HYDROXYCINNAMIC ACID AMIDES FROM *LYCIUM BARBARUM* AND
THEIR ANTI-INFLAMMATORY MECHANISMS

By

Siyu Wang

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ABSTRACT OF THE DISSERTATION

Hydroxycinnamic Acid Amides from *Lycium barbarum* and their anti-inflammatory mechanisms

by SIYU WANG

Dissertation Director:
Professor Chi-Tang Ho

Over the past few decades, much research has identified chronic inflammation as a critical component in many human diseases. Due to the intimate relationship between chronic inflammation and human diseases and conditions, an accumulating number of studies aimed to identify and discover anti-inflammatory molecules from natural resources because these molecules are able to interact with inflammatory mediators as well as modulate inflammatory pathways. Hydroxycinnamic acid amides (HCAA) are the secondary metabolites ubiquitously exist in flowering plants, formed by condensation between hydroxycinnamates and mono or polyamines. HCAA species not only serve multiple functions in plant growth and development, but also exert significant positive effects on human health. HCAA family has been recognized as the most characteristic and abundant chemical species of *Lycium barbarum*. The plant has been recognized as traditional remedies for hyperglycemia and other health conditions. Wolfberry or Goji berry, the fruits of *Lycium barbarum*, are widely consumed in Asian cuisine due to their health-promoting properties.
In the first part of my work, we synthesized a set of HCAA compounds, including *trans*-caffeic acid, *trans*-ferulic acid, and 3,4-dihydroxyhydrocinnamic acid, with extended phenolic amine components as standards to identify and quantify the corresponding compounds from different parts of *Lycium barbarum*. With optimized LC–MS/MS and NMR analysis, nine amide compounds were identified from the fruits, and 10 new HCAA species were further identified in root barks in addition to the ones reported in the literature. HCAA species were reported in leaves for the first time. The quantification showed the amide compounds with a tyramine moiety were the most abundant.

Moreover, the method was fully validated with respect to specificity, linearity, intra- and inter-day precision and accuracy, limit of detection (LOD), limit of quantification (LOQ), recovery, and reproducibility.

The anti-inflammatory properties of identified HCAAs were also examined by nitric oxide (NO) inhibition assay. Seven HCAA compounds had a potent NO inhibitory effect with IC$_{50}$ as low as 2.381 μM (*trans*-N-cafeoyl phenethylamine). Two HCAA compounds (*trans*-N-cafeoyl tryptamine and *trans*-N-cafeoyl tyramine) were chosen to investigate their anti-inflammatory molecular mechanisms by both *in vitro* and *in vivo* assays. These two HCAAs inhibited NF-κB signaling pathway in murine macrophage RAW264.7, accompanied by inhibition of PI3K/Akt/IKK pathway. *In vivo* mouse ear edema model indicated that with treatment of the two HCAAs, TPA-induced ear edema was significant reduced by showing as reduction of ear weight and thickness, pro-inflammatory enzyme expression as well as immune cells infiltration.
The developed analytical method largely improved analytical sensitivity of HCAAs species that potentially contributes to plant metabolomics and drug discovery studies. HCAAs demonstrated promising anti-inflammatory properties that could be used as preventive agent for inflammation and inflammation-related diseases.
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1. Literature Review

1.1 Chronic inflammation

1.1.1 Introduction about inflammation

Inflammation is an adaptive response that is triggered by noxious stimuli and conditions, such as microbial infections, tissue injuries and chemical irritations.

The inflammatory response is coordinated by a large range of mediators that form complex regulatory networks. The generic inflammatory response consists four components: inducers, sensors, mediators and effectors [1]. Inducers that initiate stimulus signals can be exogenous, such as microbial infections (pathogen-associated molecular patterns, PAMPs) and virulence factors of pathogens, or endogenous those are produced by stressed, damaged or malfunctioning cells and tissues. The uncontrolled and accidental cell death caused by extreme physicochemical stress leads to loss of plasma membrane integrity and cellular collapse and results in release of cell contents and exposure of damage-associated molecular patterns (DAMPs) [2]. These molecules include molecules that perform non-inflammatory functions in living cells and acquire immunomodulatory properties when released, secreted, modified, or exposed on the cell surface during cellular stress, damage, or injury, or alamins that are stored in cells and released upon cell lysis, whereupon they contribute to the danger signals as well as inflammatory response [3, 4]. These stimuli are sensed by certain receptors on the immune cells, which transduces the signals and triggers further inflammatory responses. Toll-like receptors that characterized by leucine-rich repeat in extracellular domain and striking homology with that of interleukin receptors [5]. Both PAMPs and DAMPs could be recognized by the same group
of receptors signaling. Toll signaling has been shown to play an essential role in the
immune response to microbial infection. Triggering of toll-like receptors recruits
interleukin-1 receptor-associated kinase (IRAK) to the receptor complex via adaptor
myeloid differentiation primary response 88 (MyD88), then TNF receptor associated factor
(TRAF6) is activated, which finally results in activation of downstream inflammatory
transcriptional activities [5]. Inducers of inflammation trigger the production of numerous
inflammatory mediators, which in turn alter the functionality of many tissues and organs,
the downstream effectors of the inflammatory pathway. The mediators can be produced by
specialized leukocytes (particularly tissue-resident macrophages and mast cells) or by cells
present in local tissue [6]. These mediators play a role in inflammatory response that
conduct various functions, including alternation of vascular permeability, activation of the
acute-phase response, control of leukocytes chemotaxis towards the affected tissues,
enhancement of host defense, and restoration tissues and help with leukocyte migration [7].
The effectors of an inflammatory response are the tissues and cells that may change the
functionalities in response to specific functions according to mediators that leads ultimately
to the restoration of tissue structure and homeostatic state. In general, controlled
inflammation is beneficial for the host defense system.

1.1.2 Acute inflammation and chronic inflammation

The initial recognition of infection is mediated by tissue resident macrophages and
mast cells, leading to the production of a variety of inflammatory mediators with the
functions mentioned in previous sections [1]. These mediators activated the endothelial
cells to produce adhesion factors and change their permeability that allow leukocytes and
plasma protein migrate from blood vessels to the extravascular tissues at the site of
infection or injury [8]. In acute inflammation response, neutrophils play an essential role in eliminating pathogen infections. When they reach the afflicted tissue site, neutrophils become activated, and attempt to kill the invading agents by NADPH oxygenase-dependent mechanisms that produce reactive oxygen species in neutrophil granules or antibacterial proteins, such as cathepsins, defensins, lactoferrin and lysozymes. Highly activated neutrophils secrete neutrophil extracellular traps that contain core DNA elements with histone and enzymes, in order to immobilize pathogens, facilitating further phagocytosis [9]. If the acute inflammatory response is successful to eliminate infectious agents, tissue-resident and recruited macrophages will initiate and mediate the resolution and repair phase [10]. A successful acute inflammation response is strong but often short-lived, resulting in elimination of the pathogen followed by termination of the response, and the organism lives.

However, when the abnormal conditions sustained, the inflammation response become prolonged and dysregulated, leading to maladaptive physiological responses, which are showed in many human diseases. Some new characteristics are developed while inflammation response sustaining. For instance, macrophages infiltration replaces neutrophils and may also involves T cells [11]. During chronic inflammation, a wide array of intracellular signaling pathways are often dysregulated, leading to abnormal and continuously expression of pro-inflammatory genes [12], including the effectors and mediators involved in acute inflammation. These highly potent effectors and do not discriminate between microbial and host targets, leading to collateral damage to host tissues that cause toxicity, loss of barrier function, abnormal cell proliferation, inhibiting normal function of tissues and organs, and finally leading to systemic disorders [13, 14].
1.1.3 Inflammatory signaling pathway: NF-κB pathway

At cellular level, inflammation response coordinates activation of highly regulated signaling pathways, involving cell surface receptors, kinases, and transcription factors of both pro- and anti-inflammatory processes in resident tissue cells and leukocytes recruited from the blood. These cellular cascades of inflammation is closely associated with various human diseases caused by chronic inflammation [12, 15]. Many transcription factors have been identified to participate in inflammation responses, including signal transducers and activators of transcriptions (STATs), nuclear factor-κB (NF-κB), nuclear factor of activated T-cells (NFAT), activator protein-1 (AP-1), CCAAT- enhancer binding protein (C/EBP), cAMP response element binding protein/p300 (CBP/p300), and activator transcription factor (ATF) [16].

