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Identification and *in vitro* biological activities of flavonols in garlic leaf and shoot: inhibition of soybean lipoxygenase and hyaluronidase activities and scavenging of free radicals

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Abstract: We isolated four flavonols from garlic (*Allium sativum* L) leaf and shoot and measured the *in vitro* antioxidant activity of the isolated flavonols and their aglycones. The chemical structures of the compounds were shown to be quercetin 3-*O*-*β*-D-glucopyranoside (isoquercitrin), quercetin 3-*O*-*β*-D-xylopyranoside (reynoutrin), kaempferol 3-*O*-*β*-D-glucopyranoside (astragalin) and isorhamnetin 3-*O*-*β*-D-glucopyranoside based on FAB-MS and NMR analyses. Assays of DPPH (1,1-diphenyl-*β*-picryl hydrazyl) and hydroxyl radical scavenging, inhibition of linoleic acid peroxidation, and soybean lipoxygenase (LO) and hyaluronidase (HYA) inhibition were used to evaluate the antioxidant activity. Quercetin and its glycosides showed the highest antioxidant activity among the compounds. In the LO assay, the IC_{50} values of quercetin, isoquercitrin and reynoutrin were 16.9, 40.1 and 32.9 μM respectively. Quercetin was the most effective among the flavonols. In the HYA assay the IC_{50} values of quercetin, isoquercitrin and reynoutrin were 23.0, 20.9 and 22.1 mM respectively. Isoquercitrin had the most potent inhibitory activity on HYA. The inhibition patterns of the flavonols on LO and HYA were elucidated as mixed types of competitive and non-competitive inhibition according to Lineweaver–Burk plot results. Although most garlic shoots and leaves are discarded and not used at present, our results suggest that ancillary garlic parts could be utilised as functional foods or ingredients.

Keywords: garlic leaf; shoot; isoquercitrin; reynoutrin; astragalin; isorhamnetin-3-*O*-*β*-D-glucopyranoside; radical scavenging; soybean lipoxygenase; hyaluronidase

INTRODUCTION

Garlic (*Allium sativum* L) has been used throughout the world not only as a spice but also as a traditional medicine for several thousand years.[1] Numerous studies have shown that garlic contains reactive sulphur-containing compounds that regulate many metabolic diseases.[2–7] In addition, it has been reported that allium vegetables contain high levels of quercetin and its derivatives.[8] Alfonso *et al*[9] isolated five flavonoid glycosides from wild garlic (*Allium ursinum* L). It is anticipated that there are biological activities induced by these flavonols as well as sulphur-containing compounds in garlic bulb. During the cultivation period of garlic, shoots are removed to promote bulb growth. Young shoots are used as a pickled vegetable in Asian countries such as Korea, Japan and China. Although there has been little study of the chemical composition and biological activities of ancillary garlic parts, this bioresource has been studied to some extent and the chemical composition[10] and antioxidant activity[11] of garlic shoot have been revealed.

In the present study we investigated the *in vitro* antioxidant activity of four flavonols and their aglycones isolated from garlic leaf and shoot. The aims of this study were to confirm the phenolic compounds in garlic leaf and shoot and to measure the antioxidant activity of these compounds by various assay methods such as DPPH (1,1-diphenyl-*β*-picryl hydrazyl) and hydroxy radical scavenging, inhibition of lipid peroxidation (ferric thiocyanate method), and lipoxygenase and hyaluronidase inhibition (spectrophotometric method). We also compared the antioxidant activities of the glycosides and their corresponding aglycones and investigated the enzyme inhibition patterns of the flavonols by constructing Lineweaver–Burk plots.

MATERIALS AND METHODS

Garlic (*Allium sativum* L) leaves and shoots were harvested in Kunwi, Kyungpook Province, Korea in May 1999 and dried in a convection

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oven at $50 \pm 2\,^{\circ}C$. Sephadex LH-20 gel, CD_3OD, tetramethylsilane (TMS), DPPH, 2-deoxyribose, ferric chloride, ammonium thiocyanate, soybean lipoxygenase type V, linoleic acid, nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole (BHA), α-tocopherol, hyaluronic acid, hyaluronidase, compounds 48/80, ρ-dimethyl aminobenzaldehyde, quercetin and kaempferol were purchased from Sigma Chemical Co (St Louis, MO, USA). The methanol used in this experiment was of HPLC grade (Merck, Darmstadt, Germany). All other chemicals used were of analytical grade.

