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Quercetin as a Protease (á-Chymotrypsin) Inhibitor

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Abstract
Quercetin, isolated from the skin of red onion bulb, was methylated to afford quercetin 3,3',4',7-tetramethyl ether in 83.3% yield. Quercetin has displayed á-chymotrypsin inhibitory effect (IC_{50} = 88.9 ± 2.5 µg/ml), DPPH free radical scavenging activity (IC_{50} = 14.87 ìg/ml) and ferric reducing power (EC_1 = 112.94 ìM/l equivalent to 1 mM/l $FeSO_4.7H_2O$), whereas no enhancement of these biological activities was observed using its tetramethyl ether derivative.

Key words: *Allium cepa*, á-chymotrypsin, DPPH assay, FRAP assay, quercetin 3,3',4',7-tetramethyl ether

Introduction
Protease is a large family of enzymes, which catalyses the hydrolysis of proteins. Proteases not only catalyse the destruction of cellular proteins and peptides, but also help in the replication of some viruses by cleaving their precursor proteins. Proteases of HCV (NS3 protease) and HIV are key enzymes that accelerate the replication of viruses and are thus recognised as valid targets for anti-HCV and anti-HIV drugs. The excessive activity of proteases may cause several diseases such as pulmonary emphysema, arthritis, pancreatitis etc. Proteases also help in tumour growth (Patick & Potts 1998).

α–Chymotrypsin (EC 3.4.21.1), a serine protease mainly secreted from pancreas, catalyses the breakdown of polypeptide and proteins. This enzyme not only digests proteins from foods but also catalyses the degradation of body's own tissues in uncontrolled manner in diseases such as pancreatitis and cirrhosis (Wilcox 1970). α-Chymotrypsin activates epithelial sodium channel (EnaC) by proteolytic cleavage which results in cystic fibrosis (Rauh *et al.* 2010). Chymotrypsin along with cathepsin is responsible for the cleavage of interleukin 1-β (IL-1β) precursor into functional active IL-1â, which causes inflammatory arthritis (Stehlik 2009). Therefore, inhibition of α-chymotrypsin is important for treatment of several diseases.

Research on flavonoids has become popular since these polyphenols are ubiquitous in nature and are potential for various uses. Quercetin (**1**) is one of the most potent antioxidants among flavonoids and has displayed antiviral, antibacterial, anticarcinogenic and anti-inflammatory effects (Materska 2008, Bohm 1998). It is estimated that up to 1 g of flavonoid per day is taken by human from the dietary sources and quercetin derivatives cover 70% of the value (Formica & Regelson 1995, Hertog *et al.* 1993). In food, quercetin occurs in aglycone or bounded form with sugars, phenolic acids, alcohols etc. After ingestion, quercetin derivatives are hydrolysed in the gastrointestinal tract into aglycone and then absorbed and metabolised (Scalbert & Williamson 2000, Walle 2004). Despite, quercetin is a major natural constituent we intake, the impact of quercetin on human health and research on its bioactivity are still at the developmental stage. Recently, we have commenced a research program to study the quantitative structure-activity relationship (QSAR) of quercetin derivatives. Herein, we have isolated quercetin (**1**) from the onion skin and its tetramethyl ether derivative (**2**) was synthesized. These compounds were used to evaluate protease inhibition effect

(α-Chymotrypsin assay) as well as antioxidant capacity (DPPH and FRAP assays).

Methodology

General

Analytical thin layer chromatography (TLC) was performed on 0.2 mm pre-coated plate Kieselgel 60 F_{254}. Melting point was recorded using electrical melting point apparatus (OGAWA SEIKI Co. Ltd., Tokyo). NMR spectra in CD_3OD were recorded on Advance AV-500 at H.E.J. Research Institute of Chemistry, University of Karachi, Karachi, Pakistan. Spectramax M2, Molecular Devices was used in the measurement of enzyme inhibition and 6715 UV/Vis Spectrophotometer JENWAY was used for other spectrophotometry.