The nuclear factor NF-κB pathway has long been considered as the central pro-inflammatory signaling pathway, mainly based on the role of NF-κB in the expression of pro-inflammatory gene and downstream mediators. The activation of NF-κB pathway is initiated by recognition of both exogenous and endogenous inducers, mentioned in previous sections. Inducers are sensed by cell surface receptors, according to specific structure or cellular activity characteristics. For instance, microbial infections are usually recognized by their PAMPs, such as lipopolysaccharides, and non-microbial foreign bodies that are unable to be digested can also be sensed by labeling as “frustrated phagocytosis,” and consequently initiated further inflammation responses [1]. Endogenous inducers also express certain molecular characteristics, such as certain cellular constituents that released from necrotic cell, which will be recognized by immune-cells. Cell surface receptors mediate a series of signal transductions by various upstream kinases. Many studies have
confirmed that the induction of transcription activity of NF-κB depends on Janus kinase (JAK), extracellular signal-regulated protein kinase 1/2 (Erk1/2), p38 MAPK, Ras, and phosphoinositide-3 kinase (PI3K)/Akt pathways [17]. At deactivated state, heterodimer NF-κB is inhibited and bound to inhibitor κB, preventing it access to the nucleus. The activation of NF-κB starts from the activity of inhibitor κB (IκB) kinases (IKKs), which in turn phosphorylates IκB. The subsequent ubiquitination and proteasomal degradation of IκB release NF-κB, and the transcriptional dimer is free translocate to the nucleus, and consequently bind to κB enhancer elements of target genes. In order to reach the full transcriptional activity, p65 subunit of NF-κB will be further phosphorylated, and the transcription factor is able to activate pro-inflammatory gene expression (Fig.1). NF-κB binds to target DNA elements and positively regulates the transcription of genes involved in immune and inflammatory responses, cell growth control, and apoptosis, including genes encoding cytokines, cytokine receptors, cell adhesion molecules, chemoattractant proteins, and growth regulators. The molecules regulated by NF-κB include IL-2, IL-6, IL-8, the IL-2 receptor, the IL-12 p40 subunit, vascular cell adhesion molecule 1 (VCAM-1), Intercellular Adhesion Molecule 1 (ICAM-1), Tumor Necrosis Factor α (TNF-α), and Interferon γ (IFN-γ) [18, 19].
Figure 1 Central components of NF-κB pathway. When the cell surface receptor recognizes inducers (lipopolysaccharide in the figure), the IKK complex is activated and phosphorylated by kinase cascade (not shown in the figure). The IκB is activated by IKK and undergo further ubiquitination and degradation. The NF-κB dimer is freed and translocate into nuclei to initiate inflammatory gene expression.
1.1.4 Role of chronic inflammation in human diseases

Prolonged activation of inflammatory signaling pathway both directly and indirectly contributes to various conditions and diseases, such as cardiovascular diseases, metabolic disorders and cancers.

At molecular level, NF-κB is highly activated at sites of inflammation in diverse diseases, such as rheumatoid arthritis (RA), asthma and inflammatory bowel disease [20]. Activated NF-κB can induce transcription of proinflammatory cytokines, chemokines, adhesion molecules, MMPs, COX-2, and inducible nitric oxide (iNOS) adhesion molecules, recruit inflammatory cells, such as neutrophils, eosinophils, and T lymphocytes, from the circulation to the site of inflammation [21].

Recently, many studies focused on the close relationship between chronic inflammation and cardiovascular diseases. During inflammation response, the permeability of endothelial cells is enhanced in order to facilitate immune cells migration, which is regulated by inflammatory mediators, such as nitric oxide (NO) [22]. Prolonged alternations in vascular endothelium results in increase of oxidative stress, reduction of vasodilator capacity and the appearance of endothelial dysfunction [13]. Other oxidants that are generated by immune cells can oxidize circulating cholesterol that serves as inducers to activate endothelial cells, which subsequently secrete adhesion factors for leukocytes [23]. Attached leukocytes further differentiate to macrophages to produce chemoattractant molecules and enhance the process. The molecules secreted by monocytes and macrophages maintain an inflammatory state within the artery and largely promote proliferation of vascular smooth muscle cells [24], finally resulting in a dense extracellular matrix and fibrous plaque [25]. Chronic inflammation contributes to the pathogenesis and
progression of atherosclerosis that play a role in cardiovascular diseases. It has confirmed the crosstalk between inflammation and metabolic disorder at molecular level by large number of research. Recently, instead of focusing only energy balance as a remedy of obesity, chronic low-grade inflammation was characterized and associated with obesity [26]. Furthermore, the potent inflammatory mediator, TNF-α induces insulin resistance by downregulation of insulin receptor phosphorylation, decrease of glucose uptake and expression of GLUT4 transporter [27]. Insulin-dependent diabetes, or type I diabetes, is characterized by the severe destruction of insulin-producing β cells. Oxidative stress and pancreas-specific reactive oxygen species (ROS) production generated through activation of NF-κB are believed to play a central role in β-cell death and disease progression [28]. On one hand, inflammation leads to obesity and metabolic symptoms; on the other hand, obesity-related inducer may contribute to inflammation, and the two perspectives reinforce mutually. Macronutrients, particularly lipids, have been linked to insulin sensitivity since 1960s that lipids and fatty acids reduced insulin-induced glucose uptake in isolated heart muscle, as well as activation of inflammation pathway [29]. Toll-like receptor 4, receptors sensing inflammatory inducers, such as lipopolysaccharides, has been identified as a receptor for saturated and polyunsaturated fatty acids [30]. Fatty-acid-induced activation of protein kinase C (PKC) as well as JNK in macrophages has also been linked to the production of inflammatory cytokines and promotion of muscle insulin resistance [31].

Finally, there are much research linking chronic inflammation to cancer development, and chronic inflammation is critical for tumor promotion and progression. The pathological mechanism how inflammation gets involved in cancer is complicated and unclear, but several critical gene products have been identified to suppress apoptosis and
enhance proliferation and metastasis, including cytokines and oxidant-generating enzymes involved in inflammatory response [32]. Pro-inflammatory mediators generated from inflammation response, such as ROS and RNS induce genetic change, which enhances malignant transformation and proliferation of initiated cells. The DNA damage resulting from chronic inflammation causing stomach cancers has been reported [33]. After that, inflammation continues to promote development of cancer by creating an inflammatory microenvironment where largely facilitate tumor formation. The cytokines produced by both tumor cells and inflammatory cells not only help recruitment of more inflammatory cells, but also enhance cellular proliferation to further growth and progression. Melanoma is the example in which chemokines have been shown to exert autocrine control over neoplastic cell proliferation [34]. In addition, tumor cells use the same adhesion molecule as leukocytes to aid in migration and homing during distant metastatic spread [35]. On transformation, many cancer cells start to express chemokine receptors and thereby use chemokines to aid in their migration and survival at sites where are distant from the original tumor [36, 37]. These invasive capacities of malignant cells can increase in the presence of inflammatory cytokines, possibly resulting from the upregulation of chemokine-receptor expression induced by these cytokines [38].

