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Paper microzone plate based on DPPH as a simple colorimetric assay of the total antioxidant content of herbal extracts

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Abstract The human body needs an antioxidant-rich diet that comes from foods, beverages, and herbal products to support the physiological antioxidant systems. Thus, the development of an analytical tool for a simple assay of the total antioxidant capacity (TAC) of the rich antioxidant samples is crucial. The current work demonstrates a simple colorimetric assay of TAC of the herbal extract on the paper microzone plate (PμZP) that was constructed in the 70-well of patterned paper using the screen printing technique. The PμZP was constructed by immobilizing DPPH (2,2-diphenyl-1-picrylhydrazyl) onto 70-well of PμZP as a sensing zone for colorimetric detection. The purple-sensing zone exhibited a good response to gallic acid (GA), by producing slightly gray to pale yellow color that can be captured using a scanner and then analyzed using the ImageJ program. The paper-based sensor showed a linear response toward GA at 0.05–0.6 mM ($r = 0.9895$), reproducible response (RSD < 4%), and accurate measurement with 91 to 106% recovery for measuring TAC of herbal extract, presented as mM gallic acid equivalent and showed a good agreement with the standard DPPH method. The results suggested that the proposed method can be applied for simple TAC measurement in the herbal extract.

Keywords Paper microzone plate · Paper-based sensor · Colorimetric · DPPH · Total antioxidant capacity · Herbal extract

Introduction

Antioxidants contained in food, beverages, and herbal products may be particularly critical for protection from the diseases associated with reactive oxygen species (ROS) (Halliwell 1996). ROS associated diseases appear owing to the oxidative damage of macromolecules, such as proteins, DNA, and lipids, that might be related to the development of heart and blood vessel disease, the nervous system degeneration, and cancer. Naturally, ROS are produced in the human physiology system. However, since the antioxidant defense systems are not strong enough to neutralize and tackle ROS completely. Therefore, our body needs an antioxidant-rich diet that comes from foods, beverages, and herbal products. In this context, the development of an analytical tool for a simple assay of TAC of the diet that rich with antioxidants content is crucial.

Currently, many antioxidant methods are based on the spectrophotometric, such as 1,1'-DPPH (diphenyl-2-picrylhydrazyl), DMPD (*N,N*-dimethyl-*p*-phenylendiamine), ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid), and metal complex, e.g. CUPRAC (cupric-neocuproine) and FRAP (ferric-triipyridyltriazine), are generally employed to determine activity of antioxidant of natural and synthetic antioxidant, including for measuring total antioxidant capacity (TAC) of our diet. The DPPH is one of the popular assays used to determine the TAC of foods, beverages, and herbal products (Musa et al. 2013). Firstly, the DPPH assay was proposed by Blois (1958), and then further developed by others for antioxidant assay of various compounds and measuring TAC of various food, beverage and herbal samples (Bondet et al. 1997; Sánchez-Moreno et al. 1998). Briefly, the DPPH method is based on the reduction of radical compound (DPPH) by an antioxidant, e.g. a herbal extract that makes the radical change

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color, where this even can be analyzed spectrophotometrically at 515–520 nm (Molyneux 2004). This radical is stable so that it does not need to be prepared freshly prior to the analysis, like in other radical scavenging assays (Bobo-García et al. 2015).

In the standard DPPH method, a large volume (1.0–5.0 ml) of freshly prepared reagent solution in a cuvette is needed (Seo et al. 2014; Nirmal and Panichayupakaranant 2015; Nisar et al. 2015; Pękal and Pyrzynska 2015; Bener et al. 2016; Dolinsky et al. 2016). In order to reduce the reagent used, a microwell plate has been proposed in this assay (Lee et al. 1998). Here, it was found that no significantly different in terms of analytical characteristics between cuvette and microwell-based methods within intra-lab validation (Bobo-García et al. 2015). Then, the latter method is developed by others as high throughput screening (HTS) for antioxidant capacity (Cheng et al. 2006; Abderrahim et al. 2013; Musa et al. 2013; Condezo-Hoyos et al. 2015). In order to create a simpler and faster method as HTS, the microwell-based method was further developed for DPPH in dry reagent format (Musa et al. 2013). The dry-reagent assay exhibited a good correlation with the DPPH microwell-based assay for various food samples (e.g. green tea, banana, pink guava, and honeydew). Furthermore, in order to make this method suitable for field analysis and low-cost assay, the paper-based sensors have been proposed (Nuchtavorn and Macka 2016; Sirivibulkovit et al. 2018).

