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# Evaluation of antihypertensive polyphenols of barley (*Hordeum vulgare* L.) seedlings via their effects on angiotensin-converting enzyme (ACE) inhibition

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## Abstract

Angiotensin-converting enzyme (ACE) is an important therapeutic target in the regulation of high blood pressure. This study was conducted to investigate the alterations in blood pressure associated with ACE inhibition activity of the polyphenols (**1–10**), including 3-*O*-feruloylquinic acid (**1**), luteonarin (**2**), saponarin (**3**), isoorientin (**4**), orientin (**5**), isovitexin (**6**), isoorientin-7-*O*-[6-sinapoyl]-glucoside (**7**), isoorientin-7-*O*-[6-feruloyl]-glucoside (**8**), isovitexin-7-*O*-[6-sinapoyl]-glucoside (**9**), and isovitexin-7-*O*-[6-feruloyl]-glucoside (**10**), isolated from barley seedlings (BS). All the isolated polyphenols exhibited comparable IC<sub>50</sub> values of ACE inhibition activity (7.3–43.8 μM) with quercetin (25.2 ± 0.2 μM) as a positive control, and their inhibition kinetic models were identified as noncompetitive inhibition. Especially, compound **4** was revealed to be an outstanding ACE inhibitor (IC<sub>50</sub> = 7.3 ± 0.1 μM, K<sub>i</sub> = 6.6 ± 0.1 μM). Based on the compound structure–activity relationships, the free hydroxyl groups of flavone-moieties and glucose connections at the A ring of the flavone moieties were important factors for inhibition of ACE. The alcohol extract of BS also demonstrated potent ACE inhibition activity (66.5% ± 2.2% at 5000 μg mL<sup>-1</sup>). The polyphenols from BS had strong inhibitory activity on ACE and this study results suggest that BS can be used as an effective blood pressure regulator through ACE inhibition.

**Keywords:** Angiotensin-converting enzyme, Barley seedlings, Enzyme kinetic, Flavone-glucosides, Hypertension, *Hordeum vulgare* L.

## Introduction

Hypertension is a severe health problem, and its prevalence is increasing across many countries. The angiotensin I-converting enzyme (ACE) (EC 3.4.15.1) is the primary component of the renin–angiotensin–aldosterone system whose primary known functions are to convert precursor angiotensin I (Ang I) into angiotensin II

(Ang II) [1]. Ang II stimulates the synthesis and release of aldosterone from the adrenal cortex, which increases blood pressure [2]. Therefore, the inhibition of ACE has been considered as one of the effective remedies for the treatment of cardiovascular diseases. Consequently, ACE inhibitors, which can regulate and improve high blood pressure, are widely used for treating cardiovascular diseases.

Several studies have demonstrated that polyphenols have various biological and medicinal properties for the control of diseases in humans, such as antioxidant, anti-atherosclerotic, antihypertensive, and anti-inflammatory activities [3–6]. However, the biological activities are

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dependent on their structural aspects such as the number or position of the free –OH substituents and the process of glycosylation [7–9]. Thus, each of the compound showed different biological activities and the activities should investigate with each of the target disease model. Barley seedlings (BS, young leaves of *Hordeum vulgare* L.) contain various polyphenols such as saponarin and lutanarin that promote health beneficial properties, including antioxidant, hypolipidemic [10], antiproliferative, and proapoptotic effects [11]. Although polyphenols are known to exert potent blood pressure regulation effects, the polyphenols isolated from BS have not been clearly investigated for their biological activity. In these reasons, this study was conducted to investigate the blood pressure regulation effect of BS extract and the isolated polyphenols through ACE inhibition.

## Materials and methods

### Plant material

Barley (*H. vulgare* L.) seeds used in this study was cultivated in 2014 at the experimental field of the National Institute of Crop Science, Rural Development Administration, Miryang, Korea. BS were prepared as described previously [12]. In briefly, 200 g of barley seeds were imbibed in water for 1 day and germinated for 2 days in the dark. Germinated seeds were spread on commercial soil bed, and put in a growth chamber (DS-GC 768, Dongseo Science, Republic of Korea) at 22–23 °C and 60% humidity. Barley seedlings were collected 15 days later. The collected leaves were freeze-dried immediately after sampling. Prior to extraction, the leaves were pulverized in a 100 mesh. All sample masses were calculated on the basis of dry weight.

### Instruments and reagents

For measuring antihypertensive activity, 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt (water-soluble tetrazolium salt, WST-1) was obtained from Dojindo Laboratories (Kumamoto, Japan). ACE from rabbit lung and aminoacylase (EC 3.5.1.14) were obtained from Sigma–Aldrich (MO, USA). Fluorescence spectrophotometer were used SpectraMax M5 (Molecular Devices, CA, USA).

