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A novel assay for evaluation of the total antioxidant capacity using a nontoxic probe

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ABSTRACT

Introduction: Total antioxidant capacity (TAC) is the measure of the amount of free radical scavenged by a test solution being used to assess the antioxidant activity of biological samples. In this work, a novel and simple method for measuring TAC was established. The new assay is based on generating the deep blue imipramine (IMI) free radical by oxidising IMI with ammonium tetra vanadate (NH_4VO_3) in acid medium. **Method:** Once the generated blue IMI radical is scavenged by the antioxidant compounds present in the samples, the blue colour of the oxidant disappears. Consequently, dropping in the absorption spectrum at 620 nm can be detected by the spectrophotometric method. Also, sample throughputs and precision of the assay were enhanced by cooperating the novel analyse with the flow injection analysis (FIA) technique. Result and **Conclusion:** The linearity of the assay was found to be up to 2,800 mg trolox equivalent L^{-1}. The developed method was then applied to evaluate TAC in three different fractions of the *G. Sootepensis's* extract. The proposed method was reliable, robust, inexpensive, environmentally friendly and suited for measuring TAC.

INTRODUCTION

Plants contain various phytochemical compounds which are useful for human health. In the past decade, exploring medicinal plants which rich of new compounds possessing therapeutic value is encouraged to be used in drug development, especially antioxidant compounds.[1] These active compounds prevent and recover the status of imbalance between oxidant formation and antioxidant or so-called "oxidative stress." In the situation of oxidative stress, there is an overproduction of reactive oxygen species such as H_2O_2, $OH^{\bullet}$, and $O_2^{-\bullet}$ and reactive nitrogen species such as NO and NO^{v}.[2] Oxidative stress causes macromolecular damage. Thus, it is implicated in various disease disorders such as atherosclerosis, diabetes, cancer, neurodegeneration, as well as inflammation and ageing.[3] As a result, screening and interpreting active ingredients of herbal plants are of great importance for drug discovery.

There are a variety and diversity of plants, especially in tropical countries like Thailand. All of them are packed with various antioxidant compounds which are potential sources for drug discovery. However, the complexity of the composition of a plant extract, isolating, characterizing, and testing each antioxidant compound activity is time consuming and costly. Moreover, studying individual compound separately is inefficient and unworthy because of a synergistic or an antagonism effect among the antioxidant compounds in the sample. Therefore, it is very attractive for researchers to develop a convenient method for rapid and effective screening of active antioxidants.

Total antioxidant capacity (TAC) is the measurement of the synthetic free radicals scavenged by a test solution. This technique is frequently used because of its ability which can evaluate the capacity close to the actual antioxidant capacity.[4,5] Basically, in TAC assay, an exactly known concentration of free radical is generated and is scavenged by a test solution. Currently, numerous techniques have been developed for monitoring the antioxidant status such as spectrophotometric,[6,7] Fourier-transform infrared spectroscopy,[8] electrochemistry,[9] chemiluminescence,[10,11] or even in the *vivo* method such as the microbial test system.[12] Among them, the ultraviolet (UV)-visible spectrophotometric is the most commonly used. Typically, free radical used in TAC analysis is an azo compound such as 2,2'-azobis-2-amidopropane (ABTS) which generates $ABTS^{\bullet+}$radical. The reducing amount of $ABTS^{\bullet+}$ can be determined by UV-visible spectroscopy technique. The

advantage of this technique is that ABTS can be dissolved in both polar and non-polar solvents. However, to generate the $ABTS^{\bullet+}$radical, ABTS needs to be activated before the test, which causes the technique more multifaceted. Another common method is 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay which is well-known and frequently employed for assessing antioxidant capacity. However, activating DPPH to $DPPH^{\bullet}$ is slower than ABTS,[6] thus the assay is time consuming. Even though using of spectroscopic probes is easy,[13] the color of the sample can be interfered with the color of the generated radical. Cupric reducing antioxidant capacity seems to be an ideal probe as the capacity depends on the change of the absorbance at 230 nm instead of recording the spectrum at visible region; however, there might be interference from other organic material.[14] The blue CrO_5 assay has been proposed for the evaluation of antioxidant.[15] Even the assay do not cause interference from the sample color because it is not naturally found in plants and fruits, but Cr is a toxic element.