The discovery of the relationship between chronic inflammation and many human diseases attract attentions to the research that develops novel therapies targeting at inflammatory pathway and resulting in alleviation of these human diseases and conditions. Many therapeutic approaches target at inhibition of NF-κB. For instance, corticosteroids, used in the treatment of inflammatory bowel disease, asthma, and RA, are mediated through repression of NF-κB activation. After getting in to cells, glucocorticoid-receptor
complexes bind to the p65 subunit, and this prevents transcription factor activation of inflammatory genes. Synthesis of IκBα is stimulated by the binding of glucocorticoid–receptor complexes to a glucocorticoid response element in the promoter region of the IκBα gene [39]. Although glucocorticoids are effective inhibitors of NF-κB, but they have endocrine and metabolic side effects when subjected systemically. Other non-steroid anti-inflammatory molecules may target at blocking nuclear translocation NF-κB through inhibition of IκBα degradation, such as sulfasalazine, leflunomide and aspirin [39]. This might be caused by a direct effect on IKK or upstream signals. Yin et al. reported that the inhibitory effects of aspirin and sodium salicylate result from the specific inhibition of ATP-binding to IKKβ, which markedly decreases IKKβ-dependent phosphorylation of IκBα, preventing its degradation by the proteasome and activation of the NF-κB signaling pathway [39].

1.2 Hydroxycinnamic acid amides

1.2.1 Biosynthesis of hydrocinnamic acid amides

Hydroxycinnamic acid amides (HCAAs) are secondary metabolites that exist abundantly in plants, forming upon conjugation between hydroxycinnamic acid and either mono- or polyamines [40]. They are derived from phenylalanine and tyrosine pathways (Fig. 2) which are composed of C3-C6 carbon skeleton with a series of hydroxylations and O-methylations on the aromatic ring, yielding distinct structural patterns [41].

Hydroxycinnamates are generated through phenylpropanoid pathway by the activity of enzymes that converted phenylalanine to coumarates. The first reaction occurs when the enzyme, L-phenylalanine ammonium lyase, catalyzes the deamination of L-
alanine to *trans*-cinnamic acid, followed by the second reaction which is regulated by cinnamate 4-hydroxylase known to catalyze the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid [42]. Then, 4-coumarate:CoA ligase, generates a high-energy molecular form from coumerate to the hydroxycinnamate-CoA thioester. *p*-coumaroyl-CoA serves as a central biosynthetic precursor leading to the formation of a variety of phenolic species[43]. Hydroxycinnamoyl-CoA thioester will undergo sequential modification of cinnamic acid through series of enzymes. As a result, cinnamic acid is changed into hydroxycinnamoyl-CoA thioesters such as CoA thioesters of cinnamic acid, caffeic acid, coumaric acid, sinapic acid, and ferulic acid [41]. The condensation, which results in formation of the amide bond, requires two components: the energy-rich molecule, CoA thioesters, and the amine, catalyzed by hydroxycinnamoyl-CoA:tyramine N-hydroxycinnamoyltransferase (THT). THT is a 28-kDa soluble protein catalyzing the condensation via the hydroxycinnamoyl Co-A thioesters in combination with tyramine to their respective hydroxycinnamic acid tyramine amides. (Fig. 2). The amino acid 125 to 160 in THT were found to be critical structural determinants for amine substrate specificity [44]. Several hydroxycinnamate CoA thioester and amines can be substrates of THT catalyzed amide formation, including the CoA thioester derivatives mentioned before and dopamine, doradrenaline, tyramine and tryptamine, which largely extend the diversity of hydroxycinnamic acid amide compounds in the plants [41].
Figure 2 Biosynthesis of hydroxycinnamic acid amides. 4CL: 4-coumarate:CoA ligase; C4H: Cinnamate 4-hydroxylase; HCT: Hydroxycinnamoyl-CoA:shikimate/quinate hydroxycinnamoyltransferase; PAL: L-phenylalanine ammonium lyase; THT: Hydroxycinnamoyl-CoA:tyroamine N-(hydroxycinnamoyl)transferase; CCoAOMT: caffeoyl CoA 3-O-methyltransferase
1.2.2 HCAA Functions in plants

HCAAs serve a wide array of functions in plant with presence in flowers, pollen grains, seeds, leaves and the roots of plants [45]. HCAA species have been reported to have various functions in plants. Key enzymes activities that involve in HCAA biosynthesis and accumulation of metabolites in plants associate HCAA compounds with plant development and growth. They are found at high level in Arabidopsis seeds [46]. Concentrations of HCAAs were also higher in immature tubers with enhanced phenylpropanoid expression [47]. Both studies indicated that HCAA participate or regulate plant growth, such as seed germination and tuber formation.

It has been postulated that HCAA species play a role in plant defense system to both biotic and abiotic stress. Hydroxycinnamic acids serve as lignin precursors [48]. Water stress causes decrease of lignification, which is considered as an adaptive response to water stress. An enhancement of HCAA biosynthesis was detected in water-stressed sample as a defensive response of plants to high amount of reactive oxygen species produced by water stress or reduction of lignin biosynthesis [49, 50]. HCAAs are the polyaromatic domain of suberin that is known to be associated with cell wall reinforcement for plant protection and wound healing. HCAAs as a plant defense response requires the deposition of amide conjugates in the cell wall as a means to reduce the ability of fungal pathogens to penetrate and infect cells [51]. Several candidate genes in the phenylpropanoid pathway were augmented against plant pathogens, including 4-coumarate:CoA ligase [52]. Augmentation of HCAA biosynthesis upon pathogen infection creates a thicker cell wall by crosslinking, in order to protect plants from invading pathogens. Zacares et al. challenged Rutgers tomato with *P. syringae pv.* tomato DC3000 rapidly
induced the expression of the hydroxycinnamoyl-CoA:tyramine hydroxycinnamoyltransferase (THT) gene, which coded the key enzyme of the synthesis of hydroxycinnamoyl amides [53]. Accumulation of HCAAs in the cell wall ensures a stronger barrier against pathogens invasion by enhancing the cell wall strength against hydrolytic enzymes of the pathogen as well as directly inhibiting further proliferation of the pathogen.

However, the contribution of HCAAs to various plant developmental processes is still not fully clear and under debate that if HCAAs are storage molecules or actual bioactive species [54]. A great deal of effort is still needed in order to gain a detailed understanding of the biosynthesis, function and biotechnological applications of HCAA species in the plants.

1.2.3 Analytical techniques to study and separate HCAAs from plants

Traditional purification using extraction and open column filled with C\textsubscript{18}, silica gel and Sephadex material has successfully isolated several HCAA compounds from plants [55, 56]. Advances in analytical techniques greatly facilitate biomedical researches by expediting identification and separation of compounds from complex matrices. Directly coupling high performance liquid chromatography with mass spectrometer (LC-MS) provides high sensitivity and enhances quantitative analysis accuracy. Nuclear magnetic resonance (NMR) is the widely used detection tool for structure elucidation of unknown compounds. A nontarget metabolomics based on liquid chromatography and mass spectrometry aimed at identification of resistance-related metabolites including phenylpropanoid pathway and HCAA against pathogen stress [57, 58].
In particular, one of the difficulties from an analytical perspective of metabolomics studies is to precisely define the chemical and the physical compartmentation of the HCAAs, aiming to explore the molecular mechanism in relation to developmental and environmental effects. Thus, quantitative procedures with high sensitivity and specificity are required to overcome this challenge to progress the research in this area.

