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Original Research Article

Potential activities for constituents from *Vicia faba* L.

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ABSTRACT

A survey of phytochemical and biological investigation of *Vicia faba* L. flowers resulted in isolation of polyphenolic and nitrogenous compounds; kaempferol-3-*O*-α-D-galactopyranosyl 7-*O*-α-L-rhamnopyranoside **1**, kaempferol-3-*O*-(3"-*O*-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside **2**, kaempferol-3-*O*-α-L-rhamnopyranosyl (1→6)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside **3**, allantoin **4** and adenosine **5**. For **1-3**, it is the first report of their isolation from *V. faba* L.

The structure of the isolated compounds has been characterized on the basis of spectroscopic methods in addition to comparison with literature data. These compounds along with methanol extract were tested to evaluate their antioxidant activity estimated by oxygen radical absorption capacity (ORAC) assay and anti-allergic activities through inhibition of β-hexosaminidase enzyme. For the antioxidant activity, both methanolic extract and the isolated compounds exhibited a high activity ranged from 3.1-9.6 mg TE/mg extract. For the antiallergic activity, it was noticed that all the tested compounds and methanol extract have good inhibition for β-hexosaminidase release without affecting the cell viability where the production of β-hexosaminidase was decreased to about 69-82%. These results revealed that the methanol extract and its isolated compounds of *V. faba* L. flowers had high antioxidant and high anti-allergic activities. It is expected that *V. faba* L. flowers could be used for the treatment of oxidative stress and will find new and high-value-added uses in cosmetics.

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1. Introduction

In folk medicine systems, a number of plants have been widely used for the treatment of various disorders since ancient times. Plants are a great source of bioactive metabolites, including phenolics, nitrogenous compounds, polysaccharides, alkaloids, essential oils, steroids, lignins, resins, tannins, etc. (Mohammadhosseini et al., 2016; Aidi Wannes et al., 2017; Camilo et al., 2017; Frezza et al., 2017; Gvazava et al., 2017; Mohammadhosseini, 2017a, 2017b; Mohammadhosseini et al., 2017a; Mohammadhosseini et al., 2017b; Sarker et al., 2017; Sultana et al., 2017). Among them, polyphenolics obtained from plants have various applications, especially in the health, agriculture, food, and cosmetic industries (María et al., 2015). Previous scientific studies clearly revealed that polyphenols possess various pharmacological properties such as antioxidant, antimicrobial, antiviral, antimutagenic, anticancer, anti-inflammatory, and immunomodulatory activities and as a potential source of drugs for the treatment of ischaemic heart disease (Du et al., 2016).

Among the plants rich in polyphenols, *Vicia faba* L. (broad bean) is a legume belonging to the plant family *Fabaceae*. Fava beans (*V. faba* L.) is a popular food in many countries. It is an important winter crop in Mediterranean areas and is mostly a spring crop in other regions of Europe and South America and is one of the major plant food item for the Nile River

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populations (Seif et al., 2015).

Fava beans are very high in protein content as in other beans and lentils. The beans, however, compose plentiful of health benefiting antioxidants, vitamins, minerals, and plant-sterols. Broad beans are rich in phyto-nutrients such as isoflavone and plant-sterols. Isoflavone such as genistein and daidzein have been found to protect breast cancer in laboratory animals. Phytosterols, especially β-sitosterol, help lower cholesterol levels in the body (Rocha et al., 2016).

In human nutrition only the seeds are used, while the whole plant is used as feed for animals however; it is used in folk medicine as antihyperlipidimic to control cholesterol (Mulvihill and Huff, 2010; Bouchenak and Lamri-Senhadji, 2013; Du et al., 2016).

Many reports have focused on the *V. faba* L. seed, as it contains a high number of bioactive compounds such as proteins (protease inhibitors, α-amylases, lectins), glycosides (α-galactosides, vicine and convicine), tannins, saponins, and alkaloids (Piotr et al., 2014). In contrast to the *V. faba* L. seed, little research has focused on the *V. faba* L. flower, with the exception of a study that isolated the tyramine, jasmonic acid and some flavonoid monoside derivatives from *V. faba* L. flowers (Kapinová et al., 2015). Moreover, the antioxidant capacity was evaluated by the DPPH radical scavenging capacity, total antioxidant capacity, and oxygen uptake inhibition. The previous results (Boukhanouf et al., 2016) showed that the immature faba bean fractions had significantly higher phytochemical contents and displayed a better antioxidant activity than those of mature ones; the highest level of phytochemicals and the strongest antioxidant activity were recorded in the seed coat. This finding encouraged us to study the constituents of the flowers as well as some potential activities including antioxidant and antiallergic activity of the isolated compounds. This study is the first of its kind to evaluate antioxidant and antiallergic abilities of *V. faba* L. flowers and its isolated compounds.