Imipramine (IMI) is a tricyclic antidepressant [Figure 1] which is the most effective drug for the treatment of depression. The blue oxidation product of IMI can be formed with different oxidants, for example, $Ce(SO_4)_2$, $K_2Cr_2O_7$, KIO_4, $(NH_4)_2S_2O_8$, and NH_4VO_3.[16,17] IMI is a nontoxic compound and is easy to be oxidized into a radical form. As a potential to be a "green oxidant," IMI is a good candidate for TAC assay development.

The aim of this research was to explore and develop a novel method for the evaluation of TAC status in *Gardenia sootepensis* extract. The new radical probe was generated from IMI. The deep blue probe encourages the technique to be a unique method as it is not interfering from the color of the samples. IMI oxidant is nontoxic to the researcher and environment. In addition, the probe is relatively cheap. In this study, the method was cooperated with the flow injection analysis (FIA) or FIA to improve sample throughput and the precision of the technique. The new approach could be used for screening natural extracts which always have several fractions. The essay can reduce the time of finding new active compounds from plant extracts.

MATERIALS AND METHODS

Reagents

Trolox, ascorbic acid, gallic acid, caffeic acid, DPPH, ABTS, IMI hydrochloride, and folin-ciocalteu reagent (FCR) were purchased from Sigma-Aldrich (Germany). All chemicals and solution were analytical grade and were prepared in deionization water throughout the experiment.

Preparation of the Stock Solution

The stock solution of NH_4VO_3 was prepared by dissolving NH_4VO_3 in 10 M H_2SO_4, and then the final volume was adjusted with water. The stock solution of IMI solution was prepared by dissolving IMI and adjusted the volume with water. All antioxidant compound solutions, which were ascorbic acid, gallic acid, and caffeic acid, were prepared by dissolving the compound in water. The serial dilution of the antioxidant solutions was achieved by diluting each stock solution with water.

Sample Preparation

In this work, sample extraction procedure was originally developed before by Nuanyai *et al*.[18] Briefly, the fresh apical buds of *G. sootepensis* were extracted with methanol. After soaking for 2 days, the solvent was evaporated, and then crude extract was separated into a fraction by silica gel column chromatography.

Procedure

Preliminary antioxidant analysis by the novel assay, IMI probe

A stock solution of IMI was mixed with NH_4VO_3 in H_2SO_4 with different concentration of ascorbic acid, and then the final volume was adjusted with water. The mixture was mixed by vortex for 5 s and was recorded for the spectrum at 15 s.

FIA

FIA cooperated with IMI assay to improve the convenient and the precision of the test. The laboratory-made FIA analyzer was depicted in Figure 1. The set-up consists of a peristaltic pump, three carrier solutions, and two mixing coils. The spectrum of the solution was recorded using a spectroscopy method.

DPPH assay

DPPH assay originally from Ukeda *et al*.[19] was applied with slight modification. Briefly, 1 mM of DPPH was prepared by weighing 0.0400 g of DPPH then was dissolved in 80% v/v methanol in water. The solution was stirred until all solid was completely dissolved. 25 ml the stock solution was dilute with 80% v/v methanol in water. The spectrum of the solution was recorded at 517 nm, and the absorbance should be around 1. Then, 50 μL of different concentration of ascorbic acid was added to 2.95 mL of DPPH solution. The mixture was mixed by vortex for 15 s then allows the solution to react for 30 min in the dark condition with the aim of protecting ascorbic acid from decomposing. The decreasing of the solution spectrum at 517 nm was recorded.

