and DPPH method at $r^2 = 0.9848$ and moderate relationship with FCR method at $r^2 = 0.3393$. There is a fair relationship between FCR and DPPH method at $r^2 = 0.3835$. These relationships among the three methods can be explained by considering the mechanism behind the assays. Both imipramine and DPPH are based on the scavenging of the free radical by the antioxidant compound while FCR is quantitative of the polyphenol compounds. As a result, it is not surprising that the strong correlation was observed between the proposed method and the DPPH method rather than FCR method.

Fig. 3 The developed method was applied for the evaluation of TAC a) example of flow profile from fruit extracts which were injected for TAC analysis three times ($n = 3$) b) TAC results in 22 fruit extracts (Sample code and scientific names of fruits sample can be found in electronic supplementary material, Table S2)

Conclusion

The proposed assay has been achieved for developing a novel total antioxidant capacity assay to fulfill the following implications, i.e., (i) no hazardous chemicals are required in the analytical procedure, and only a tiny reagent was consumed (15 μ L of reagents per analysis); (ii) the semi-automatic system provided satisfactorily high sample throughput of up to 200 analysis/h, therefore, the system is suitable for high mass screening for drug discovery; (iii) the chemical reaction used in this work occurs immediately, thus the analysis time is shorter than other antioxidant assays; (iv) due to the advantage of the blue probe, the color from samples did not interfere with the detection. The results of this study also provided useful data regarding the capacity of the antioxidant of Thai tropical fruit extracts.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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