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Inhibition and Interaction with Hyaluronidase by Compounds from Hop (*Humulus lupulus* L) Flowers

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Hop (*Humulus lupulus L.*) flowers are widely used in the brewing industry and folk medicine. To study the antiallergic activity of hop flowers, the bioactivity-tailored isolation and detailed chemical characterization were used. The results showed that the active compounds are quercetin, rutin, kaempferol and isorhamnetin, which possessed hyaluronidase inhibitory activity in a concentration-dependent manner *in vitro*. Fluorescence and circular dichroism spectra were further employed to elucidate the interactions between these four bioactive compounds and hyaluronidase. The results revealed that these compounds could quench the intrinsic fluorescence, reduce the hydrophobicity and induce conformational changes of the hyaluronidase and therefore inhibit the catalytic activity of hyaluronidase. This study suggests that compounds from hop flowers are potential inhibitors of hyaluronidase, indicating a promising strategy for the development of natural antiallergic medicines or functional foods.

Key Words: Hop flowers, *Humulus lupulus L*, Hyaluronidase, Antiallergic.

INTRODUCTION

Allergic diseases, such as pollen allergy, atopic dermatitis and food allergy, are becoming more prevalent but no curative treatments are currently available. These allergic disorders are usually classified as type I allergy, in which the symptoms are caused by the activation of mucosal mast cells and/or basophiles, release of chemical mediators from these cells and activation of hyaluronidase, when triggered by various antigens[1]. Current research focus on exploring a wide variety of therapeutic agents (*e.g.*, antispastic drugs, sympathomimetic drugs and histamine release inhibitors) and strategies to prevent or reduce allergic disease[2]. However, current treatments present limitations regarding efficacy and safety. Hence, alternative treatments can achieve better control of allergic symptoms are highly demanded.

Hyaluronidase (HAase, EC 3.2.1.35), an enzyme which cleaves the polysaccharide hyaluronic acid in the extracellular matrix of connective tissue, is mainly known to be involved in allergic reaction[3,4]. Inhibiting the function of hyaluronidase was reported to show antiallergic effect[5,6]. Some natural compounds, such as polyphenols, flavonoids and polysaccharides, were reported as antiallergic agents or inhibitors of hyaluronidase[7-9]. In addition, compounds or extracts from food materials have also been reported to possess antiallergic activity[10,11]. For their manageable safety profile and reduced overall cost, natural products from plants, especially from food materials, continue to hold significant promise for the treatment and prevention of allergic disease[12,13].

Female hop (*Humulus lupulus L.*) flowers are widely used in the brewing industry to add both bitterness and aroma of the beer. Various studies revealed that extracts from hops possess many bioactivities, such as inhibition on bone resorption[14], anticancer[15], estrogenic activity[16] and inhibition on nitric oxide production[17]. Recently, hop water extract has been reported to have antiallergic activity. For example, hop water extract, with the major fraction of flavonoid glycosides, has been observed to inhibit histamine release from human basophilic KU812 cells[18]. In addition, by evaluating the Evans blue leakage from ICR mice caused by compound 48/80 stimulation and the histamine release from ovalbumin-sensitized BALB/c mice, the same research group reported that this hop water

extract could also significantly reduce the vascular permeability and histamine release *in vivo*[19]. Further clinical trials showed that an oral administration of the water extract could obviously alleviate the allergic symptoms related to Japanese cedar pollinosis[20]. While all these studies indicated that extract from hops contains antiallergic compositions, the active ingredients of the extract and the underlying mechanisms need to be further investigation.

In our screening program for identifying potential anti-allergic agents with adequate safety and fewer side effects from traditional medicinal herbs, we found that the ethyl acetate/ethanol crude extract of hop flowers could inhibit hyaluronidase activity. In order to investigate main active fractions of the extract, we performed a bioactivity-tailored isolation to identify the bioactive compounds and evaluated their hyaluronidase inhibition activity *in vitro*.