FCR assay

Folin-ciocalteu procedure was conducted by the reference method[20] with slight modification. Briefly, 10% v/v FCR was mixed with Na_2CO_3 and samples which contain polyphenolic compounds and then allow to react at room temperature for 60 min. The mixture solution will change from yellow to blue color. The spectrum of the solution was recorded at 765 nm. Total phenolic acid contents in the sample can be easily calculated as mg gallic acid/g dry sample.

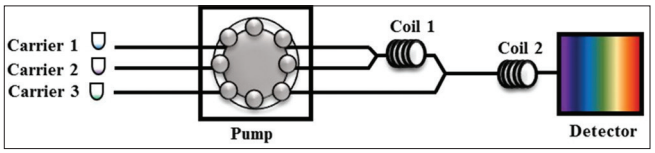

Figure 1: Flow injection analysis manifold for total antioxidant capacity analysis by imipramine (IMI) assay. Three carrier solutions were pumped into the system by the peristatic pump. Carrier 1 is IMI solution which was merged with NH_4VO_3 in H_2SO_4, carrier 2 at coil 1 to generate IMI radical. Next IMI radical was scavenged by antioxidants, carrier 3, after mixing in coil 2

RESULTS AND DISCUSSION

IMI probe

Herein, NH_4VO_3 was used as an oxidant to yield colored blue stable oxidized products as the reactions equilibrium state was gained very rapidly.[16] After mixing the IMI solution with the oxidant, NH_4VO_3, the absorbance spectrum of the blue radical was recorded with maximum wavelength (λ_{max}) at 610 nm. The decrease in absorption was found when 20–80 μM of ascorbic was added to scavenge IMI radical as shown in Figure 2a. The linear regression between % radical scavenging and ascorbic acid concentration was illustrated in Figure 2b which pointed toward that there was a strong correlation between antioxidant capacity and the decreasing of the blue product at 610 nm. This finding has important implications for developing a novel assay.

Optimization of the condition for IMI probe

The experimental condition of the proposed assay was optimized to obtain the greatest results. The influence of various parameters on the optimal conditions was investigated as follow.

The concentration of reagents

NH_4VO_3

The oxidation reaction of IMI was strongly influenced by the type and concentration of oxidant. Herein, NH_4VO_3 was chosen as an oxidant as it provided the intense and more stable colored probe at 620 nm in IMI-NH_4VO_3 system.[16] The effect of the concentration of NH_4VO_3 was studied by recording the absorbance of the solutions containing a fixed amount of IMI and various amounts of NH_4VO_3. The results were given in Figure 3. There was no significant difference in absorbance from NH_4VO_3 ranging from 0.004 to 0.80 mM.

H_2SO_4

Despite the fact that there was no significant in absorbance of the colored IMI probe from the various concentrations of NH_4VO_3, the absorbance was considerably increased when increasing the concentration of the H_2SO_4 medium [Figure 4]. The effect of the concentration of H_2SO_4 on color intensity was studied from 4 M to 8 M. A significant increase in the intensity and stability of absorbance was observed. At 7 M of H_2SO_4, IMI probe provided intense blue color and very stable more than 250 s which is long enough for the further experiment. Thus, 7 M of H_2SO_4 was used for further investigation.

Interferences study

The new approach was tested for its tolerance by investigating the signal of IMI in the presence of various interferents which were NaCl, Na2CO3 and NaOH. A level of interferent was considered to be acceptable if the error was not longer than 2%. According to the results, there is no significant change in the absorbance of the IMI radical when the concentration of the interferents was less than 0.2 mol/L.

A novel blue IMI method was applied to assess TAC. After the study, the optimum condition was chosen and was presented in Table 1.

Study of the stability of IMI probe

After the method was validated, the stability of the free radical probe was tested as it is the most important parameter in antioxidant evaluation assay. The ideal radical probe should stable enough to detect the signal but not too inert as it is not a good candidate for the radical presence in the human body which is reactive species. According to the result in Figure 5, IMI radical was stable up to 3500 s. Moreover, it reacted very fast with antioxidant compounds.