Extraction, isolation and identification of flavonoids

Dried garlic leaves and shoots were extracted twice with methanol, filtered and concentrated into a small volume *in vacuo*. The methanolic extract was evaporated in a rotary evaporator and suspended in 10% methanol solution. The aqueous solution was successively extracted with diethyl ether, ethyl acetate and *n*-butanol. After evaporation the ethyl acetate extract was subjected to amberlite XAD-2 (Organo Co Ltd, Tokyo, Japan) column chromatography by elution with an MeOH/H_2O solvent system under gradient conditions. Four fractions (H_2O, 30% MeOH, 70% MeOH and 100% MeOH, v/v) were collected. The 70% MeOH fraction was subjected to Sephadex LH-20 column chromatography with 60% MeOH as eluent, separating into five fractions. Further separation/purification of fraction IV was performed using an HPLC (LC-10A, Shimadzu Co Ltd, Tokyo, Japan) with a reverse phase Develosil ODS-5 semi-preparative column (8 mm × 250 mm, Nomura Chemical Co Ltd, Seto, Japan) and a UV detector set at 254 nm. The solvent system was isocratic with 40% MeOH (0.1% TFA) at a flow rate of 2.0 ml min^{-1}.

NMR analysis of isolated flavonoids

^1H NMR (125 MHz) and ^{13}C NMR (500 MHz) spectra were obtained with an NMR spectrometer (Varian Unity Plus 500, Varian Co, Palo Alto, CA, USA) in CD_3OD containing TMS as an internal standard. The chemical shifts are given as δ values. FAB-MS spectra were obtained using a mass spectrometer (JEOL JMS-DX 705L, JEOL, Tokyo, Japan) with glycerol as the mounting matrix.

Hydrolysis of flavonols

Acid hydrolysis of flavonol glycosides was carried out according to the method of Markham[12] with slight modification. Each flavonol fraction was concentrated to dryness and then dissolved in 2 ml of methanol. Next it was mixed with 2 ml of 1 M HCl in 50% methanol. The aglycone and sugar portion from the flavonol glycosides was obtained after hydrolysis at 80 °C for 1 h. The hydrolysate was extracted with ethyl acetate (3 × 10 ml) to separate aglycone, which was identified by HPLC.

DPPH radical-scavenging activity

A 0.2 ml aliquot of flavonol solution and 0.8 ml of 0.4 mM ethanolic solution of DPPH radical were added to 3 ml of ethanol and shaken vigorously. The mixture was left to stand for 10 min.[13] The control was prepared in the same way but without the test compound. BHA and α-tocopherol were also prepared under the same conditions. Changes in the absorbance of samples were measured by a spectrophotometer (UV-1601, Shimadzu Co Ltd) at 525 nm. DPPH radical-scavenging activity was calculated using the following formula:

$$\%\ \text{radical-scavenging activity} = \frac{\text{control OD} - \text{sample OD}}{\text{control OD}} \times 100$$

All tests were performed in triplicate and the results averaged.

Hydroxyl radical-scavenging activity

The hydroxyl radical-scavenging activity of flavonols was assayed by the 2-deoxyribose oxidation method.[14] 2-Deoxyribose is oxidised by hydroxyl radical formed by the Fenton reaction and is degraded to malondialdehyde. A solution (0.2 ml) of flavonol in 0.1 M phosphate buffer (pH 7.4), 0.2 ml of 10 mM 2-deoxyribose, 0.1 mM Fe^{2+}/EDTA and 30% H_2O_2 were individually added to 0.1 M phosphate buffer (pH 7.4) solution to a final volume of 2 ml. The mixture was incubated at 37 °C for 4 h. After incubation, 1 ml of 2.8% trichloroacetic acid and 1 ml of 1% thiobarbituric acid in 50 mM NaOH were added to the reaction mixture. The solution was heated at 100 °C for 10 min, cooled in ice and its absorbance was measured with a spectrophotometer at 532 nm. The controls were divided into two groups by reaction temperature as follows: Tc, incubation at 37 °C; To, incubation at −4 °C for 4 h.

On the basis of the above experiments, hydroxy radical-scavenging activity was calculated using the following formula:

$$\%\ \text{radical-scavenging activity} = \left(1 - \frac{\text{Sample OD} - \text{To OD}}{\text{Tc OD} - \text{To OD}}\right) \times 100$$

All tests were performed in triplicate and the results averaged.

Linoleic acid peroxidation

A solution (120 µl) of flavonol in methanol and 2.88 ml of 2.51% linoleic acid in 80% EtOH were added to 40 mM phosphate buffer (pH 7.0) to a final volume of 12 ml. The prepared solution was incubated in a conical flask at 40 °C and the degree of hydroperoxide production was measured by the ferric thiocyanate method.[15] To do this, 0.1 ml of 30% ammonium thiocyanate and 0.1 ml of 2×10^{-2} M freshly prepared $FeCl_2$ (in 3.5% aqueous HCl) were added. Precisely

3 min after addition the absorbance of the red complex $[Fe(SCN)]^{2+}$ was measured at 500 nm. The control for the assay was prepared in the same manner but without the test compound. BHA and α-tocopherol were used as reference compounds. All tests were performed in triplicate and the results averaged.