### Preparation of ACE inhibitors

One gram of dried leaves powder (1 g) was extracted with 20 mL of solvent at 37 °C for 24 h. Three different solvent extraction systems were used (water, methanol, and hexane), and then the extract was filtered. The filtered crude extract was concentrated using an evaporator under reduced pressure condition at 50 °C. In the previous study [13], the isolated polyphenolic

compounds were identified using spectroscopic data including 2D-NMR and by comparison with previously published data.

### ACE inhibition assay procedure

The ACE inhibition assay was performed according to a procedure previously described in the literature with some modifications [14, 15]. The synthesized substrate 3-hydroxybutyrylglycyl-glycyl-glycine (3HB-GGG) was applied in the assay of ACE inhibition activities. 3HB-GGG was synthesized according to a previously described procedure [14]. The sample solutions, which included the extracts and the compounds isolated from BS, were dissolved in 200 mM sodium borate buffer with pH 8.3. Then, 20 µL of the sample solution was pre-mixed with 20 µL of 1.2 mM 3HB-GGG, which was earlier dissolved in 50 mM sodium borate buffer (including 300 mM NaCl, pH 8.3). Then, 20 µL of an enzyme working solution, which was dissolved in aminoacylase (86 kU mL<sup>-1</sup>) with 50 mM sodium borate buffer (pH 8.3), and ACE (0.1 U mL<sup>-1</sup>) were added and mixed well to start the reaction at 37 °C. The reaction resulted in the production of 3HB, which is a 3-hydroxybutyrate derived from 3HB-GGG. The 3HB levels were determined using 200 µL of an indicator working solution. This indicator working solution was composed of 0.333 mM NAD<sup>+</sup>, 0.333 mM WST-1, 0.1 mM EDTA, diaphorase (0.633 U mL<sup>-1</sup>), and 3-hydroxybutyrate dehydrogenase (0.700 U mL<sup>-1</sup>). WST-1 formazan was measured at 490 nm for 60 min by spectrophotometer. The ACE inhibition rate (%) was calculated according to the following Eq. (a) [16, 17]:

$$\text{Inhibition rate (\%)} = 100 [1/(1 + ([I]/IC_{50}))] \quad (\text{a})$$

where [I]: inhibitor concentration and IC<sub>50</sub>: concentration of an inhibitor that resulted in 50% inhibition of an enzyme.

The dose-dependent enzyme inhibition activity was determined using four concentrations of each compound. The IC<sub>50</sub> value was calculated using a linear regression analysis plot of ACE inhibition % vs. concentration of each compound. The inhibition constant (K<sub>i</sub>) values were calculated using Eq. (b) [16, 17]:

$$K_i = IC_{50}/[1 + ([S]/K_m)] \quad (\text{b})$$

where [S]: substrate concentration, K<sub>m</sub>: Michaelis constant, the substrate concentration that provides a reaction velocity that is half of the maximal velocity obtained under saturating substrate conditions. The natural product quercetin was used as a positive control.

### Enzyme kinetic analysis

The inhibition kinetics of the enzyme by the isolated compounds was determined according to the Michaelis–Menten kinetic model,  $V = V_{\max} [S]/(K_m + [S])$ . Lineweaver–Burk plots ( $1/V$  vs.  $1/[S]$ ) and Dixon plots ( $V_{\max}$  vs.  $[I]$  with varying concentrations of substrate), which were derived using the saturation curves, were used to determine the inhibitor type according to the following Eq. (c) [16, 17]:

$$1/V = (K_m/V_{\max}) [S] + 1/V_{\max} \quad (c)$$

where  $V$ : reaction rate,  $K_m$ : Michaelis constant,  $[S]$ : substrate concentration,  $V_{\max}$ : maximum reaction rate.

Kinetic parameters such as  $K_m$ ,  $V_{\max}$ , and  $K_i$  were calculated using the Sigma Plot (SPSS Inc., Chicago, IL, USA) software.

### Statistical analysis

All measurements were made in triplicate. The results were subjected to analysis of variance using the sigma plot to analyze the differences. Differences were considered to be significant at  $p < 0.05$ . Multiple comparisons were analyzed using one-way analysis of variance, followed by Dunnett's post hoc test, and the results are expressed as mean values with their respective standard deviations.

