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# Anti-oxidative assays as markers for anti-inflammatory activity of flavonoids

Wasaporn Chanput [a,*], Narumol Krueyos [a], Pitiporn Ritthiruangdej [b]

[a] Department of Food Science and Technology, Faculty of Agro-Industry, Kasetsart University, 50 Ngamwongwan Rd., Ladyoaw, Chatuchuck, Bangkok 10900, Thailand
[b] Department of Product Development, Faculty of Agro-Industry, Kasetsart University, 50 Ngamwongwan Rd., Ladyoaw, Chatuchuck, Bangkok 10900, Thailand

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ABSTRACT

The complexity of *in vitro* anti-inflammatory assays, the cost and time consumed, and the necessary skills can be a hurdle to apply to promising compounds in a high throughput setting. In this study, several antioxidative assays *i.e.* DPPH, ABTS, ORAC and xanthine oxidase (XO) were used to examine the antioxidative activity of three sub groups of flavonoids: (i) flavonol: quercetin, myricetin, (ii) flavanone: eriodictyol, naringenin (iii) flavone: luteolin, apigenin. A range of flavonoid concentrations was tested for their antioxidative activities and were found to be dose-dependent. However, the flavonoid concentrations over 50 ppm were found to be toxic to the THP-1 monocytes. Therefore, 10, 20 and 50 ppm of flavonoid concentrations were tested for their anti-inflammatory activity in lipopolysaccharide (LPS)-stimulated THP-1 monocytes. Expression of inflammatory genes, IL-1β, IL-6, IL-8, IL-10 and TNF-α was found to be sequentially decreased when flavonoid concentration increased. Principle component analysis (PCA) was used to investigate the relationship between the data sets of antioxidative assays and the expression of inflammatory genes. The results showed that DPPH, ABTS and ORAC assays have an opposite correlation with the reduction of inflammatory genes. Pearson correlation exhibited a relationship between the ABTS assay and the expression of three out of five analyzed genes; IL-1β, IL-6 and IL-8. Our findings indicate that ABTS assay can potentially be an assay marker for anti-inflammatory activity of flavonoids.



## 1. Introduction

Antioxidants have been proven to prevent injury caused by free radicals in different mechanisms such as; inhibition of radical formation, promoting free radical decomposition and free radical scavenging [1]. For example, flavonoid antioxidants can directly scavenge superoxide and peroxynitrite. They can also inhibit the reaction of free radicals in the nitric oxide signaling pathway in different types of cells [2]. Resveratrol has been shown to have an impact on NADPH oxidase activity and NF-κB which contribute to vascular oxidative stress and low-grade chronic inflammation in diabetic patients [3]. Mitigation of oxidative-stress diseases by antioxidants, for example Alzheimer's, Crohn's, chronic kidney, airway inflammatory and coronary artery disease have been reported [4–6]. It has been shown that a high concentration of ROS triggers the expression of NF-κB, a transcription factor of pro-inflammatory genes, namely, TNFα, IL-6, IL-1β, COX-2 and iNOS. Oxidative stress causes expression of the cyclooxygenase (COX) and lipoxygenase (LOX) genes which play a role in initiating inflammatory mediators, in which flavonoid antioxidants have been proven to act as anti-inflammatory compounds [7,8]. Therefore, interaction exists between oxidative stress and inflammation and between antioxidative and anti-inflammatory activity.

In order to determine anti-inflammatory activity, *in vitro*, *ex-vivo* or *in vivo* studies have to be performed. Though an *in vitro* assay is the least complex, it is still relatively expensive, time consuming and requires advanced operator skill. Another obstacle is to have lipopolysaccharide (LPS) free-test compounds to prevent undesirable immune responses which limit an application range of food materials. Most of antioxidative assays rely on the basis of a chemical reaction. The determination is based on changes in color using a spectrophotometer. Since there are many studies have shown the relation between antioxidants and inflammatory disorders in animal models [4,6,9,10], thus antioxidative assays might be able to be a marker for anti-inflammatory activity.

The basic structure of a flavonoid is composed of three rings as shown in Fig. 1. Flavonoids can be classified into 6 subgroups in which they differ in substitution of the C ring [11]. Flavonoids are well recognized for their pharmacological activities, for example, as antioxidant, antitumor, antibacterial and anti-inflammatory agents. Such activities of flavonoids are structure dependent [12]. In this study, we examined antioxidative activity and anti-inflammatory activity of three flavonoid subgroups; (i) flavonol: quercetin, myricetin, (ii) flavanone: eriodictyol, naringenin, (iii) flavone: luteolin, apigenin (structure illustration are

* Corresponding author.
*E-mail address:* wasaporn.c@ku.ac.th (W. Chanput).

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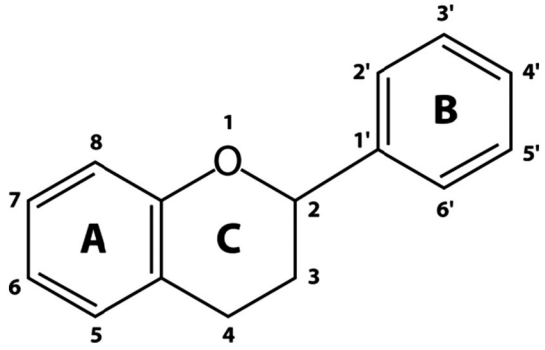


**Fig. 1.** Basic flavonoid structure.

shown in the Discussion). The criteria for selection of flavonoids were based on the abundance in either fruits, vegetables, herbs or spices [13].