Soybean lipoxygenase inhibitory activity

The soybean lipoxygenase assay was performed using a slightly modified version of the method reported by Block *et al*.[16] Tris buffer (0.1 M, 2.0 ml, pH 8.5) and a solution of inhibitor in ethanol (20 µl) were added to a cuvette at 18 °C, followed by the addition of soybean lipoxygenase (type V, 500 units final concentration) solution in buffer (30 µl). After a 5 min equilibration period an ethanolic solution of linoleic acid (50 µl, 110 µM final concentration) was added to start the reaction. The absorbance at 234 nm was recorded as a function of time on a spectrophotometer. The rates were measured from the initial slopes of the linear portions of the curves obtained for 3 min. The positive control for the assay was prepared in the same manner but without the test compound. The negative control for the assay was prepared in the same manner but without lipoxygenase. The IC_{50} value (concentration in µM required for 50% inhibition of enzyme activity) was determined by plotting a graph relating percentage inhibition to different concentrations of test compounds. BHA, α-tocopherol and NDGA were used as reference compounds. All tests were performed in triplicate and the results averaged.

Hyaluronidase inhibitory activity

The hyaluronidase assay was performed by the method reported by Ingo *et al*.[17] A 50 µl aliquot of hyaluronidase in 0.1 M acetic acid buffer (4 mg ml^{-1}, pH 4.0) was added to the test compound in distilled water (100 µl) to activate the hyaluronidase at 37 °C for 20 min, followed by the addition of compounds 48/80 solution in acetic acid buffer (100 µl). The incubation mixture was equilibrated at 37 °C for 20 min before the reaction was started by the addition of hyaluronic acid in buffer (0.8 mg ml^{-1}, 250 µl). It was then reincubated at 37 °C for 40 min. A 300 µl aliquot of 0.2 M NaOH and borate solution was added to the mixture, which was then heated for 3 min at 100 °C. The borate solution was prepared by dissolving 2.24 g of KOH in 100 ml of 0.8 M H_3BO_3. After cooling the mixture, 3 ml of ρ-dimethyl aminobenzaldehyde solution was thoroughly mixed with the solution and then held for 20 min at 37 °C. To prepare this reagent, 5 g of ρ-dimethyl aminobenzaldehyde was dissolved in a mixture of 44 ml of glacial acetic acid and 6 ml of 5 M HCl; the solution was diluted with 10 volumes of glacial acetic acid immediately before use. The generated *N*-acetyl glucosamin from hyaluronic acid was analysed by its absorbance at 585 nm. A mixture containing all the reagents except for the enzyme was used as the blank for the sample. The IC_{50} value (concentration in mM required for 50% inhibition of enzyme activity) was determined by plotting a graph relating percentage inhibition to different concentrations of inhibitors.

Enzyme kinetics

The inhibition pattern of flavonols on lipoxygenase and hyaluronidase was studied by determining the Michaelis–Menten constant (K_m) and the maximum velocity (V_{max}) and then plotting $1/V$ vs $1/S$ according to Lineweaver and Burk.[18]

RESULTS

Characterisation of isolated flavonoids

To isolate the antioxidant compounds from garlic leaves and shoots, a crude methanolic extract was fractionated by diethyl ether, ethyl acetate and *n*-butanol. The ethyl acetate fraction, with strong antioxidant activity, was subjected to amberlite XAD-2 and Sephadex LH-20 column chromatography. Fig 1 shows the HPLC chromatogram of fraction IV eluted from the Sephadex LH-20 column. Further purification of this fraction was carried out by preparative HPLC. The four compounds obtained were characterised by UV, FAB-MS and NMR spectroscopy as follows.