## Results and discussion

### Quantitative isolation and structure identification

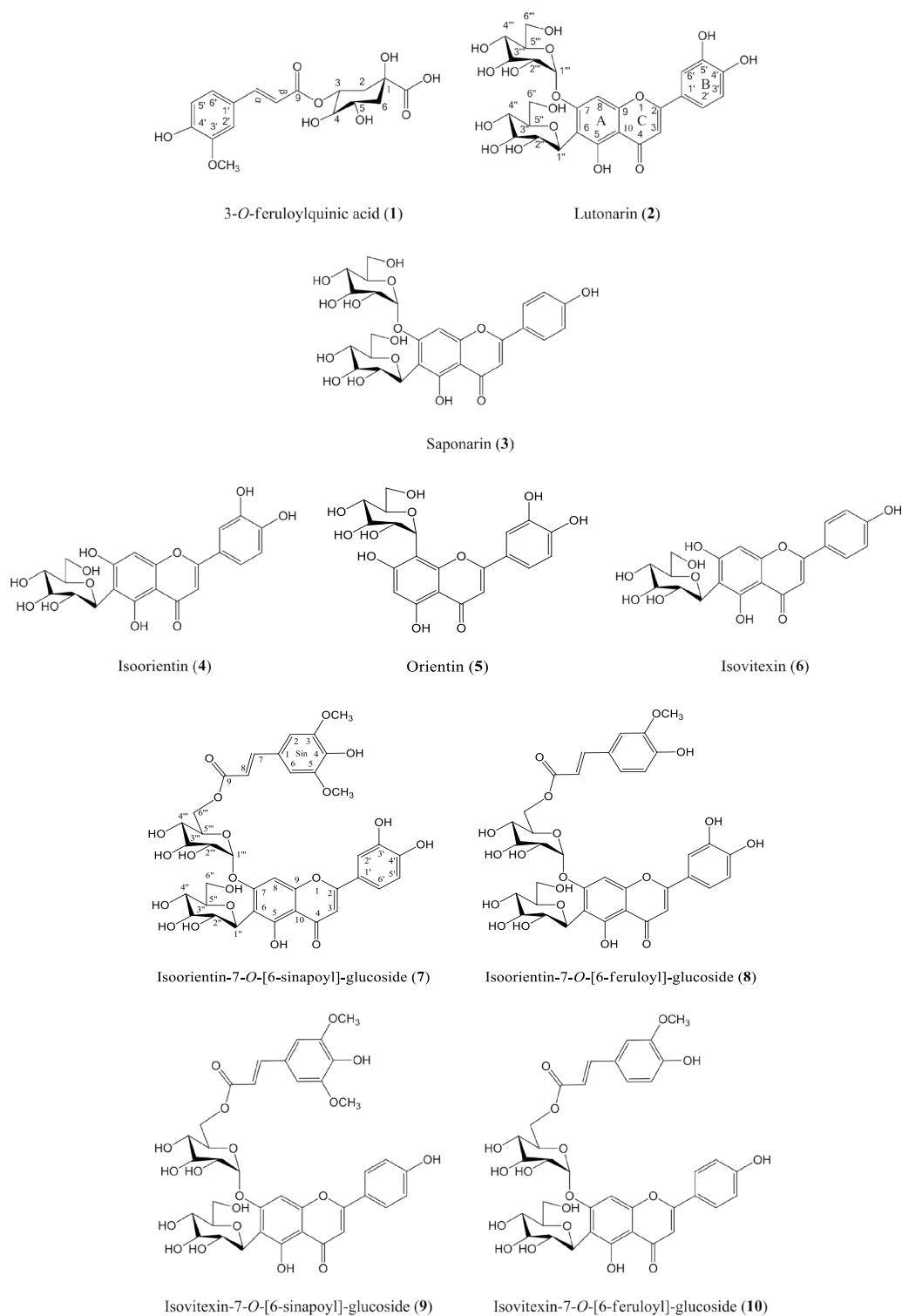
In the present study conducted to determine a natural therapeutic approach for the treatment of high blood pressure through the inhibition of ACE, three different BS extracts were prepared using water, methanol, and hexane and their ACE inhibition activities were compared (Additional file 1: Table S1). The methanol extract of BS showed the dominant ACE inhibition activity of  $66.5\% \pm 2.2\%$  than the water extract with the ACE inhibition activity of  $56.1\% \pm 0.8\%$ , whereas the lowest activity of  $32.1\% \pm 1.1\%$  was obtained with the hexane extract at  $500 \mu\text{g/mL}$ . In the previous study [13], 10 polyphenolic compounds (1–10) were isolated and confirmed as major polyphenolics in barley seedlings. Therefore, we isolated 10 polyphenolic compounds from 150 g of BS extract as described preciously and identified by spectroscopic data (NMR spectrum are available as Additional file 1: Figs. S1–60). These 10 polyphenolic compounds (1–10) were 3-*O*-feruloylquinic acid (1), lutanarin (2), saponarin (3), isoorientin (4), orientin (5), isovitexin (6), isoorientin-7-*O*-[6-sinapoyl]-glucoside (7), isoorientin-7-*O*-[6-feruloyl]-glucoside (8), isovitexin-7-*O*-[6-sinapoyl]-glucoside (9), and

