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Anti-inflammatory activity of *Barleria lupulina*: Identification of active compounds that activate the Nrf2 cell defense pathway, organize cortical actin, reduce stress fibers, and improve cell junctions in microvascular endothelial cells

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Abstract

Ethnopharmacological relevance—Hot aqueous extracts of the plant *Barleria lupulina* (BL) are used for treating inflammatory conditions and diabetic vascular complications.

Aim of the Study—The goal was to identify active compounds in hot aqueous extracts of BL (HAE-BL) that are consistent with a role in reducing inflammation and reducing the vascular pathology associated with diabetes. In particular, we examined activation of the Nrf2 cell defense pathway because our initial findings indicated that HAE-BL activates Nrf2, and because Nrf2 is known to suppress inflammation. Activation of Nrf2 by HAE-BL has not been described previously.

Materials and Methods—Human endothelial cells, real-time PCR, western blotting, cytoskeletal analyses, and assay-guided fractionation with HPLC were used to identify specific compounds in HAE-BL that activate the Nrf2 cell defense pathway and reduce markers of inflammation in vitro.

Results—HAE-BL potently activated the Nrf2 cell defense pathway in endothelial cells consistent with its traditional use and reported success in reducing inflammation. Assay guided

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Chemical compounds: 4-methylcatechol (PubChem CID: 9958), 4-vinylcatechol (PubChem CID: 151398), 4-ethylcatechol (PubChem CID: 70761), poliumoside (PubChem CID: 6442411), shanzhiside methyl ester (PubChem CID: 13892722), caffeic acid (PubChem CID: 689043)

Conflict of interest

The authors confirm that there are no known conflicts of interest associated with this publication.

fractionation with HPLC identified three alkyl catechols: 4-ethylcatechol, 4-vinylcatechol, and 4-methylcatechol, that are each potent Nrf2 activators. In addition to activating Nrf2, HAE-BL and alkyl catechols each profoundly improved organization of the endothelial cell actin cytoskeleton, reduced actin stress fibers, organized cell-cell junctions, and induced expression of mRNA encoding claudin-5 that is important for formation of endothelial tight junctions and reducing vascular leak.

Conclusions—HAE-BL contains important alkyl catechols that potently activate the Nrf2 cell defense pathway, improve organization of the endothelial cell cytoskeleton, and organize tight cell junctions. All of these properties are consistent with a role in reducing inflammation and reducing vascular leak. Because activation of the Nrf2 cell defense pathway also prevents cancers, neuro-degeneration, age-related macular degeneration, and also reduces the severity of chronic obstructive pulmonary disorder and multiple sclerosis, HAE-BL warrants additional consideration for these other serious disorders.

Graphical Abstract

Keywords

Barleria lupulina; traditional medicine; Nrf2; 4-methylcatechol; 4-vinylcatechol; 4-ethylcatechol; microvascular endothelial cells; endothelial barrier function; actin cytoskeleton

1. Introduction

Barleria lupulina Lindl. (Acanthaceae family), also known as hophead Philippine violet and Vishallakarani, is a well-known historical herb used in folklore medicine of Southeast Asia, India, and South China. The plant is cultivated both as an ornamental and medicinal plant; and, for medicinal use, a hot aqueous extract of the aerial parts of the plant is taken orally. *Barleria lupulina* (BL) is traditionally used for insect and snakebites, inflammation, allergic responses, rheumatoid arthritis, and diabetes (Mazumder PM, 2012; Suba et al., 2004; Suba et al., 2005; Wanikiat et al., 2008). In particular, the Thailand Ministry of Public Health has categorized BL as an essential medicinal plant for primary healthcare (Wanikiat et al., 2008). Phytochemical studies on this plant have focused on a number of iridoid glycosids as main components (Kanchanapoom et al., 2001; Kim et al., 2015; Suksamrarn et al., 2003). However, the compounds in BL that provide medicinal benefits towards reducing inflammation and the vascular complications of diabetes have not been defined.

An important possibility, not considered previously, is that BL exerts its medicinal benefits through activation of the Nrf2 (NFE2L2) cell defense pathway (Kensler et al., 2007;

Motohashi and Yamamoto, 2004). The Nrf2 transcription factor, through binding to antioxidant response elements in ~ 200 genes, induces expression of anti-oxidant and detoxifying enzymes that protect against oxidative damage and also provide protection against toxic foreign chemical substances through phase II enzyme modification (Dinkova-Kostova and Abramov, 2015; Holmstrom et al., 2013). Compelling evidence for the importance of the Nrf2 pathway for disease prevention and control of tissue damage associated with inflammation comes from numerous studies with mice lacking the Nrf2 gene (reviewed in (Senger et al., 2016)). Also, consistent with folklore use of BL for diabetic complications, Nrf2 has been implicated in protection from diabetic pathology (de Haan, 2011; Evans and Goldfine, 2016; Li et al., 2012; Long et al., 2016). Consequently, in studies described here, we investigated regulation of the Nrf2 pathway by BL in human microvascular endothelial cells, which are pivotal “gatekeepers” of inflammation (Dejana, 2004; Granger DN, 2010) and closely involved in diabetic complications (Avogaro et al., 2011; Brownlee, 2005). In addition, we investigated BL activity towards the microvascular endothelial cytoskeleton that, at the sub-cellular level, exerts critical barrier control over blood vessel leakiness, tissue edema, and the extravasation of inflammatory cells (Bogatcheva and Verin, 2008; Lampugnani, 2010; Lum and Malik, 1994; Millan et al., 2010).