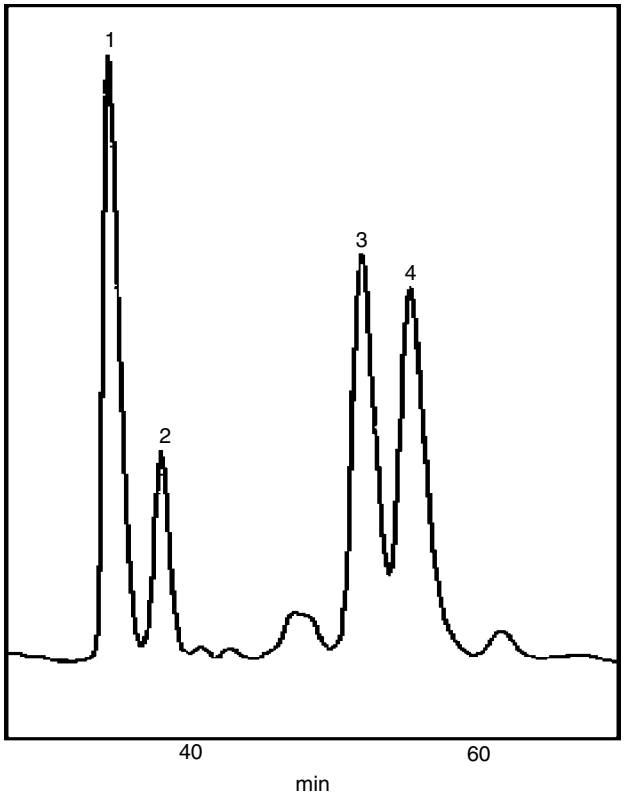

Figure 1. HPLC chromatogram of fraction IV of Sephadex LH-20 eluents from XAD-2 70% methanol fraction of garlic leaf and shoot: 1, isoquercitrin; 2, reynoutrin; 3, astragalin; 4, isorhamnetin 3-*O*-β-D-glucopyranoside.

Compound 1
UV (MeOH), λ_{max} 256, 357 nm; FAB-MS, *m/z* 465 (M + H); ^{1}H NMR (in CD_3OD) δ 7.61 (1H, m, H2′), 7.48 (1H, d, H6′), 6.76 (1H, d, H5′), 6.27 (1H, m, H8), 6.08 (1H, m, H6), 5.13 (1H, d, H1″), 3.62 (1H, dd, H6″a), 3.61 (1H, dd, G6b), 3.38 (1H, t, G2), 3.34 (1H, t, G3), 3.20 (1H, t, G4), 3.13 (1H, ddd, G5); ^{13}C NMR (in CD_3OD) δ 179.40 (C4), 166.52 (C7), 162.98 (C5), 158.94 (C2), 158.48 (C9), 149.87 (C4′), 145.90 (C3′), 135.59 (C3), 123.18 (C6′), 123.03 (C1′), 117.52 (C2′), 115.99 (C5′), 105.52 (C10), 104.34 (C1″), 100.05 (C6), 94.82 (C8), 78.37 (C5″), 78.08 (C3″), 75.69 (C2″), 71.18 (C4″), 62.51 (C6″). Given these results and considering the UV shift spectrum (data not shown), compound 1 was identified as quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin), consistent with the assignment of Markham and co-workers.[19,20]

Compound 2
UV (MeOH), λ_{max} 256, 357 nm; FAB-MS, *m/z* 435 (M + H); ^{1}H NMR (in CD_3OD) δ 7.60 (1H, m, H2′), 7.57 (1H, d, H6′), 6.85 (1H, d, H5′), 6.38 (1H, m, H8), 6.19 (1H, m, H6), 5.17 (1H, d, H1″), 3.77 (1H, dd, H5″a), 3.51 (1H, dd, H2″), 3.50 (1H, ddd, H4″), 3.39 (1H, t, H3″), 3.09 (1H, dd, H5″b); ^{13}C NMR (in CD_3OD) δ 179.37 (C4), 166.12 (C7), 163.05 (C5), 158.88 (C2), 158.90 (C9), 149.89 (C4′), 146.05 (C3′), 135.39 (C3), 123.27 (C6′), 123.01 (C1′), 117.19 (C2′), 115.98 (C5′), 105.59 (C10), 104.59 (C1″), 99.92 (C6), 94.72 (C8), 77.55 (C3″), 75.28 (C2″), 70.99 (C4″), 67.22 (C5″). Given these results and considering the ^{13}C-DEPT, two-dimensional NMR, HSQC and HMBC data (not shown), compound 2 was identified as quercetin 3-*O*-β-D-xylopyranoside (reynoutrin), consistent with the assignment of Markham and co-workers.[19,20]