isovitexin-7-*O*-[6-feruloyl]-glucoside (10) (Fig. 1). The weight contribution of the total isolated polyphenols and each isolated compound 1–10 were 1.3%, 16.7%, 71.4%, 1.5%, 1.5%, 1.1%, 1.4%, 1.8%, 1.2%, and 2.1%, respectively (LC chromatogram of the BS extract and the content of the isolated polyphenols are available as Additional file 1: Fig. S61 and Table S2, respectively).

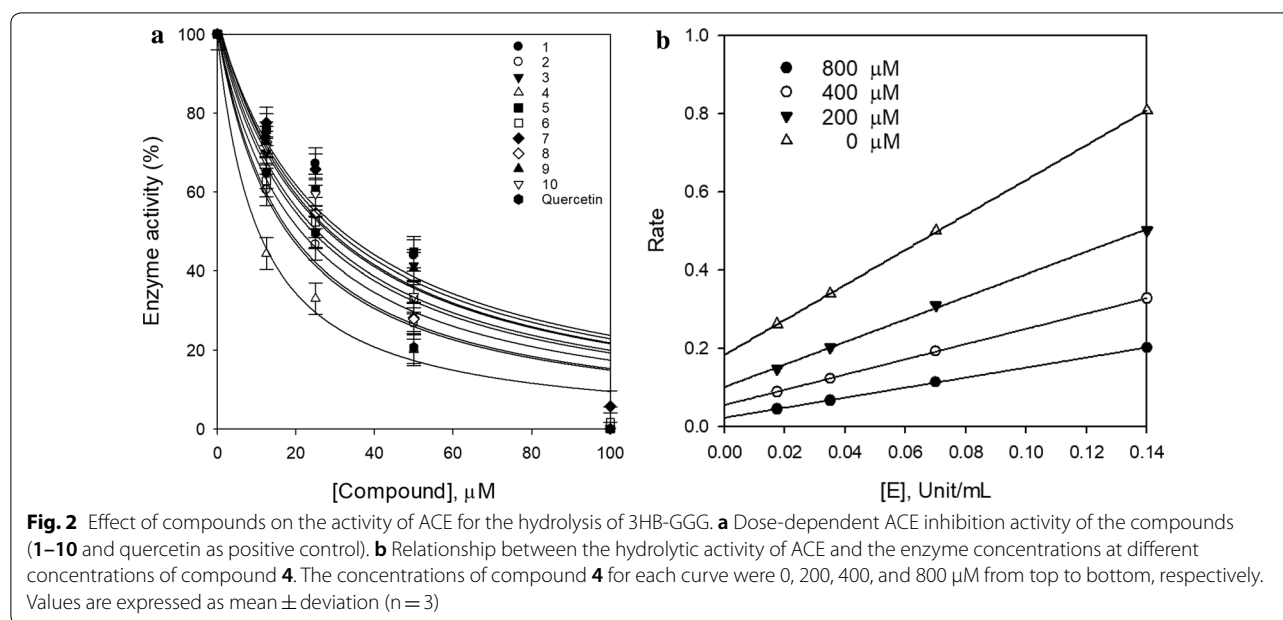
### ACE inhibition activity

The inhibitory activities of the compounds 1–10 on ACE were investigated using a fluorometric method [14, 15]. The reaction kinetic parameters of the ACE inhibition effect for the hydrolysis of 3HB-GGG were obtained using the substrate concentration (200–800  $\mu\text{M}$ ) versus the enzyme concentration of ACE (0.5–0.8 U/mL) with aminoacylase (86 kU/mL). And the formation of 3HB was compared with quercetin as a positive control which is a well-known potent inhibitor of ACE among polyphenols [18–20]. Compounds 1–10 exhibited significant dose-dependent inhibition of ACE (Fig. 2a); the inhibition concentration ( $IC_{50}$ ) values ( $\mu\text{M}$ ) of compounds 1–10 are presented in Table 1. All compounds exhibited excellent ACE inhibition activities, with the  $IC_{50}$  values ranging between 7.3 and 43.8  $\mu\text{M}$  (Table 1), compared with quercetin ( $IC_{50} = 25.2 \mu\text{M}$ ,  $K_i = 24.9 \mu\text{M}$ ). As shown in Fig. 2b, as the concentration of compound 4 was increased, the enzyme activity was rapidly diminished and resulted in lowering of the slope of the line. Furthermore, the generation of 3HB by the action of ACE increased with the increase in ACE concentration. Therefore, the inhibition of compound 4 was reversible against ACE [16, 17, 21].

