Radical Scavenging Activity Assay and Red Fluorescence Microscopy Studies: Antioxidant Properties of Selected Young and Mature Leaves for Application in Pharmaceutical Industry

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https://doi.org/10.5109/4055222

出版情報：Evergreen. 7 (2), pp.216-220, 2020-06. 九州大学グリーンテクノロジー研究教育センター
バージョン：

Radical Scavenging Activity Assay and Red Fluorescence Microscopy Studies: Antioxidant Properties of Selected Young and Mature Leaves for Application in Pharmaceutical Industry

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(Received October 4, 2019; Revised May 20, 2020; accepted May 21, 2020).

Abstract: A fast screening method for antioxidant capacity of plant leaves based on red fluorescence was studied. The red fluorescence of young and matured leaves of *A. occidentale*, *M. indica*, *S. dulcis*, belonging to *Anacardiaceae*, and *P. betle* Linn., *P. sarmentosum*, and *P. crocatum* of *Piperaceae* family using fluorescence imaging microscopy was investigated. The total antioxidant capacity of methanol extracts of plant leaves was determined using the 2,2-diphenyl-1-picrylhydrazine (DPPH) free radical scavenging assay. From young to matured leaves of the plants belonging to the *Anacardiaceae* and *Piperaceae*, the red fluorescence and the total antioxidant capacity show the same trend.

Keywords: antioxidant; *Anacardiaceae*; DPPH assay; *Piperaceae;* Red fluorescence

1. Introduction

Free radicals which are mainly associated with the reactive oxygen and nitrogen species could be produced by pollutants, radiations, chemicals, and toxins in the environment, and they can be beneficial or toxic depending on their concentration. At low concentrations, the reactive species enhances cellular responses and functions, but at high concentrations the reactive species could generate destructive oxidative stress to cells. As a result, the free radicals accelerate the oxidation process, destructing the human immune system, accelerating aging process, and causing many health problems associated to several chronic and degenerative diseases[1-3)], such as cancer, aging, autoimmune disorders, and arthritis[4)].

To eliminate the oxidation reaction, the immune system in human body generates antioxidants which encounter the free radicals. To a certain extent, however, the free radicals are excessive and outnumber the antioxidants generated by human body[5)]. Therefore, it is necessary to consume foodstuffs and beverages naturally having high antioxidants, such as fruit, vegetable, tea, coffee, and wine. In general, the natural antioxidants contained in the foodstuffs and beverages include various compounds of phenolic, flavonoid, and flavanol derivatives[6)]. With high contents of the natural antioxidants, mainly their phenolics and catechins[7)], plant leaves have been gaining more interest and popularity as the source of antioxidants[8)]. Moreover, the flavonoids of the plant leaves have been related to their antimicrobial activities such as antibacterial activity in ethanol extract and essential oil of Citrus sinensis[9)]. Due to the growing interest on the natural antioxidants, the quantification of the antioxidant capacity of different fruits, vegetables, and plant leaves has become indispensable[10-12)].

The most common and reliable methods to evaluate the antioxidant capacity of plant leaves are centered on the radical scavenging assays, including 2,2-diphenyl-1-picrylhydrazine (DPPH) and ferric reducing antioxidant power (FRAP), combined with spectroscopic analysis[13)]. Another important approach to quantify the antioxidant capacity of leave infusions or extracts in solution is electrochemical analysis, including cyclic voltammetry and differential pulse voltammetry[14-16)]. However, all the radical scavenging assays and electrochemical analysis are time-consuming, and require chemicals, mainly the organic solvents, reagents, and buffers.

Inventing non-invasive method without using any

organic solvents is pursued. In this sense, fluorescence imaging spectroscopy has been proposed to evaluate the fluorescence properties of the plant leaves belonging to *Apiaceae*, *Rubiaceae*, and *Anacardiaceae* family, respectively. The red fluorescence intensity which was attributed to the phenolic compounds bound to the protein complexes along with the chlorophylls in chloroplasts[17] can be related to the antioxidant capacity of the plant leaves. The antioxidant capacity of the plant leaves which was determined using DPPH radical scavenging assay was then related to their red fluorescence intensity. With this method, the antioxidant capacity of the plant leaves can be qualitatively screened[18]. However, this method has only been applied on several plants[18]. In order to prove the applicability of this method in general, in this study the relation between red fluorescence and antioxidant capacity of leaves was extended across different plant families, including *Anacardiaceae* and *Piperaceae*. This antioxidant properties of selected young and mature leaves can be used for application in pharmaceutical industries.

2. Materials and method

2.1 Materials

DPPH and gallic acid monohydrate were purchased from Sigma-Aldrich (United States). Methanol was purchased from Merck (Germany). All of the chemicals were of analytical grade, and they were used without any purification. *Anacardium occidentale, Mangifera indica, Spondias dulcis, Piper betle* Linn., *Piper sarmentosum* and *Piper crocatum* leaves were provided from Brunei Darussalam.