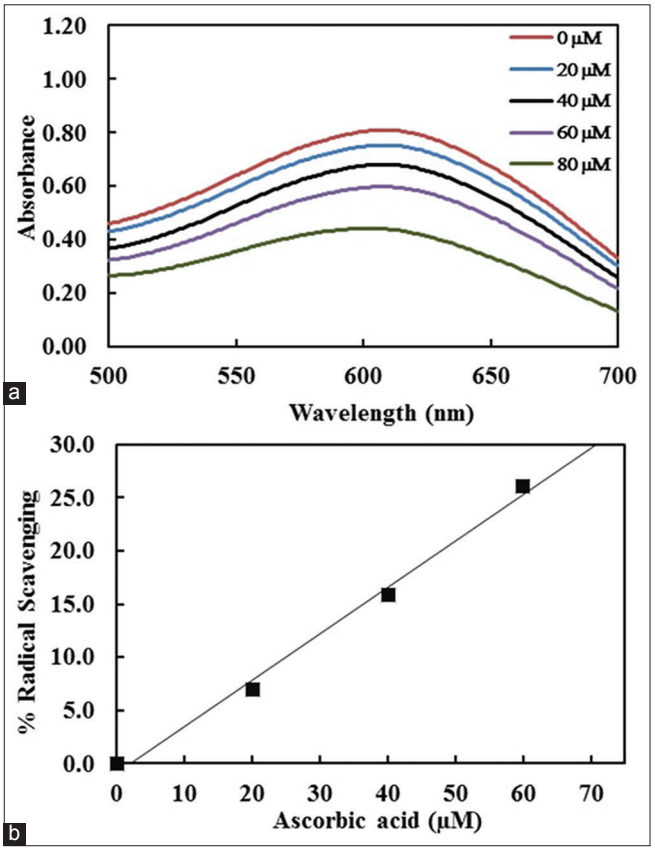

Figure 2: (a) Imipramine radical probe was scavenged by ascorbic acid. The absorbance spectrum at 610 nm was decrease depending on the concentration of antioxidant, (b) the relationship between % radical scavenging and ascorbic acid concentration

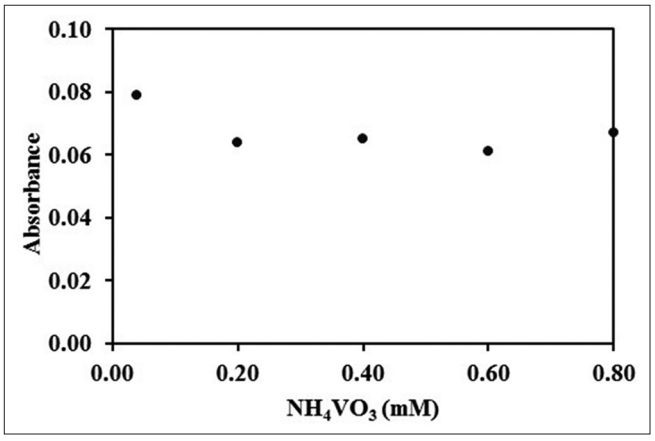

Figure 3: The effect of NH_4VO_3 concentrations on the absorbance intensity was studied using concentration ranged from 0.004 to 0.80 mM. The higher fold excess of NH_4VO_3 did not further increase the oxidation of imipramine and constant absorbance reading was obtained

FIA for IMI probe and TAC calculation

Even using spectroscopy method is simple, but recording unstable and rapid decompose of the IMI probe was not convenient and can reduce the accuracy and precision of the method. As a result, FIA technique was introduced. The manifold of FIA for IMI assay was shown in Figure 1. By cooperating with FIA, the mixture was recorded at the same condition with the same result as the mixture was continuous mixing. TAC can be accessed by plotting % radical scavenging versus concentration of the concentration of the antioxidant compound. The % radical scavenging was calculated as the equation in the following.