EXPERIMENTAL

The hop flowers were provided by Tsingtao Brewery Group (Qingdao, China), sodium salt of hyaluronic acid was the product of Solarbio Ltd. (Beijing, China) and bovine testicular hyaluronidase (EC3.2.1.35, 801 U/mg) was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Other reagents were reagent grade available and purchased from J & K Chemical Ltd. (Beijing, China).

Extraction, isolation and characterization of the hop flowers-derived hyaluronidase inhibitor: The hop flowers (1.6 Kg) were extracted with ethyl acetate (3 × 7 L) at room temperature (25ºC for average) for 24 h to obtain crude extract (0.6 Kg) after filtrating and concentrating at 40 ºC. The extract was subjected to normal-phase Silica gel (200-300 mesh; PE/EtOAc 1:0, 15:1, 1:1, 0:1, EtOAc/MeOH 4:1) and fractions displaying hyaluronidase inhibitory effect were further chromatographed over a C-18 reversed-phase column to obtain pure compounds. All compounds were characterized using ^{1}H and ^{13}C NMR spectra.

Colorimetric Morgan-Elson assay for the inhibition against hyaluronidase activity: Effect on hyaluronidase enzyme activity was measured by the modified Morgan-Elson method with slight modifications[21]. Briefly, hyaluronidase enzyme (500 U/mL) was incubated with the isolated compounds for 20 min at 37 ºC. Then, calcium chloride (12.5 mM) was added to the mixture and again incubated for 20 min at 37 ºC. The hyaluronic acid sodium solution (0.5 mg/mL) was added and incubation at 37 ºC for 40 min. After the addition of sodium hydroxide and sodium borate, the reaction mixture was heated in a boiling water bath for 5 min to stop the enzyme reaction. After cooling to room temperature, *p*-dimethylaminobenzaldehyde (DMAB) reagent was added and incubated at 37 ºC for 0.5 h when colour developed. The absorbance at 530 nm of the clear supernatant was measured. The hyaluronidase inhibition rate was calculated using the following formula:

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

Intrinsic fluorescence measurements: Hyaluronidase (5 μM) was pretreated with certain concentrations of quercetin, rutin, kaempferolm and isorhamnetin (0-200 μM) for 10 min at 37 ºC. The intrinsic fluorescence spectra (300-400 nm) were measured using a Cary Eclipse fluorescence spectrophotometer (Varian Inc. USA) with the excitation wavelength was 295 nm.

Hydrophobic analysis of hyaluronidase using *bis*-8-anilinonaphthalene-1-sulfonate: Hyaluronidase (5 μM) was incubated at 37 ºC for 10 min in the absence or presence of certain concentrations of quercetin, rutin, kaempferolm and isorhamnetin (0-200 μM). *Bis*-8-anilinonaphthalene-1-sulfonate (15 μM) was then added and fluorescence was measured after incubation at 37 ºC for 15 min (λ_{ex} = 395 nm, λ_{em} = 450-600 nm) using FlexStation II microplate spectrofluorometer (Molecular Devices, USA).

Circular dichroism spectroscopy for the secondary structure analysis of hyaluronidase: Hyaluronidase (3 μM) treated with different concentrations (0-200 μM) of quercetin, rutin, kaempferolm and isorhamnetin for 10 min. CD spectra (190-250 nm) of hyaluronidase were measured with a JASCO 715 spectropolarimeter (JASCO, Tokyo, Japan). The spectra were collected and corrected by subtraction of a blank containing 20 mM potassium phosphate buffer (pH 5.8), reduction of noise and smoothing. The program JWSSE (JASCO) was used for estimation of the secondary structure percentage of hyaluronidase according to the method of Yang *et al*[22].