1.2.4 Bio-activities exhibited by HCAA family

Research in natural sources of antioxidant has been advanced rapidly because incorporation of natural antioxidants not only extend shelf life of food products by inhibiting generation of off-flavor and nutritional loss caused by oxidation, but also deliver potential health benefits to human health by reduction of oxidative stress. Studies involved in vitro antioxidant assay by using free radical quenching and lipid oxidation found that HCAAs are important class of antioxidants with potential application in prevention of human diseases [59, 60]. For instance, Zhou et al. identified a set of dicaffeoylspermidine derivatives that provide effective antioxidant activities and protection against Alzheimer’s disease [61]. The nature of amide species not only enhances the antioxidant activities of the molecules, but also improves the stability in physiological conditions and delivery applications [62]. Unlike esters that are readily hydrolyzed by the rich variety of hydrolase enzymes present in the human body, amides have the advantages of being more suitable for oral use [63].

HCAA family has been found to exhibit various biological activities. Some HCAA compounds, including dihydro-N-caffeoyltyramine, trans-N-feruloyloctopamine, trans-N-caffeoyltyramine, and cis-N-caffeoyltyramine demonstrated anti-fungal properties by impeding the dimorphic transition of Candida albicans [64]. trans-N-caffeoyltyramine was
also showed to have anti-cancer activities, along with other HCAA compounds, \textit{trans}-N-cinnamoyltyramine, \textit{trans}-N-feruloyltyramine, and \textit{trans}-N-snapoyltyramine. The treatment of the cells with HCAA compounds activated caspase-3 activity, and inhibited the growth of cells via decreasing in protein tyrosine kinase activity [65]. Anti-inflammatory properties of food-sourced components draw a lot of attention because of the connection between chronic inflammation and human diseases and conditions. By following a bioactivity-guided method, \textit{trans}-N-caffeoyltyramine was identified to have anti-inflammatory properties by inhibiting NF-κB [66]. \textit{trans}-N-feruloyltyramine was found to have an inhibitory effect on LPS (lipopolysaccharide)-induced inflammation response [67].

The large diversity and ubiquitous presence of HCAA species lays great potential to exploit the chemical diversity offered in them to obtain analogs with improved potency.

1.3 \textit{Lycium barbarum}

1.3.1 Introduction of \textit{Lycium barbarum}

\textit{Lycium barbarum} belongs to Solanaceae family. The genus \textit{Lycium} encompasses approximately 80 species unevenly distributed throughout South America, southern Africa, North America, Eurasia, Australia, and several islands in the Pacific Ocean [68]. The plant is currently widely cultivated in China. Wolfberries, the fruits of \textit{Lycium barbarum}, are 1–2 cm-long, bright orange-red ellipsoid berries processing a bitter to sweet taste. Its harvest period begins in August and lasts till October [69]. Berries are generally dried and then consumed. They are widely used in Asian cuisine such as, soups, congee and herbal tea. Recently, they have been marketed as dietary supplements and functional foods in multiple
regions [70]. There are many wolfberries derived-products on market, such as dried fruits, juice, wine and goji chocolate. China, the main supplier of wolfberry products in the world, had total exports generating US$120 million in 2004 [70]. Along with the fruits, the leaves and root barks of the plants were used as traditional medicine in Asian countries. *Lycium* root bark (Jikoppi) is formulated in the Kampo formula, and included as one of the active ingredients approved by the Japanese government [70].

### 1.3.2 Major chemical components identified from *L. barbarum*

The health benefits associated with plants have led to intensive research in identification phytochemical composition of the plant. In addition, new constituents were identified from diverse plant parts, most of which possess the novel chemical structures. Qian et al. provided comprehensive review about chemical constituents of genus *Lycium* that alkaloid, amides and steroids are the most abundant components [71]. Wolfberry have led to investigations into isolating and identifying several categories of compounds including polysaccharides [72], polyphenols [73], phenolic amides [61], carotenoids [74], flavonoids, organic acids, and their derivatives [75]. Polysaccharides present high abundance in fruits, which counts approximately 5%-8% of the dry weight of wolfberries [72]. A large number of studies reported the antioxidant, immunomodulatory and anti-aging effects of the fruits, which majorly attributed to the polysaccharides components from the fruits [76, 77]. These polysaccharides extensively branched and covalently bind to proteins with molecular weight ranging from 10 to 2300 kDa [78]. Gas chromatography (GC) analysis revealed the major monosaccharides include rhamnose, arabinose, galactose, glucose, mannose, fructose and uronic acids [79-81]. There are inconsistencies among different studies identifying polysaccharides linkages. Many studies pointed out the
presence of α-(1 → 6)-D-glucans and α-(1 → 3) or α-(1 → 5) substitution [70, 78]. Carotenoid, which deliver red-orange color of wolfberries, serves as the second essential bioactive component, exists in the dried fruit in approximately 2-4 mg per gram by weight [72, 82]. Among the carotenoids present in the fruits, zeaxanthin dipalmitate (Fig. 3) comprises at highest concentration, about 56% of the total carotenoid amount in the fruit. Besides zeaxanthin dipalmitate, the fruits also contain β-cryptoxanthin palmitate, zeaxanthin monopalmitate, and low amounts of free zeaxanthin and β-carotene. The fruits have uniquely high amounts of dipalmitates and the higher bioavailability of esterified zeaxanthin (1143.7 µg/g dried fruit) compared to free carotenoid [82]. Flavonoids serve as large compositional group of leaves, and rutin is the dominant flavonoid, following by quercitrin and quercetin [83-85]. In the root barks, phenolic amides [86], alkaloids [64], peptides [87, 88], flavonoids [89], terpenoids [90] have been identified that potentially contributed to the beneficial properties of the root barks. Kukoamine A, which was spermine amide conjugated with caffeic acid, was found as the principal hypotensive component of the root bark of the plant [91].
1.3.3 Health benefits associated with different parts of the plant

Foods affect human health far beyond just providing essential mineral and nutrients, some food molecules have established pharmacological effects affecting health by modulating biomedical pathways. Recently, increasing attention focuses on preventive and curative foods. *Lycium barbarum* has recognized as the most researched herbal nutritional sources [92]. Positive health effects of associated with *Lycium barbarum* included antioxidant, anticancer, immunomodulatory, hypoglycemic and antiaging properties [93].

The plant extracts showed radicals scavenging properties which are attributed to carotenoid pigments, flavonoids, polysaccharide fractions and $2-O-\beta$-D-glucopyranosyl-L,
a vitamin C analogue [94]. The polysaccharides have been shown to enhance the expression of various cytokines including IL-2 and TNF-α, as well as activation of transcription factors, such as NF-κB and AP-1 [70, 95]. Other investigations focused on the neuroprotective properties of the polysaccharide that protected neurons against β-amyloid peptide toxicity in vitro, which possible counter neuronal loss in neurodegenerative diseases [96]. The wolfberries gained increase in popularity due to numerous health benefits that improve kidney and liver function, immune system modulation, as well as provide anti-aging and cytoprotective effects [97, 98]. The root bark also demonstrated pharmacological activities to alleviate hematemesis, hypertension and hyperglycemia[91, 99]. However, very limited information about either chemical composition or the pharmacological effects about the leaves.

