Cumhuriyet Science Journal

e-ISSN: 2587-246X
ISSN: 2587-2680

Cumhuriyet Sci. J., 41(1) (2020) 185-192
http://dx.doi.org/10.17776/csj.642223

Can an abts antioxidant test be performed without a spectrophotometer?

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Abstract

ABTS$^{\bullet+}$ (2,2-azinobis- (3-ethylbenzothiazoline-6-sulphonic acid)) radical scavenging assay is widely used to determine the antioxidant activity of natural and synthetic substances. As other antioxidant activity determination methods, a spectrophotometer device is needed to determine the numerical value of the color formed in ABTS method. Therefore, the dependence on the device is disadvantageous due to especially the high cost and restriction of workspace. To overcome this disadvantage, a new colorimetric method in the determination of antioxidant activity for ABTS method was developed with the help of a scanner device and free software (Image J). Mixtures (plant extracts and antioxidant standards) in this new colorimetric method were prepared as in the spectrophotometric method and, after the incubation period, each of these reagent mixtures was dropped onto the thin layer chromatography (TLC) plate. Then the colors that appeared on the thin layer chromatography were transferred to the computer with the help of a scanner and CSC_{50} values (the color value of the antioxidant concentration required to scavenge 50% of the ABTS radical in the test solution using Image J software) were measured with free software of Image J. The same measurements were performed simultaneously on the spectrophotometer. The results of both methods were compared. There was a strong correlation between the new colorimetric method and the spectrophotometric method for ABTS. Thus, the new colorimetric method for the ABTS test has become easily applicable since no spectrophotometer device is needed and in all environments.

Article info

History:
Received:04.11.2019
Accepted:05.01.2020
Keywords:
ABTS,
Antioxidant,
Colorimetric,
Thin layer chromatography.

1. Introduction

Free radicals are high-energy atoms or molecules containing one or more unpaired electrons in the outer orbital [1]. The radicals can occur during cellular metabolism or through external factors and also damage cellular components. Thus, they cause many serious diseases such as cancer [2-3]. Antioxidants play an important role in protecting the organism from damage caused by free radicals and thus preventing such diseases. There is a natural balance between free radicals and antioxidants. For this reason, it is important to define the antioxidant content of the foods offered for consumption and to consume those with high content. Especially, numerous studies have been conducted assessment of the antioxidant activities of synthetic and natural compounds and there are still ongoing studies on this topic nowadays [4-5].

Antioxidant activity determination methods are classified according to the type of antioxidants measuring, the character of solvent, reagent and reaction mechanism (electron transfer and hydrogen atom transfer) [6]. There are many methods having different reaction mechanisms for the determination of antioxidant activity substances; for example, commonly used ABTS$^{\bullet+}$ (2,2-azinobis- (3-ethylbenzothiazoline-6-sulphonic acid) radical scavenging [7]. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging [8], ferric reducing/antioxidant power (FRAP) [9], cupric reducing antioxidant capacity (CUPRAC) [10], and determination of total phenolics with Folin – Ciocalteu reagent [11]. In the ABTS method [7] to be modified for this study ABTS$^{\bullet+}$ radical is formed in the oxidation of 2,2 3-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) with persulfate. The radical is used to measure the total radical scavenging capacity. As a result, the activity values of substances in the method are defined based on the loss of color of ABTS$^{\bullet+}$ by antioxidant compounds. This method is widely used in antioxidant activity determination studies for many reasons such as easy to apply, stable ABTS radical over a wide pH range, low redox potential of the radical, soluble in water as well as organic solvents, and therefore it is