2.2. Fluorescence imaging

Based on texture, colour, and size, young leaves from all of samples plants were separated from matured leaves. A small piece of specimen (3×3 cm^2) was taken from the young and matured leaves, and it was sandwiched between a glass slide and a cover glass. The specimen was mounted on the sample stage of a microscope (Nikon Eclipse 50iPOL, Japan). The bright field microscopic images of the specimen were obtained by focussing white light from halogen lamp at 380–750 nm onto the specimen through a condenser lens. (Nikon Achr, NA 0.40). The transmitted light was then passed through and an objective lens (Nikon; 20×; NA 0.40) into a camera (Nikon; DS-Fi1C).

Fluorescence images of the specimen was detected after 365-nm light excitation of high pressure mercury lamp. The excitation wavelength was selected by using a band pass filter between 360 and 390 nm. The excitation light was focused onto the specimen by the objective lens, and the backward emission from the specimen was passed through a dichroic mirror at 415 nm. The elastic scattering was then eliminated by putting a long pass filter at 435 nm before the signal was finally passed through into the camera. The captured bright-field and fluorescence images were instantaneously analysed and the images were carefully compared side by side. Both images were then cropped with the same width and height. This is necessary to ensure the same area is selected for analyses.

The fluorescence intensities were analysed based on the brightness. In this case, the dark segment of the fluorescence images were used as the internal standard. The fluorescence images were assessed by RGB (red, blue, green) colour analysis using NIH ImageJ software. The red fluorescence intensity was used in the qualitative analysis of the antioxidant of the leaves.

2.2 DPPH assay

In the present study, the antioxidant properties of the plant leaves were evaluated by DPPH radical scavenging assay.

The separated young and matured leaves were dried in dehumidified room at ambient temperature, ground into powder, and then was subjected to Soxhlet extraction. It is similar method used to obtain the ethanol extract of *C. sinensis (L.)* peels[9]. Typically, the powder was refluxed in methanol with the ratio of 1:30 w/v for 5 h. The methanol extracts were then filtered through a Whatman No.1 filter paper, and the supernatant was dried using rotary evaporation. The crude extract was then scraped off and collected. The crude extract was dissolved in methanol with concentrations ranged from 1 to 500 mg L^{-1}. The absorbance of the solutions at wavelength of 517 nm was measured using a single beam UV-VIS spectrophotometer (Thermo Scientific™, Pittsburgh). Radical scavenging activity (RSA) was calculated using Eqn. (1):

$$RSA\ (\%) = \frac{(A_0 - A_S)}{A_0} \times 100\ \% \qquad (1)$$

Here, A_0 and A_s is the absorbance of DPPH solution in the absence and in the presence of the extract. The RSA values were then plotted as a function of concentration of the extract, from which the IC_{50} was determined[19].

To obtain the total antioxidant capacity (TAC) of the extract, the IC_{50} values of the plant leaves extracts were compared with the IC_{50} of gallic acid, as a standard antioxidant, which was determined with the same experimental conditions. The TAC of the extract was expressed in mmol gallic acid equivalent (GAE) per g extract.

3. Results and discussion

Fig. 1 shows the bright-field and fluorescence images of young and matured leaves of *A. occidentale, M. indica, S. dulcis*, belonging to *Anacardiaceae* as well as those of *P. betle* Linn., *P. sarmentosum*, and *P. crocatum* of *Piperaceae* family. The results clearly indicated that the size of cells in young leaves in this study are much less compared in matured leaves, suggesting that the young leaves have a higher density of plant cells. Upon UV light

excitation, the various compounds bound in the cells would emit different fluorescence wavelengths. Typically, the blue and green fluorescence have been attributed to ferulic and hydroxycinnamic derivatives, which are bound to the cell wall or mesophyll, whereas red fluorescence has been assigned to be due to the chlorophylls inside the chloroplast[20-23]. By ImageJ software, the distribution of RGB fluorescence was obtained and the red fluorescence intensity was considered as an indicative of the antioxidant activity.