Isolation of quercetin (1)

Dried skin of red onion bulb (*Allium cepa*) (500 g), collected from a local shop, was extracted with MeOH (3 l) using a Soxhlet extractor. The methanolic extract was concentrated using a rotary evaporator under reduced pressure. To the obtained concentrate was added distilled water (300 ml) and extracted with Et_2O (500 ml × 3). Organic solvent was evaporated under vacuum to obtain a solid residue (5 g). This residue was fractionated by column chromatography on silica gel (60-120 mesh) with an increasing polarity gradient of EtOAc/MeOH (from 100:0 to 80:20) collecting 17 fractions of 100 ml. Fractions (F1-F7) were found to possess a distinct yellow spot with R_f = 0.88 together with other spots on TLC (EtOAc/MeOH, 9:1), which were combined. The pooled fraction was concentrated to obtain a yellow residue (2 g). This residue was further purified on silica gel column eluting with hexane/EtOAc (from 10:1 to 10:9) to afford a chromatographically pure yellow compound, which after recrystallisation in MeOH gave crystals of quercetin (3,3',4',5,7-pentahydroxy flavone; 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one (**1**), 500 mg, 0.1%). UV-Vis λ_{max} nm: (MeOH) 232, 255, 372; (MeOH + NaOAc) 232, 272, 377; (MeOH + NaOAc + H_3BO_3) 230, 262, 386; (MeOH + $AlCl_3$) 232, 273, 452; (MeOH + $AlCl_3$ + HCl) 229, 271, 359 (inf), 429; (MeOH + NaOH) 230, 288, 328 (inf), 425. ^{1}H NMR (CD_3OD) ä ppm: 6.2 (d, *J* = 1.9 Hz, H-8), 6.42 (d, *J* = 1.9 Hz, H-6), 6.9 (d, *J* = 8.5 Hz, H-5'), 7.56 (dd, *J* = 2.2, 2.2 Hz, H-6'), 7.68 (d, *J* = 2.2 Hz, H-2'), 9.33 (s, OH-3'), 9.35 (s, OH-4'), 9.63 (s, OH-3), 10.85 (s, OH-7), 12.49 (s, OH-5). These data are in close agreement with the findings of (Tuladhar and Bajracharya 1999).

Synthesis of quercetin 3,3',4',7-tetramethyl ether (2)

Dehydration of acetone was carried out in prior by treating with excess of K_2CO_3 for overnight followed by filtration and distillation at normal atmospheric pressure.

In a two-necked flask of 50 ml capacity equipped with magnetic stirrer, reflux condenser and $CaCl_2$ guard tube was added **1** (0.3 mmol, 92.2 mg) and dehydrated acetone (12 ml). To the stirred reaction mixture was introduced excess of anhydrous K_2CO_3 (1.21 g) and MeI (1.1 ml). After refluxing at 65 ÚC for 5 h, complete consumption of **1** was observed on TLC and the reaction mixture was cooled to room temperature followed by quenching with water (6 ml). The reaction mixture was neutralised by addition of 2N H_2SO_4 drop wisely and then extracted with EtOAc (15 ml × 3), washed with brine, dried with Na_2SO_4, filtered and finally concentrated. The residue was purified by column chromatography (200-400 mesh) using an increasing polarity gradient of hexane/EtOAc (from 100:0 to 25:75) to afford quercetin 3,3',4',7-tetramethyl ether (5-hydroxy-3,3',4',7-tetramethoxy flavone; 2-(3,4-dimethoxyphenyl)-5-hydroxy-3,7-dimethoxy-4*H*-chromen-4-one (**2**), 90.0 mg, 83.3% yield). Melting point = 156-158Oº C (Reported 156-161^{o}C) (Web 1). UV-Vis λ_{max} nm: (MeOH) 232, 256, 355; (MeOH + NaOAc) 232, 257, 350; (MeOH + NaOAc + H_3BO_3) 230, 256, 350; (MeOH + $AlCl_3$) 230, 272, 360, 402; (MeOH + $AlCl_3$ + HCl) 232, 272, 358, 402; (MeOH + NaOH) 231, 284, 374. ^{1}H NMR (CD_3OD) δ ppm: 3.82 (s, OMe), 3.89 (s, OMe), 3.917 (s, OMe), 3.924 (s, OMe), 4.53 (s, OH-5, interchangeable), 6.35 (d, *J* = 2.0 Hz, H-8), 6.66 (d, *J* = 2.0 Hz, H-6), 7.12 (d, *J* = 8.5 Hz, H-2'), 7.74 (d, *J* = 2.0 Hz, H-5'), 7.78 (dd, *J* = 2.0, 8.5 Hz, H-6'). The ^{1}H NMR data are in close agreement with the literature (Yoshioka *et al.* 2004).