2. Experimental

2.1. Material and methods

2.1.1. Plant material

Flowers of *V. faba* L. were collected in full bloom stage at March 2016 from the upper Egypt about 500 Km far from Cairo and a voucher specimen (V-22) was deposited at the Laboratory of Systemic Forest and Forest Product Sciences, Department of Agro-Environmental Science, Kyushu University, Japan.

2.1.2. Extraction and isolation

Air-dried *V. faba* L. flowers (100 g) were extracted three times with MeOH (5 L of each) at room temperature to yield the methanol extract (16 g), which was suspended in distilled water and partitioned between chloroform, ethyl acetate and *n*-butanol (1.0 L each) to give the chloroform fraction (3.5 g), ethyl acetate fraction (4 g), *n*-butanol fraction (5.5 g), and the remaining aqueous fraction (2.5 g). Based on chemical screening of the methanolic extract of *V. faba* L. most of flavonoid glycosides and nitrogen containing compounds (to which the biological activity is attributed) is present in the ethyl acetate fraction (Piotr et al., 2014) and hence, the ethyl acetate fraction was subjected to further bioassays in this research and sub-fractionated on a silica gel column using $CHCl_3$-MeOH gradient elution (25%, 50%, 75% and 100%; 2 L each). The fraction eluted by 50% methanol (2.1 g) was further separated by chromatography on an ODS column (80×200 mm; Cosmosil 140 C_{18} PREP, Nacalai Tesque, Kyoto, Japan) using six mobile phase systems of MeOH-H_2O (10%, 25%, 40%, 50%, 70% and 90% v/v; elution volume: 1.5 L of each) to give six corresponding fractions. The fraction eluted with 40% MeOH (850 mg) was further chromatographed by column chromatography on silica gel and eluted on a stepwise gradient of $CHCl_3$-MeOH (ratios of 9:1, 6:1, 4:1, 3:1 and 1:1; v/v elution volume: 200 ml each) to give five corresponding fractions. The fraction eluted with 4:1 resulted in isolation of **1** (25 mg) and **2** (16 mg), and that eluted with 3:1 $CHCl_3$-MeOH resulted in elution of compounds **3-5** (12, 15 and 8 mg respectively).

2.2. Apparatus

^1H, ^{13}C NMR and 2D spectra of the isolated compounds were recorded using a Bruker DRX 600 NMR spectrometer (Bruker Daltonics, Billerica, MA). HR-ESI-MS was determined using LC-MS-IT-TOF (Shimadzu, Tokyo, Japan). The instrument was fitted with an Inertsil ODS-3, 5 µm, 4.6×150 mm column (GL Science, Tokyo, Japan), using a mobile phase composed of solvents A (water) and B (methanol). The total flow rate was 0.5 mL/min. Based on the previous result of HPLC-PDA analysis, the LC chromatogram of compound (**1**) was obtained at UV 250 nm and 280 nm. Using isocratic elution of water-methanol (60: 40), R_t of compound (**1**) was 7.5 min. The MS instrument was operated using an ESI source in both positive and negative ionization modes with survey scans acquired from *m/z* 100-2000 for MS and *m/z* 50-1500 for MS/MS. The ionization parameters were as follows: probe voltage, ±4.5 kV; nebulizer gas flow, 1.5 L/min; CDL temperature, 200 ºC; heat block temperature, 200 ºC. Dimethylsulfoxide (DMSO) and other organic solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sephadex LH-20 was purchased from GE Healthcare (Uppsala, Sweden). Silica gel (75-120 mesh) and RP-18 silica gel (38-63 µm) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Thin layer chromatography (TLC) silica gel 60 F_{254} was purchased from Merck (Darmstadt, Germany). The developed chromatograms were visualized under

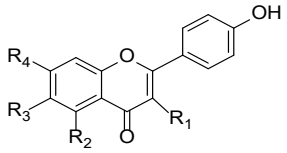
254 nm UV light and the spots were made visible by spraying with1% w/v vanillin/H_2SO_4 reagent before warming in an oven preheated to 110 ºC for 5 min.