1.3.4 HCAAs that have been identified from Lycium barbarum as well as their anti-inflammatory activities

By using ultra-high performance liquid chromatography coupled with LTQ-Orbitrap mass spectrometry, a set of HCAA compounds has been identified from the root barks, including cis-N-feruloylputrescine, cis-N-feruloyloctopamine, trans-N-feruloylputrescine, dihydro-N-caffeoyltymamine, cis-N-caffeoyltymamine, trans-N-feruloyloctopamine, trans-N-caffeoyltymamine, cis-N-p-hydroxycinnamoyltymamine, dihydro-N-feruloyltymamine, dihydro-N-sinapoyltymamine, cis-N-feruloyltymamine, N-cis-sinapoyltymamine, trans-N-p-hydroxycinnamoyltymamine, N-cis-feruloyl-3-methyldopamine, trans-N-feruloyltymamine, N-trans-sinapoyltymamine and N-trans-feruloyl-3-methyldopamine [86] and trans-N-isoferuloyltymamine [89]. Lee et al. reported that cis-N-caffeoyltymamine was also isolated and identified from the root barks [100].
HCAA species identified from the root barks possessed potent ant-inflammatory activities. Following a bioactivity-guided method, *trans*-N-caffeoyltyramine was identified as a NF-κB inhibitor with an IC$_{50}$ value of 18.4 μM, which is known as a major transcription factor activated in response to inflammation and contributes to pro-inflammatory mediator production [101]. Han et al. reported that dihydro-Ν-caffeoyltyramine downregulated cyclooxygenase-2 expression through inhibition of the transcription factor activity of C/EBP and AP-1 in murine macrophages[102]. *trans*-N-feruloyltyramine was found to have an inhibitory effect on LPS-induced activation of transcription factor AP-1 and the MAPK signaling pathway as well as downstream NO and prostaglandin E2 production [67].
2. Development of analytical method to identify and quantify HCAAs from different parts of *Lycium barbarum*

As of submission of this dissertation, part of the work in this chapter has been submitted in the title of “Identification and quantification of potential anti-inflammatory hydroxycinnamic acid amides from wolfberry” to *Journal of Agricultural and Food Chemistry* for consideration of publication.

2.1 Introduction

As discussed in previous section, there are diverse groups of chemical species identified from different parts of the plants. Among these species, hydroxycinnamic acid derivatives are found to be abundant and characteristic in the plants [75]. By using preparative high performance liquid chromatography, Zhou et al. identified a set of dicaffeoylspermidine derivatives that provide effective antioxidant activities and protection against Alzheimer’s disease [61]. Other bioactive HCAA compounds have been identified from wolfberry. These include *cis*-N-feruloyltyramine, *trans*-N-feruloyltyramine, and its dimer through the use of an activity-guided method and NMR-based identification [103, 104].

The anti-inflammatory properties of natural products have attracted more attention due to the large body of scientific evidence that supports the close relationship between chronic inflammation and many human diseases and conditions, as well as the potential health beneficial properties exerted by these food-sourced components [105]. The identified HCAA species identified from wolfberry have been shown to possess putative anti-inflammatory properties. Furthermore, the biosynthesis of HCAA compounds
involves conjugation of cinnamoyl thioester and amine, catalyzed by the promiscuous enzyme THT. In previous discussion, various cinnamoyl derivatives and amines could be substrates of THT. The bioactive HCAA species identified from wolfberry lead to the hypothesis that there are potentially more HCAA species present in the different parts of *Lycium barbarum* with possible anti-inflammatory properties. This prompted us to synthesize a series of amide compounds with similar extended amine components that potentially exist in wolfberry. In our investigation, we designed and synthesized three sets of HCAA compounds according to different hydroxycinnamic acid species, *trans*-caffeic acid, *trans*-ferulic acid, and 3,4-dihydroxyhydrocinnamic acid. The objective was to develop the methodology that combined organic synthesis with NMR and UHPLC-TripleQ, aiming to use these synthetic amide compounds as references to identify and quantify compounds extracted from different part of the plants.

**2.2 Materials and Method**

**2.2.1 Materials**

*trans*-caffeic acid, *trans*-ferulic acid, 3,4-dihydroxyhydrocinnamic acid, phenethylamine, tryptamine, tyramine, dopamine hydrochloride, 3-phenylpropylamine, *N*-((3-(dimethylamino)propyl)-*N*-ethylcarbodiimide hydrochloride, sulfanilamide, naphthylethlenenediamine dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Triethylamine and 3,4-dimethoxyphenethylamine were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Dimethylformamide (DMF), ethyl acetate, and hexane were purchased from Pharmco-AAPER (Brookfield, CT, USA). Dimethyl sulfoxide-d6 was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA).
LC–MS grade methanol, acetonitrile, water, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

2.2.2 Synthesis procedure

Hydroxycinnamic acid (5 mM; *trans*-caffeic acid, *trans*-ferulic acid, or 3,4-dihydroxyhydrocinnamic acid) was mixed with 5 mM triethylamine in 10 mL of DMF and placed on ice for 15 min. Then 7.5 mM phenolic amine (phenethylamine, tryptamine, tyramine, 3-phenylpropylamine, dopamine hydrochloride, or 3,4-dimethoxyphenethylamine) and 5 mM of N-(3-(dimethylamino)propyl)-N'-ethylcarbodiimide hydrochloride were added to DMF under nitrogen atmosphere at room temperature for 12 h (Fig. 4). The reaction solution was then mixed with 100 mL of distilled water and extracted three times using 100 mL of ethyl acetate. The organic layer was next washed with 0.2 M hydrochloric acid and brine, dried, evaporated, and purified by using silica gel (standard grade, pore size 60 Å, 230–400 mesh particle size, 40–63 μm particle size) column chromatography (ethyl acetate and hexane), then finally freeze-dried, which resulted in the target compounds. The purity of synthetic compounds was determined by TLC and NMR.

Figure 4 Synthesis schemes of HCAAs
2.2.3 NMR Analysis

NMR Analysis. Proton nuclear magnetic resonance spectra (1 H NMR) were recorded on a Varian VNMRS-500 MHz, and Varian VNMRS 400 MHz instrument and reported in ppm using solvent containing TMS as an internal standard (CDCl₃ at 7.26 ppm, (CD₃)₂SO at 2.50 ppm, CD₃OD at 3.31 ppm). Data are reported as s = singlet, d = doublet, t = triplet, dd = doublet of doublets, m = multiplet; integration; coupling constant(s) in Hz.

2.2.4 Preparation of standard solutions and plant extract

Stock solutions were prepared at a concentration of 1,000 µg/mL, filtered through 0.45 µm nylon membrane filters, and stored at -20 °C until use. 1, 2, 3, 4, 6, 7, 8 and 9 were dissolved in dimethyl sulfoxide, 12, 13, 14 and 15 were dissolved in dimethyl sulfoxide:methanol (1:1) mixture, and 5, 10, 11, and 16 were dissolved in dimethyl sulfoxide:methanol (1:4) mixture. Standard working solutions were prepared by diluting and mixing each stock solution with methanol to obtain proper concentrations. For compound 17 (internal standard), a stock solution was prepared in dimethyl sulfoxide:methanol (1:4) mixture at a concentration at 1,000 µg/mL, and a working solution was prepared by diluting the stock solution with methanol. Each sample contained 10 ng/mL of internal standard.

Dried wolfberries were finely ground into powder, and then extracted using one of two different methods. For compounds 9 and 10, which were found in relatively high quantities in the sample, 10 mg of the powder was extracted using 4.5 mL of methanol and 0.5 mL of internal standard solution in an ultrasonic bath at ambient temperature for 40 min. After vigorous agitation by using a multi-tube vortexer for 1 h, the resultant solution was filtered through a 0.45 µm membrane filter, and injected into the LC-MS. For all other...
compounds, an evaporation procedure was added during the extraction. 100 mg of the powder was extracted using 4.5 mL of methanol and 0.5 mL of internal standard solution using the same procedure previously described. Then, 3 mL of the filtrate was vaporized under nitrogen, and the residue was suspended in 0.3 mL of methanol before injection into the LC-MS/MS system.

Ten to one hundred milligrams of leaves and roots were extracted with 5 mL methanol containing internal standard (N-trans-feruloyl 3-phenylpropylamine, 17) by ultrasonic assisted extraction for 40 min followed by agitation for 60 min. After filtered with a 0.22 μm filter, samples were optionally concentrated by evaporation with nitrogen gas or directly injected into UHPLC system.