The paper-based sensor is simple, low-cost, and easy to produce as well as needs a low reagent or sample volume. The paper-based sensor color change as an antioxidant response can be scanned or captured using a flatbed scanner or camera. The paper-based antioxidant sensors based on DPPH were successfully developed (Nuchtavorn and Macka 2016; Sirivibulkovit et al. 2018). Lately, other methods (e.g. ABTS, CUPRAC, and Folin–Ciocalteu) were impregnated separately on the paper-based devices that enabled for determination of TAC and total phenolic content simultaneously (Puangbanlang et al. 2019). The paper microzone plate (PμZP) as a sensing platform was first introduced by Carrilho et al. (2009) using a 96-microzone plate and fabricated using the photolithography technique. Then, they were developed by others for the detection of total phenolic content (Aid et al. 2015), tumor markers (Wang et al. 2012), foodborne pathogens (Jokerst et al. 2012), etc. By considering the DPPH assay could also be performed on the 96-microwell plate (Musa et al. 2013; Condezo-Hoyos et al. 2015), it is open-up the potential that the DPPH assay could be employed using the PμZP platform.

In this context, a simple and low-cost sensing platform for TAC determination in herbal extracts by immobilizing DPPH onto the microzone sensing area of PμZP has been developed. Practically, it has a simpler fabrication compare

to the previous paper-based sensor based on DPPH (Nuchtavorn and Macka 2016; Sirivibulkovit et al. 2018) since, in this work, the screen printing technique has been used to create 70-well of microzone areas of PμZP using rubber-based ink. Furthermore, the scanometric method has been used for colorimetric TAC assay in our recent work (Hidayat et al. 2019) that create simpler, faster, and a low-cost assay of TAC. As far as PμZP as a concern, our work might be the first DPPH method using the PμZP platform. The PμZP was successfully used for the TAC assay of herbal extracts, and the results have a good correlation with the standard DPPH method.

Materials and methods

Chemicals

Gallic acid (GA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (USA). Methanol was purchased from Merck (UK). The GA stock solutions were prepared in methanol and working solutions were prepared daily by dilution of the stock solution using distilled water. All chemicals used were analytical grade.

Herbal samples

Herbal samples used in this study, such as West Indian elm leaf (*Guazuma ulmifolia*), red and white guava leaf (*Psidium guajava*), stonebreaker leaf (*Phyllanthus niruri*), and green tea leaf (*Camellia sinensis*) were obtained from Materia Medika, Batu, Indonesia and submitted as voucher specimens (MM 1901–1905) at Pharmacognosy Laboratory, Faculty of Pharmacy, University of Jember, Indonesia. Each of the herbal leaves was dried, ground, and filtered via #100 sieves. Plant powder (0.2 g) was sonicated (Elmasonic S180H, Germany) using a 10 ml solvent for 10 min. Methanol was employed as an extraction solvent for herbal leaf samples. Then, they were filtered using Whatman filter paper #1 (Merk, UK) and the extract solutions were placed in glass tubes in stored a refrigerator. For standard DPPH assay, all herbal extracts were diluted 100 times using methanol.

