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Anti-inflammatory and Antioxidant Activity of Quercetin-3, 3', 4'-Triacetate

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ABSTRACT

Quercetin (3,5,7,3',4'-pentahydroxyflavone) was chosen as the lead compound in development of the anti-inflammatory agent due to its low gastric ulcer side effect in addition to its COX-2 and iNOS inhibitory activities. This study aims to obtain data showing antioxidant potency and anti-inflammatory activity of quercetin-3, 3' and 4'-triacetate. Antioxidant potency was determined by DPPH radical scavenging and lipid peroxidation inhibitory methods, while anti-inflammatory activity was determined using carrageenan induced-rat paw oedema test. The results showed that the anti-inflammatory activity of quercetin-3, 3' and 4'-triacetate was significantly higher than that of quercetin. Percentage of oedema inhibition of quercetin-3, 3', 4'-triacetate and quercetin was 14-49 and 2-8%, respectively. However antioxidant activity of quercetin-3, 3' and 4'-triacetate was lower than that of quercetin. IC₅₀ of quercetin-3, 3', 4'-triacetate and quercetin applying DPPH radical scavenging method was 325.57 and 16.23, respectively, while that of lipid peroxidation inhibitory method was 161.5 and 5.25, respectively.

Key word: Anti-inflammatory, DPPH radical scavenger, lipid peroxidation, quercetin, quercetin-3, 3', 4'-triacetate, rat paw oedema

INTRODUCTION

Anti-inflammatory activity of flavonoids has been object of diverse reviews and it has been demonstrated that they are able to inhibit a series of enzymes which are activated in inflammatory process. Prostaglandins and nitric oxide biosynthesis is involved in inflammation and isoforms of inducible nitric oxide synthase (iNOS) and of cyclooxygenase (COX-2) are responsible for the production of a great amount of these mediators (Gonzalez-Gallego *et al.*, 2007). Furthermore, it is well known that Reactive Oxygen Species (ROS) generating in a situation of oxidative stress plays an important role in inflammation. The ROS released from activated neutrophils and macrophages damages macromolecules and lipid of membranes, leads to tissue injury. ROS also aggravates inflammation by stimulating various mediators of inflammation. Consequently, compounds with antioxidant properties such as some of flavonoids, could be expected to have anti-inflammatory activity (Melagraki *et al.*, 2009; Conforti *et al.*, 2009).

Quercetin (3,5,7,3',4'-pentahydroxyflavone, 1) is one of the most abundant flavonoid in the human diet. Quercetin blocked both the cyclooxygenase and lipooxygenase pathways at relatively high concentrations, while at lower concentrations it blocked mainly the lipooxygenase pathway

(Guardia *et al.*, 2001). *In vitro* studies have confirmed that the flavonoid quercetin inhibits nitric oxide production in IL-1 β -stimulated hepatocytes through the inhibition of iNOS expression (Martinez-Florez *et al.*, 2005).

In addition of its anti-inflammatory activity, quercetin also has gastric ulcer protective effect, by the reduction in the lipid peroxidation and an increase in the activity of antioxidant enzymes (Coskun *et al.*, 2004). These properties make quercetin as anti-inflammatory agent with no gastrointestinal side effect. However, quercetin has not been used widely in therapeutic medicine because it is practically insoluble in water or in oil and many other reasons such as low of oral absorption and bioavailability (Peng *et al.*, 2008). It is therefore important for the molecular modification to enhance the solubility and bioavailability of quercetin.

Some acetic acid esters of quercetin were reported enhanced the anti-inflammatory activity of quercetin. Chen *et al.* (2001) reported that quercetin pentaacetate showed the stronger inhibitory activity on prostaglandin E2 (PGE2) production and COX-2 protein expression, compared to quercetin. We have reported that product of mono acetylation of quercetin, i.e., quercetin-3-monoacetate showed higher anti-inflammatory activity compared to the parent compound (Herowati *et al.*, 2008).

Following our previous result in development of quercetin as anti-inflammatory agent, this study aims to obtain data showing antioxidant potency and anti-inflammatory activity of quercetin-3, 3', 4'-triacetate. Antioxidant potency was determined by 2,2-diphenyl-2-picrylhydrazyl DPPH radical scavenging and lipid peroxidation inhibitory methods, while anti-inflammatory activity was determined using carrageenan induced-rat paw oedema test.

MATERIALS AND METHODS

The research was conducted at Medicinal Chemistry Laboratory and Pharmacology and Toxicology Laboratory of School of Pharmacy, Institut Teknologi Bandung, Indonesia during February to December 2009.

Materials: Quercetin, λ -carrageenan, DPPH (Sigma-Aldrich), acetic anhydride, dimethyl formamide, sodium acetate, sodium chloride, ethyl acetate, MgSO₄, ethanol, methanol, tris-HCl buffer (0.01 M, pH 7.4), FeSO₄, trichloroacetic acid, HCl, thiobarbituric acid, sodium diclofenac.