2.3. Antioxidant (ORAC) assay

In terms of antioxidant activity, samples were estimated by oxygen radical absorption capacity (ORAC) assay. The ORAC assay was conducted as described previously (Garrett et al., 2010; Roy et al., 2010). Each sample (the methanolic extract and compounds: **1-5**) was dissolved in 75 mmol/L phosphate buffer and diluted the buffer at a moderate concentration. This concentration of the buffer should be appropriate for the different concentrations of the trolox solution used for making the standard curve. The standard curve was made with 50, 25, 12.5, and 6.25 µmol/L trolox solution. Mixtures of fluorescein with the sample solution or trolox were incubated at 37 ºC for 10 min. Then, AAPH (2.2´-azobis(2-amidinopropane) dihydrochloride) was added and the absorbances at excitation and emission wavelengths of 485 and 515 nm respectively were measured with a fluorescence spectrophotometer for 90 min. The results were expressed as mg of trolox equivalents (TE) on the basis of the extract (mg TE/mg extract).

2.4. Procedure: RBL-2H3 Cell Line Assay

RBL-2H3 cells are the tumor analog of mast cells, which after being sensitized with mouse monoclonal IgE or ionophore A23187 respond by releasing inflammatory mediators such as β-hexosaminidase (Qianqian et al., 2015; Taehun et al., 2015). As a result, the sample is considered to have anti-allergic activity if it can inhibit mast cells degranulation and produce a significant reduction in β-hexosaminidase release.



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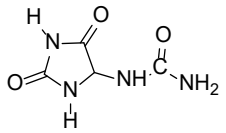

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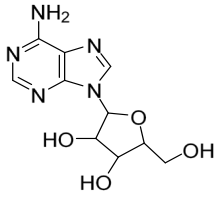

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1	R_1= -*O*-α-D- galactopyranosyl, R_2= OH, R_3= H, R_4= -O-α-l-rhamnopyranoside
2	R_1= 3-*O*-(3"-*O*-α-l-rhamnopyranosyl)-α-L-rhamnopyranoside , R_2 = -OH, R_3= H, R_4= OH
3	R_1=3-*O*-α-l-rhamnopyranosyl(1→6)-β-D-galactopyranoside , R_2= OH, R_3= H, R_4= -*O*-α-L-rhamnopyranoside

Fig. 1. Structure of compounds **1-5**.

2.4.1. Cell culture

RBL-2H3 cells (100 µL, 1×105 cells/well) were cultured with EMEM in a 96-well plate for 24 h. Then samples (1 µL/well; DMSO 1 µL as control) were added.

2.4.2. Cell viability assay

A cell viability assay (using MTT) was performed to ensure that the activity of the sample at the used concentration was related to the inhibition of histamine release rather than to the cytotoxicity of the RBL-2H3 cells. The cell viability assay was done as follows: After 24 h incubation of cultured cells in a CO_2 incubator at 37 ºC, MTT (10 µL, 5 mg/mL in PBS) was added to each well, and the plate was incubated for another 4 h. The medium was then removed, acid iso-propanol (100 µL) containing HCL (0.04 N) reagent was added to each well, the plate was incubated overnight at room temperature, and the absorbance was read at 570 nm using a microplate reader.

2.4.3. Anti-allergy assay

The anti-allergy assay was determined as described by (Yun et al., 2010), with minor modification, as follows: RBL-2H3 cells (1×106 cells/well) were inoculated with EMEM in a 96-well plate for 48 h, then EMEM medium was replaced by tyroid buffer with the composition; [100 µL, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2.6H_2O$, 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 5.6 mM glucose, 0.1% BSA, pH 7.2/well], after preparing tyroid medium, sample (1 µL/well) was added, and the plate was incubated for 30 min in CO_2 incubator at 37 ºC. A23187 (Calcimycin, Calcium Ionophore, Antibiotic A23187) (10 µg/mL, 2 µL/well) was added after removal of the sample and the addition of new tyroid buffer (100 µL/well). After 30 min incubation, 50 µL from each well was collected and transferred to another 96-well plate. An equal volume of substrate solution (l mM), *p*-nitrophenyl-*N*-acetyl-β-glucosaminide was added to each well, and the plate was left at room temperature on the shaker for 1 h. Finally, the reaction was terminated by adding 100 µL of stopping buffer (Na_2CO_3, 100 mM, pH=10) (which is a solution used to terminate the peroxidase reaction for ELISA applications), and the absorbance was measured at 405 nm using a microplate reader. The statistical difference between the control and each sample was determined by student's t-test.