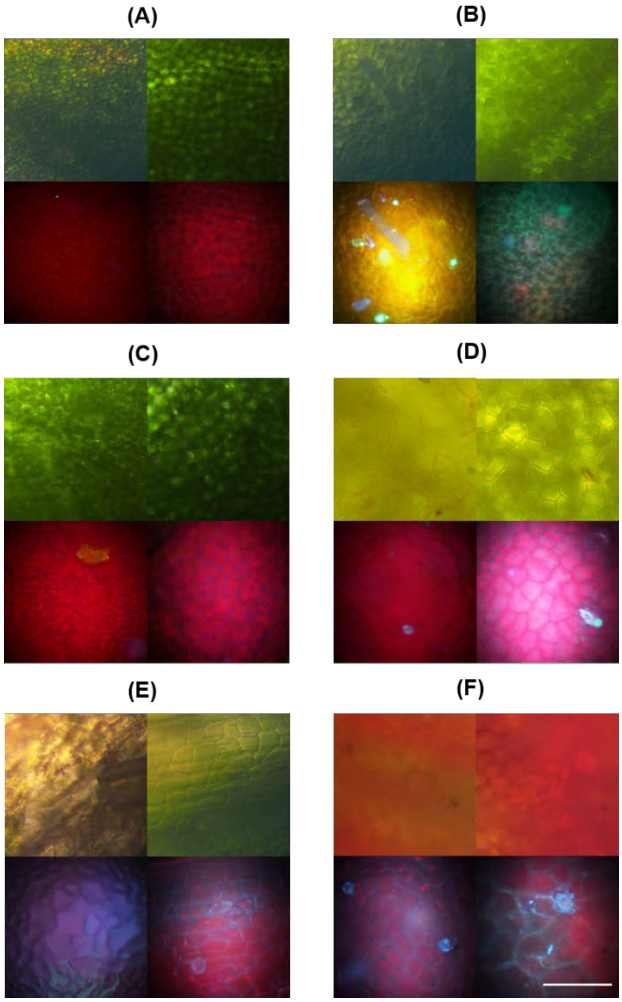

Fig. 1: Bright-field and fluorescence images of young leaves (left column) and matured leaves (right column) of *A. occidentale* (A) *M. indica* (B) *S. dulcis* (C) *P. betle* Linn. (D) *P. sarmentosum* (E) *P. crocatum* (F). The scale bar of 5 mm is applied for all images.

The antioxidant and fluorescence characteristics of the leaves were found to be sensitive to the maturity of the leaves. The red fluorescence intensity of young leaves was higher than matured leaves, as it has also been pointed out by Meyer et al.[24] One might anticipate that the higher red fluorescence intensity of the young leaves could be related to their higher number density of cells [25]. As TAC of plant leaves can be considered to be due to phenolic, flavonoid, and flavanol compounds occurred in their cells. These compounds can be used as an active compound for developing new adsorbents that further can be used as nitrogen removal for pharmaceutical industry. This finding strongly indicates that the antioxidants along with other bio- and photo-active compounds are bound to the protein complexes inside the chloroplasts of the leaves [17]. It can be rationalized that TAC decreases with the number density of cells per unit volume [25]. This is clearly reflected by the decrease of both TAC and red fluorescence of by young and matured leaves of plants of Anacardiaceae and Piperaceae in Fig. 1(A) – (F). It is explained by considering the size of cell and the total chlorophylls in the cell. One might consider that from young to matured leaves, the number of chlorophylls in the cell increased but less than the increase in the cell size. This resulted in lower TAC and red fluorescence intensity, as it was observed for the leaves of *P. betle* Linn. The decrease in the fluorescence of leaves of grasses with their maturity has also been reported [26]. It is interesting to note that the red fluorescence intensity of young *P. crocatum* leaves is much higher than the matured leaves due to the contribution of back reflection from the natural red color of pigments in the leaves, rather than the antioxidants bound in the leaves. Therefore, even the red fluorescence intensity and TAC of young leaves of *Anacardiaceae* and *Piperaceae* were found to have the same trend with the matured ones, but their relationship yet cannot be precisely and straightforwardly quantified. In this sense, further empirical and numerical analyses on fluorescence and the antioxidant capacity of large number of leaves from different plants and specific chemical compounds isolated from the leaves are essentially of interest. Although antioxidants might be mainly bound in the chloroplasts, the spatial distribution and relative intensity of red fluorescence characteristic may be further utilized as probe for the locations of the antioxidants in leaves [25,27].

Since the antioxidant capacity of plant leaves is most probably due to compounds inside the chloroplast which might be related to the red fluorescence, the red fluorescence intensity of the leaves is shown in Fig. 2. It is clearly observed that the young leaves of *Anacardiaceae* show higher fluorescence intensity than the matured leaves, meanwhile the opposite trend was detected for *Piperaceae* leaves. This implies that the fluorescence intensity, which is an indication for the different antioxidant capacities of the leaves, depends on plant species and maturity of their leaves. It is noted that the red fluorescence of plant leaves can be used to distinguish the antioxidant capacity of the different maturity of leaves from the same plants. In general, the TAC values roughly show the same and across the plant family. Thus, this method would be potentially applicable as a green and non-invasive of lab-on-chip device[28] to screen the antioxidant capacity of leaves from various plant families and other possible agricultural applications[29].