The PμZP fabrication

The PμZP (paper microzone plates) as the paper-based sensor was fabricated by patterning paper sheets (Whatman filter paper No 1, Merck UK) using a screen-printed technique according to our recent work (Hidayat et al. 2019). Using this fabrication technique, several numbers of microzones or well plate shapes could be printed based on the desired purposes. In this case, the microzone as the

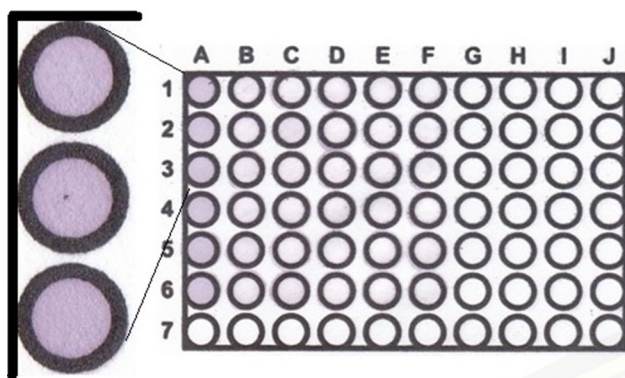


Fig. 1 The P μ ZP with violet color microzones that acting as the antioxidant sensor

antioxidant sensor was constructed by immobilizing DPPH reagent (3 μ l), followed by the drying process for 10 min at ambient condition and the result can be given in Fig. 1. Finally, the P μ ZP was kept in the container at 4 °C before it is used.

TAC determination

The general procedure can be described as follows: GA as a standard antioxidant or sample solution is taken 3 μ l using a pipette and introduce into the microzone plate as a sensing zone. Then, a flatbed scanner (CanoScan, LIDE 110, Japan) is used to scan the microzone color change (300 dpi resolution in a color mode). The scanned images color intensity (JPEG format) was then measured using the ImageJ to calculate the mean value of R (red), G (green), B (blue), or the combined color as RGB (red–green–blue). For quantitative measurement, each color value of standard or herbal extract was subtracted with the blank values. TAC of the herbal sample was calculated using the constructed calibration curve of GA. All of the measurements were conducted in triplicate.

TAC assay using the standard DPPH method

The standard DPPH method was performed using the UV/Vis spectrophotometric method for assaying antioxidant capacity of herbal extracts using GA equivalent, as reported elsewhere (Lu et al. 2014). Here, the spectrophotometer was used with absorbance at 515 nm to make a calibration curve of GA as a gallic acid equivalent in mM (mM GAE). The TAC of the herbal samples were calculated using this calibration curve. All of the measurements were conducted in triplicate. Then the TAC value of all herbal samples measured by the P μ ZP and the standard DPPH methods were compared using the *t* test. In order to evaluate the correlation of the obtained TAC value of both methods, the analysis of correlation (*r*) was also used.

Results and discussion

Sensing mechanism

Here, radicals DPPH is employed as the basic sensing scheme for colorimetric detection of the antioxidant activity (Sánchez-Moreno et al. 1998). Owing to DPPH has been most frequently used for the assay of antioxidant activity of phytochemicals, e.g. polyphenols and flavonoids (Musa et al. 2013). Here, the chromogen radical (DPPH) that is purple color is reduced to produce pale yellow of hydrazine (DPPH-H) by antioxidants, such as GA. In this case, the reduction of DPPH by GA as a hydrogen-donating compound or antioxidant is detected colorimetrically via its color change to the pale yellow. Here, there is no required for the radicals regeneration in situ, which creates the antioxidant sensor simpler and easy to use the method. Moreover, for the determination of this reduced product (DPPH-H) quantitatively, the scanometric method was applied that used a common flatbed scanner and free color image analysis that makes this method even cheaper.

Color value selection

In order to obtain the optimum response of the P μ ZP toward antioxidants in term of color value, the different color response as R, G, B, and RGB, were evaluated and compared. Here, the optimum P μ ZP response was determined by calculating the color intensity change as an antioxidant sensor with the introduction of GA in a serial concentration (0.1–0.5 mM). Here, it was found that the blue color value (Δ Mean Blue) gave the highest response and showed the best correlation coefficient (*r*) among others. Based on this value, Δ Mean Blue was selected and used for further measurements.