2. Materials and Methods

2.1 Plant material

The aerial parts of *B. lupulina* Lindl. (the accepted name, see: <http://www.theplantlist.org/tpl1.1/record/kew-2670049>) were purchased from Vung Tau City, Ba Ria – Vung Tau Province, Vietnam (Coordinates: 10°35'N 107°15'E), from March 2012 to March 2014. Voucher specimens (Nos. 101–103) are deposited at Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA.

2.2 Preparation of hot aqueous extract of *Barleria lupulina* (HAE-BL)

200 grams of dried, chopped, and uniformly mixed aerial portions of *Barleria lupulina*, consisting of wood and leaf fragments, was boiled in purified, deionized water (1.2 L) for 6 hours in a stainless steel teapot on an electric hot plate with surface temperature of 550°F (288°C). After 6 hours, aqueous extract typically was condensed to a final volume of 100 mL. Boiling time or final volume was adjusted, as necessary, to achieve final volume of 100 mL. However, boiling time never differed from 6 hours by more than 30 min. The condensed extract was filtered through a funnel lined with fluted filter paper and then centrifuged at 15,000×g for 30 min. Finally, it was filter-sterilized and stored under sterile conditions at 4 degrees C. Aliquots of the extract were evaporated to dryness, and dry weight of the extract was determined to be 150 mg/mL.

2.3 Analytical and Preparative HPLC

Agilent analytical HPLC 1100 system: Column = C18, 100 Å, 100 × 4.6 mm, 5 µm; Flow-rate = 0.7 mL/min; Solvents: from 100% water to 100% acetonitrile in 20 min, and then 100% acetonitrile for 10 min. Agilent preparative HPLC 1100 system: Column = C18, 100

Å, 100 × 21.2 mm, 5 µm; Flow-rate = 10 mL/min; Solvents = from 100% water from to 100% acetonitrile in 20 min, and then 100% acetonitrile for 10 min.

2.4 LC/MS condition for the analysis of 4-EC (4-ethylcatechol), 4-MC (4-methylcatechol), 4-vinylcatechol (4-VC) and HAE-BL

Agilent 1290 uHPLC coupled to a Bruker Impact HD ESI-q-TOF mass spectrometer in negative mode. Column: Phenyl, 150 Å~ 4.6 mm, 5 µm; Flow-rate: 0.5 mL/min; Solvent A: water 0.1% formic acid, Solvent B: methanol 0.1% formic acid, 0–2 minutes loading at 1 % B, then increase gradient over 20 minutes to 100% B, re-equilibrate over 7 minutes to 1% B.

2.5 Chemicals

Shanzhiside methyl ester was purchased from ChromaDex, Y-27632 from Tocris, 4-ethylcatechol and 4-methylcatechol from Sigma Aldrich, and 4-vinylcatechol (from Toronto Research Chemicals). 4-Vinylcatechol is highly unstable and was preserved with 1% w/w butylated hydroxytoluene. In control experiments, we determined that this proportion of butylated hydroxytoluene had no effect on results.

2.6 Antibodies

The following antibodies (Abcam, Inc., Cambridge, MA) were used for western blotting: heme oxygenase-1 (rabbit monoclonal, clone EP1391Y), Nrf2 (rabbit monoclonal, clone EP1808Y), CD31 (rabbit monoclonal, clone EPR3094). Secondary antibody used for western blotting was polyclonal goat anti-rabbit (H+L), conjugated to horseradish peroxidase (Life Technologies, cat. # G-21234). The following antibody was used for immunohistochemical staining of Nrf2 in cells (Paupe et al., 2009): Nrf2 (H-300), sc-13032 rabbit polyclonal raised against amino acids 37–336 of human Nrf2 (Santa Cruz Biotechnology Inc.). Secondary antibody used for immunohistochemical staining was goat anti-rabbit IgG (H+L) Alexa Fluor 488 conjugate (ThermoFisher).

2.7 Cells and cell culture experiments, cell viability assays, RNA isolation from cells, immunohistochemical staining of cells, and cell lysates for western blotting

Primary human dermal microvascular endothelial cells were purchased from Lonza Inc, Walkersville MD, and cultured in Lonza EGM-2MV medium. For short-term experiments lasting 4 hours, cells were shifted to serum-free EBM-2 (Lonza), containing 25 ng/ml human VEGF165 (R&D Systems). In experiments lasting 24 hours, cells were fed complete EGM-2MV medium at time zero. RNA was isolated from cells cultured in 6 well plates with the Qiagen RNeasy Plus Mini Kit that provides on-column removal of genomic DNA. For immunohistochemical staining of Nrf2, cells were fixed and stained exactly as described (Paupe et al., 2009). In addition, cells were co-stained for F-actin with Alexa Fluor 488 phalloidin (Molecular Probes, #A12379) or Alexa Fluor 594 phalloidin (Molecular Probes, #A12381), as indicated. All images used in comparisons were captured with identical exposures. For western blotting applications, cells were cultured in 24 well plates, treated as described in Figure legends, washed 3x with ice cold PBS, and harvested in 120 µL of 1.5x Laemmli SDS sample buffer containing protease inhibitor cocktail (Sigma-Aldrich, #P8340).

2.8 Western blotting

Cell lysates subjected to SDS polyacrylamide electrophoresis on pre-cast 4–20% gradient gels (GenScript) and separated proteins transferred to PVDF immunoblotting membrane (Bio-Rad). PVDF membrane was blocked in 5% w/v skim milk (from powder, EMD Millipore), and stained with primary antibodies and secondary horseradish peroxidase-conjugated antibody (see Antibodies, above). Signal was developed with ECL western blotting substrate (Pierce Chemical) and captured on x-ray film. For re-probing with additional antibodies, blots were stripped (Re-Blot Plus, Millipore) and blocked again with skim milk, as above. Blots were scanned digitally and bands were quantified with Image J. All experiments were repeated three times with similar results.