Compound 3
UV (MeOH), λ_{max} 266, 347 nm; FAB-MS, *m/z* 449 (M + H); ^{1}H NMR (in CD_3OD) δ 8.05 (2H, d, H2′, 6′), 6.88 (2H, d, H3′, 5′), 6.39 (1H, d, H8), 6.20 (1H, H6), 5.24 (1H, d, H1″), 3.69 (1H, dd, H6″a), 3.58 (1H, dd, H6″b), 3.43 (1H, dd, H2″), 3.35 (1H, t, H3″), 3.29 (1H, t, H4″), 3.20 (1H, ddd, H5″); ^{13}C NMR (in CD_3OD) δ 179.52 (C4), 165.91 (C7), 163.02 (C5), 161.53 (C4′), 159.11 (C9), 158.55 (C2), 135.53 (C3), 132.30 (C2′, 6′), 122.81 (C1′), 116.13 (C3′, 5′), 105.72 (C10), 104.13 (C1″), 99.91 (C6), 94.83 (C8), 78.43 (C5″), 75.72 (C3″), 73.10 (C2″), 71.43 (C4″), 62.61 (C6″). Given these results and considering the ^{13}C-DEPT, two-dimensional NMR, HSQC and HMBC data (not shown), compound 3 was identified as kaempferol 3-*O*-β-D-glucopyranoside (astragalin), consistent with the assignment of Markham and co-workers.[19,20]

Compound 4
UV (MeOH), λ_{max} 254, 354 nm; FAB-MS, *m/z* 479 (M + H); ^{1}H NMR (in CD_3OD) δ 7.82 (1H, m, H2′), 7.48 (1H, d, H6′), 6.91 (1H, d, H5′), 6.27 (1H, m, H8), 6.09 (1H, m, H6), 5.29 (1H, d, H1″), 3.84 (3H, s, −OC*H*3), 3.74 (1H, dd, H6″a), 3.57 (1H, dd, H6″b), 3.45 (1H, t, H3″), 3.31 (1H, t, H4″), 3.25 (1H, ddd, H5″), 3.21 (1H, dd, H2″); ^{13}C NMR (in CD_3OD) δ 179.71 (C4), 166.62 (C7), 163.42 (C5), 158.93 (C9), 158.82 (C2), 151.10 (C3′), 148.63 (C4′), 135.52 (C3), 123.94 (C6′), 123.32 (C1′), 116.21 (C2′), 114.64 (C5′), 105.80 (C10), 103.81 (C1″), 100.11 (C6), 94.83 (C8), 78.63 (C3″), 78.11 (C5″), 75.93 (C2″), 71.54 (C4″), 62.62 (C6″), 56.84 (−O*C*H3). Given these results and considering the UV shift spectrum (data not shown), compound 4 was identified as isorhamnetin 3-*O*-β-D-glucopyranoside, consistent with the assignment of Markham and co-workers.[19,20]

Antioxidant activity

Table 1 shows the antioxidant activities of the isolated flavonols and their aglycones determined by DPPH and hydroxyl radical-scavenging activity and the ferric thiocyanate method, compared with those of the well-known antioxidants BHA and α-tocopherol at the same concentrations.

For DPPH radical-scavenging activity the aglycones and their glycosides were compared at a concentration of 10 mg kg^{-1} under the same conditions. Quercetin, isoquercitrin and reynoutrin showed DPPH radical-scavenging activities of about 88, 55 and 40% respectively, kaempferol and astragalin about 62 and 3% respectively, and isorhamnetin and its glycopyranoside about 55 and 18% respectively.

Hydroxyl radical-scavenging activity was measured by the 2-deoxyribose oxidation method. This antioxidant assay using 2-deoxyribose is effectively because 2-deoxyribose is sensitive to free radical-mediated oxidation. The radical-scavenging activity of the

Table 1. Antioxidant activity of flavonols isolated from garlic leaf and shoot

	Activity (%)[a]		
Compound	DPPH radical scavenging[b]	Hydroxy radical scavenging[c]	Linoleic acid peroxidation inhibition[b]
Isoquercitrin	54.76 ± 3.3	94.19 ± 2.1	42.09 ± 1.3
Reynoutrin	39.58 ± 2.2	95.11 ± 2.9	62.28 ± 1.7
Astragalin	3.11 ± 0.4	81.96 ± 2.3	42.49 ± 1.3
Isorhamnetin 3-G[d]	17.94 ± 1.2	80.45 ± 1.8	58.62 ± 1.5
Quercetin	88.38 ± 3.3	90.83 ± 1.7	89.93 ± 3.4
Kaempferol	61.87 ± 2.7	81.11 ± 1.3	61.90 ± 2.1
Isorhamnetin	55.03 ± 2.1	80.45 ± 1.9	63.01 ± 2.3
BHA	24.01 ± 2.3	90.11 ± 1.7	90.12 ± 2.7
α-Tocopherol	44.12 ± 0.9	82.20 ± 1.5	88.37 ± 2.2

[a] Values are mean ± standard deviation (n = 3).
[b] Test concentration 10 ppm.
[c] Test concentration 1 ppm.
[d] Isorhamnetin 3-*O*-β-D-glucopyranoside.

compounds at a concentration of 1 mg kg^{-1} was determined. Quercetin, isoquercitrin and reynoutrin showed hydroxyl radical-scavenging activities of about 91, 94 and 95% respectively, kaempferol and astragalin about 81 and 82% respectively, and isorhamnetin and its glycopyranoside both about 80%.