RESULTS AND DISCUSSION

Extraction, isolation and characterization of hop flowers-derived hyaluronidase inhibitor: The hop flowers (1.6 Kg) were extracted three times with ethyl acetate at room temperature for 24 h to obtain crude extract (0.6 Kg) after filtrating and concentrating. The crude extract showed a moderate inhibitory effect. To isolate the active fractions, the extract was subjected to normal-phase Silica gel (200-300 mesh; PE/EtOAc1:0, 15:1, 1:1, 0:1, EtOAc/MeOH 4:1) to afford 5 fractions. Fraction 3 and 4 (12.2 g), displaying the highest hyaluronidase inhibitory effect, were combined and then chromatographed over a C-18 reversed-phase column (acetonitrile/H_2O, 30:70) to obtain rutin (8 mg), quercetin (8 mg), kaempferol (6.2 mg), isorhamnetin (5 mg). However, other fractions showing no obviously inhibitory activities against hyaluronidase were not isolated further more. ^{1}H and ^{13}C NMR spectra of all compounds (Fig. 1) isolated were as following.

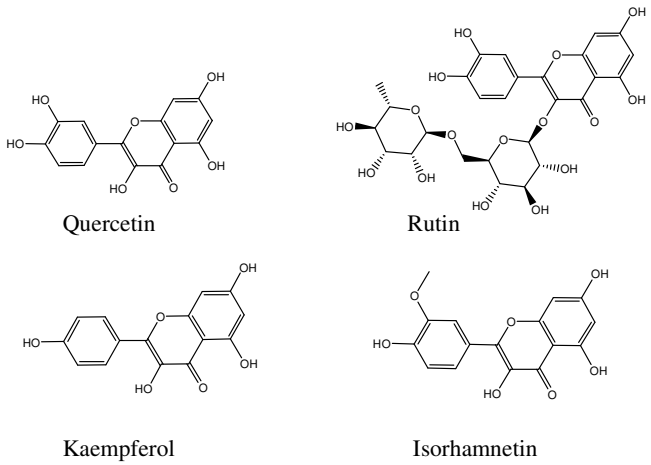

Fig. 1. Chemical structures of quercetin, rutin, kaempferol and isorhamnetin

Rutin: Yellow amorphous powder,[1]H NMR (500 MHz, DMSO-d_6, δ, ppm): 12.59 (1H, s, OH-5), 10.77 (1H, s, OH-7), 9.29-9.80 (2H, brs, OH-3′, 4′), 7.55 (1H, d, *J* = 8.3 Hz, H-6′), 7.53 (1H, s, H-2′), 6.83 (1H, d, *J* = 8.3 Hz, H-5′), 6.38 (1H, d, *J* = 2.1 Hz, H-8), 6.19 (1H, d, *J* = 2.1 Hz, H-6), 5.34 (1H, d, *J* = 7.5 Hz, H-1′′, Glc), 4.39 (1H, s, H-1′′, Rha), 0.99 (3H, d, *J* = 6.2 Hz, H-6′′, Rha); [13]C NMR (125 MHz, DMSO-d_6, δ, ppm): 177.3 (C-4), 164.1 (C-7), 161.2 (C-5), 156.5 (C-9), 156.4 (C-2), 148.4 (C-3′), 144.7 (C-4′), 133.3 (C-3), 121.5 (C-1′), 121.1 (C-6′), 116.2 (C-2′), 115.2 (C-5′), 103.9 (C-10), 101.2 (C-1, Glc), 100.7 (C-1, Rha), 98.6 (C-6), 93.5 (C-8), 76.4 (C-3, Glc), 75.9 (C-5, Glc), 74.0 (C-2, Glc), 71.8 (C-4, Rha), 70.5 (C-3, Rha), 70.3 (C-2, Rha), 69.9 (C-4, Glc), 68.2 (C-5, Rha), 67.0 (C-6, Glc), 17.7 (C-6, Rha).