Reagent optimization

Here, the DPPH was used as a colorimetric reagent in a paper-based sensor as previously reported (Nuchtavorn and Macka 2016; Sirivibulkovit et al. 2018). Here, in order to develop the P μ ZP for the TAC assay, the DPPH was immobilized onto the surface of the microzone plate. Therefore, the concentration of DPPH needs to be optimized. In this case, various concentrations (2, 4, 5 and 7 mM) of DPPH immobilized and tested to obtain the best response toward antioxidants. Three microliters of each DPPH concentration was immobilized onto different microzone plates to produce the antioxidant sensor. Here, a serial concentration of GA (0.1–0.5 mM) was introduced to each DPPH concentration as the sensing zone. The constructed calibration curves presented as Δ Mean Blue.

Here, it was found that 5 mM of DPPH exhibits the highest response toward GA, in terms of its coefficient correlation (r) and slope. Therefore, the DPPH concentration at 5 mM was chosen as the sensor reagent.

Response time

The response time of the P μ ZP as an antioxidant sensor toward a GA solution (0.2 mM) presented as mean blue (Δ mean B) was found at 12 min, where the response was relatively constant. Based on this response time (12), the color change of the sensing zone in the P μ ZP toward serial concentrations of GA is given in Fig. 2a. Here, the violet sensing zone changed into slightly gray, after the addition of the GA solution. While the pale yellow was found at the GA concentration of 15 mM forward.

Linearity and detection limit

Although the microzone color change can be easily viewed by the nude eye, for the quantitative measurements, they need to be calculated using the scanometric method as reported elsewhere (Hidayat et al. 2016). The P μ ZP has a linear response toward GA in the range of 0.05–0.6 mM ($r = 0.995$) (Fig. 2a). The sensitivity of the P μ ZP response based on the calibration slope was found to be 24.912 Δ mean Blue/mM GA ($\alpha = 0.05$, $n = 3$). While the limit of

detection calculated from the three times of the standard deviation of the blank was found to be 0.035 mM.

Reproducibility and accuracy

The reproducibility of the P μ ZP response in the TAC assay of herbal extracts was conducted according to Yuwono and Indrayanto (2005). Here, TAC of the green tea extract was assayed in several measurements ($n = 9$) as depicted in Fig. 2b. It was found that the relative standard deviation (RSD) values of the nine replicate measurements were 3.737%, indicating the P μ ZP has good reproducibility of TAC measurement in the herbal extracts. While the recovery study as the P μ ZP response accuracy in the TAC assay of the herbal extract (green tea), was conducted by spiked individually with GA to give 30, 45, and 60% of the initial TAC value of the extracts. Based on this study, the TAC recovery of the extract was found between 91 and 106%, indicated that the P μ ZP has good accuracy as an antioxidant sensor.

Stability of sensor

The P μ ZP stability was studied in two storage temperatures, i.e. at room (25 °C) and chiller (4 °C) condition by stored the P μ ZP in a sealed container separately at these two conditions. Here, the P μ ZP response was observed to

Fig. 2 **a** The calibration curve of GA constructed by the P μ ZP at 12 min ($n = 3$). **b** The typical color change of antioxidant sensor after green tea extract addition ($n = 9$). A1–A3 = blank, B1–J1 = green tea extracts, and B2–J3 = empty micro zones (no added reagent)