Animals: Health male Wistar albino rats, weighing 150-200 g and averaging 12-16 weeks old were utilized in this study. Twenty five of rats were randomly divided into groups of six, i.e., control, quercetin, quercetin-3-3'4'-acetate dan sodium diclofenac group. They were housed in cages under standard laboratory conditions (12 h of light period, 27 \pm 3 $^{\circ}$ C). The animals were given standard rat pellets and tap water ad libitum. All animals received human care according to the protocol approved by local Health Research Ethics Committee (Faculty of Medicine Padjajaran University-Dr. Hasan Sadikin General Hospital).

Chemistry: Figure 1 outlines the synthesis of quercetin-3, 3' and 4'-triacetate. Thin Layer Chromatography (TLC) was performed by using precoated silica gel plates (Merck 60 GF254) which were detected by short UV light and ammonia vapor. Melting points were measured on melting point apparatus and are uncorrected. UV spectra were performed on spectrophotometer UV-Vis Beckman DU 6501. IR spectra were performed on Spectrophotometer JASCO FT/IR NMR spectra were performed on spectrometer NMR JNMECA-500, JEOL (500 MHz), using DMSO-d₆ as solvent

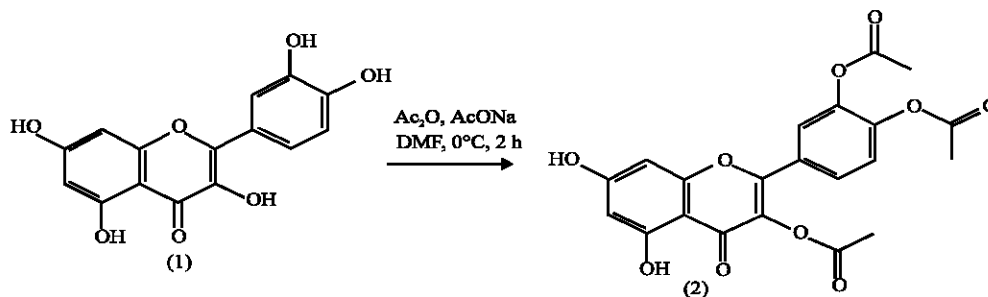


Fig. 1: Synthesis of Quercetin-3, 3', 4'-triacetate

and TMS as an internal standard. Chemical shifts (δ) are expressed in ppm downfield from TMS. Column chromatography was performed on 70-230 mesh silica gel from Merck.

Quercetin-3, 3', 4'-triacetate (2) was synthesized based on procedure by Herowati *et al.*, (2008). Anhydrous quercetin (3.38 g, 10 mmol) was dissolved in 10 mL of dimethyl formamide. Dry sodium acetate (1.64 g, 20 mmol) and acetic anhydride (1.2 mL, 12 mmol) were added. The mixture was reacted at 0°C for 2 h and then 10 mL of aqueous saturated sodium chloride solution was added. The mixture was extracted 3 times using 10 mL of ethyl acetate. Ethyl acetate phase was washed twice using water and dry MgSO_4 was added to remove the water. The crystal was obtained after vacuum evaporation of the solvent. The product was separated using flash chromatography. This yielded 0.85 g (19.9%) of yellowish white crystal. Melting point 167.5-168.5°C, $^1\text{H NMR}$ (CDCl_3), δ (ppm): 2.12 (s, 3H, $\text{CH}_3\text{-C}=\text{O}$), 2.40 (s, 6H, $\text{CH}_3\text{-C}=\text{O}$), 6.74 (d, 1H, H_6), 7.10 (d, 1H, H_8), 7.44 (d, 1H, H_9), 7.61 (d, 1H, H_2), 7.87 (dd, 1H, H_9); MS spectrum (m/z) : 428, 386, 344, 302.

Biology

DPPH radical scavenging test: The stable 2, 2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of the test compounds were determined according to the method by Orhan *et al.* (2009) at 5, 12.5, 25, 50 and 100 μM concentrations. Each solution (1.5 mL) was mixed with DPPH solution (1.5 mL of 0.1 M) and vortexed. Remaining DPPH amount, which is characterized by a decrease in absorption, was measured at 520 nm. Inhibition of DPPH in percent (I%) was calculated as:

$$\text{I\%} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100$$

where, A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample) and A_{sample} is the absorbance of the test compounds.