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suitable for determining the antioxidant activity capacity of lipophilic, and hydrophilic compounds [12]. A spectrophotometer device was used for the determination of activity values in the ABTS method, as in other antioxidant determination methods. In addition, HPLC or additional devices were used [13-16]. In this study, it was shown that ABTS method can be applied without using a device such as a spectrophotometer and HPLC. Bioactivity studies are performed using a smooth surface (for example TLC and filter paper) without using a spectrophotometer [17-21]. Material extracts studied in the bioautographic studies are applied to drop onto TLC plates or chromatography papers. The plates/papers are then submerged in the solvent mixtures tank (mobile phase) [22-23]. Finally, the images formed on flat surfaces are transposed to the computer and assessed with using image processing software. The analysis of the images is determined as structural or color value [24-29]. Many programs have been developed for the analysis of images transferred to the computer. Image J software used in many studies [30-32] was used in this study. As in our study, to evaluate the color of the extracts dropped on the filter paper, enzymatic phenol analysis studies were performed by using the Image J program [29, 33]. In this study, a new method has been developed with the modification of ABTS antioxidant detection method. It is dependent on easily available materials (scanner, TLC plate and free software) without using spectrophotometer in any laboratory. In other words, it is aimed to eliminate the need for spectrophotometric devices. This will reduce the cost of determination antioxidant activity and remove device limitation. Similar colorimetric studies were carried out for DPPH and CUPRAC antioxidant activity methods [24-25]. This new colorimetric method ABTS will be an alternative application for spectrophotometric measurements.

2. Materials and Methods

2.1. Chemicals and instrumentation

$ABTS^{\bullet+}$ radical, potassium persulfate ($K_2S_2O_8$) and all standards (BHT, caffeic acid, catechin, and gallic acid), Whatman® Filter Paper ashless blue ribbon and black ribbon were supplied with Sigma Aldrich Chemical Company. Methanol ≥ 99.9% in purity, ethanol ≥ 99.5% in purity and thin layer chromatography silica gel 60 plates were provided from Merck Company. UV-1800 (Shimadzu, Japan) spectrophotometer was used to measure absorbance. The printer used to scan TLC plates is the HP Deskjet Ink Advantage 2060.

2.2. Selection and collection of samples

The plants selected to cover a variety of herbs rich in terms of antioxidant content and widely consumed in Turkey. The herbs used as samples in this study (agarwood, bilberry, centaury, curcuma, ginger, green tea, linden, peppermint, rosehip, and rosemary) were purchased from an herbalist's trade Trabzon, Turkey.

2.3. Preparations of solutions

Trolox® antioxidant standard was prepared in absolute ethanol. Besides, solutions of other standards (BHT, caffeic acid, catechin, and gallic acid), were prepared in methanol.

$ABTS^{\bullet+}$ reagent solution was prepared according to the ABTS method [7]. Briefly, 7 mM $ABTS^{\bullet+}$ radical cation solution was prepared by dissolving 0.038 g ABTS reagent in 1 mL distilled water and adding 5 mL ethanol. 2.45 mM potassium persulfate solution was prepared by dissolving 0.0066 g potassium persulfate in 3 mL distilled water and adding 1 mL ethanol. Then, they were mixed with each other in a test tube. The lid of the mixture tube was closed and wrapped with aluminum foil. Then the tube left in dark for 18 h. After 18 h, $ABTS^{\bullet+}$ reagent solution was diluted with ethanol until the absorbance read as 0.70 (±0.02) at 734 nm.

2.4. Preparation of sample extracts

To extract, dried plant material (10 g) was added to methanol (100 mL) and the process was continued at room temperature with a magnetic stirrer for 2 hours. Thereafter these extracts of plants were subjected to filtration by means of black-ribbon and blue-ribbon filter papers, respectively. The solvents were completely taken away from these extracts in a rotary evaporator. Then the extracts were pre-tested with 1500 µM $ABTS^{\bullet+}$ radical solution to determine the initial stock concentrations, in common with standards. The clear extracts were stored at room temperature and in dark until analyses. In analyses, each standard/sample studied in triplicate.

2.5. Determination of antioxidant activity

In the current research, a routine ABTS method in which the measurements are taken spectrophotometrically and a new ABTS radical scavenging method (non-spectrophotometric) called "new colorimetric ABTS method", were applied to antioxidant standards and plant extracts.

Simultaneously the plant samples and antioxidant standards were studied in spectrophotometric and the new colorimetric ABTS method. The same concentrations of samples/standards were used in spectrophotometric and colorimetric methods.