$$\% \text{ Radical scavenging} = ([A_{control}-A_{sample}]/A_{control})\times 100\ldots \quad (1)$$

Where, $A_{control}$ is an absorbance in the absence of sample or antioxidant compound and A_{sample} is an absorbance in the presence of sample or antioxidant compound at different concentration. To validate the proposed method, four antioxidant compounds were used to estimate the antioxidant capacity. According to the slope, ascorbic acid was the most sensitive to IMI probe follow by trolox, gallic acid, and caffeic acid, respectively. The linear regression was illustrated in Table 2. In this work, TAC was express as trolox equivalent as trolox is the most common standard used in antioxidant research. Graphs demonstrated the linear regression of ascorbic acid, gallic acid, and caffeic acid can be found in the supplementary information.

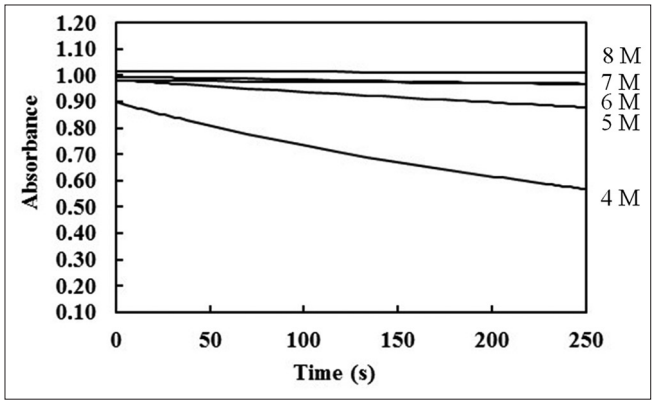

Figure 4: Effect of the excess of H_2SO_4 on the absorbance of colored product of imipramine. An intense and stable absorbance was obtained when H_2SO_4 concentration was more than 6 M. 7 M solution of H_2SO_4 was selected for further study

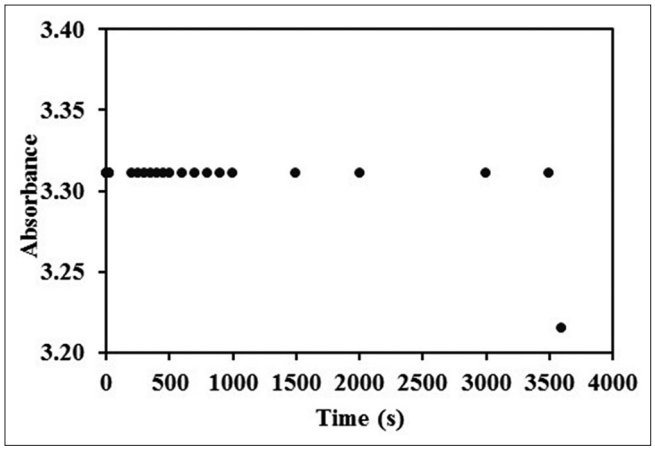

Figure 5: Effect of stability time on absorbance intensity induced due to of oxidation of imipramine in the optimal condition

Antioxidant Analysis in *G. sootepensis's* Extract Compare with the DPPH˙ Method and FCR Assay

Total soluble polyphenolic compounds were determined by a spectrophotometric method using folin-ciocalteu method. However, it can be considered as an antioxidant capacity method. The other method used to validate the proposed method was DPPH. The antioxidant capacity contents of the three fraction of the *G. sootepensis*'s extract were shown in Figure 6 with three different assays (a) the proposed method, (b) DPPH method, and (c) FCR analyze. The capacity status of the examined extract was in the order of S1>S2>S3 which a strong correlation of TAC from three methods was found from the data in Figure 6.