The most potential inhibitor to ACE was compound 4 whose  $IC_{50}$  value was 7.3  $\mu\text{M}$  ( $K_i = 6.6 \mu\text{M}$ ), whereas compound 1 that does not have the flavone moiety exhibited the least activity with an  $IC_{50}$  value of 43.8  $\mu\text{M}$  ( $K_i = 41.4 \mu\text{M}$ ). The number of hydroxyl groups in the B-ring had a positive effect on the inhibitory activity (compound 4:  $IC_{50} = 7.3 \mu\text{M}$  vs. compound 6:  $IC_{50} = 26.1 \mu\text{M}$ , and compound 2:  $IC_{50} = 15.9 \mu\text{M}$  vs. compound 3:  $IC_{50} = 24.4 \mu\text{M}$ ). This result suggested that the free hydroxyl groups of flavone moieties in polyphenols formed chelates with zinc ions, which are a part of the ACE active site [22]. As a consequence of these reactions, the activity of ACE was interfered. A previous study also reported similar results wherein the number of hydroxyl groups in hydroxybenzoic acid plays a role as the key factor on the activity of ACE [23]. The glucose connections at the A-ring of the flavone moieties were important factors that also had an influence on the inhibition potential. For example, the flavone 6-*C*-glucose moiety at the A-ring of flavones-glucosides 4 ( $K_i = 6.6 \mu\text{M}$ ) was more effective than 8-glucosylated-flavone 5 ( $K_i = 26.8 \mu\text{M}$ ).



**Fig. 1** The structures of compounds **1–10** isolated from BS used in this study. The polyphenolic compounds **1–10** were 3-*O*-feruloylquinic acid **1**, lutonarin (**2**), saponarin (**3**), isoorientin (**4**), orientin (**5**), isovitexin (**6**), isoorientin-7-*O*-[6-sinapoyl]-glucoside (**7**), isoorientin-7-*O*-[6-feruloyl]-glucoside (**8**), isovitexin-7-*O*-[6-sinapoyl]-glucoside (**9**), and isovitexin-7-*O*-[6-feruloyl]-glucoside (**10**); these isolated compounds have been identified with spectroscopic data in a previous study [13]



**Table 1** Comparisons of an angiotensin-converting enzyme (ACE) inhibition effects on the compounds (1–10) from barley seedlings (BS)

Compound	ACE inhibition	
	$\text{IC}_{50}$ ( $\mu\text{M}$ ) <sup>a</sup>	Type of inhibition, $K_i$ ( $\mu\text{M}$ ) <sup>b</sup>
3- <i>O</i> -feruloylquinic acid (1)	43.8 $\pm$ 0.6	Noncompetitive, 41.4 $\pm$ 1.2
Lutonarin (2)	15.9 $\pm$ 1.1	Noncompetitive, 15.9 $\pm$ 0.3
Saponarin (3)	24.4 $\pm$ 1.2	Noncompetitive, 24.1 $\pm$ 0.9
Isoorientin (4)	7.3 $\pm$ 0.1	Noncompetitive, 6.6 $\pm$ 1.6
Orientin (5)	24.4 $\pm$ 1.6	Noncompetitive, 26.8 $\pm$ 1.7
Isovitexin (6)	26.1 $\pm$ 0.5	Noncompetitive, 21.1 $\pm$ 0.3
Isoorientin-7- <i>O</i> -[6-sinapoyl]-glucoside (7)	36.2 $\pm$ 0.8	Noncompetitive, 32.1 $\pm$ 1.3
Isoorientin-7- <i>O</i> -[6-feruloyl]-glucoside (8)	29.8 $\pm$ 0.5	Noncompetitive, 28.4 $\pm$ 0.9
Isovitexin-7- <i>O</i> -[6-sinapoyl]-glucoside (9)	40.7 $\pm$ 0.1	Noncompetitive, 37.2 $\pm$ 1.1
Isovitexin-7- <i>O</i> -[6-feruloyl]-glucoside (10)	35.4 $\pm$ 0.1	Noncompetitive, 33.2 $\pm$ 0.4
Quercetin <sup>c</sup>	25.2 $\pm$ 0.2	Noncompetitive, 24.9 $\pm$ 1.7