2.5.1. Spectrophotometric ABTS assay

The ABTS assay, developed by Re et al. [7] was used with a minor modification. This method is based on the determination of the antioxidant capacities of various substances by using the radical $ABTS^{\bullet+}$, in terms of Trolox equivalent capacity (TEAC) [7].

The stock solution of $ABTS^{\bullet+}$ was produced by mixing 2.45 mM potassium persulfate solution to 7 mM $ABTS^{\bullet+}$ radical cation solution and waited in the dark environment and at about 20 ºC to 25 ºC during 18 h. Then the blue-green solution was diluted with ethanol until the absorbance adjusted to 0.70 AU at 734nm. After that, to obtain a standard calibration graph, Trolox® standard was diluted to various concentrations (0.03125 - 0.625 - 0.125 - 0.5 - 1 mg/mL) with ethanol. For analysis, firstly 50 µL standard/extract was mixed with 1950 µL of $ABTS^{\bullet+}$ solution, vortexed, and left at about 20 ºC to 25 ºC, in dark for 20 min. At the end of the incubation period, the reagent blank solution was diluted until the absorbance read as 0.70 (± 0.02) AU at 734 nm. This dilution rate was also applied to other test tubes. After diluting, the absorbance were read at 734 nm. The standard calibration curve was drawn at Microsoft Office Excel program. The standard calibration curve equation (y = ax + b) was used to calculate the µmol/L TEAC values of the samples. Then, the results were calculated and expressed as µM TEAC and SC_{50} values. There is an inverse relationship between the absorbance value and antioxidant activity.

2.5.1. Colorimetric ABTS assay

The volume of drop/spot for colorimetric method improved in this research was tested under the same optimization conditions as the colorimetric CUPRAC and DPPH tests studied previously [24-25]. According to this, the drop/spot volume which minimizes the amount of chemicals and provides the best color formation was determined as 15 µL. Therefore, after determining the absorbance values by spectrophotometrically, 15 µL of standard/sample was taken by the help of a micropipette and dropped onto a TLC plate as triplicates.

Each plant sample extract, and the standard was studied in repetitive, blank of the sample and reactive. After dropping the standard/sample on the TLC plate, it was left to dry for 5 min. Then it was transposed to the computer with the help of the HP Deskjet Ink Advantage 2060 scanner and the scanned files were saved with the appropriate contrast (85%) and brightness (100) [24-25].

Later on, the color ring volume was adjusted to the value of 1572 on the saved images and the color values of each sample were measured at the Image J program and the mean values were calculated in the Microsoft Office Excel program. The graphs were plotted. With the equality equation (y=ax + b) of the standard calibration curve of Trolox®, the values of TEAC (µmol/L, CTEAC) were calculated. Then, CSC_{50} values were calculated from these "color value-concentration" graphs. CSC_{50} can be defined as the sample concentration, which reduces the color value (intensity) by half. When calculating the CSC_{50} value, it is necessary to work at different concentrations, as in the calculation of the SC_{50} value [24-25].

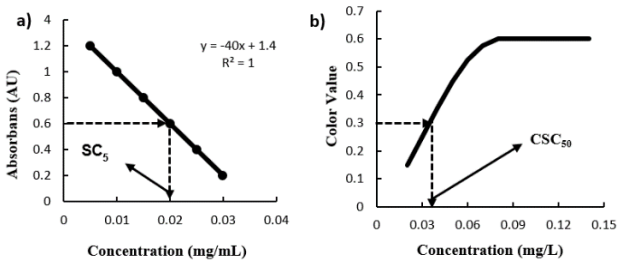

Figure 1. Concentration and absorbance graphics for SC_{50} values (a) and for CSC_{50} (b)

3. Results and Discussion

3.1. Determination of the concentration of ABTS radical solution

A spectrophotometer device is needed in most antioxidant detection methods. Due to the need for the device increases both the cost and restricts working area. Considering this situation, in the study, a new colorimetric ABTS antioxidant determination method has been developed. There are DPPH radical scavenging activity and CUPRAC test studies which are adapted to the colorimetric method without using a spectrophotometer [20-25]. Similarly, in this study, the standards and samples treated with ABTS radical solution were dropped onto the TLC and color changes on the TLC were transferred to the computer. These colors were converted to numerical values by using the Image J program and the spectrophotometric (SC_{50}) and colorimetric (CSC_{50}) values of standard and plant extracts were compared. The concentration of $ABTS^{\bullet+}$ radical was determined based on the concentration-color value of the experiments with Trolox® (Figure

2a) and gallic acid (Figure 2b) antioxidant standards in the same concentrations. Thus, concentrations of $ABTS^{\bullet+}$ radical for the colorimetric method was determined in concern with these standards.

