LWT - Food Science and Technology xxx (2014) 1–8

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Inhibitory effect of daylily buds at various stages of maturity on nitric oxide production and the involved phenolic compounds

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ARTICLE INFO

Article history:
Received 19 April 2014
Received in revised form
21 October 2014
Accepted 9 November 2014
Available online xxx

Keywords:
Daylily flower bud
Maturity
Nitric oxide
Flavonoids
Phenolic compounds

ABSTRACT

The objective of this study was to investigate the antioxidant activity and anti-inflammatory ability of daylily buds during their various developmental stages, from green (stage 1) to ripe maturity (stage 4). Thus, the nitric oxide (NO) scavenging effects of the bud extract in sodium nitroprusside (SNP) system and the inhibitory effects on NO production in RAW 264.7 macrophages were determined. The highest total antioxidant activity was observed at stage 1, along with the highest content of total phenolics and flavonoids. Nevertheless, the NO scavenging activity of bud extracts increased from stage 1 to its maximum at stage 4; the data indicated that rutin might be the vital compound that was responsible for this activity. In the macrophage model system, the inhibitory effect of bud extracts on NO production was concentration-dependent. The strongest NO-suppressing activity was observed at stage 1 and decreased with advancing maturity as buds were extracted at a concentration of 400 μg/mL. Flavan-3-ol EGCG and (-)-epicatechin might be the primary active constituents in the extracts that inhibited NO production in lipopolysaccharide (LPS)-activated cells. Daylily buds are an effective NO scavenger and suppressor that may be favorable for the alleviation of inflammatory conditions and beneficial for human health.

1. Introduction

Under physiological conditions, NO is a crucial regulatory molecule that exhibits an enormous range of beneficial functions in organisms, including regulation of blood pressure, platelet function, and neurotransmission (Jagetia, Rao, Baliga, & Babu, 2004). NO plays an essential role in the host defense mechanism and is generated in large amounts during infection and inflammation through immunological stimulation and may cause some adverse effects on host cells (Marcocci, Maguire, Droy-Lefaix, & Packer, 1994). NO is synthesized from L-arginine by a family of nitric oxide synthase (NOS), which are either constitutive (cNOS) or inducible (iNOS). iNOS is not detected in healthy tissues, but is induced quantitatively by inflammatory stimuli in various cells, including macrophages and smooth muscle cells, hepatocytes and fibroblasts (Mayer & Hemmens, 1997). Excess NO production caused by overexpressed iNOS accelerates the formation of reactive nitrogen species, triggers several deleterious cellular responses, and causes immunopathological disorders such as inflammation, sepsis, and atherosclerosis (Beckman & Koppenol, 1996). Therefore, inhibition of NO production by inhibiting the expression or activity of iNOS and its scavenging might ameliorate such injuries.

Interest in botanical phytochemicals has recently increased because they are considered promising therapeutic drugs for the treatment of free radical pathologies. Plants that are used in traditional medicine can be a source of therapeutic constituents for the cure and prevention of chronic diseases such as cancer, cardiovascular disease, and aging related diseases. Daylilies have been harvested for several centuries in eastern Asia, where they are used as ornamental plants, medicine, and food. Daylilies have been reported to be used in the nursing for depression, inflammation, and promote digestion (Cichewicz & Nair, 2002). Several constituents, such as phenolic glycoside (Cichewicz & Nair, 2002), anthraquinone (Cichewicz, Lim, McKerrow, & Nair, 2002), and lactams (Inoue, Konishi, Kiyosawa, & Fujiwara, 1994) have been identified in

Abbreviations: NO, nitric oxide; SNP, sodium nitroprusside; LPS, lipopolysaccharide; NOS, nitric oxide synthase; cNOS, constitutive nitric oxide synthase; iNOS, inducible nitric oxide synthase; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide; ABTS, 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; TEAC, Trolox equivalent antioxidant capacity; QE, quercetin equivalent; IS, internal standards; EGCG, (-)-epigallocatechin-3-gallate; BCRC, Bioresource Collection and Research Center; ANOVA, analysis of variance.

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http://dx.doi.org/10.1016/j.lwt.2014.11.023
0023-6438/

daylilies. These compounds exhibit a variety of pharmacological activities, including antioxidant, anti-tumor (Cichewicz, Zhang, Seeram, & Nair, 2004), and schistosome inhibitory effects (Cichewicz et al., 2002). Its edible flower has been shown to exhibit pronounced antioxidant activities and marked inhibitory effects on free radicals (Fu, He, Zhao, Yang, & Mao, 2009); this might be due, at least in part, to the action of antioxidant compounds, such as phenolics and ascorbic acid (Fu et al., 2009). However, maturity plays a crucial role in the variation of antioxidant activity and involved antioxidant compounds. At the stage of the flower opening, the content of ascorbic acid and the antioxidant activity are highest among all maturity stages from bud development to flower senescence (Fu et al., 2009). Bor, Chen, and Yen (2006) reported that extracts from dried mature daylily flowers (yellow) indicated a more favorable scavenging effect on NO derived from SNP in an in vitro system than that of fresh immature daylily flowers (green). Nevertheless, the extracts from green immature daylily flowers expressed stronger inhibition of NO generation in LPS-activated RAW 264.7 macrophages. Bor et al. (2006) suggested that the components that provided the inhibitory effect on NO generation in LPS-stimulated RAW 264.7 cells differed from those of the scavenging effect on NO that was derived from SNP. The composition and distribution of the effective components that were responsible for NO inhibition in RAW 264.7 macrophages and NO scavenging might have varied during flower development and therefore require identification and verification.