Quercetin: Yellow amorphous powder, [1]H NMR (500 MHz, DMSO-d_6, δ, ppm): 9.54 (1H, s, OH-3), 12.49 (1H, s, OH-5), 6.20 (1H, d, *J* = 2.0 Hz, H-6), 10.75 (1H, s, OH-7), 6.41 (1H, d, *J* = 2.0 Hz, H-8), 7.68 (1H, d, *J* = 2.1 Hz, H-2'), 9.31 (1H, s, OH-3'), 9.33 (1H, s, OH-4'), 6.89 (1H, d, *J* = 8.5 Hz, H-5'), 7.54 (1H, dd, *J* = 2.1, 8.5 Hz, H-6'); [13]C NMR (125 MHz, DMSO-d_6, δ, ppm): 147.6 (C-2), 135.7 (C-3), 175.8 (C-4), 160.7 (C-5), 98.1 (C-6), 163.8 (C-7), 93.3 (C-8), 156.1 (C-9), 102.9 (C-10), 121.9 (C-1'), 115.0 (C-2'), 145.0 (C-3'), 147.6 (C-4'), 115.6 (C-5'), 119.9 (C-6').

Kaempferol: Yellow amorphous powder, [1]H NMR (500 MHz, DMSO-d_6, δ, ppm): 6.20 (1H, d, *J* = 2.0 Hz, H-6), 6.46 (1H, d, *J* = 2.0 Hz, H-8), 6.98 (2H, d, *J* = 8.5 Hz, H-3′, 5′), 8.10 (2H, d, *J* = 8.5 Hz, H-2′, 6′), 9.35 (1H, s, OH-4′), 10.07 (1H, s, OH-3), 10.74 (1H, s, OH-7), 12.48 (1H, s, OH-5); [13]C NMR (125 MHz, DMSO-d_6, δ, ppm): 146.8 (C-2), 135.6 (C-3), 175.9 (C-4), 160.7 (C-5), 98.2 (C-6), 163.9 (C-7), 93.4 (C-8), 156.1 (C-9), 103.0 (C-10), 121.6 (C-1′), 129.4 (C-22, 6′), 115.4 (C-3′, 5′), 159.1 (C-4′).

Isorhamnetin: Yellow amorphous powder, [1]H NMR (500 MHz, DMSO-d_6, δ, ppm): 12.46 (1H, s, OH-5), 10.73 (1H, s, OH-7), 9.69 (1H, s, OH-3), 9.38 (1H, s, OH-4′), 7.76 (1H, d, *J* = 2.1 Hz, H-2′), 7.69 (1H, dd, *J* = 2.1, 8.5 Hz, H-6′), 6.95 (1H, d, *J* = 8.5 Hz, H-5′), 6.48 (1H, d, *J* = 2.1 Hz, H-8), 6.20 (1H, d, *J* = 2.1 Hz, H-6), 3.86 (3H, s, H-OCH_3); [13]C NMR (125 MHz, DMSO-d_6, δ, ppm): 147.3 (C-2), 135.8 (C-3), 175.8 (C-4), 160.6 (C-5), 98.2 (C-6), 163.9 (C-7), 93.5 (C-8), 156.1 (C-9), 102.9 (C-10), 121.9 (C-1′), 111.8 (C-2′), 148.8 (C-3′), 146.6 (C-4′), 115.5 (C-5′), 121.7 (C-6′), 55.8 (C-OCH_3).

Compounds from hop flowers inhibit hyaluronidase activity: Hyaluronidase plays an important role in allergic diseases and inhibition on the hyaluronidase would be an effective antiallergic therapy[5]. To evaluate the antiallergic activities of the compounds extracted from hop flowers, we investigated their inhibitory effect on hyaluronidase and found that these compounds showed different levels of inhibition against hyaluronidase. As shown in Table-1, over a range of 50-200 μM, quercetin, rutin, kaempferol and isorhamnetin showed obvious inhibition against hyaluronidase, with the inhibition rate of 54.63 ± 3.16, 61.87 ± 5.48, 50.75 ± 3.78 and 51.04 ± 4.48 % at 200 μM, respectively. However, other fractions and compounds, such as β-sitosterol and daucosterol, show no obvious inhibitory activity under the present conditions (data not shown). The inhibition effect against hyaluronidase activity of these four compounds was consistent with results of previous studies that quercetin, rutin, kaempferolm and isorhamnetin all showed inhibitory effect on hyaluronidase activity[23-27]. However, the reported inhibition rate of each compound varies a lot, possibly due to the different hyaluronidase sources and activities in those experiments. In addition, it has been reported that, in KU812 cell, quercetin and kaempferolm could inhibit the release of histamine, which is a key chemical mediator in the allergic disease[18]. Their inhibition against both hyaluronidase and the release of histamine indicated the potential application of quercetin, rutin, kaempferolm and isorhamnetin in the treatment of allergic diseases.