The lipid peroxidation inhibitory activity of the compounds was determined in an aqueous linoleic acid system. The value obtained without test samples was taken as 100% lipid peroxidation. At a concentration of 10 mg kg^{-1}, quercetin, isoquercitrin and reynoutrin showed lipid peroxidation inhibitory activities of about 90, 42 and 62% respectively, kaempferol and astragalin about 62 and 42% respectively, and isorhamnetin and its glycopyranoside about 63 and 59% respectively.

Soybean lipoxygenase (LO) inhibitory activity

Fig 2 shows the inhibitory activities of the isolated flavonols and their aglycones on soybean lipoxygenase-induced oxidation. This antioxidant assay using soybean lipoxygenase (type V) *in vitro* is meaningful because soybean LO uses arachidonic acid as a substrate; the product of arachidonic acid oxidation is 12- or 15-hydroperoxyarachidonic acid and the active site of LO is similar to that of human 5-LO,[16,21,22] which mediates the production of inflammatory intermediates such as leukotrienes from the arachidonic acid cascade.

In the course of screening LO inhibitors, it was found that the ethyl acetate fraction from garlic ancillary parts showed fairly high inhibitory activity towards LO (data not shown). Therefore the LO inhibitory activity of the four isolated flavonoid glycosides and three aglycones was determined spectrophotometrically.

The inhibitory activity of the flavonols was determined by the IC_{50} value, equivalent to the concentration required for 50% inhibition of LO activity *in vitro*, and compared with that of BHA, α-tocopherol and NDGA. Quercetin, isoquercitrin and reynoutrin showed IC_{50} values of 16.9, 40.1 and 32.9 μM respectively, kaempferol and astragalin 54.4 and 78.3 μM respectively, and isorhamnetin and its glycopyranoside 67.4 and 79.1 μM respectively. The IC_{50} values of BHA and α-tocopherol were 315 and 418 μM respectively, indicating slight LO inhibitory activity, although they are strong antioxidants in the lipid peroxidation system.

Fig 3 shows the results of the enzymatic kinetic studies with the lipoxygenase system. To investigate the inhibition pattern of the flavonols on LO, the effect was evaluated at a fixed aglycone concentration, modulating the concentration of linoleic acid. The enzymatic kinetic studies showed a significant change in the maximum velocity (V_{max}) but only a slight

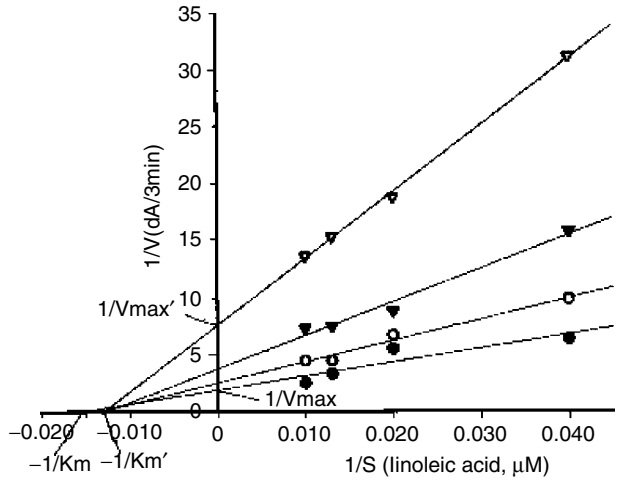

Figure 3. Substrate-dependent kinetics of soybean lipoxygenase inhibition by flavonols: full circles, control; open circles, kaempferol; full triangles, isorhamnetin; open triangles, quercetin.