2.2.5 UHPLC-MS/MS analysis

UHPLC analyses were carried out with an Ultimate 3000 ultra high performance liquid chromatography (UHPLC) system from Dionex (Sunnyvale, CA, USA). The instrument was equipped with a XRS Open autosampler, a binary RS pump and a RS column compartment. Separations were conducted with a Phenomenex (Torrance, CA, USA) Synergi Fusion-RP column (2.0 mm × 100 mm, 2.5 μm particle size). The mobile phases were prepared by adding 0.1% formic acid to water (mobile phase A) and to acetonitrile (mobile phase B), respectively. Mobile phase B was linearly increased from 20 to 100% for 5 min and maintained for 2 min before re-equilibration to the initial condition. The flow rate was 0.4 mL/min, and the injection volume was 10 μL. Column temperature was set at 25 °C.

The UHPLC system was hyphenated with a TSQ Quantiva (Thermo Fisher Scientific, San Jose, CA, USA) triple quadrupole mass spectrometer equipped with
heated-electrospray ionization (HESI) source. Electrospray ionization was operated in positive or negative polarity mode depending on the analytes, and spectra were acquired through selected reaction monitoring (SRM) measurements. Nitrogen gas was employed as sheath and auxiliary gases, and argon gas was used as collision gas. The source parameters were as follows: The positive spray voltage was set to 3,500 V and the negative spray voltage was 2,500 V. The sheath, aux and sweep gases were set to 45, 15 and 1 Arb, respectively. The ion transfer tube and vaporizer temperatures were both set at 350 °C. The following MS/MS parameters were used: The collision gas pressure was set to 2 mTorr, the dwell time was adjusted to 100 msec, and the chrom filter was set to 3 sec. Data collection and processing were performed using Xcaliber software (Ver. 3.0).

2.3 Results and Discussion

2.3.1 Synthetic standards

By using the method mentioned in previous section, there were total 17 HCAA compounds successfully synthesized (Fig. 5). The name of and corresponding numbers are listed in Table 1. Compound 17, Trans-feruloylphenepropylamine, was used as internal standard for LC-MS/MS analysis.
Figure 5 Chemical structures of 17 synthetic hydroxycinnamic acid amides
Table 1 Synthetic HCAA standard numbers and corresponding compound names

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Compound Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N-trans-caffeoyl phenethylamine</td>
</tr>
<tr>
<td>2</td>
<td>N-trans-caffeoyl 3,4-dimethoxyphenethylamine</td>
</tr>
<tr>
<td>3</td>
<td>N-trans-caffeoyl tryptamine</td>
</tr>
<tr>
<td>4</td>
<td>N-trans-caffeoyl tyramine</td>
</tr>
<tr>
<td>5</td>
<td>N-trans-caffeoyl dopamine</td>
</tr>
<tr>
<td>6</td>
<td>N-trans-feruloyl phenethylamine</td>
</tr>
<tr>
<td>7</td>
<td>N-trans-feruloyl 3,4-dimethoxyphenethylamine</td>
</tr>
<tr>
<td>8</td>
<td>N-trans-feruloyl tryptamine</td>
</tr>
<tr>
<td>9</td>
<td>N-trans-feruloyl tyramine</td>
</tr>
<tr>
<td>10</td>
<td>N-trans-feruloyl 3-methoxytyramine</td>
</tr>
<tr>
<td>11</td>
<td>N-trans-feruloyl dopamine</td>
</tr>
<tr>
<td>12</td>
<td>N-3,4-Dihydroxyhydrocinnamoyl phenethylamine</td>
</tr>
<tr>
<td>13</td>
<td>N-3,4-Dihydroxyhydrocinnamoyl 3,4-dimethoxyphenethylamine</td>
</tr>
<tr>
<td>14</td>
<td>N-3,4-Dihydroxyhydrocinnamoyl tryptamine</td>
</tr>
<tr>
<td>15</td>
<td>N-3,4-Dihydroxyhydrocinnamoyl tyramine</td>
</tr>
<tr>
<td>16</td>
<td>N-3,4-Dihydroxyhydrocinnamoyl dopamine</td>
</tr>
<tr>
<td>17</td>
<td>N-trans-feruloyl 3-phenylpropylamine</td>
</tr>
</tbody>
</table>

2.3.2 $^1$H and $^{13}$C NMR spectrum of synthetic standards and NMR spectral characteristics

N-trans-caffeoyl phenethylamine (1, Figure 5). Yellow powder; HESIMS $m/z$ 284.1 [M + H] (calcd for C_{17}H_{17}NO_{3}, 283.33); $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.22 (d, $J = 8.1$ Hz, 2H), 8.04 (t, $J = 5.7$ Hz, 1H), 7.31–7.14 (m, 6H), 6.93 (d, $J = 2.1$ Hz, 1H), 6.81 (dd, $J = 8.2$, 2.1 Hz, 1H), 6.73 (d, $J = 8.1$ Hz, 1H), 6.31 (d, $J = 15.7$ Hz, 1H), 3.41–3.33 (m, 2H), 2.75 (t, $J = 7.4$ Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta$ 165.80, 147.70, 145.96, 139.97, 139.47, 129.06, 128.76, 126.82, 126.51, 120.82, 118.93, 116.18, 114.23, 40.76, 35.68.

N-trans-caffeoyl 3,4-dimethoxyphenethylamine (2, Figure 5). Yellow powder; HESIMS $m/z$ 344.1 [M + H] (calcd for C_{19}H_{21}NO_{5}, 343.38); $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta$ 9.20
(s, 2H), 7.99 (t, J = 5.7 Hz, 1H), 7.21 (d, J = 15.6 Hz, 1H), 6.91 (d, J = 2.1 Hz, 1H), 6.84 (d, J = 8.2 Hz, 1H), 6.81−6.79 (m, 2H), 6.73−6.69 (m, 2H), 6.31 (d, J = 15.7 Hz, 1H), 3.71 (s, 3H), 3.69 (s, 3H), 3.37−3.33 (m, 2H), 2.67 (t, J = 7.3 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-d$_6$) δ 165.76, 149.03, 147.68, 147.65, 145.95, 139.40, 132.40, 126.83, 120.87, 120.79, 118.99, 116.17, 114.21, 112.97, 112.33, 55.95, 55.80, 40.90, 35.21.

$N$-trans-caffeoyl tryptamine (3, Figure 5). Dark Yellow powder; HESIMS m/z 321.1 [M - H] (calcd for C$_{19}$H$_{18}$N$_2$O$_3$, 322.36); $^1$H NMR (500 MHz, DMSO-d$_6$) δ 10.78 (s, 1H), 9.32 (s, 1H), 9.09 (s, 1H), 8.07 (t, J = 5.8 Hz, 1H), 7.54 (d, J = 7.9 Hz, 1H), 7.33 (s, 1H), 7.23 (d, J = 15.7 Hz, 1H), 7.14 (d, J = 2.3 Hz, 1H), 7.08−7.01 (m, 1H), 6.99−6.92 (m, 1H), 6.93 (d, J = 2.1 Hz, 1H), 6.82 (dd, J = 8.2, 2.0 Hz, 1H), 6.73 (d, J = 8.1 Hz, 1H), 6.32 (d, J = 15.7 Hz, 1H), 3.48−3.39 (m, 2H), 2.86 (t, J = 7.4 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-d$_6$) δ 165.72, 147.66, 145.95, 139.37, 136.68, 127.67, 126.88, 123.06, 121.34, 120.77, 119.12, 118.71, 118.65, 116.17, 114.24, 112.30, 111.79, 40.02, 25.77.