TABLE-1
INHIBITORY EFFECT OF COMPOUNDS ISOLATED FROM HOP FLOWERS ON HYALURONIDASE. VALUES ARE GIVEN AS MEAN ± SD

Compounds	Inhibition (%)		
	50 (μM)	100 (μM)	200 (μM)
Quercetin	29.39 ± 1.94	40.74 ± 2.07	54.63 ± 3.16
Rutin	41.06 ± 4.21	55.31 ± 2.69	61.87 ± 5.48
Kaempferol	17.34 ± 1.01	31.91 ± 2.11	50.75 ± 3.78
Isorhamnetin	12.15 ± 1.85	19.44 ± 2.27	51.04 ± 4.48

Intrinsic fluorescence quenching induced by quercetin, rutin, kaempferolm and isorhamnetin: Our present results and previously reports showed that quercetin, rutin, kaempferolm and isorhamnetin possess inhibitory activities on hyaluronidase *in vitro*. These results suggest that these compounds may bind to the enzyme by directly interacting with the hyaluronidase molecule. To test this hypothesis, we first investigated the changes in intrinsic tryptophan fluorescence of the enzymes in the presence of quercetin, rutin, kaempferolm and isorhamnetin, respectively. When excitated at 295 nm, quercetin, rutin, kaempferolm and isorhamnetin had no obvious fluorescence emission and contributed negligible florescence interference (Fig. 2). Fluorescence intensity of hyaluronidase was quenched gradually in a concentration dependent manner, with the treatment of quercetin (Fig. 2A), rutin (Fig. 2B), kaempferolm (Fig. 2C) and isorhamnetin (Fig. 2D). Among these four compounds, at the high concentration (100 and 200 μM), rutin quenched the intrinsic fluorescence more significantly than the other compounds. These results established that there is an interaction between quercetin, rutin, kaempferolm, isorhamnetin and hyaluronidase and this interaction induces a microenvironment variation for Trp residues in hyaluronidase. Possibly, this variation hinders the formation of active centre and/or the binding of the substrates. The intrinsic fluorescence changes in hyaluronidase are similar to the fluorescence quenching in other enzymes, induced by their corresponding inhibitors[28,29].

Quercetin, rutin, kaempferolm and isorhamnetin reduced hydrophobicity of hyaluronidase: The hydrophobic characteristics and environmental sensitivity of *bis*-8-anilinonaphthalene-1-sulfonate (*bis*-ANS) allow it to be widely used as a probe to measure protein surface hydrophobicity in compound-protein interaction studies[30]. To investigate whether the compounds isolated from hop flowers could induce changes in hydrophobicity of hyaluronidase, *bis*-ANS was employed and the fluorescence intensities were shown in Fig. 3. When incubated with hyaluronidase, *bis*-ANS gave a remarkable