2.9 Quantitative real-time PCR (RT-PCR)

Multi-gene transcriptional profiling with quantitative RT-PCR was performed with SYBR Green I and the Applied Biosystems 7500 Fast Real-Time PCR System, as described (Wada et al., 2011). cDNA was prepared with SuperScript III reverse transcriptase (Life Technologies) (Wada et al., 2011). For primer design and validation, gene-specific sequences were selected based on NCBI Nucleotide BLAST searches to eliminate any homology to other genes (Shih and Smith, 2005). In addition, primers satisfied standard design parameters of Primer Express Software (Applied Biosystems). Primers were synthesized by Integrated DNA Technologies (Coraville, IA). Human claudin-5 primers were as follows: AGGCGTGCTCTACCTGTTTTG (forward); AACTCGCGGACGACAATGTT (reverse). For all other primer sequences see ref. (Senger et al., 2016). All PCR reactions were performed in duplicate and copy numbers were calculated from standard curves generated from a master template (Shih and Smith, 2005). Mean and standard deviation (S.D) were calculated from at least three different experimental samples (see below). Data were expressed as copy number/ 10^6 copies of 18S rRNA that is superior to using housekeeping genes for normalizing across multiple cell types (Aerts et al., 2004; Bas et al., 2004; Wada et al., 2011). Also, mRNAs encoding control housekeeping genes were also measured. Thus, two levels of normalization control are provided for RT-PCR data: (i) mRNA copy number for gene of interest/ 10^6 copies 18S rRNA, and (ii) direct comparisons between mRNA copy number for genes of interest vs. relevant housekeeping genes, each expressed as a function of mRNA copy number/ 10^6 copies 18S rRNA.

2.10 Statistical analyses and assignment of statistical significance

RT-PCR data are presented as mean $\pm$ S.D. Statistical analyses were performed with GraphPad software using the unpaired t-test. In all cases, an individual experimental group was compared with the appropriate vehicle control group; and calculated p-values are based on direct comparisons between the two groups. All experiments were repeated three times with similar results. For assignment of statistical significance based on p values, standard threshold limits were used: $p < 0.001$ extremely significant, $p = 0.001$ to < 0.01 very significant, $p = 0.01$ to < 0.05 significant, $p > 0.05$ not significant.

3. Results

3.1 Activation of the Nrf2 pathway by the hot aqueous extract of *Barleria lupulina* (HAE-BL)

First, we performed multi-gene transcriptional profiling of human microvascular endothelial cells (MVECs) with real-time PCR to analyze Nrf2 target gene transcripts induced by HAE-BL. Three separate lots of HAE-BL markedly induced expression of heme oxygenase-1 (HO-1) and NAD(P)H:quinone oxidoreductase1 (NQO1); and, additionally, but more modestly, induced expression of glucose 6-phosphate dehydrogenase (G6PD) within 24 hours (Figure 1A). In contrast, HAE-BL had no significant effects on transcripts encoding two endothelial cell structural “housekeeping” genes, CD31 (PECAM-1) and VE-cadherin (Figure 1A). HO-1, NQO1, and G6PD are each well-established target genes of the Nrf2 transcription factor (Cho et al., 2002; Holmstrom et al., 2013; Li et al., 2004; Mitsuishi et al., 2012), whereas CD31 and VE-cadherin are not regulated by Nrf2 (Senger et al., 2016). Thus, HAE-BL selectively activates Nrf2-mediated transcription. In contrast, we did not detect induction of Nrf2 target genes with two compounds previously isolated from *Barleria lupulina* and which we specifically identified in HAE-BL: the iridoid glycoside, shanzhiside methyl ester (Kanchanapoom et al., 2001; Kim et al., 2015; Suksamrarn et al., 2003), and the phenolic glycoside, poliumoside (Kanchanapoom et al., 2001) (Figure 1A). We also investigated induction of Nrf2 target genes by HAE-BL at 4 hours. As shown in Figure 1B, induction of HO-1 was considerably greater at 4 hours in comparison with 24 hours. NQO1 and G6PD were also induced by 4 hours but more modestly than at 24 hours, suggesting that there are gene-specific differences in the time course of induction by Nrf2.

In the absence of Nrf2 pathway activation, Nrf2 protein is retained in the cytosol by the actin-binding protein Keap1 that promotes rapid Nrf2 degradation by proteasomes (Itoh et al., 2010; Itoh et al., 2003; Kang et al., 2004; Kensler et al., 2007; Motohashi and Yamamoto, 2004). Upon activation, Nrf2 is released from Keap1 and rapidly moves to the nucleus to induce expression of anti-oxidant and detoxifying enzymes. The “redox sensor” mechanism that releases Nrf2 from Keap1, thereby allowing Nrf2 transport to the nucleus, involves oxidation-sensitive sulfhydryl groups in cysteine residues of Keap1 (Dinkova-Kostova et al., 2002; Holland and Fishbein, 2010; Wakabayashi et al., 2004; Zhang and Hannink, 2003). Our findings with RT-PCR, that HAE-BL induced transcripts encoded by Nrf2 target genes, suggested that HAE-BL promotes Nrf2 translocation to the nucleus. Therefore, to test for nuclear translocation of Nrf2, we stimulated MVECs with HAE-BL and stained for Nrf2 with immunohistochemistry. As shown in Figure 2, HAE-BL promoted nuclear accumulation of Nrf2, consistent with induction of Nrf2 target genes (Figure 1). In contrast, shanzhiside methyl ester and poliumoside, two compounds isolated from HAE-BL that do not induce expression of Nrf2 target genes (Figure 1), did not induce nuclear accumulation of Nrf2.