To verify the abovementioned finding, the IC_{50} of the

methanol extracts of the plant leaves was quantified by the radical scavenging assay, as presented in Table 1. The IC_{50} values of *A. occidentale*, *M. indica*, and *S. dulcis* young and matured leaves were in the range of 1.8 to 6.2 mg L^{-1}, related to the TAC of 2.55 to 8.52 mmol GAE per g extract. The IC_{50} values of *P. betle* Linn., *P. sarmentosum* and *P. crocatum* young and matured leaves were ranged 18.29 to 473.16 mg L^{-1}, equivalent to 0.03 to 0.84 mmol GAE per g extract.

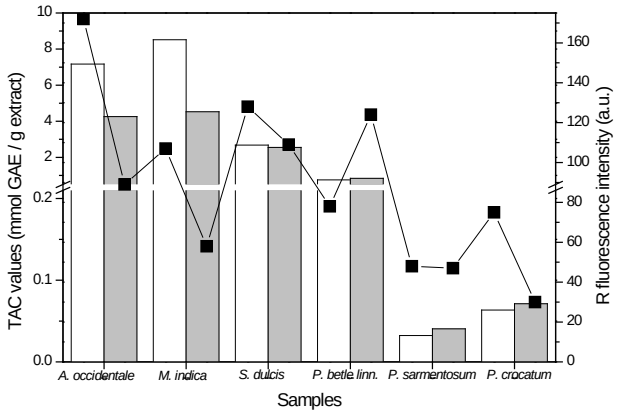

Fig. 2: The TAC and R fluorescence intensity of young leaves (white bars) and matured leaves (grey bars) of *A. occidentale, M. indica, S. dulcis, P. betle* Linn., *P. sarmentosum* and *P. crocatum* and their respective R fluorescence intensity (■). The solid line in the graph is only for guidance

It may be noted that the TAC of methanolic extracts of *A. occidentale* leaves is higher than that of water-methanol (20:80) infusion[1)], suggesting that methanol can extracts more antioxidants from leaves as compared with water. Interestingly, as presented in Table 1, the young leaves exhibit higher TAC compared to matured leaves for the plants belonging *Anacardiaceae*, whereas the opposite trends was found for the plants belonging *Piperaceae*. This finding is generally in agreement with the trends for red fluorescence intensity of the leaves (see Fig. 2). This confirms unambiguously the correlation between the red fluorescence intensity and TAC for the leaves of plants belonging to both *Anacardiaceae* and *Piperaceae*.

Table 1. The IC_{50} values, TAC values and red fluorescence intensity of leaves of *Anacardiaceae* and *Piperaceae* plants

Sample		IC_{50} [a)]	TAC [b)]	R [c)]
A. occidentale	Young	2.2	7.17	172
	Matured	3.7	4.26	89
M. indica	Young	1.9	8.52	107
	Matured	3.5	4.53	58
S. dulcis	Young	5.9	2.68	128
	Matured	6.2	2.55	109
P. betle Linn.	Young	20.3	0.75	78
	Matured	18.3	0.84	124
P. sarmentosum	Young	473.2	0.03	48
	Matured	377.8	0.04	47
P. crocatum	Young	240.8	0.06	75
	Matured	214.7	0.07	30

[a)] in mg L^{-1}; [b)] in mmol GAE/g extract; [c)] red fluorescence intensity (a.u.).

4. Conclusion

Conventionally, the methods to determine the antioxidant capacity are based on chemical and electrochemical analyses. These analyses involve long processes of sample preparations and consume chemicals. In the present study, the red fluorescence characteristics of different maturity of leaves of *A. occidentale, M. indica, S. dulcis* belonging to *Anacardiaceae* and *P. betle* Linn., *P. sarmentosum*, and *P. crocatum* of *Piperaceae* family were analyzed using fluorescence imaging microscopic method. The total antioxidant capacity of the methanol extracts of the respective plant leaves has also been evaluated by DPPH radical scavenging assay. From young to matured leaves of plants belonging to *Anacardiaceae* and *Piperaceae*, the red fluorescence and the total antioxidant capacity show the same trends. This highlighted that along with the chlorophyll the antioxidants most likely are occurred and bound to the protein complexes inside the chloroplasts of the plant leaves. This work implies that fluorescence imaging micro spectroscopy would be a promising green and non-invasive technique to screen the antioxidant capacity of any plant leaves of different species and families. This also can be used as preliminary study for developing the active compound for preparing of adsorbents and also can be used as nitrogen removal for pharmaceutical industry.

Acknowledgements

EK thankful for the financial support from Kemenristek/BRIN, Indonesia through through Tesis Magister Project-2020 year entitled," Design of adsorbent synthetic zeolite- and pectin-based as nitrogen removal for pharmaceutical industry". The authors are also grateful for all facilities provided by Universiti Brunei Darussalam.

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