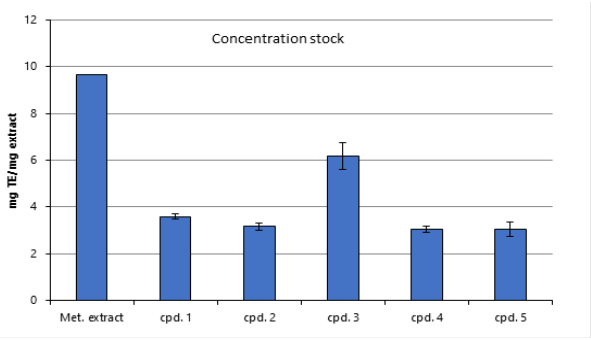

Fig. 2. ORAC values of the methanol extract and isolated compounds (**1-5**) of *Vicia faba* L. flowers, values are expressed as mg Trolox eq. /mg compound or extract and presented as means ±SD (n=3).

3. Results and Discussion

3.1. Identification of compounds

The aerial parts of *V. faba* L. flowers were extracted with methanol and then fractionated with $CHCl_3$, EtOAc, *n*-BuOH and H_2O. By using combined chromatographic separation of the EtOAc fraction, three polyphenolic compounds; Kaempferol-3-*O*-α-D-galactopyranosyl 7-*O*-α-L-rhamnopyranoside **1** (Xu et al., 2009; Halabalaki et al., 2011), kaempferol-3-*O*-(3"-*O*-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside **2** (Jiaju et al., 2011), kaempferol-3-*O*-α-L-rhamnopyranosyl(1→6)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside **3** (Petpiroon et al., 2015), and two nitrogenous compounds; allantoin **4** (Shubashini et al., 2011) and adenosine **5** (Jiang et al., 2011) (Fig. 1) were isolated.

3.2. Antioxidant (ORAC) assay

The methanolic extract showed a high antioxidant activity (9.6 mg TE/mg extract) which means that the activity of 1 mg of the methanolic extract is equivalent to that of 9.6 mg TE/mg.

For the isolated compounds, it was noticed that compound **3**, kaempferol-3-*O*-α-L-rhamnopyranosyl (1→6)-β-D-galactopyranoside-7-*O*-α-L-rhamnopyranoside, has the highest antioxidant activity (6.1 mg TE/mg extract). This may be due to the presence of a higher number of hydroxyl groups compared to other isolated flavonoids. Followed by compound **1**, Kaempferol-3-*O*-α-D-galactopyranosyl 7-*O*-α-L-rhamnopyranoside (3.6 mg TE/mg extract) then compound **2**, kaempferol-3-*O*-(3"-*O*-α-L-rhamnopyranosyl)-α-L-rhamnopyranoside, (3.1 mg TE/mg extract). For compounds **4**, allantoin and compound **5**, adenosine, they have nearly the same effect (3.0 mg TE/mg extract). This result supports the high antioxidant activity of flavonoids over that of purine basis products (Jakub and Karel, 2016) (Table 1, Fig. 2).

A previous recent study was carried out on phytochemicals and antioxidant activity of different *V. faba* L. bean parts (except flowers) at immature and mature stages (Boukhanouf et al., 2016). This study showed that phytochemical contents and antioxidant activity of different immature and mature faba bean parts were reduced significantly by steam cooking and boiling. As boiling caused higher loss in phytochemicals than steaming, this latter should be the best method in retaining these bioactive compounds. Moreover, that work supported that immature faba beans have higher amounts of phenolic compounds and exhibited a better antioxidant activity than mature ones.

3.3. Antiallergic (RBL-2H3 Cell Line Assay) assay

After establishing the structures, compounds (**1-5**) were investigated using RBL-2H3 Cell Line Assay. It was noticed that all tested compounds and methanol extract exhibited inhibition for β-hexosaminidase release without affecting the cell viability. Among the isolated compounds, compound (**1**) exhibited inhibition activity 21.4%, while compound (**5**) exhibited 21.3% and compound (**3**) exhibited 21.1% and compound (**2**) exhibited 20.7% while compound (**4**) exhibited 18% for β-hexosaminidase activity at a final concentration of 20 μM the same as the positive control quercetin. For the methanol extract, it exhibited about 30.7% inhibition activity in 100 μg/mL, without effect on cell viability (Table 2, Fig. 3). The suggested mechanism of the polyphenols on the mast cell-mediated allergic inflammation was studied previously (Hyo-Hyun et al., 2008), as they inhibited IgE or phorbol-12-myristate 13-acetate and calcium ionophore A23187 (PMACI)-mediated histamine release in RBL-2H3 cells. The pharmacological actions of polyphenols suggest their potential activity for the treatment of allergic inflammatory diseases through the down-regulation of mast cell activation.