To confirm that nuclear translocation of Nrf2 and induction of Nrf2 target gene mRNAs correlated with increased protein expression of an Nrf2 target gene, we stimulated MVECs with HAE-BL, performed western blotting for HO-1. As shown in Figure 3, HAE-BL strongly induced (> 12-fold) expression of HO-1 protein, consistent with induction of HO-1 mRNA as determined with real-time PCR (Figure 1). In addition, HAE-BL increased total

Nrf2 protein (>2.5-fold), consistent with Nrf2 stabilization and Nrf2 pathway activation. Again, two compounds isolated from HAE-BL, shanzhiside methyl ester and poliumoside, that did not induce Nrf2 target gene expression and did not induce nuclear translocation of Nrf2, also did not increase Nrf2 protein or induce expression of HO-1.

3.2 Organization of cortical actin, reduction of stress fibers, and organization of endothelial cell junctions by HAE-BL; and preventing stress fiber induction by hydrogen peroxide

Next, we examined the activity of HAE-BL towards the actin cytoskeleton of MVECs. The endothelial cell cytoskeleton serves a critical role in control of vascular permeability and tissue edema (Bogatcheva and Verin, 2008; Lampugnani, 2010; Lum and Malik, 1994; Millan et al., 2010). We found that HAE-BL markedly re-organized actin filaments to the peripheral, cortical regions of MVECs and also greatly reduced actin stress fibers (Figure 4A). In contrast, neither shanzhiside methyl ester nor poliumoside exhibited similar activity. Consistent with organization of cortical actin and reduction in stress fibers, HAE-BL also strongly improved organization of cell-cell (adherens) junctions as indicated by staining for VE-cadherin (Figure 4B). Thus, all of these findings are consistent with improved microvascular endothelial cell barrier function (Bogatcheva and Verin, 2008; Lampugnani, 2010; Lum and Malik, 1994; Millan et al., 2010). Finally, hydrogen peroxide induces actin stress fibers in endothelial cells by causing oxidative stress (Zhao and Davis, 1998). Because HAE-BL activates the Nrf2 pathway that protects against oxidative stress (Itoh et al., 2010; Kensler et al., 2007), we tested HAE-BL to determine if it prevented hydrogen peroxide-induced stress fibers and found that, indeed, HAE-BL did prevent marked induction of actin stress fibers by oxidative stress (Figure 4C).

3.3 Assay-guided identification of compounds in AE-BL that activate the Nrf2 pathway

In addition to shanzhiside methyl ester and poliumoside, we screened a variety of other compounds isolated from HAE-BL, including p-coumaric acid, caffeic acid, chlorogenic acid, ferulic acid, sinapic acid, vanillic acid, and syringic acid. All of these compounds tested negative in the RT-PCR assay for Nrf2 activation (as in Figure 1, and at a concentration > 30 micro-molar), and they also tested negative in the cytoskeletal assay illustrated in Figure 4 (at > 30 micro-molar). Nonetheless, while reviewing the known structures of potent Nrf2 activators (Prochaska et al., 1985; Senger et al., 2016) and by comparing them with the structures of compounds we isolated from HAE-BL, important clues emerged. In particular, catechol (also known as pyrocatechol or 1,2-dihydroxybenzene) is well-characterized Nrf2 activator, and the 1,2-hydroxyls present on the benzene ring are essential for its activity (Prochaska et al., 1985). Catechol moieties are contained in several of the compounds we identified HAE-BL, including caffeic acid, chlorogenic acid, and poliumoside, although the native compounds containing these catechol moieties all tested negative for Nrf2 activation in our assays (see Figures 1–3 for poliumoside and ref. (Senger et al., 2016) for chlorogenic acid and caffeic acid). Nonetheless, the presence of a catechol moiety in these compounds suggested the possibility that fragmentation of larger molecules might liberate smaller catechol-related compounds with structural characteristics consistent with Nrf2 activators. Another important clue was suggested by previous reports that thermolysis (pyrolysis) of caffeic and chlorogenic acids can generate catechols as thermal

degradation products (Frank et al., 2007; Moon and Shibamoto, 2010; Terpin et al., 2011). Collectively, this evidence suggested the hypothesis that HAE-BL contains catechols that activate the Nrf2 pathway and possibly also regulate the actin cytoskeleton.

To test this hypothesis, we performed Nrf2 assay-guided HPLC fractionation of HAE-BL, combined with mass spectrometry (MS). For these analyses we eliminated formic acid from the solvent phase to prevent chemical modification of catechols in the isolated fractions during evaporation of the solvent (see Methods). HAE-BL was passed through a C18 open column, and then eluted with acetonitrile (Figure 5A). The pass-through was inactive in Nrf2 assays, whereas the acetonitrile eluent was active. The acetonitrile eluent was separated using a C18 prep-HPLC column, and sixteen fractions were collected (Figure 5B). Nrf2 assays indicated that fraction 11 (t_R 20–22 min) contained the majority of activity. Three alkyl catechols: 4-methylcatechol (4-MC, t_R 20.1 min), 4-vinylcatechol (4-VC, t_R 20.8 min), and 4-ethylcatechol (4-EC, t_R 21.5 min) were identified in fraction 11, and the identities (Figure 5C) confirmed by LC/MS. Importantly, each of these alkyl catechols satisfies the well-defined electrochemical characteristics of a classical Nrf2 activator (Prochaska et al., 1985). Most importantly, we have documented previously that each of these alkyl catechols (5 micro-molar to 30 micro-molar) induces HO-1, NQO1, and G6PD mRNAs, as measured with quantitative RT-PCR (Senger et al., 2016). We also have shown previously that each of these alkyl catechols induces nuclear translocation of Nrf2 as determined with immunohistochemistry and that each induces expression of HO-1 protein as measured with western blotting (Senger et al., 2016). Thus, we conclude that 4-methylcatechol, 4-vinylcatechol, 4-ethylcatechol are each present in HAE-BL and that each contribute to the Nrf2 activity illustrated in Figures 1, 2, and 3. In contrast, and as summarized above, larger molecules present in HAE-BL such as poliumoside, chlorogenic acid, and caffeic acid were all found to be inactive despite the presence of the catechol moiety in each of these compounds.