According to Taiwanese dietary preferences, the edible parts of daylily flowers are their flower buds, particularly those expected to bloom on the next one or two, even three days. The blooms are edible as well; nevertheless, fully opened flowers are not easy for plucking and processing such as soaking in sulphite solution, drying, and packing. Most commercial products are closed flower buds including fresh and dried ones. The objective of this present study was to study the effects of extracts of daylily buds at various maturity stages on NO scavenging in SNP system and NO suppression in LPS-stimulated macrophages. Moreover, the involved phenolic components of daylily buds were analyzed using HPLC to evaluate their contributions to the anti-inflammatory activities.

2. Materials and methods

2.1. Chemicals

SNP, LPS, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), Folin-Ciocalteu phenol reagent, and gallic acid were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Pinocembrin, procyanidin A-2, pelargonidin chloride, and authentic standards of phenolic compounds were sourced from Chromadex Co. (Irvine, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Life Technologies (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO) and all extraction and HPLC solvents (LiChrosolv, gradient grade) were sourced from Merck KGaA (Darmstadt, Germany). All other chemicals were of analytical grade purity.

2.2. Plant material and preparation

The daylily flowers (*Hemerocallis fulva* Linn.) were plucked from a pilot farm in Hualien, Taiwan, at the bud development stage. The collected flower buds were classified into four groups according to their length and stored in a freezer within 30 min of harvest; they were then transported to the lab. The frozen flower buds were lyophilized. The freeze-dried flower buds were then ground and sieved through a 70 mesh screen to obtain the daylily bud powder, which was tightly sealed in glass bottles and stored at −20 °C.

2.2.1. Daylily flower bud growth stages

The length of the flower buds increased with maturity; hence, the growth of the flower buds can be divided into four distinct stages, as follows:

Stage 1. The length of the flower bud is equal to or shorter than 3 cm (bud length ≦ 3 cm).
Stage 2. The length of the flower bud is between 3.1 and 6 cm (bud length 3.1–6 cm).
Stage 3. The length of the flower bud is between 6.1 and 8 cm (bud length 6.1–8 cm).
Stage 4. The length of the flower bud is between 8.1 and 10 cm (bud length 8.1–10 cm).

2.3. Sample extraction

Ethanol was chosen as the extraction solvent because it is safer and more environmentally friendly than other organic solvents. Moreover, ethanol is efficient in the extraction of phenolic compounds from daylily flowers (Que, Mao, & Zheng, 2007).

Sample extract was prepared according to a modified method of Que et al. (2007). The sieved and freeze-dried bud powder was extracted using 95% ethanol (25 mL/g), stirred using a magnet for 24 h, and then centrifuged at 15,000 × *g* for 30 min. The supernatants were concentrated at 37 °C at a reduced pressure and then freeze-dried to obtain the dry extracts. These ethanolic extracts were weighed to assess the extraction yields. The ethanol extracts were sealed in glass bottles and stored at −20 °C for further use.

2.4. Evaluation of antioxidant activity by using an ABTS assay

The antioxidant activity was determined using the procedure described by Arnao, Cano, and Acosta (1999), with modifications. Briefly, the $ABTS^{\bullet+}$ solution was prepared by mixing 0.25 mL of 0.1 mmol/L ABTS, 0.25 mL of 0.05 mmol/L H_2O_2, 0.25 mL of peroxidase (4.4 unit/mL), and 1.5 mL of deionized water. The mixture was then reacted at 25 °C for 1 h. After 0.25 mL of optimal diluted bud extract was added, the mixture was reacted at room temperature for 10 min. The absorbance was then measured at 734 nm. Quantification was conducted based on the standard curve of Trolox. Antioxidant capacity was presented as millimolars of Trolox equivalent antioxidant capacity (TEAC) per gram of dry extract.

2.5. Determination of total phenolics

The amount of total phenolics in the bud extracts was determined using the Folin-Ciocalteu colorimetric method described by Shi, Xu, Hu, Na, and Wang (2011), with modifications. Each optimal diluted 50 μL extract was mixed with 0.5 mL of 50% Folin-Ciocalteu reagent and 1 mL of deionized water, before being incubated at room temperature for 3 min. Following the addition of 2.5 mL of 20% aqueous sodium carbonate, the mixture was mixed thoroughly. Absorbance of the resultant solution was measured at 738 nm after 1 h. Quantification was conducted based on the standard curve of gallic acid. The results were expressed as milligrams of gallic acid per gram of dry extract.

2.6. Determination of total flavonoids

The amount of total flavonoids in the bud extracts was determined using the method described by Huang, Chang, and Shao

Please cite this article in press as: Kao, F. J., et al., Inhibitory effect of daylily buds at various stages of maturity on nitric oxide production and the involved phenolic compounds, *LWT - Food Science and Technology* (2014), http://dx.doi.org/10.1016/j.lwt.2014.11.023