4. Concluding remarks

The previous studies which focused on parts of *V. faba* L. other than flowers, showed that the immature faba bean fractions had significantly higher

Table 1
ORAC values of the methanol extract and the isolated compounds from the flowers of *Vicia faba* L.[a]

Compound/Extract	ORAC value [b]
1	3.59 ± 0.09
2	3.17 ± 0.15
3	6.17± 0.57
4	3.05 ± 0.11
5	3.03 ± 0.30
Methanol extract	9.67 ± 0.37

[a] The results are expressed as mean values ± SD (n=3).
[b] The concentration is mg Trolox eq. /mg compound or extract

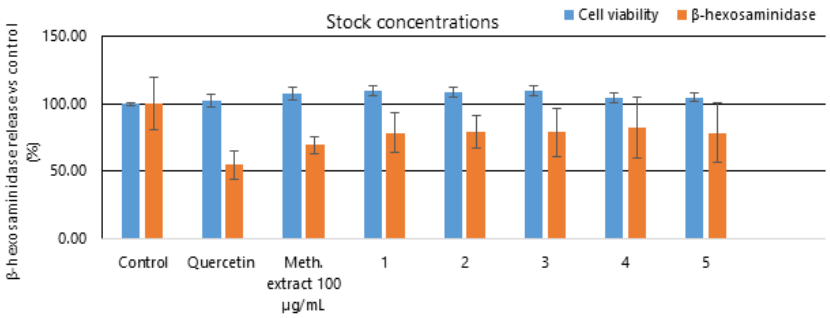

Fig. 3. Cell viability and effects of the isolated compounds (**1-5**) and methanol extract on ionophore A23187-stimulated β-hexosaminidase release from RBL-2H3 basophilic leukemia cells, all the values are the mean ± SD (n=3).

Table 2
Effects of the methanol extract and the isolated compounds on β-hexosaminidase production[a].

Compound/extract	β-Hexosaminidase production (%)	Cell viability (%)
Control	100.00 ± 8.055	100.00 ± 4.40
Positive control (Quercetin) [b]	54.92 ± 10.46	102.42 ± 3.17
1	78.66 ± 14.97	109.61 ± 4.21
2	79.35 ± 12.29	109.18 ± 4.45
3	78.92± 18.03	110.03 ± 5.79
4	82.13 ± 22.62	104.64 ± 2.14
5	78.75 ± 22.27	104.79 ± 4.25
Methanol extract (100 µg/mL)	69.38 ± 6.56	108.03 ± 3.15

[a] The results are expressed as mean values ± SD (n=3).
[b] Final concentration of the compounds and the positive control (Quercetin) was 10 µM.

phytochemical contents with strong antioxidant activity which recorded in the seed coat and hence, this study is the first report to study the constituents of the flowers as well as some potential activities including antioxidant and antiallergic activity of the isolated compounds.

In the present study, from *V. faba* L. flowers, many compounds including flavonoid glycosides, allantion and adenosine were found to have antioxidant and antiallergic propereties. All fractions and the isolated compounds were tested against these activities which resulted in that both methanol extract and the isolated compounds have high antioxidant activity ranged from 3.1-9.6 mg TE/mg extract. For the antiallergic activity, it was noticed that all the tested compounds and methanol extract have good inhibition for β-hexosaminidase release without affecting the cell viability where the production of β-hexosaminidase was decreased to about 69-82%.

This finding could be a good opportunity for researchers to provide such compounds for the treatment of oxidative stress and allergic conditions and in cosmetic purposes. Moreover, other potential activities should be studied for that rich source of polyphenols.

Statistical analysis

The results are expressed as mean values ± SD. For ORAC antioxidant calculations, the concentration is mg Trolox eq. /mg compound or extract.

Conflict of interest

The authors declare that there is no conflict of interest.

Supplementary material

Spectroscopic data of compounds **1-5** are available as electronic supplementary material. The online version of this article contains supplementary material, which is available to all researchers free of charge.

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