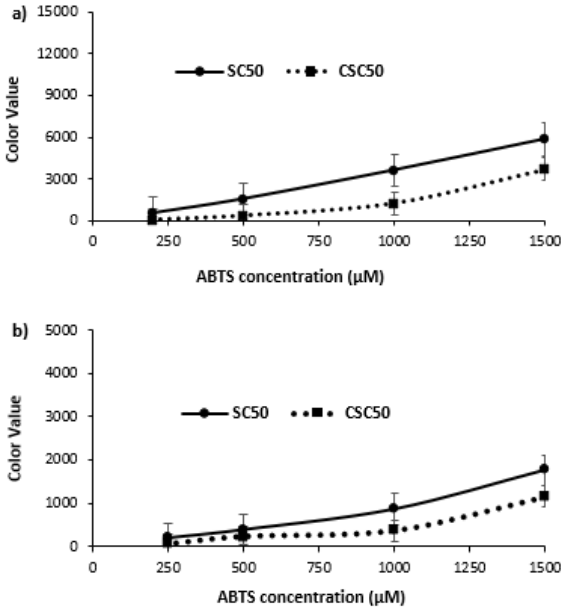

Figure 2. The linearity of CSC_{50} and SC_{50} as an effect of ABTS reactive concentration the depending on standards of Trolox® (a) and gallic acid (b).

The concentration of the $ABTS^{\bullet+}$ radical was 500 µM in the experiments performed with both standards but the blue-green color from ABTS was more dominant when it increased above 1000 µM. For this reason, it has been determined that the concentration of $ABTS^{\bullet+}$ can be run at a concentration between 1000 and 1500 µM.

3.2. Evaluation of spectrophotometric ABTS assay

The activity values of plant extracts and antioxidant standards studied at different concentrations were calculated from "concentration-absorbance" graphs in mg/ml SC50 values as spectrophotometric and the graphs obtained for caffeic acid antioxidant standard and peppermint plant extract are shown in Figure 3.

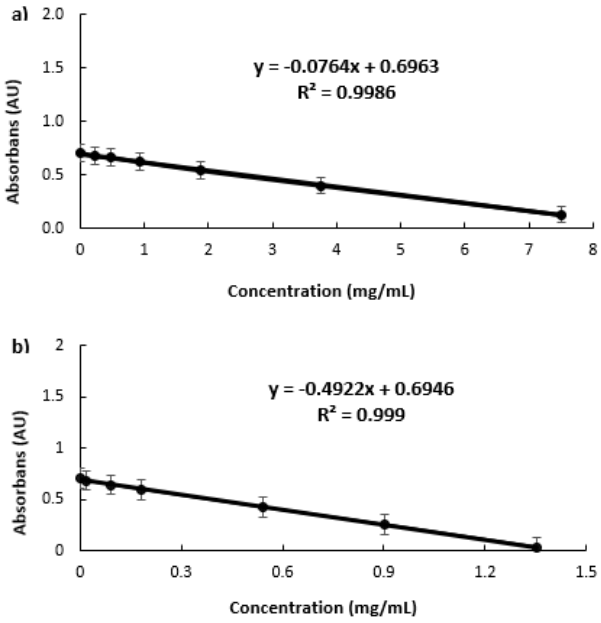

Figure 3. Comparison of SC_{50} values of caffeic acid standard (a), and peppermint extract (b) with spectrophotometric method.