Lipid peroxidation inhibitory test: Lipid peroxidation was assayed by the method of Selvam and Kurien (1987) in which malondialdehyde (MDA) released served as the index of lipid peroxidation. Liver homogenate (10%) was made in Tris-HCl buffer (0.01 M, pH 7.4). To each tube, 2.2 mL of buffer, 0.2 mL of FeSO_4 (10 mM) and 0.6 mL of the tissue homogenate were added. The tubes were incubated on a mechanical shaker for 60 min. After the incubation 0.5 mL of 40% trichloroacetic acid (TCA) and 0.25 mL of 5 N HCl were added and the contents mixed thoroughly. This was followed by the addition of 0.5 mL of 2% thiobarbituric acid. The tubes were shaken and incubated in a water bath at 90°C for 20 min. They were then cooled and 3 mL of chloroform was added. After thorough mixing they were centrifuged for 15 min at 3000 g. The supernatant was aspirated and its absorbance at 532 nm determined using water blank.

Carrageenan induced-rat paw oedema test: Carrageenan induced rat paw oedema was done by modified technique of Posadas *et al.* (2004). Animals were fasted 18 h prior to the experiment. Test compounds were administrated orally (equivalent dose with 20 mg kg⁻¹ of quercetin) 30 min before subplantar injection of 0.1 mL of a 1% solution of λ-carrageenan in 0.9% saline on the left hind paw. Paw volumes were measured plethysmographically immediately before induction of oedema and hourly from 1-6 h. Sodium diclofenac (4.5 mg kg⁻¹) was used as reference drug. The increase in paw volume was calculated by subtracting the initial paw volume (basal) to the paw volume measured at each time point.

RESULTS

DPPH radical scavenging test: Scavenging activity of quercetin was raised up from lowest concentration (5 μM) and reached the 93% of inhibitory activity at 50 μM. The IC₅₀ value of quercetin was 16.23 μM. However, the inhibitory activity of quercetin-3, 3' and 4'-triacetate at concentration of 100 μM was only under 20%, significantly decrease compared to that of quercetin. The IC₅₀ value of quercetin-3, 3', 4'-triacetate was 325.57 μM, indicated that the antioxidant activity of these compounds was very low (Fig. 2, Table 1).

Lipid peroxidation inhibitory test: At concentration of 5 μM quercetin showed 37% of lipid peroxidation inhibitory activity and reached the 83% of inhibitory activity at 100 μM. The IC₅₀ value of quercetin was 5.25 μM. However, the inhibitory activity of quercetin-3, 3' and 4'-triacetate at concentration of 100 μM was only under 30%, significantly decrease compared to that of quercetin. The IC₅₀ value of quercetin-3, 3' and 4'-triacetate was 161.5 μM (Fig. 2, Table 1).

Carrageenan induced-rat paw oedema test: Figure 3 showed the percentage of oedema inhibitory of test compounds. Inhibitory activity after oral administration of 20 mg kg⁻¹ b.wt. of

Table 1: Antioxidant activity of the test compounds

Compound	IC ₅₀ (μM)	
	DPPH test	Lipid peroxidation test
Quercetin	16.23	5.25
Quercetin-3,3',4'-triacetate	325.57	161.5

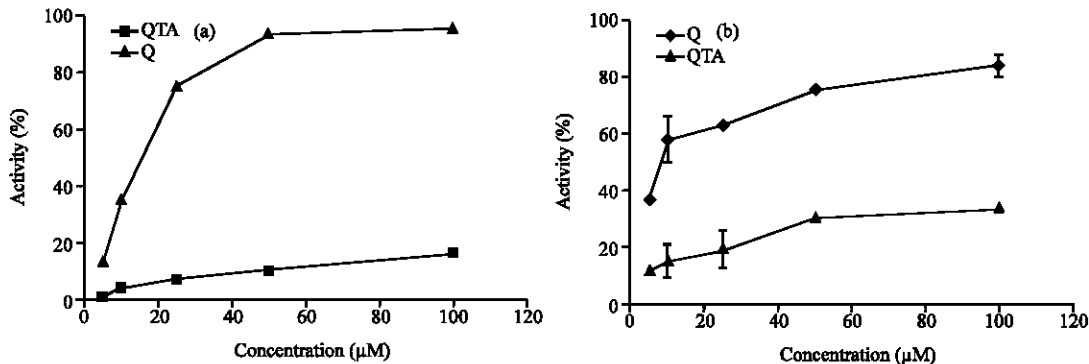


Fig. 2: DPPH Radical Scavenger Activity (left) and Lipid Peroxidation Inhibitory Activity (right) of quercetin and quercetin -3, 3', 4'-triacetate (QTA)

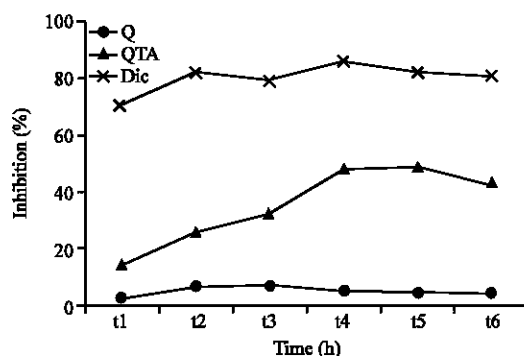


Fig. 3: Percentage of oedema inhibition of the test compounds

quercetin only below of 10%. At equimolar dose of quercetin, quercetin-3, 3' and 4'-triacetate showed higher oedema inhibitory activity than quercetin. Sodium diclofenac showed the highest inhibitory activity.