Chanput et al. (2014) [14], has reviewed the extensive use of THP-1 cell line to study modulation of monocytes and macrophages functions and responses from external stimuli, for instance, nutrients and drugs. Signaling pathways and mechanisms of monocytes and macrophages have been successfully performed using the THP-1 cell line. Similarities between the THP-1 cell line and human peripheral blood mononuclear cells (PBMCs) were found to be comparable [15–17], which makes THP-1 cell line to be a reliable model for immune modulation approach.

In this study, flavonoids, as a set of model compounds, were used with the aim to investigate whether a relation between antioxidative assays and anti-inflammatory activity using THP-1 cell line could be found.

## 2. Materials and methods

### 2.1. Chemicals

All chemicals were procured from Sigma-Aldrich (St. Louise, MO, USA.). Six flavonoids with a purity ≥95%; luteolin (L9283), apigenin (A3145), quercetin (Q4951), myricetin (M6760), naringenin (N5893) and eriodictyol (89061) were dissolved in 95% ethanol to make stock solutions. RNA isolation and cDNA synthesis kits were purchased from Thermo Fisher Scientific (Waltham, MA, USA.). iQ™ SYBR® Green Supermix was purchased from Biorad (Hercules, *CA*., USA.).

### 2.2. Anti-oxidative assays

#### 2.2.1. 2,2-diphenyl-1-picrylhydrazyl (DPPH assay)

The modified method of Du et al. (2009) was used [18]. Four μl of selected flavonoid concentrations were mixed with 300 μl of 25 ppm DPPH solution in a microtiter plate. After incubation for 30 min in the dark, the absorbance was measured at 517 nm. The % DPPH scavenging activity of samples and Trolox standard are calculated as:

$$\%\text{DPPH} = \left(1 - \text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}\right) \times 100$$

#### 2.2.2. 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS assay)

The modified method of Nenadis et al. (2004) was used [19]. Briefly, 195 μl of 7 mM ABTS solution was added to 5 μl of defined flavonoid concentrations in a microtiter plate. After incubating for 6 min in the dark, the absorbance was measured at 734 nm. The % ABTS scavenging activity of samples and Trolox standard are calculated as:

$$\%\text{ABTS} = \left(1 - \text{Abs}_{\text{sample}}/\text{Abs}_{\text{control}}\right) \times 100$$

#### 2.2.3. Oxygen radical absorbance capacity (ORAC assay)

Flavonoid stock solution in 95% ethanol was further diluted with phosphate buffer pH 7.0 to obtain the defined concentrations. The assay is carried out by the modified method of Li and Beta (2011) [20]. Briefly, the peroxyl radical was generated by 2′2-azobis(2-amidinopropane) dihydrochloride (AAPH). The plate containing 200 μl fluorescence working solution and 20 μl flavonoid compounds was incubated at 37 °C for 15 min. After 75 μl AAPH was added into the solution, the reaction was immediately monitored. The relative fluorescence intensity with an excitation wavelength of 485 nm and an emission wavelength of 535 nm was measured every minute. The total reaction time was 50 min. Trolox was used as a standard. The ORAC values were calculated as:

$$\text{Net AUC} = \text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}}$$

#### 2.2.4. Xanthine oxidase (XO)

The modified method from xanthine oxidase activity colorimetric assay of Biovision® (Cat. No. K710-100) was used. The $H_2O_2$ is produced from the synthetic xanthine-xanthine oxidase (X-XO) reaction system. Each reaction contained 44 μl assay buffer, 2 μl substrate mix (xanthine), 2 μl enzyme (xanthine oxidase), 2 μl OxiRedTM Probe and 40 μl flavonoid compound. The amount of $H_2O_2$ was measured at 570 nm. The assay was based on the scavenged $H_2O_2$ after 35 min of incubation with flavonoids.

### 2.3. Anti-inflammatory activity

The human monocytic leukemia cell line THP-1 (ATCC, USA) was grown in RPMI 1640 culture medium (Lonza, Switzerland) supplemented with fetal bovine serum (FBS, Invitrogen, UK) and penicillin/streptomycin (Invitrogen) at respectively 10% and 1%, in a 5% $CO_2$ humidified incubator at 37 °C. THP-1 monocytes at the exponential growth phase were simultaneously stimulated with 50 ng/ml LPS and flavonoids for 3 h [21]. Cells were harvested for RNA isolation.

#### 2.3.1. Cytotoxicity assay

Cytotoxicity was determined by trypan blue and the MTT assay as described by Chanput et al. (2012) [22]. For the MTT assay, THP-1 monocytes were exposed to flavonoid compounds in the concentration of 10–250 ppm for 6 and 24 h. After that cells were lysed with 10 μl DMSO:ethanol (1:1) and the absorbance was measured at 570 nm. The results were expressed relatively to the control (non-stimulated cells).

#### 2.3.2. Gene expression of inflammatory genes

Total RNA was isolated from the harvested THP-1 cells using the GeneJET RNA purification kit (Thermo Fisher Scientific). The purity and quality of the isolated RNA were determined using NanoDrop and agarose gel electrophoresis under UV detection. 200 μg of the isolated RNA was used to make complementary DNA (cDNA) using the cDNA synthesis kit (Thermo Fisher Scientific). The synthesized cDNA was mixed with IQ™ SYBR Green Supermix (Bio-Rad) according to manufacturer's protocol. The real-time qPCR (CFX96 Touch™, Bio-Rad) condition was used as previously published [21]. All primer sequences (IL-1β, IL-6, IL-8, IL-10 and TNF-α genes) in this study were obtained from Chanput et al. (2010), which have been validated prior use by dilution series of cDNA from LPS stimulated THP-1 monocytes to analyze PCR efficiency. Relative gene expression was calculated using the 2^-ΔΔCt method [21]. Unstimulated cells and GADPH gene-expression were used for normalization. The experiments were performed with two technical and two biological replicates.