3.3. Evaluation of new colorimetric ABTS method

The color values of different concentrations of the standards determined by using the new colorimetric method were plotted with Image J program and CSC_{50} values were calculated from these graphs. Each standard was diluted 1:1 starting from different concentrations and dropped onto the TLC layer in six different concentrations as three replicates (Figure 4). CSC_{50} values of caffeic acid antioxidant standard are shown in Figure 5a. Similarly, CSC_{50} values for different concentrations of each plant extract were determined by the new colorimetric method. Among the plants, the values of peppermint extract are given as examples in Figure 5b.

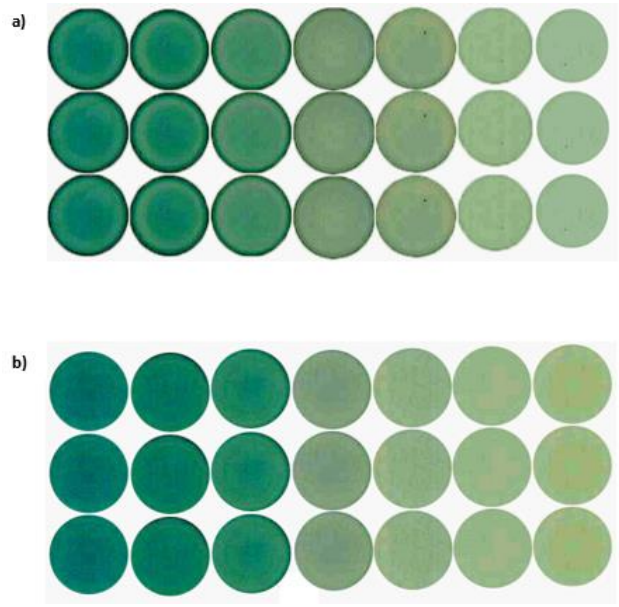

Figure 4. Image of caffeic acid standard (starting from 20 mg/ml of concentration and diluted 1:1) dropped onto the TLC layer (a) Image of peppermint extract (starting from 0.03 mg/ml of concentration and diluted 1:1) dropped onto the TLC layer (b).

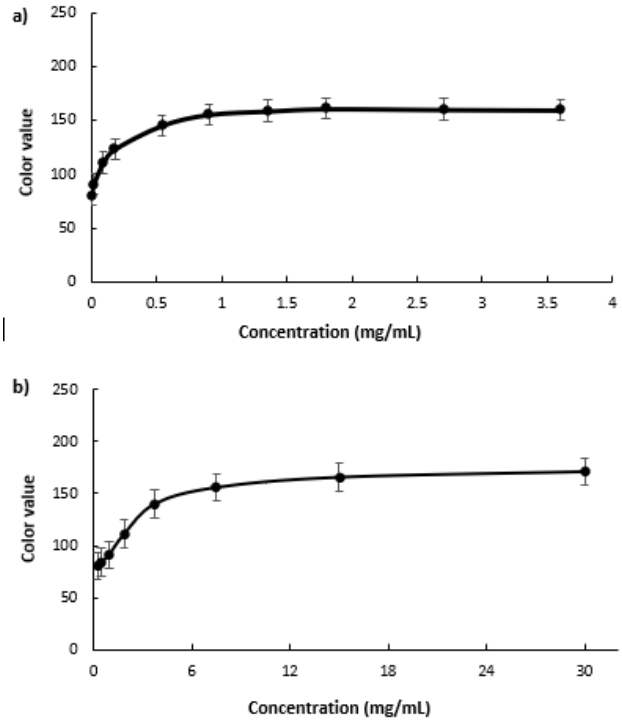

Figure 5. Comparison of CSC_{50} values of caffeic acid standard (a), and peppermint extract (b) with new colorimetric method.

3.4. Comparison of spectrophotometric and new colorimetric methods

The SC_{50} and CSC_{50} values of plant extracts and antioxidant standards used in spectrophotometric and colorimetric methods were compared and the new colorimetric ABTS method and the widely used ABTS spectrophotometric method showed a strong positive correlation for both plant extracts and antioxidant standards (Figure 6).