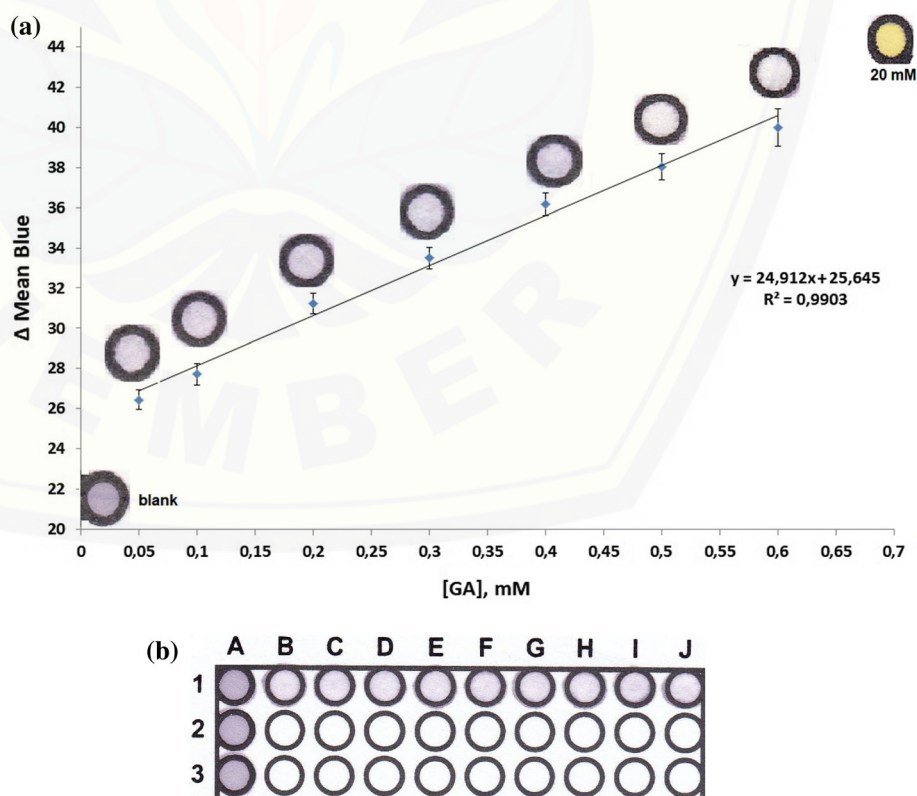


Table 1 The comparison results of the TAC of the herbal extracts obtained by the P μ ZP and the standard DPPH method (n = 3, α = 0.05)

Sample extracts	Sensor	UV–Vis	Sig. value (p)
West Indian elm leaf	0.307 \pm 0.039	0.335 \pm 0.019	0.069
Red guava leaf	0.374 \pm 0.125	0.417 \pm 0.017	0.075
White guava leaf	0.427 \pm 0.019	0.542 \pm 0.051	0.144
Stonebreaker leaf	0.327 \pm 0.058	0.352 \pm 0.041	0.113
Green tea leaf	0.126 \pm 0.026	0.113 \pm 0.011	0.143

decrease more than 15% after 5 h at room temperature. While at a chiller condition, the P μ ZP response decreases more than 15% was found in 3 days. The P μ ZP stability was less than the paper-based DPPH antioxidant sensor (Sirivibulkovit et al. 2018). This short stability might be due to the immobilized DPPH employed in this case was stored without plastic lamination as in the previous sensor, that might increase vaporization of the immobilized DPPH along with the effect of ambient condition. In this case, an attempt to laminate the P μ ZP with a plastic layer was carried out, and it could extend the stability up-to 5 days when it stored in the chiller condition.

Application in herbal samples

The comparison results of TAC values between the P μ ZP and the standard DPPH method are given in Table 1. Here, the TAC values of the herbal extracts resulted using the P μ ZP are similar to the result by the standard DPPH method. Hence, these results demonstrated that the P μ ZP was similar to the standard DPPH method in measuring TAC of herbal extracts. Furthermore, the resulted TAC values of both methods were in good agreement ($r = 0.9887$). Here, the P μ ZP was showed to be simpler, faster and cheaper than the standard DPPH method, or even with the microplate-based DPPH method (Krylova et al. 2016) in terms of method and cost, and also with more recently the paper-based sensor based on DPPH (Puangbanlang et al. 2019). The P μ ZP is also suitable for the field analysis as HTS for the TAC assay, and it is compatible to be used in the remote area or low resource-setting. Moreover, it can be used directly with a smartphone camera coupled with a color analysis application, such as Color Lab or Color Grap, that also freely available.

Conclusion

The P μ ZP as a paper-based antioxidant sensor for TAC assay of herbal extract combined with the scanometric method has been developed based on immobilized DPPH. The response time of the P μ ZP was found in 12 min that is faster compared to the standard DPPH method, and the TAC values of the herbal extracts measured by both

methods were in good agreement. In P μ ZP as the antioxidant sensor, the reagent and sample need were reduced drastically, including the low-cost for performing the TAC assay.

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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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