CONCLUSIONS

There is the need for the new method for the evaluation of TAC to improve the drawback from the traditional approach. Our new antioxidant probe, IMI was demonstrated to be a good candidate for the antioxidant probe. There were several

Table 1: Optimization condition

Parameter	Optimal results
Reagent concentrations	
NH_4VO_3	2.000 mg/L
IMI	100 mg/L
H_2SO_4	7 mol/L
Antioxidant compounds (mg/L)	
Ascorbic acid	0–2.000
Gallic acid	0–1.700
Caffeic acid	0–150
Trolox	0–2.800
Flow rate (mL/min)	
NH_4VO_3	1.5
IMI	1.5
Sample	2.0
Length of mixing coil (cm^3)	
Coil 1	30
Coil 2	30

IMI: Imipramine

Table 2: Linear regression between % radical scavenging and antioxidant concentration

Antioxidant compounds	Linear equation	r^2
Ascorbic acid	y=0.0085x±0.0633	0.9966
Gallic acid	y=0.0368x±0.9314	0.9877
Caffeic acid	y=0.1708x±0.8431	0.9728
Trolox	y=0.0256x±0.0093	0.9995

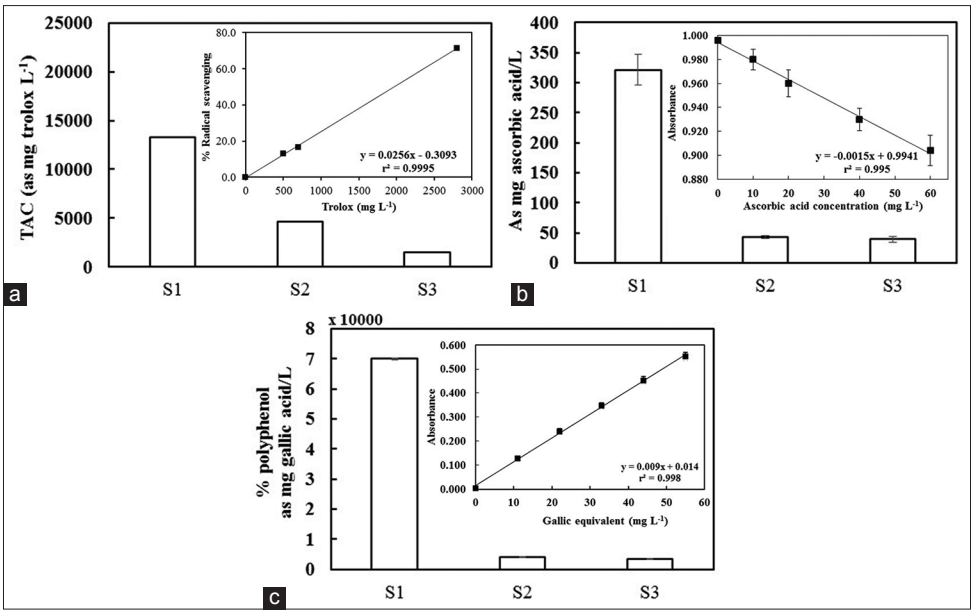

Figure 6: Bar graph showing total antioxidant capacity results from (a) the proposed imipramine probe, (b) 2,2-diphenyl-1-picrylhydrazyl, and (c) folin-ciocalteu reagent assay. The results of both standard methods were significantly correlated with the novel method

advantages of the developed method over the traditional methods such as IMI probe was sensitive, and the reaction between the probe and the antioxidant compounds was relatively fast. The blue free radical generated was not interfere with the color of samples. The reagents used in this assay were cheap and nontoxic. The calibration curve of IMI and antioxidant compounds was linear which was simple to calculate TAC. The probe can be operated with FIA technique which provided the method more precise, fast, and accurate.