All extracts were examined in a set of experiments repeated three times

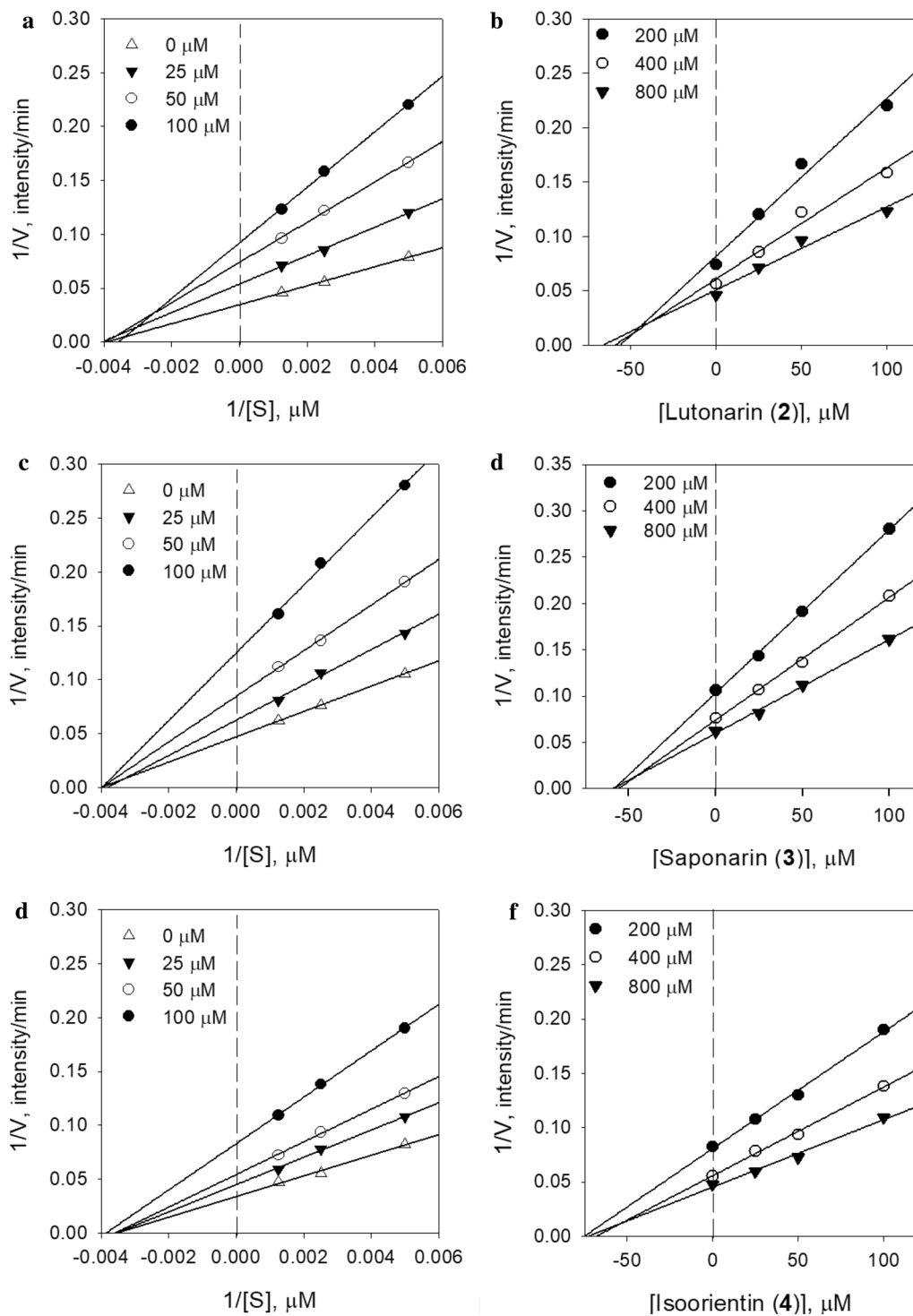
<sup>a</sup>  $\text{IC}_{50}$  values of compounds represent the concentration that caused 50% enzyme activity loss