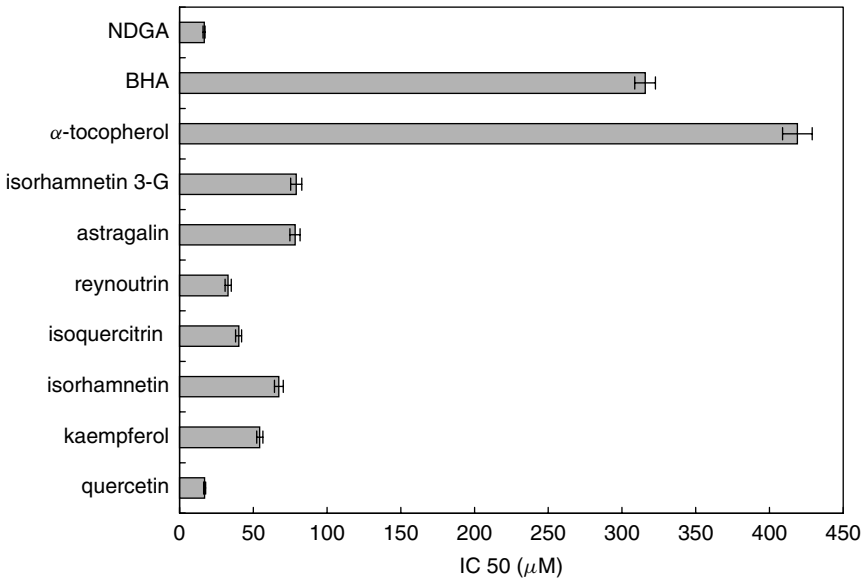

Figure 2. Soybean lipoxygenase inhibitory activity of flavonol glycosides and their aglycones isolated from garlic leaf and shoot. Data represent mean ± standard deviation ($n = 3$).

change in the K_m value. The kinetics indicates that the inhibition pattern of the flavonols was a mixed type of competitive and non-competitive inhibition (ie the appearance K_m value is increased or decreased in the presence of an inhibitor, but V_{max} is always decreased in such a case).

Hyaluronidase (HYA) inhibitory activity

The HYA inhibitory activities of the isolated flavonols and their aglycones are shown in Fig 4. Recent reports suggest that HYA, an enzyme known to be involved in tissue inflammation, participates in a type I allergic reaction. The IC_{50} value, equivalent to the concentration required for 50% inhibition of HYA activity *in vitro*, was used to compare the inhibitory activity. Quercetin, isoquercitrin and reynoutrin showed IC_{50} values of 23.0, 20.9 and 22.1 mM respectively, kaempferol and astragalin 36.3 and 26.5 mM respectively, and isorhamnetin and its glycopyranoside 55.4 and 50.4 mM respectively.

The inhibition pattern of quercetin and isoquercitrin on HYA by Lineweaver–Burk plot is shown in Fig 5. The enzymatic kinetic studies with the hyaluronidase system demonstrated a considerable change in the maximum velocity (V_{max}) of hyaluronic acid degradation. However, there was only a slight change in the K_m value, which again indicates a mixed type of competitive and non-competitive inhibition, similar to the LO inhibition pattern.

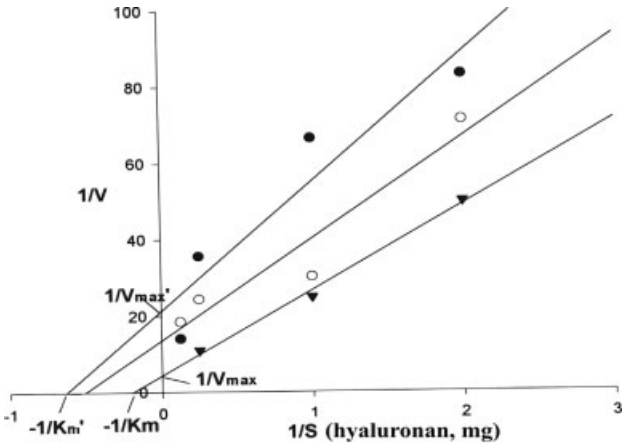

Figure 5. Substrate-dependent kinetics of hyaluronidase inhibition by flavonols: triangles, control; open circles, quercetin; full circles, isoquercitrin.

DISCUSSION

In the present study the antioxidant compounds of garlic leaf and shoot were identified and the *in vitro* antioxidant activities of the glycosides and their aglycones were evaluated as shown in Table 1. The chemical structures of the compounds were determined to be isoquercitrin, reynoutrin, astragalin and isorhamnetin glucopyranoside.

First, the radical-scavenging activity of the isolated compounds was evaluated, because it is known that free radicals are associated with many diseases. In the model systems of DPPH free radical and hydroxy radical scavenging, quercetin and its glycosides showed the highest scavenging activity, superior to that of BHA and α-tocopherol under the same conditions, although their hydroxy radical-scavenging activities were almost the same. In the assay of linoleic acid peroxidation inhibitory activity, quercetin was the most effective among the tested flavonols, comparable to BHA and α-tocopherol. Comparing the activities of the glycosides with those of their aglycones, it is suggested that the aglycone forms have higher radical-scavenging activities than the glycoside forms. It is reported that flavonols work as antioxidants by donating electrons and breaking radical chains.[23] Taking these results into consideration, it may be recognised that the flavonols from garlic ancillary parts could lessen the severity of damage induced by active oxygen radicals in living systems when used as dietary antioxidants.