DISCUSSION

The DPPH is a stable free radical which accepts an electron or hydrogen radical to become a stable diamagnetic molecule. The reduction capability of DPPH radicals was determined by the decrease in its absorbance (Londonkar and Kamble, 2009). Several studies reported the strong DPPH radical scavenging activity of quercetin (Johnson and Loo, 2000; Amic *et al.*, 2003). However, this study resulted that selective acetylation of quercetin, produced quercetin 3, 3', 4'-triacetate and significantly decreased its activity. Amic *et al.* (2003) and Zhang (2005) summarized the structural criteria that modulate the free radical scavenging activity of flavonoids, i.e., 3',4'-dihydroxy (catechol structure) in the B ring, the 3-OH moiety of the C ring and the C2-C3 double bond conjugated with a 4-keto group. In case of quercetin 3,3',4'-triacetate, the absence of the catechol structure in the B ring decreased the antioxidant potency, which this moiety was needed for electron donating properties and being a radical target.

It is well known that the free radical scavenging activity of flavonoids is highly dependent on the presence of 3-OH group. Thus the substitution of 3-OH by a methyl or glycosyl group decreased the antioxidant activity (Soobrattee *et al.*, 2005). However, Saija *et al.* (2003) reported that DPPH radical scavenging activity of 3-O-acetylquercetin was comparable to that of quercetin. In case of quercetin-3,3',4'-triacetate, acetylation of 3-OH group together with O-cathecol group, perfectly decrease the antiradical activity of quercetin.

Antioxidant activity of flavonoids, such as quercetin, can prevent the development of degenerative diseases, due to their protection effect against reactive oxygen species generated in the human body causing lipid peroxidation. High reactivity of hydroxyl substituent plays an important role in flavonoid antioxidant activity due to the hydroxyl substituent donates a hydrogen atom to radicals, stabilizes them and produces a relatively stable flavonoid radical. By trapping free radicals, flavonoids inhibit lipid peroxidation, thus prevent the formation of malondialdehyde, because they convert lipid peroxidation to stable products (Liao *et al.*, 2006). LPO inhibitory activity of quercetin-3,3',4'-triacetate was also significantly decreased, similarly to their DPPH radical scavenging activity,

Carrageenan-induced rat paw oedema is a widely used test to determine the anti-inflammatory activity of. Inflammation induced by carrageenan is acute, nonimmune, well-researched and highly reproducible (Morris, 2003). As shown in Fig. 2a and b, percent of oedema of quercetin was slightly decrease compared to the control group and the oedema inhibition of quercetin was only below 10%. However, quercetin 3,3',4'-triacetate significantly enhanced the oedema inhibitory.

The antioxidant and anti-inflammatory of such compounds were closely related based on the fact that reactive oxygen species (primarily superoxide anion and H₂O₂) were produced during the inflammation process by phagocytic cells. Furthermore, reactive oxygen species may are involved in the cyclooxygenase- and lipoxygenase-mediated conversion of arachidonic acid into proinflammatory intermediates. The role of reactive oxygen species in inflammatory process induced several investigation of anti-inflammatory activity of antioxidant compounds. Increasing of anti-inflammatory and decreasing of antioxidant activity of partial acetylated quercetin derivatives indicated that antioxidant property did not underlie the anti-inflammatory activity of quercetin and quercetin derivatives.

We suggested that acylation protected the hydroxyl group of quercetin from metabolism reaction such as methylation, sulfonation or glucuronidation, whereas these metabolites have no anti-inflammatory activity. Quercetin was rapidly glucuronated, sulfated and methylated after diffusing into the cytosol, resulted only very low levels of original compound was founded in blood or plasma, mostly as metabolites. Biasutto *et al.* (2007) reported that ester-based derivatives enhanced bioavailability of quercetin. Ester derivatives of quercetin may increase systemic quercetin concentration thus increase the anti-inflammatory activity.

It was concluded that acetylation of hydroxyl group at C3, C3' and C4' of quercetin enhanced its anti-inflammatory activity but decreased its antioxidant activity. *In vitro* experiment of COX inhibitory activity is ongoing to prove the mechanism action of quercetin-3,3',4'-triacetate; the results will be reported later.

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