<sup>b</sup> Values of inhibition constant

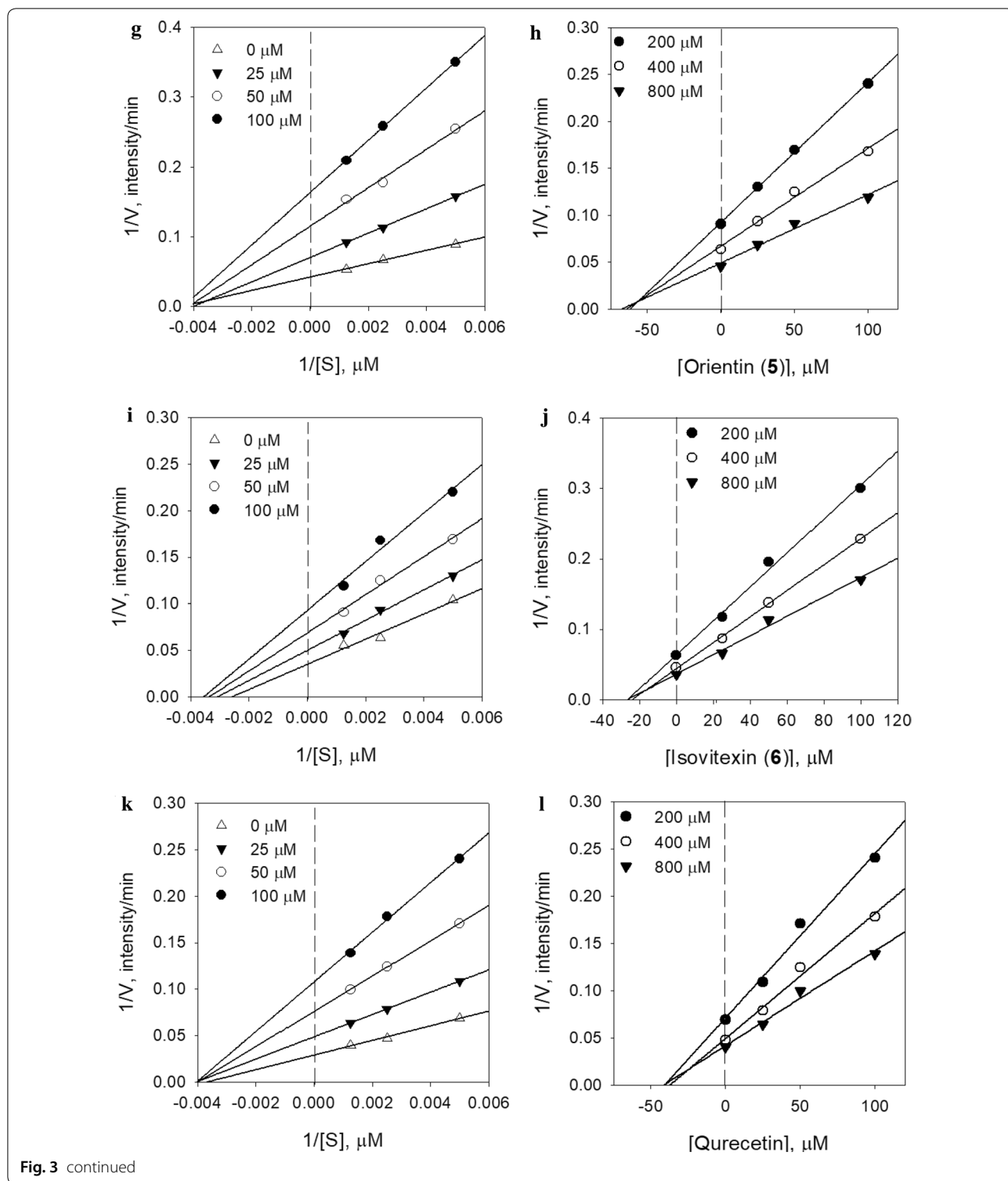
<sup>c</sup> This compound was used as a positive control

And, the inhibition activity on ACE tended to decrease with the presence of sinapoyl-glucoside or feruloyl-glucoside at C-7 of the A-ring (compound 4:  $\text{IC}_{50}=7.3 \mu\text{M}$  and compound 6:  $\text{IC}_{50}=26.1 \mu\text{M}$  vs. compound 7:  $\text{IC}_{50}=36.2 \mu\text{M}$ , compound 8:  $\text{IC}_{50}=29.8 \mu\text{M}$ , compound 9:  $\text{IC}_{50}=40.7 \mu\text{M}$ , and compound 10:  $\text{IC}_{50}=35.4 \mu\text{M}$ ). Also, compounds 2 and 3, major polyphenols in BS [24], exhibited the excellent ACE inhibition activities, with their  $\text{IC}_{50}$  values being 15.9 and 24.4  $\mu\text{M}$ , respectively.