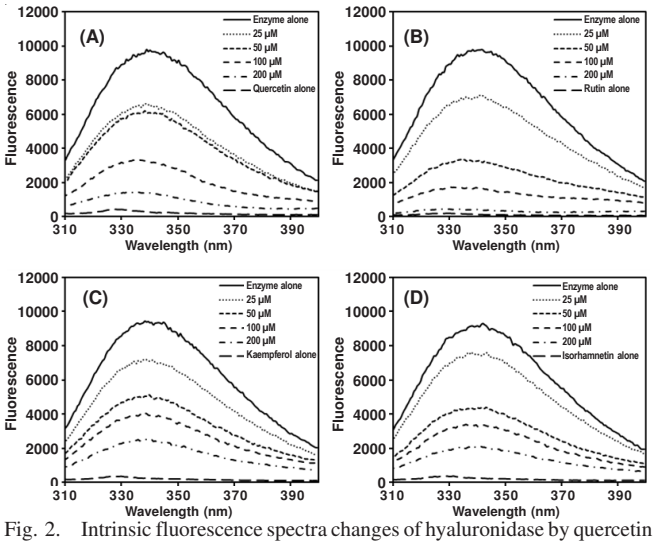

Fig. 2. Intrinsic fluorescence spectra changes of hyaluronidase by quercetin (A), rutin (B), kaempferolm (C) and isorhamnetin (D). The enzyme (5 μM) was incubated with specified concentrations of compounds tested (0-200 μM) for 10 min at 37 ºC. The excitation wavelength was 295 nm and emission spectra were acquired by scanning from 300-400 nm

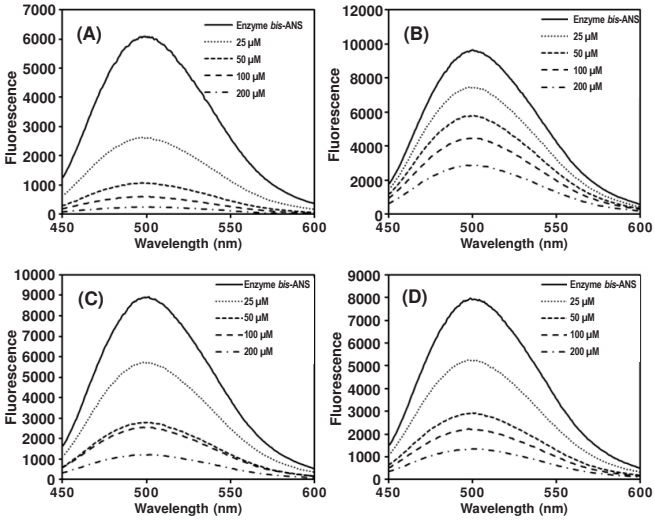

Fig. 3. Changes of hyaluronidase-*bis*-ANS complex fluorescence by quercetin (A), rutin (B), kaempferolm (C) and isorhamnetin (D). Hyaluronidase (5 μM) was incubated for 10 min at 37 ºC in the absence or presence of specified concentrations of compounds tested (0-200 μM). *Bis*-ANS (15 μM) was then added and fluorescence was measured after 15 min of incubation at 37 ºC (excitation at 395 nm, emission at 450-600 nm) using a Cary Eclipse fluorescence spectrophotometer

fluorescence (Enzyme *Bis*-ANS). However, the enzyme-*bis*-ANS fluorescence was reduced in the presence of quercetin, rutin, kaempferolm and isorhamnetin. As shown in Fig. 3A, with increased concentrations of quercetin, the enzyme-*bis*-ANS fluorescence was reduced in a concentration-dependent manner; quercetin at 200 μM almost abolished the fluorescence completely. Rutin (Fig. 3B), kaempferolm (Fig. 3C) and isorhamnetin (Fig. 3D) also reduced the enzyme-*bis*-ANS fluorescence in concentration dependent manner. These results suggested that quercetin, rutin, kaempferolm and isorhamnetin could interact with hyaluronidase, reduce the hydrophobic surface of hyaluronidase molecules and therefore affect the catalytic activity. In our previous study of screening for α-glucosidase inhibitors, such reduced enzyme hydrophobicity were also observed when α-glucosidase was incubated with its inhibitors[31,32]. The inhibitor-induced hydrophobicity reduction of different enzyme molecules supports the notion that poor hydrophobic surface usually hinder the formation of active center in the enzyme molecules.