$N$-trans-caffeoyl tyramine (4, Figure 5). Yellow powder; HESIMS m/z 300.1 [M + H] (calcd for C$_{17}$H$_{17}$NO$_4$, 299.33); $^1$H NMR (500 MHz, DMSO-d$_6$) δ 9.31 (s, 1H), 9.14 (s, 1H), 9.08 (s, 1H), 7.98 (t, J = 5.7 Hz, 1H), 7.20 (d, J = 15.6 Hz, 1H), 6.99 (d, J = 8.4 Hz, 2H), 6.91 (d, J = 2.1 Hz, 1H), 6.80 (dd, J = 8.2, 2.1 Hz, 1H), 6.72 (d, J = 8.1 Hz, 1H), 6.66 (d, J = 8.3 Hz, 2H), 6.29 (d, J = 15.7 Hz, 1H), 3.32−3.25 (m, 2H), 2.62 (t, J = 7.4 Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-d$_6$) δ 165.72, 156.06, 147.67, 145.95, 139.37, 129.98, 129.90, 126.84, 120.79, 119.01, 116.17, 115.54, 114.23, 41.12, 34.90.

$N$-trans-caffeoyl dopamine (5, Figure 5). Yellow powder; HESIMS m/z 316.1 [M + H] (calcd for C$_{17}$H$_{17}$NO$_5$, 315.33); $^1$H NMR (500 MHz, DMSO-d$_6$) δ 8.92 (s, 4H), 7.98 (t, J =
5.7 Hz, 1H), 7.21 (d, $J = 15.7$ Hz, 1H), 6.92 (d, $J = 2.0$ Hz, 1H), 6.81 (dd, $J = 8.2, 2.1$ Hz, 1H), 6.72 (d, $J = 8.1$ Hz, 1H), 6.62 (d, $J = 7.9$ Hz, 1H), 6.58 (d, $J = 2.0$ Hz, 1H), 6.43 (dd, $J = 8.0, 2.1$ Hz, 1H), 6.30 (d, $J = 15.6$ Hz, 1H), 3.28 (dd, $J = 14.0, 6.5$ Hz, 2H), 2.59–2.52 (m, 2H).

$^{13}$C NMR (125 MHz, DMSO) δ 165.73, 147.67, 145.95, 145.48, 143.95, 139.37, 130.70, 126.85, 120.79, 119.65, 119.04, 116.41, 116.18, 115.93, 114.23, 41.16, 35.19.

$N$-trans-feruloyl phenethylamine (6, Figure 5). Colorless powder; HESIMS m/z 298.1 [M + H] (calcd for C$_{18}$H$_{19}$NO$_3$, 297.35); $^1$H NMR (500 MHz, DMSO-$d_6$) δ 9.39 (s, 1H), 8.01 (t, $J = 5.6$ Hz, 1H), 7.34–7.24 (m, 3H), 7.24–7.16 (m, 3H), 7.12–7.07 (m, 1H), 6.96 (dd, $J = 8.0, 0.8$ Hz, 1H), 6.42 (dd, $J = 15.6, 0.8$ Hz, 1H), 3.78 (s, 3H), 3.44–3.28 (m, 2H), 2.75 (t, $J = 7.3$ Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 165.78, 148.68, 148.24, 139.95, 139.35, 129.05, 128.75, 126.84, 126.50, 121.94, 119.39, 116.07, 111.19, 55.95, 40.73, 35.66.

$N$-trans-feruloyl 3,4-dimethoxyphenethylamine (7, Figure 5). Colorless powder; HESIMS m/z 358.2 [M + H] (calcd for C$_{20}$H$_{23}$NO$_5$, 357.41); $^1$H NMR (500 MHz, DMSO-$d_6$) δ 9.39 (s, 1H), 7.96 (t, $J = 5.7$ Hz, 1H), 7.30 (d, $J = 15.6$ Hz, 1H), 7.09 (d, $J = 1.9$ Hz, 1H), 6.96 (dd, $J = 8.3, 1.9$ Hz, 1H), 6.83 (d, $J = 8.2$ Hz, 1H), 6.80 (d, $J = 2.0$ Hz, 1H), 6.77 (d, $J = 8.1$ Hz, 1H), 6.71 (dd, $J = 8.1, 2.0$ Hz, 1H), 6.43 (d, $J = 15.7$ Hz, 1H), 3.78 (s, 3H), 3.72 (s, 3H), 3.69 (s, 3H), 3.43–3.34 (m, 2H), 2.68 (t, $J = 7.3$ Hz, 2H). $^{13}$C NMR (125 MHz, DMSO-$d_6$) δ 165.76, 149.04, 148.66, 148.24, 147.65, 139.31, 132.36, 126.86, 121.91, 120.86, 119.45, 116.08, 112.95, 112.32, 111.18, 55.95, 55.93, 55.80, 40.86, 35.19.

$N$-trans-feruloyl tryptamine (8, Figure 5). Pale yellow powder; HESIMS m/z 337.2 [M + H] (calcd for C$_{20}$H$_{20}$N$_2$O$_3$, 336.39); $^1$H NMR (500 MHz, DMSO-$d_6$) δ 10.80 (s, 1H), 9.39
(s, 1H), 8.04 (t, J = 5.7 Hz, 1H), 7.54 (d, J = 8.0 Hz, 1H), 7.35−7.32 (m, 2H), 7.16 (d, J = 2.0 Hz, 1H), 7.11 (d, J = 2.0 Hz, 1H), 7.05 (ddd, J = 8.1, 7.0, 1.2 Hz, 1H), 6.99−6.94 (m, 2H), 6.78 (d, J = 8.1 Hz, 1H), 6.45 (d, J = 15.7 Hz, 1H), 3.79 (s, 3H), 3.46 (dd, J = 13.1, 7.3 Hz, 2H), 2.87 (t, J = 6.9 Hz, 2H). 13C NMR (125 MHz, DMSO-d6) δ 165.79, 148.65, 148.25, 139.28, 136.70, 127.68, 126.91, 123.07, 121.93, 121.36, 119.60, 118.72, 118.66, 116.09, 112.28, 111.80, 111.19, 55.96, 39.99, 25.75.

N-trans-feruloyl tyramine (9, Figure 5). Colorless powder; HESIMS m/z 314.1 [M + H]+ (calcd for C18H19NO4, 313.35); 1H NMR (500 MHz, DMSO-d6) δ 9.38 (s, 1H), 9.14 (s, 1H), 7.95 (t, J = 5.7 Hz, 1H), 7.29 (d, J = 15.7 Hz, 1H), 7.09 (d, J = 2.0 Hz, 1H), 6.99 (d, J = 8.4 Hz, 2H), 6.96 (dd, J = 8.2, 2.0 Hz, 1H), 6.77 (d, J = 8.1 Hz, 1H), 6.66 (d, J = 8.4 Hz, 2H), 6.41 (d, J = 15.8 Hz, 1H), 3.78 (s, 3H), 3.33−3.29 (m, 2H), 2.63 (t, J = 7.4 Hz, 2H). 13C NMR (125 MHz, DMSO-d6) δ 165.72, 156.06, 148.64, 148.23, 139.27, 129.95, 129.89, 126.86, 121.92, 119.47, 116.07, 115.54, 111.17, 55.95, 41.09, 34.87.