SC_{50} value is inversely proportional to antioxidant activity. Considering this situation, ABTS radical scavenging antioxidant activities (TEAC) of all standards were sorted from highest value to lowest value for spectrophotometric as following: gallic acid>catechin> caffeic acid> BHT. It was sorted in plant extracts: green tea>peppermint>ginger> bilberry > rosemary> linden> centaury> curcuma> rosehip> agarwood. In the new colorimetric method, the order of activity values for the standards was formed as follows: gallic acid> catechin> caffeic acid> BHT. This order for plant extracts: green tea> mint> linden> bilberry> ginger> centaury> rosemary> turmeric> rosehip>agarwood. When the activity sequence was evaluated, it was observed that there was a correlation between the spectrophotometric and colorimetric methods. While the order of antioxidant standards was identical in both methods, there was little difference between in order of plant extracts. Akar et al. [24] conducted a study in which the 2,2-diphenyl-1-picrylhydrazyl test (DPPH) was adapted for the same colorimetric method as our study. They applied the DPPH test to some antioxidant standards and plant extracts and compared the obtained SC_{50} and CSC_{50} values as well as the TEAC and CTEAC values. They reported a high correlation between both methods for DPHH. Akar and Burnaz [25] showed that the values of colors formed on TLC layers by cupric reducing antioxidant capacity method (CUPRAC) were correlated strongly positive with the spectrophotometric values. In addition, there are some colorimetric studies on the ABTS method.

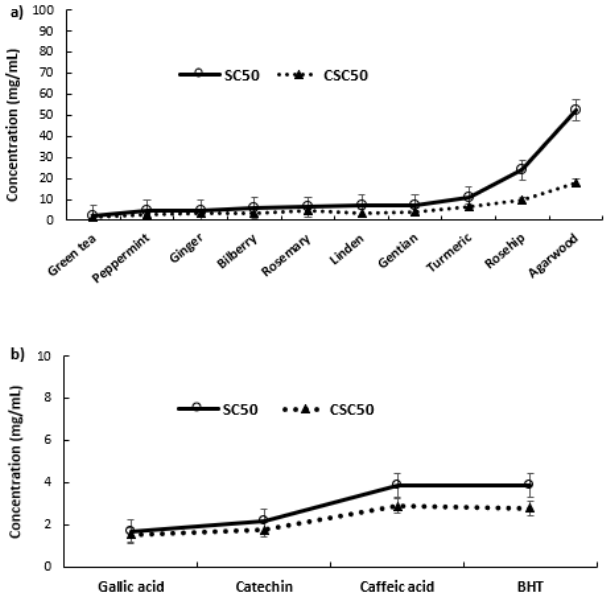

Figure 6. The SC_{50} and CSC_{50} values of standards (R^2=0.9793) (a) and extracts (R^2=0.9953) (b).

Zampini et al. [34] used a different colorimetric method autographic analysis method for the rapid determination of antioxidant activity with ABTS analysis. However, numerical values were not obtained in this study and only the presence of activity was determined based on the evaluation of color change with UV lamp. In addition, since different phenolic standards were used in this study, no comparison could be made with our present study.

4. Conclusions

There are two general types of analysis commonly used for antioxidant studies. One of them is an analysis of lipid peroxidation and the others are related to the electron or radical scavenging including ferric reducing antioxidant power test (FRAP), DPPH test and ABTS test. Antioxidant capacity measurements of the samples can vary with the analysis method used reaction medium (solvent and pH) and reaction time. Generally, these assays are used for reactions in aqueous media. However, the ABTS test, also known as the TEAC test, can be applied in both the aqueous and lipid phases, and no pH adjustment is required, and the reaction time is very short. For these reasons, it is one of the commonly used methods in determining the antioxidant activity of food and beverages. The results of the new colorimetric method of the ABTS test showed a strong correlation with the results obtained by the spectrophotometric method. Thus, the applicability of the ABTS test independently of the spectrophotometer will provide advantages such as easy, fast and low cost and reliability.

Acknowledgment

This research was supported by Gümüşhane University Scientific Research Fund (Project code: 15.F5119.02.01).

Conflicts of interest

The authors state that did not have conflict of interests**.**

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