Quercetin, rutin, kaempferolm and isorhamnetin changes the secondary structures of hyaluronidase: Quercetin, rutin, kaempferolm and isorhamnetin could quench the intrinsic fluorescence and decrease the hydrophobicity of hyaluronidase, strongly indicates that these four compounds could change the secondary structure of hyaluronidase. To confirm this assumption, we measured the circular dichroism (CD) spectra (190-250 nm) of hyaluronidase in the absence and presence of quercetin, rutin, kaempferolm and isorhamnetin, respectively. As shown in Fig. 4, quercetin (Fig. 4A), rutin (Fig. 4B), kaempferolm (Fig. 4C) and isorhamnetin (Fig. 4D) triggered a minor change in the CD spectrum of hyaluronidase. Quercetin, rutin, kaempferolm and isorhamnetin (100 μM) could decrease the β-helix content and increase the β-sheet content of hyaluronidase, however, rutin and kaempferolm induced a converse changes at the concentration of 200 μM (Table-2). The conformational changes in hyaluronidase may contribute to the mixed type of competitive and non-competitive inhibition mode observed in previous studies[24]. However, the reason that different concentration of rutin and kaempferolm induce contrary changes in the secondary structure of hyaluronidase remains unknown. Nonetheless, this finding further confirmed that quercetin, rutin, kaempferolm and isorhamnetin could bind to the enzyme, slightly change the secondary conformation of hyaluronidase and therefore hamper active center formation or prevent substrates binding to the enzyme.

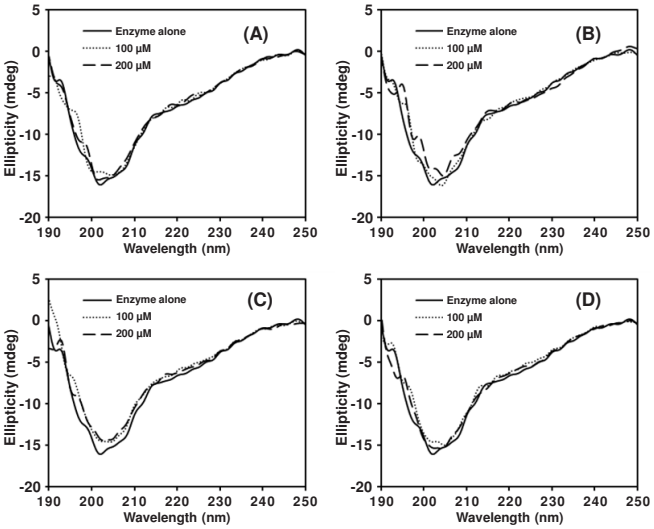

Fig. 4. CD spectra of hyaluronidase in the absence or presence of quercetin (A), rutin (B), kaempferolm (C) and isorhamnetin (D). Hyaluronidase (3 μM, dissolved in 20 mM potassium phosphate buffer, pH 5.8) alone or incubated with quercetin, rutin, kaempferolm and isorhamnetin (100, 200 μM) at 37 ºC for 0.5 h. Spectra were acquired from 190-250 nm

Conclusion

In summary, compounds with potential antiallergic activity were isolated from hop flowers and investigated for

TABLE-2
SECONDARY STRUCTURE CONTENT OF HYALURONIDASE INFLUENCED BY QUERCETIN, RUTIN, KAEMPFEROLM AND ISORHAMNETIN

Compounds	Conc. (μM)	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Random (%)
Control	0	2	52	4.6	41.4
Quercetin	100	1.8	55	4.4	38.9
	200	1.9	53.2	4.4	40.6
Rutin	100	1.2	56.1	2.8	39.9
	200	3.4	51.1	5.8	39.8
Kaempferolm	100	1.5	54.7	1.8	42
	200	3	50.5	6.1	40.5
Isorhamnetin	100	1.8	54.9	3.8	39.5
	200	1.1	55.5	3.4	40.1

The secondary structure content of hyaluronidase is predicted by program JWSSE (JASCO).

their inhibition and interaction with hyaluronidase. The experimental results showed that quercetin, rutin, kaempferol and isorhamnetin could interact with hyaluronidase, induce conformational changes and therefore inhibit the catalytic activity of hyaluronidase. This study further proved the biochemical and medical significance of the flavonoids in hop flowers and implied that hop flowers are a source of potential inhibitors of hyaluronidase, indicating a promising strategy for the future development of natural antiallergic medicines or functional foods.

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