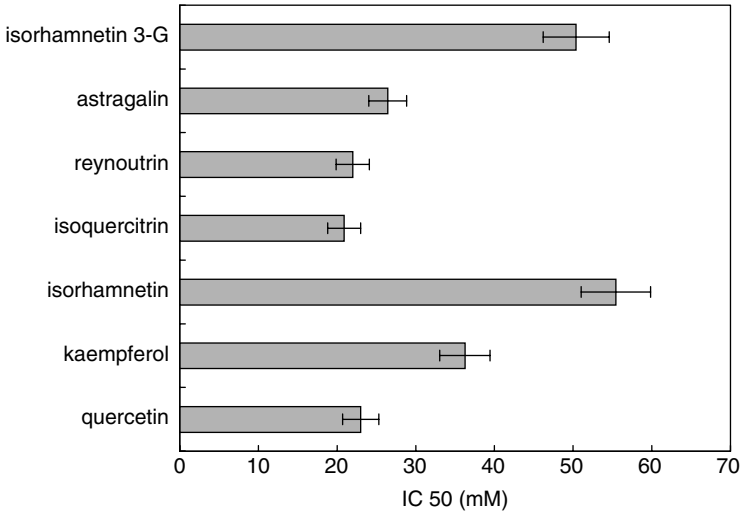

Figure 4. Hyaluronidase inhibitory activity of flavonol glycosides and their aglycones isolated from garlic leaf and shoot. Data represent mean ± standard deviation ($n = 3$).

Next, lipoxygenase (LO) and hyaluronidase (HYA) *in vitro* inhibitory activities of the compounds were measured because they are thought to induce inflammatory intermediates.[17,24] In the LO assay, quercetin was the most active among the test aglycones, comparable to NDGA, known as a potent inhibitor, and reynoutrin was the most effective among the glycosides, as shown in Fig 2. The fact that the aglycones were more active than the glycosides in the LO assay corresponded to the findings of the radical-scavenging assays. Luiz da Silva *et al*[25] reported that quercetin has stronger inhibitory activity on LO than quercitrin. In addition, this result suggests that the *ortho*-diOH of the B-ring in the flavonoid structure plays an important role in inhibiting LO, which is consistent with the radical-scavenging activity. Lipid peroxides are highly reactive molecules capable of altering amino acid side chains, modifying DNA and releasing inflammatory mediators.[24,26,27] It was suggested that the flavonols isolated from garlic ancillary parts have an inhibitory activity on enzymatic and non-enzymatic lipid peroxidation systems *in vitro*. The inhibition pattern of the flavonols on LO was a mixed type of competitive and uncompetitive inhibition according to the Lineweaver–Burk plot. The inhibitory mechanism of flavonols has been reported to be due to their ability to act as electron donors to the peroxy radical, thus inhibiting the oxidation of fatty acids by chain radical termination.[28] It was reported that the inhibition activity of a flavonol was not due to inactivation of free enzymes or to interference of its reaction with its normal substrate, but due to a reduction of intermediates.[29] Therefore it is suggested that LO inhibitory activity is closely related to the radical-scavenging activity of flavonols isolated from garlic leaf and shoot.

HYA inhibitory activity was measured by the colorimetric determination of the products of hyaluronan hydrolysis. HYA has been used to denote a group of enzymes from different sources that catalyse the depolymerisation of certain acidic glycosaminoglycans[30] involved in tissue inflammation. Relatively high HYA activity seems to be associated with prostate cancer progression,[31,32] breast cancer metastasis[33] and other malignancies.[34] Isoquercitrin had the lowest IC_{50} among the tested flavonols. Meanwhile, the flavonol glycosides exhibited smaller IC_{50} values for HYA inhibition than the corresponding aglycones. Flavonol glycosides of the quercetin type were found to have higher inhibitory activity than kaempferol glycoside, which is compatible with the LO inhibition pattern of the flavonols. The inhibition pattern of the flavonols on HYA was elucidated as a mixed type of competitive and non-competitive inhibition according to the Lineweaver–Burk plot results shown in Fig 5. Considering the LO and HYA inhibitory activities, it can be suggested that the flavonols isolated from garlic leaf and shoot may play an inhibitory role in the formation of inflammation-related mediators.

In conclusion, it was confirmed that discarded garlic leaves and shoots are a useful resource, and the antioxidants isolated from them could act as chemopreventive agents against reactive radicals and inflammatory mediators in living systems when used as dietary ingredients.

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