In plant flavonoids, a family of polyphenolic compounds, including anthocyanins (e.g., delphinidin-3-*O*-sambubioside and cyanidin-3-*O*-sambubioside) [25], flavonols (e.g., quercetin glucuronide and quercetin-3-*O*-(6''-galoyl)-galactoside) [26], and flavan-3-ols (e.g., epicatechin dimer, epicatechin tetramer, and epicatechin hexamer) [27, 28], have been investigated for their ACE inhibition effect. However, they exhibited less



**Fig. 3** Determination of the inhibition type for compound 2–6, which showed the predominant ACE inhibition activity, and quercetin as a positive control. **a, c, e, g, i, and k** represent the Lineweaver–Burk plots for compounds 2–6 and quercetin on the ACE inhibition activity for the hydrolysis of 3HB-GGG in the presence of four different concentrations of compounds, 0, 25, 50, and 100 μM, **a** for lutanarin (2), **c** for saponarin (3), **e** for isoorientin (4), **g** for orientin (5), **i** for isovitexin (6) and **k** for quercetin, respectively. **b, d, f, h, j, and l** represent the Dixon plot for compound 2–6 and quercetin on the ACE inhibition activity for the hydrolysis of 3HB-GGG in the presence of four different concentrations of compounds, 0, 25, 50, and 100 μM, **b** for lutanarin (2), **d** for saponarin (3), **f** for isoorientin (4), **h** for orientin (5), **j** for isovitexin (6) and **l** for quercetin, respectively



ACE inhibition activity compared to that of other isolated flavonoids investigated in the present study.

The Lineweaver–Burk plot is useful in diagnosing inhibitor modality as a means of estimating the kinetic

parameters  $K_m$  and  $V_{max}$ . The Dixon plot is also useful in determining the inhibitor  $K_i$ , which is the dissociation constant, when the inhibitor binds to the enzyme [16, 17]. These two kinetic plots of compounds 2–6,

including quercetin, which exhibited the predominant ACE inhibition activity, are depicted in Fig. 3. In the Dixon plot (Fig. 3b, d, f, h, j, and l), when the concentrations of compounds 2–5 and 6 were increased, the  $V_{\max}$  values were decreased without changing the  $K_m$  value. In addition, as shown in Lineweaver–Burk plot (Fig. 3a, c, e, g, i, and k), three different concentrations of compounds 2–6 converged at one point on the negative x-axis, respectively. These data indicated that compounds 2–6 were noncompetitive inhibitors of ACE and have affinity for both the free ACE and the ACE–substrate complex. The inhibitor constant  $K_i$  values, which represent the degree of binding of the inhibitor to the enzyme, were calculated using the Dixon plot. The  $K_i$  values for compounds 2–6 and quercetin were 15.9, 24.1, 6.6, 26.8, 21.1, and 24.9  $\mu\text{M}$ , respectively. Among these compounds, the affinity of compound 4 was the highest. The detailed information of other compounds has been described in Additional file 1: Fig. S62. Because of the structural nonsimilarity with the natural substrate of ACE, this noncompetitive inhibition for ten compounds (1–10) was accepted in this experiment.

The ACE inhibitory activity was highest in methanol extract of barley seedlings, and isolated polyphenols from methanol extract were exhibited notable ACE inhibition activity. This result implied that the methanol extract contains high levels of ACE inhibitors. This was consistent with a previous investigation, wherein methanol was found to be an appropriate solvent for the maximum extraction of polyphenols from natural sources [29]. Therefore, the ACE inhibition activities of BS methanol extracts were derived from the polyphenolic compounds present in BS. In conclusion, the results of our study have highlighted the therapeutic potential of BS, which can be used as a healthy food and additive, for lowering blood pressure in humans.

## Supplementary information

**Supplementary information** accompanies this paper at <https://doi.org/10.1186/s13765-020-00519-9>.

**Additional file 1.** NMR spectrum, LC chromatogram and content of the 10 isolated compounds, ACE inhibition activity of BS extracts, inhibition kinetic mode of compounds 1, 7, 8, 9, and 10 are available as supplementary materials.

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## Authors' contributions

J-ER were contributors in writing the manuscript and performing major data analysis. S-YW was performing data analysis of enzyme assay. H-J, HYK and HH performed the minor experiments and prepared the raw materials. MJL and IMC data were contributed in discussion of experimental results, JHL was contributed in design a major experiment, WDS planned and led this research. All authors read and approved the final manuscript.

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## Availability of data and materials

All data generated or analysed during this study are included in this published article and its additional files.

## Competing interests

The authors declare that they have no competing interests.

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