Next, we tested the hypothesis that alkyl catechols are generated by thermolysis of these larger molecules present in HAE-BL, such as caffeic acid. For this purpose, we performed thermolytic analysis of caffeic acid under conditions similar to those used for preparation of HAE-BL. Four milligrams of caffeic acid was dissolved in 10 mL deionized water, which was heated to 200°C; and deionized water was added constantly to keep the solution from drying out. LC/MS showed that about 5% of caffeic acid was converted to 4-vinyl catechol within 30 minutes (Figure 5D).

3.4 The alkyl catechols identified in HAE-BL each promote organization of cortical actin and reduction of stress fibers in MVECs and also induce expression of claudin-5 mRNA

Next, we searched for compounds in HAE-BL that are responsible for organization of cortical actin and reduction of stress fibers, as illustrated in Figure 4. To this end, we followed the same HPLC fractionation scheme shown in Figure 5. We found that the three alkyl catechol Nrf2 activators identified in HAE-BL, 4-ethylcatechol, 4-vinylcatechol, and 4-methylcatechol, each induced cortical re-arrangement of actin filaments and reduced stress fibers similarly to unfractionated HAE-BL (Figure 6). Thus, these findings indicate that the three alkyl catechols identified in HAE-BL not only induce activation of the Nrf2 pathway

but also re-organize the MVEC actin cytoskeleton in a manner consistent with improved endothelial barrier function and reduced vascular leak. Moreover, these findings are consistent with a direct connection between reduced oxidative stress by Nrf2 and improved cytoskeletal organization.

Finally, claudin-5 is a key protein involved in the formation of microvascular endothelial cell tight junctions and control of vascular leak (Kluger et al., 2013). Because endothelial cell-cell adhesion mediated by VE-cadherin regulates expression of claudin-5 (Gavard and Gutkind, 2008; Taddei et al., 2008), and because we found that HAE-BL improves organization of VE-cadherin at cell-cell junctions (see Figure 4B), we examined claudin-5 expression in response to stimulation with HAE-BL and each of the alkyl catechols. As determined with RT-PCR, we found that each significantly induced expression of claudin-5 mRNA in MVECs by more than 100% (Figure 7). Thus, increased expression of claudin-5 enhancement of tight junctions is likely another mechanism by which HAE-BL improves microvascular endothelial barrier function, reduces vascular permeability, and thereby improves vascular homeostasis during inflammation and in diabetes. A model summary diagram is presented in Figure 8.

4. Discussion

Hot aqueous extracts of *Barleria lupulina* (HAE-BL) have been used traditionally as an oral medicine for reducing inflammation associated with snake and insect bites, allergic reactions, and rheumatoid arthritis; however, cellular mechanisms and biologically active compounds consistent with this activity have not been identified. As reported here, we identified potent activation of the Nrf2 cell defense pathway by HAE-BL. We also identified three different chemical activators of the Nrf2 cell defense pathway in HAE-BL, specifically: 4-ethylcatechol, 4-vinylcatechol, and 4-methylcatechol. Each of these compounds has been shown previously to be potent activators of Nrf2 (Senger et al., 2016). Activation of Nrf2 cell defense by HAE-BL has not been considered previously, although there are striking connections between activation of the Nrf2 pathway and reduced inflammation. Much evidence comes from mice engineered to lack the Nrf2 gene. These Nrf2 null mice are more susceptible to airway inflammation and asthma (Rangasamy et al., 2005), UVB-induced skin inflammation (Saw et al., 2014), LPS-induced inflammation (Wang et al., 2010), increased rheumatoid arthritis (Wruck et al., 2011), and development of lupus-like autoimmune kidney disease (Yoh et al., 2001). In addition, Nrf2 null mice are more sensitive to chemical toxins, resulting in increased inflammation and damage to lung, brain, and kidney (Kensler et al., 2007; Liu et al., 2009). Thus, our finding that HAE-BL activates the Nrf2 cell defense pathway is consistent with the traditional medicinal use of HAE-BL for treating inflammation.

Our finding that HAE-BL activates the Nrf2 cell defense pathway is also consistent with the traditional medicinal use of HAE-BL for treating diabetic complications (de Haan, 2011; Li et al., 2012). In particular, the enzyme HO-1 that is a key Nrf2 target gene induced by HAE-BL (Figure 3) protects from vascular injury caused by diabetes. Also, in mouse models of diabetes, HO-1 null mice exhibit more severe vascular injury than corresponding wild type mice (Ryter et al., 2006); and, conversely, over-expression of HO-1 in diabetic models is

vaso-protective (Abraham et al., 2004; Ryter et al., 2006). Furthermore, mice lacking the HO-1 gene exhibit increased inflammation (Kapturczak et al., 2004), whereas over-expression of HO-1 in mice reduces inflammation in response to inflammatory stimuli (Zampetaki et al., 2003).

Key to the mechanism by which the Nrf2 pathway and its target genes such as HO-1 control inflammatory and diabetic complications likely involves neutralization of dangerously high levels of reactive oxygen species (ROS) (Chen and Kunsch, 2004; de Haan, 2011; de Vries et al., 2008; Dinkova-Kostova and Abramov, 2015). Inflammatory cells release copious amounts of ROS; and, if not adequately controlled, ROS cause serious endothelial cell dysfunction and collateral tissue damage (Chen and Kunsch, 2004; Lum and Roebuck, 2001; Mittal et al., 2014; Wruck et al., 2011). Also, high levels of ROS are typical in diabetes and thereby cause serious diabetic complications (Avogaro et al., 2011; Brownlee, 2005; Giacco and Brownlee, 2010).