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REFERENCES

1. Song HP, Wu SQ, Qi LW, Long F, Jiang LF, Liu K, *et al*. A strategy for screening active lead compounds and functional compound combinations from herbal medicines based on pharmacophore filtering and knockout/knockin chromatography. J Chromatogr A 2016;1456:176-86.
2. Birben E, Sahiner UM, Sackesen C, Erzurum S, Kalayci O. Oxidative stress and antioxidant defense. World Allergy Organ J 2012;5:9-19.
3. Gupta RK, Patel AK, Shah N, Chaudhary AK, Jha UK, Yadav UC, *et al*. Oxidative stress and antioxidants in disease and cancer: A review. Asian Pac J Cancer Prev 2014;15:4405-9.
4. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem 2005;53:1841-56.
5. Pisoschi AM, Negulescu GP. Methods for total antioxidant activity determination: A review. Anal Biochem 2012;1:1000-106.
6. Prior RL, Wu X, Schaich K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. J Agric Food Chem 2005;53:4290-302.
7. Rubio CP, Hernandez-Ruiz J, Martinez-Subiela S, Tvarijonaviciute AC. Spectrophotometric assays for total antioxidant capacity (TAC) in dog serum: An update. BMC Vet Res 2016;2:1-7.
8. Leopold LF, Leopold N, Diehl HA, Socaciu C. Prediction of total antioxidant capacity of fruit juices using FTIR spectroscopy and PLS regression. Food Anal Methods 2012;5:405-7.
9. Lugonja NM, Stankovic DM, Spasic SD, Roglic GM, Manojlovic DD, Vrvic MM. Comparative electrochemical determination of total antioxidant activity in infant formula with breast milk. Food Anal Methods 2014;7:337-44.
10. Zargoosh K, Ghayeb Y, Aeineh N, Qandalee M. Evaluation of antioxidant capacity of hydrophilic and hydrophobic antioxidants using peroxyoxalate chemiluminescence reaction of the novel furandicarboxylate derivative. Food Anal Methods 2014;7:283-90.
11. Christodouleas D, Papadopoulos K, Calokerinos AC. Determination of total antioxidant activity of edible oils as well as their aqueous and organic extracts by Chemiluminescence. Food Anal Methods 2011;4:475-84.
12. Zhou D, Wang D, Yang L, Liu Z, Zhang Y. A modified and improved assay based on microbial test system (MTS) to evaluate antioxidant activity. Food Anal Methods 2016;9:895-904.
13. Jerman KT, Mozetic VB. DPPH Solution (in)stability during kinetic UV/Vis spectrometry measurements of phenols antioxidant potential. Food Anal Methods 2012;5:781-3.
14. Ozyurek M, Bektasoglu B, Guclu K, Gungor N, Apak R. Phenolic antioxidants isolated from the flowers of *Osmanthus fragrans*. J Food Comp Anal 2010;23:689-98.
15. Charalampidis PS, Veltsistas P, Karkabounas S, Evangelou A. Blue CrO5 assay: A novel spectrophotometric method for the evaluation of the antioxidant and oxidant capacity of various biological substances. Eur J Med Chem 2009;44:4162-8.
16. Misiuk W. Spectrophotometry assay of imipramine and desipramine using ammonium metavanadate and its application to pharmaceutical preparations. J Pharm Biomed Anal 2000;22:189-96.
17. Borg DC. Free radicals from imipramine. Biochem Pharmacol 1965;14:115-20.
18. Nuanyai T, Sappapan R, Teerawatananond T, Muangsin N, Pudhom K. Cytotoxic 3,4-seco-cycloartane triterpenes from

Gardenia sootepensis. J Nat Prod 2009;72:1161-4.
19. Ukeda H, Adachi Y, Sawamura M. Flow injection analysis of DPPH radical based on electron spin resonance. Talanta 2002;58:1279-83.
20. Singleton VL, Orthofer R, Lamuela-Raventos RM. Total phenolic contents and antioxidant activities of different extracts and fractions from the aerial parts of *Artemisia biennis* Willd. Iran J Pharm Res 1999;13:152-78.