á-Chymotrypsin assay

The inhibitory activity of á-chymotrypsin was performed in 50 mM *Tris*-HCl buffer pH 7.6 with 10 mM $CaCl_2$ (Choudhary *et al*. 2011). The enzyme α-chymotrypsin (12 units/ml prepared in the buffer) with the 0.5 mM sample prepared in DMSO, was incubated at 30 °C for 25 min. The reaction was initiated by the

addition of the chromogenic substrate, *N*-succinyl-L-phenylalanine-*p*-nitroaniline (SP*p*NA; 0.4 mM final concentration prepared in the buffer). The change in absorbance by release of *p*-nitroanilide was continuously monitored at 410 nm. The positive control without test compound was replaced by DMSO (final concentration 7%). The percentage of inhibition based upon initial velocity and calculated as:

$$\% \text{ Inhibition} = 100\text{-(minus)} \ (OD_{sample}/OD_{control}) \times 100$$

IC_{50} value (Inhibition of enzymatic hydrolysis of the substrate SP*p*NA by 50%) were determined by monitoring the inhibition value by the increasing the concentration of the test compounds. These IC_{50} values were calculated by using EZ-Fit enzyme kinetics program (Perellela Scientific, Inc., Amherst, Mars, USA).

2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

The DPPH assay was carried out according to the reported method (Brand-Williams *et al.* 1995). To 0.5 ml of the solution of test compounds prepared in different concentrations (5, 10, 15, 20 and 25 µg/ml in methanol) was added 0.1 mM methanolic DPPH$^{\cdot}$ solution (2.5 ml) and kept in the dark for 30 min before recording of absorbance at 517 nm against methanol. In the control tube, the sample was replaced by MeOH. The inhibition of initial absorbance of the DPPH$^{\cdot}$ solution was calculated by using the formula shown below. Regression analysis was carried out by using Microsoft Excel 2007 program to calculate IC_{50} (Concentration causing 50% inhibition) value.

$$\% \text{ of Inhibition} = (1 - A_{sample}/A_{control}) \times 100$$

Ferric reducing activity power (FRAP) assay

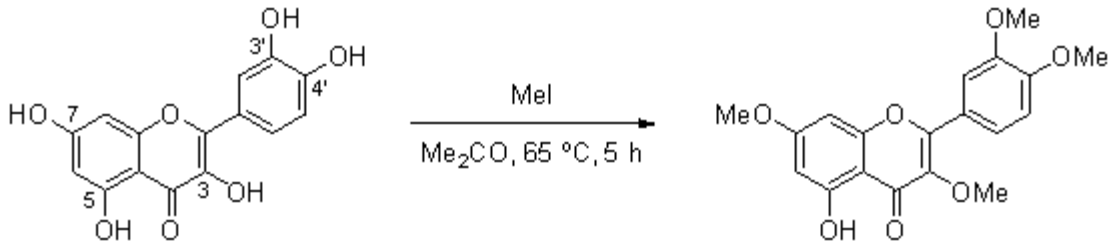
The FRAP assay measures the total antioxidant activity on the basis of the ability to reduce Fe(III) to Fe(II) ions (Benzie & Strain 1996). Working solutions of different concentrations of samples (5, 10, 15, 20, 25 µg/ml in methanol) and reference $FeSO_4.7H_2O$ (25, 100, 200, 300, 400 and 500 µg/ml in distilled water) were prepared. To each working solution (200 µl) was added 2800 µl of the FRAP reagent. After 4 min of incubation at 37 ^{o}C, the absorbance at 593 nm was measured against methanol/water (1:14) or water as a blank. The obtained data were used to calculate EC_1 (Effect concentration equivalent to 1 mM/l $FeSO_4.7H_2O$) value.

Results and Discussion

The onion bulb skin peel contains a high content of quercetin glycosides (2-10% w/w), oxidized derivatives as well as free aglycone (Griffiths *et al.* 2002, Suh *et al.* 1999). From the onion skin, we have isolated free quercetin (**1**) in 0.1% w/w. After methylation of **1**, tetramethyl ether derivative (**2**) was yielded in 83.3% (Scheme 1). Both the compounds were authenticated by UV-Vis and ^{1}H NMR spectra analyses. A comparable UV-Vis spectral analysis for the identification of the substitution pattern in these compounds was proved valuable and the results are summarised in Table 1. The UV-Vis spectra of compound **2** with $AlCl_3$ and $AlCl_3$/HCl were similar with a bathochromic shift of 47 nm in Band I indicating the presence of a free 5-hydroxyl group. We were not surprised to obtain quercetin 3,3',4',7-tetramethyl ether (**2**) rather than pentamethyl ether derivative since methylation of the 5-hydroxyl group in the structural motif is known to be difficult (Looker & Ernst 1954).