In addition to activating the Nrf2 cell defense pathway, we found that HAE-BL markedly improved cortical organization of the actin cytoskeleton, reduced actin stress fibers, and improved organization of microvascular endothelial cell adherens junctions (Figure 4). These cellular effects are important and relevant because they are all highly consistent with improved microvascular endothelial barrier control over blood vessel leakiness and tissue edema that are characteristic consequences of inflammation (Bogatcheva and Verin, 2008; Lampugnani, 2010; Lum and Malik, 1994; Millan et al., 2010; Mittal et al., 2014). Thus, improved cortical actin organization, reduction in stress fibers, and improvement in adherens junctions by HAE-BL are each consistent with the traditional use of HAE-BL in treating inflammation (summarized in Figure 8). Importantly, the Nrf2 activators we identified in HAE-BL: 4-ethylcatechol, 4-vinylcatechol, and 4-methylcatechol each copied the effects of HAE-BL in organizing cortical actin and cell junctions and in reducing actin stress fibers (Figures 6 and 8), suggesting that these cellular improvements are likely mediated by HAE-BL through activation of Nrf2. Also, consistent with a mechanism for HAE-BL that involves activation of the Nrf2 cell defense pathway to provide protection from detrimentally high concentrations of ROS, we found that HAE-BL protects against marked induction of actin stress fibers by hydrogen peroxide (Figure 4).

Yet another important finding is that unfractionated HAE-BL and each of the alkyl catechols, 4-ethylcatechol, 4-vinylcatechol, and 4-methylcatechol, that we identified in HAE-BL significantly induce expression of mRNA encoding claudin-5 (Figures 7 and 8). Claudin-5 is important for the assembly of microvascular endothelial tight junctions and therefore induction of claudin-5 is likely an additional important mechanism through which HAE-BL can improve microvascular endothelial barrier function and thereby reduce inflammation. Claudin-5 is not regulated by Nrf2-mediated transcription but rather it is induced by cell-cell adhesion through VE-cadherin (Gavard and Gutkind, 2008; Taddei et al., 2008). Thus it seems likely that induction of claudin-5 by HAE-BL is an indirect consequence of HAE-BL-mediated improvement in cell-cell junctions, as illustrated in Figure 4B.

There are other indirect mechanisms by which HAE-BL and the alkyl catechols we identified in HAE-BL may improve the integrity of endothelial cell junctions and reduce inflammation, and these require further study. In particular, ROS has been shown to activate the GTPase RhoA that, in turn, activates Rho kinase to diminish cortical actin and induce actin stress fibers (Aghajanian et al., 2009). Therefore, one possibility is that activation of the Nrf2 pathway by HAE-BL reduces ROS and thereby reduces RhoA activity, ultimately resulting in reduced Rho kinase activity and reduced stress fibers. Interestingly, the Rho kinase inhibitor Y-27632 has been shown to reduce stress fibers and improve microvascular barrier function in vitro (Hoang et al., 2004; van Nieuw Amerongen et al., 2000) and reduce inflammation in vivo (Chen et al., 2011; Henry et al., 2005; Pigati et al., 2015; Schaafsma et al., 2008; Tasaka et al., 2005). Another likely possibility is that by reducing ROS through Nrf2, HAE-BL prevents ROS-mediated inhibition of tyrosine phosphatases at adherens junctions (Mittal et al., 2014; Ostman et al., 2011). By preserving the activity of tyrosine phosphatases, Nrf2 can prevent hyper-phosphorylation of cell junction proteins on tyrosines (Roura et al., 1999) and thereby improve the integrity of adherens junctions (Andriopoulou et al., 1999; Gomez et al., 1999; Mittal et al., 2014; Rao, 2008). Finally, another important possibility involves endothelial cell expression of vascular cell adhesion molecule 1 (VECAM-1), which promotes adhesion of inflammatory cells to the blood vessel wall. Interestingly, activated Nrf2 suppresses VECAM-1 expression (Banning and Brigelius-Flohe, 2005; Chen et al., 2006), while oxidative stress induces VECAM-1 (Cook-Mills et al., 2011). Therefore, it is reasonable to speculate that HAE-BL also reduces inflammation by activating the Nrf2 pathway that reduces ROS, reduces VECAM-1 expression, and thereby reduces inflammatory cell adhesion to endothelium.

Regarding the origins of 4-ethylcatechol, 4-vinylcatechol, and 4-methylcatechol in HAE-BL, it seems likely that thermolysis of larger compounds containing catechol moieties is at least partly responsible, although we cannot rule out other possibilities. As noted above, we identified chlorogenic acid and caffeic acid in HAE-BL, and these compounds each contain the catechol moiety. We also identified poliumoside and verbascoside, as reported by others previously (Kanchanapoom et al., 2001), and these compounds also contain the catechol moiety. Thermolysis of caffeic and chlorogenic acids has been reported to generate catechols as degradation products, including 4-vinylcatechol and 4-ethylcatechol (Frank et al., 2007; Moon and Shibamoto, 2010; Terpin et al., 2011). Moreover, as demonstrated here, boiling of caffeic acid in water, under conditions similar to those we used for preparation of HAE-BL, generated 4-vinylcatechol. Thus, these studies underscore the possible significance of heat in generating Nrf2 activators from larger plant precursors.

Interestingly, in our preliminary experiments, we noticed that the Nrf2 biological activity present in HAE-BL was completely lost if methanol and formic acid were used for the assay-guided separation and fractions were evaporated to dryness. Such loss of activity is consistent with the tendency of 4-vinylcatechol to polymerize when exposed to oxygen. It is also consistent with chemical modification of alkyl catechols by formic acid during evaporation. Given that 4-vinylcatechol is highly unstable and that it is also a major pyrolysis product of caffeic acid, we speculate that the major active component in HAE-BL might be 4-vinylcatechol, which in complex solution with other components of HAE-BL is stabilized by anti-oxidants. However, further work beyond the scope of this project will be

required to evaluate all possibilities. Also, we failed to find any compounds, other than the alkyl catechols, which activate the Nrf2 pathway or reorganize the MVEC actin cytoskeleton similarly to unfractionated HAE-BL. However, we cannot rule out the possibility that others are yet to be discovered. Regardless, our findings that HAE-BL activates the Nrf2 cell defense pathway and, that this activity is embodied by 4-ethylcatechol, 4-vinylcatechol, and 4-methylcatechol, represent an important advance towards understanding the medicinal activity and bioactive molecular composition of this popular traditional medicine.

Lastly, our findings that HAE-BL potentially activates the Nrf2 cell defense pathway suggest additional important uses for this traditional medicine. In particular, activation of Nrf2 cell defense is important for preventing cancers (Kwak et al., 2004; Slocum and Kensler, 2011), disorders associated with neuro-degeneration (Johnson et al., 2008), premature aging of skin (Saw et al., 2014), age-related macular degeneration (Zhao et al., 2011), and for reducing the severity of chronic obstructive pulmonary disorder (Garber, 2012) and multiple sclerosis (Bomprezzi, 2015). Thus, going forward, it will be important to determine if there is evidence for the efficacy of this traditional medicine for treating any of these conditions, similarly to its utility in treating inflammation and diabetic complications.

5. Conclusions

As summarized in Figure 8, we have demonstrated that traditional hot aqueous extracts of *Barleria lupulina* potentially activate the Nrf2 cell defense pathway. Nrf2 defense is a critical cellular mechanism for controlling oxidative stress, and activation of Nrf2 is highly consistent with traditional use of HAE-BL for reducing inflammation and treating diabetic vascular complications. HAE-BL biological activity is embodied by three distinct but closely related Nrf2 activators, 4-ethylcatechol, 4-vinylcatechol, and 4-methylcatechol. In addition to activating Nrf2, these compounds each organize microvascular cortical actin and reduce stress fibers similarly to HAE-BL, and also induce expression of mRNA encoding the endothelial cell tight junction protein claudin-5. All of these biological activities are consistent with improved endothelial barrier function, reduced vascular leak, reduced tissue edema, and reduced inflammation. It seems probable that the active alkyl catechols in HAE-BL are generated, at least in part, by thermolysis of precursor compounds. Thus, these studies suggest new biochemical insights into the potential importance of heat in the preparation of traditional anti-inflammatory medicines, and in particular, for generating Nrf2 activators containing the catechol moiety.

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Figure 1. Induction of Nrf2 target gene RNAs by HAE-BL in MVECs, as measured with RT-PCR
Y-axis = (mRNA copies)/(10⁶ 18S rRNA copies). Nrf2 target genes = heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), glucose 6-phosphate dehydrogenase (G6PD). Control, non-NRF2 target mRNAs = CD31 (PECAM-1) and VE-cadherin (cadherin-5). HAE-BL was added to a final dilution of 1:100. Shanzhiside methyl ester (SME) and poliumoside (POL) were added to a final concentration of 30 micro-molar. (Ctrl) = vehicle control. Cells were harvested at 24 hours (panel A), and 4 hours (panel B). For all panels, error bars = $\pm$ standard deviation (S.D.); n = 3 for each data point. *Statistical significance (see Methods):* (Panel A) For HO-1, NQO1, and G6PD panels: HAE-BL vs. vehicle Ctrl, SME, or POL = all extremely significant ($p < 0.001$); for CD31 and VE-cadherin panels: no significant differences. (Panel B) For HO-1 and NQO1 panels: HAE-BL vs. vehicle Ctrl = extremely significant ($p < 0.001$); for G6PD panel: HAE-BL vs. vehicle Ctrl = very significant ($p < 0.01$); for CD31 and VE-cadherin panels: no significant differences.

Figure 2. HAE-BL induces nuclear translocation of Nrf2 in MVECs

MVECs were incubated with HAE-BL at 1:100 dilution or, alternatively with shanzhiside methyl ester (SME) or poliumoside (POL) each at 30 micro-molar concentration for 24 hours. Ctrl = vehicle control. Cells were fixed and stained for Nrf2 (green color) and F-actin (red color). Note bright green staining of Nrf2 concentrated in nuclei of cells stimulated with HAE-BL in comparison with Ctrl, SME, or POL. All samples were processed and stained in parallel; green images (Nrf2) were captured at identical exposure; and, similarly, red images (F-actin) were captured at identical exposure. Subsequently, red and green images were merged without any manipulation so that images presented here are valid for direct comparisons.

Figure 3. HAE-BL Induces HO-1 protein expression in MVECs

Western blotting of MVECs, harvested 24 hours after adding HAE-BL (1:100 dilution) or addition of compounds shanzhiside methyl ester (SME) or poliumoside (POL), each at 30 micro-molar final concentration. Ctrl lane = vehicle control. HAE-BL strongly induced expression of HO-1 protein (> 12-fold, average of 3 experiments) relative to controls, consistent with marked induction of HO-1 mRNA (Fig. 1). Also HAE-BL increased Nrf2 protein (> 2.5-fold, average of 3 experiments) relative to controls, consistent with increased Nrf2 stabilization. CD31 panel = protein loading controls. *Statistical significance (see Methods):* For HO-1 panel, HAE-BL vs. each of the controls (Ctrl, SME, POL) = extremely significant ($p < 0.001$); no significant differences between the various controls (Ctrl, SME, POL). For Nrf2 panel: HAE-BL vs. each of the controls (Ctrl, SME, POL) = all very significant ($p < 0.01$); no significant differences between each of the controls (Ctrl, SME, POL). For CD31 panel: no consistent or significant differences between HAE-BL, Ctrl, SME, or POL (3 separate experiments).