Table 1. UV-Vis spectral analyses of compounds 1 and 2

Reagent	Quercetin (1)				Quercetin 3,3',4',7-tetramethyl ether (2)			
	UV Band		Shift of Band (nm)	Substitution pattern	UV Band		Shift of Band (nm)	Substitution pattern
	I	II			I	II		
(MeOH)	372	255			355	256		
(+ NaOAc)	377	272	II (+17)	7-OH free	350	257	II (+1)	7-OMe
(+ NaOAc + H_3BO_3)	386	262	I (+14)	3',4'-di OH free	350	256	I (–5)	3',4'-di OMe
(+ $AlCl_3$)	452	273	I (+80)	5-OH and/or 3-OH free	360, 402	272	I (+47)	5-OH free
(+ $AlCl_3$ + HCl)	429	271	I (+57)	5-OH free	358, 402	272	I (+47)	5-OH free
(+ NaOH)	425	288	I (+53)	3,4'-di OH free	374	284	I (+19)	3,4'-di OMe



Quercetin (**1**) Queretin tetramethyl ether (**2**)

Scheme 1. Synthesis of compound **2**

The compounds **1** and **2** were evaluated for á-chymotrypsin inhibitory activity. Compound **1** displayed inhibitory effect with IC_{50} value of 88.9 ± 2.5 ìg/ml, whereas compound **2** showed 9.3% inhibition at 500 µM, thus found ineffective and therefore IC_{50} was not evaluated.

The % inhibition versus concentration of compounds **1** and **2** showed a linear variance in the DPPH assay (Fig. 1). It can be clearly seen from Fig. 1 that compound **2** displayed poor DPPH free radical scavenging capacity (≤5% inhibition at 5-25 ì g/ml concentration). The IC_{50} values calculated for compounds **1** and **2** were 14.87 and 283.61 ìg/ml, respectively. Since IC_{50} is a measure of inhibitory concentration, a lower IC_{50} value would reflect a greater antioxidant activity of the sample. Therefore in contrast to the parent quercetin (**1**), quercetin 3,3',4',7-tetramethyl ether (**2**) was found ineffective for the free radical scavenging potentiality.

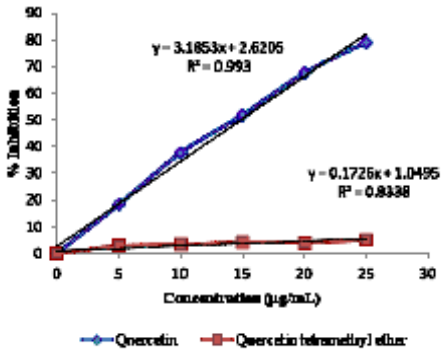


Fig. 1. The curve plotted between % inhibition versus concentration of compounds **1** and **2** in the DPPH assay.

Taking $FeSO_4.7H_2O$ as the reference, a standard curve of absorbance versus concentration in mM/l was plotted in the FRAP assay (Fig. 2). The concentration of the compound (as an antioxidant) having a ferric reducing ability equivalent to that of 1 mM/l $FeSO_4.7H_2O$ was calculated and the values for compounds **1** and **2** were found to be 112. 94 and 389.54 ì M/l, respectively. This result clearly indicated that quercetin (**1**) had 3.5 folds more ferric reducing power than quercetin 3,3',4',7-tetramethyl ether (**2**).

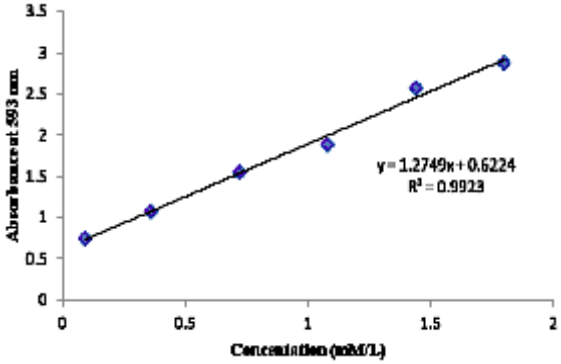


Fig. 2. Concentration-response ferrous sulphate standard curve in the FRAP assay.

In conclusion, quercetin aglycone (**1**) was isolated from the skin of red onion bulb and used for the methylation to afford quercetin 3,3',4',7-tetramethyl ether (**2**). α-Chymotrypsin inhibition, DPPH free radical scavenging activity and ferric reducing power of **1** were found to be diminished upon using its tetramethyl ether derivative **2**.

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