Figure 4. HAE-BL organizes cortical actin, reduces stress fibers, and improves microvascular endothelial cell junctions

(A) MVECs were incubated with HAE-BL at 1:100 dilution or, alternatively with shanzhiside methyl ester (SME) or poliumoside (POL) each at 30 micro-molar concentration. Ctrl = vehicle control. After 4 hours, cells were fixed and stained for F-actin (green color) and cell nuclei (blue color). Note that HAE-BL organizes cortical actin and eliminates stress fibers. All samples were processed and stained in parallel, and images were

captured at identical exposure without any manipulation so that images presented here are valid for direct comparisons.

(B) MVEC's were incubated with HAE-*BL* for 4 hours, as in Panel A, and fixed and stained for F-actin (red color), VE-cadherin (green color), and cell nuclei (blue color). Yellow color = merge of green (VE-cadherin) + red (F-actin). Note that HAE-*BL* markedly improved organization of VE-cadherin with F-actin at cell junctions, relative to vehicle control (Ctrl). As in Panel A, all images were captured at identical exposure without any manipulation and are valid for direct comparisons. **(C)** Hydrogen peroxide (H₂O₂) was added to a final concentration = 200 micro-molar. In the right panel, hydrogen peroxide was added together with a 1:00 dilution of HAE-*BL* (H₂O₂ + HAE-*BL*). Cells were fixed at 4 hours, processed, and photographed as in Panel A. Green color = F-actin; blue color = cell nuclei. All images were captured at identical exposure without any manipulation and are valid for direct comparisons. Note that hydrogen peroxide markedly induced actin stress fibers that lack cortical organization, whereas HAE-*BL* prevented induction of stress fibers.

Figure 5. Assay-guided fractionation of HAE-BL and identification of Nrf2 activators; thermolysis of caffeic acid to 4-vinylcatechol

(A) HPLC chromatogram of the acetonitrile eluent of unfractionated HAE-BL. Y-axis = absorbance at 254nm (mAU), X-axis = minutes. (B) Diagram describing assay-guided isolation of Nrf2 activators from HAE-BL. (C) Structures of active compounds identified: 4-MC = 4-methylcatechol; 4-VC = 4-vinylcatechol; 4-EC = 4-ethylcatechol. (D) HPLC analyses of caffeic acid and thermolytic conversion to 4-vinylcatechol (see text for experimental details). Y-axis = absorbance at 254nm (mAU), X-axis = minutes. Top panel: HPLC of caffeic acid and 4-vinylcatechol standards. Bottom panel: HPLC of caffeic acid

subjected to thermolysis. Retention times: caffeic acid = 8.1 minutes, 4-vinylcatechol = 10.7 minutes.

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Figure 6. Alkyl catechols isolated from HAE-BL organize cortical actin and reduce stress fibers in MVECs

MVECs were incubated with HAE-BL at 1:100 dilution or, alternatively with 4-methylcatechol (4MC), 4-vinylcatechol (4VC), or 4-ethylcatechol (4EC), each at 30 micromolar concentration. Ctrl = vehicle control. After 4 hours, cells were fixed and stained for F-actin (green color) and cell nuclei (blue color). Note that each of the alkyl catechols (4MC, 4VC, 4EC) organizes cortical actin and eliminates stress fibers similarly to unfractionated HAE-BL. All samples were processed and stained in parallel, and images were captured at identical exposure without any manipulation so that images presented here are valid for direct comparisons.

Figure 7. HAE-BL and alkyl catechols induce claudin-5 mRNA in MVECs, as demonstrated with RT-PCR

Y-axis = (mRNA copies)/(10⁶ 18S rRNA copies). HAE-BL was added to a final dilution of 1:100. 4-methylcatechol (4MC), 4-vinylcatechol (4VC), and 4-ethylcatechol (4EC) each were added to a final concentration = 30 micro-molar. (Ctrl) = vehicle control. Cells were harvested at 24 hours. For all panels, error bars = $\pm$ standard deviation (S.D.); n = 3 for each data point. *Statistical significance (see Methods)*: Differences between Ctrl and HAE-BL, 4MC, 4VC, and 4EC = all extremely significant ($p < 0.001$); for VE-cadherin panel: no significant differences.

Figure 8. Model diagram for mechanism by which *Barleria lupulina* reduces inflammation

The hot aqueous extract of *Barleria lupulina* (HAE-BL) contains 4-methylcatechol (4MC), 4-vinylcatechol (4VC), and 4-ethylcatechol (4EC) that activate the Nrf2 pathway to induce expression of Nrf2 target genes, including heme oxygenase-1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), and glucose 6-phosphate dehydrogenase (G6PD), that collectively provide the reducing power to neutralize reactive oxygen species (ROS). In microvascular endothelial cells, reduction of ROS improves cortical organization of actin, reduces stress fibers, and improves cell-cell adhesion. Improved cell-cell adhesion induces claudin-5 to form tight endothelial junctions, thereby strengthening endothelial barrier function, reducing vascular leak, and reducing inflammation. Other important connections between reduced ROS and reduced inflammation (not shown here) include reduction of GTPase RhoA activity, restoration of tyrosine phosphatase activity at cell-cell junctions, and repression of VECAM-1 expression (see Discussion for details)