N-trans-feruloyl 3-methoxytyramine (10, Figure 5). Colorless powder; HESIMS m/z 344.2 [M + H]+ (calcd for C19H21NO5, 343.38); 1H NMR (500 MHz, DMSO-d6) δ 9.38 (s, 1H), 8.68 (s, 1H), 7.95 (t, J = 5.7 Hz, 1H), 7.29 (d, J = 15.7 Hz, 1H), 7.09 (d, J = 1.9 Hz, 1H), 6.96 (dd, J = 8.3, 1.9 Hz, 1H), 6.80−6.73 (m, 2H), 6.66 (d, J = 8.0 Hz, 1H), 6.58 (dd, J = 8.0, 1.9 Hz, 1H), 6.42 (d, J = 15.7 Hz, 1H), 3.78 (s, 3H), 3.72 (s, 3H), 3.38 - 3.29 (m, 2H), 2.64 (t, J = 7.4 Hz, 2H). 13C NMR (125 MHz, DMSO-d6) δ 165.73, 148.64, 148.24, 147.82, 145.22, 139.27, 130.65, 126.86, 121.90, 121.16, 119.49, 116.08, 115.77, 113.20, 111.17, 55.95, 55.94, 40.98, 35.24.
N-trans-feruloyl dopamine (11, Figure 5). Colorless powder; HESIMS m/z 330.1 [M + H] (calcd for C_{18}H_{19}NO_{5}, 329.35); ^1H NMR (500 MHz, DMSO-d_6) δ 9.38 (s, 1H), 8.72 (s, 1H), 8.62 (s, 1H), 7.94 (t, J = 5.6 Hz, 1H), 7.29 (d, J = 15.7 Hz, 1H), 7.09 (d, J = 2.0 Hz, 1H), 6.96 (dd, J = 8.3, 1.9 Hz, 1H), 6.77 (d, J = 8.1 Hz, 1H), 6.62 (d, J = 7.9 Hz, 1H), 6.58 (d, J = 2.1 Hz, 1H), 6.48 - 6.35 (m, 2H), 3.78 (s, 3H), 3.33−3.23 (m, 2H), 2.56 (t, J = 7.4 Hz, 2H). ^13C NMR (125 MHz, DMSO-d_6) δ 165.70, 148.64, 148.23, 145.48, 143.95, 139.26, 130.68, 126.87, 121.92, 119.64, 119.50, 116.41, 116.07, 115.93, 111.17, 55.95, 41.12, 35.15.

N-3,4-Dihydroxyhydrocinnamoyl phenethylamine (12, Figure 5). Colorless liquid; HESIMS m/z 286.1 [M + H] (calcd for C_{17}H_{19}NO_{3}, 285.34); ^1H NMR (500 MHz, DMSO-d_6) δ 8.63 (s, 1H), 7.84 (t, J = 5.5 Hz, 1H), 7.29−7.21 (m, 2H), 7.17 (dt, J = 8.2, 1.8 Hz, 2H), 7.16−7.12 (m, 2H), 6.61−6.57 (m, 1H), 6.55 (d, J = 2.1 Hz, 1H), 6.40 (dd, J = 8.0, 2.2 Hz, 1H), 3.27−3.18 (m, 2H), 2.67−2.63 (m, 2H), 2.59 (t, J = 7.7 Hz, 2H), 2.24 (dd, J = 8.7, 6.9 Hz, 2H). ^13C NMR (125 MHz, DMSO-d_6) δ 171.85, 145.40, 143.72, 139.98, 132.60, 129.06, 128.71, 126.43, 119.16, 116.10, 115.82, 40.63, 38.00, 35.66, 31.04.

N-3,4-Dihydroxyhydrocinnamoyl 3,4-dimethoxyphenethylamine (13, Figure 5). Yellow amorphous powder; HESIMS m/z 346.2 [M + H] (calcd for C_{19}H_{23}NO_{5}, 345.40); ^1H NMR (500 MHz, DMSO-d_6) δ 8.69 (s, 1H), 8.59 (s, 1H), 7.81 (t, J = 5.6 Hz, 1H), 6.82 (d, J = 8.1 Hz, 1H), 6.75 (d, J = 2.0 Hz, 1H), 6.64 (dd, J = 8.2, 2.0 Hz, 1H), 6.60 (d, J = 8.0 Hz, 1H), 6.56 (d, J = 2.1 Hz, 1H), 6.40 (dd, J = 8.0, 2.1 Hz, 1H), 3.71 (s, 3H), 3.69 (s, 3H), 3.21 (dt, J = 7.7, 6.2 Hz, 2H), 2.62−2.58 (m, 4H), 2.28−2.21 (m, 2H). ^13C NMR (125 MHz, DMSO-d_6) δ 172.35, 149.53, 145.92, 144.24, 133.15, 132.97, 121.41, 121.39, 119.67, 116.61, 116.35, 113.44, 112.84, 56.46, 56.30, 41.32, 38.53, 35.74, 31.59.
N-3,4-Dihydroxyhydrocinnamoyl tryptamine (14, Figure 5). Yellow amorphous powder; HESIMS \( m/z \) 325.2 [M + H] (calcd for C_{19}H_{20}N_{2}O_{3}, 324.38); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \( \delta \) 10.76 (s, 1H), 8.63 (s, 2H), 7.88 (t, \( J = 5.8 \) Hz, 1H), 7.51 (d, \( J = 7.9 \) Hz, 1H), 7.31 (d, \( J = 8.1 \) Hz, 1H), 7.10 (s, 1H), 7.06–7.02 (m, 1H), 6.96 (t, \( J = 7.4 \) Hz, 1H), 6.60 (d, \( J = 7.9 \) Hz, 1H), 6.57 (d, \( J = 2.1 \) Hz, 1H), 6.41 (dd, \( J = 8.0, 2.1 \) Hz, 1H), 3.30 (dd, \( J = 13.8, 7.0 \) Hz, 2H), 2.77 (t, \( J = 7.5 \) Hz, 2H), 2.61 (dd, \( J = 9.8, 6.8 \) Hz, 2H), 2.30–2.21 (m, 2H). \(^{13}\)C NMR (125 MHz, DMSO-\(d_6\)) \( \delta \) 172.30, 145.90, 144.20, 137.15, 133.18, 128.17, 123.50, 121.80, 119.65, 119.18, 119.12, 116.59, 116.33, 112.83, 112.26, 40.42, 38.61, 31.57, 26.21.

N-3,4-Dihydroxyhydrocinnamoyl tyramine (15, Figure 5). Colorless amorphous powder; HESIMS \( m/z \) 302.1 [M + H] (calcd for C_{17}H_{19}NO_{4}, 301.34); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \( \delta \) 8.88 (d, \( J = 153.8 \) Hz, 3H), 7.80 (t, \( J = 5.6 \) Hz, 1H), 6.95 - 6.91 (m, 2H), 6.67 - 6.63 (m, 2H), 6.60 (d, \( J = 8.0 \) Hz, 1H), 6.56 (d, \( J = 2.1 \) Hz, 1H), 6.40 (dd, \( J = 8.0, 2.1 \) Hz, 1H), 3.20–3.12 (m, 2H), 2.63–2.56 (m, 2H), 2.57–2.50 (m, 2H), 2.27–2.20 (m, 2H). \(^{13}\)C NMR (125 MHz, DMSO-\(d_6\)) \( \delta \) 171.81, 156.01, 145.40, 143.71, 132.63, 130.01, 129.90, 119.16, 116.10, 115.83, 115.51, 41.01, 38.02, 34.89, 31.06.

N-3,4-Dihydroxyhydrocinnamoyl dopamine (16, Figure 5). Colorless liquid; HESIMS \( m/z \) 318.2 [M + H] (calcd for C_{17}H_{19}NO_{5}, 317.34); \(^1\)H NMR (500 MHz, DMSO-\(d_6\)) \( \delta \) 8.63 (s, 4H), 7.79 (t, \( J = 5.6 \) Hz, 1H), 6.63 - 6.56 (m, 2H), 6.55 (d, \( J = 2.1 \) Hz, 2H), 6.39 (ddd, \( J = 8.1, 6.0, 2.1 \) Hz, 2H), 3.15–3.11 (m, 2H), 2.61–2.57 (m, 2H), 2.48–2.45 (m, 2H), 2.25–2.21 (m, 2H). \(^{13}\)C NMR (125 MHz, DMSO-\(d_6\)) \( \delta \) 171.76, 145.46, 145.39, 143.91, 143.69, 132.65, 130.71, 119.64, 119.14, 116.37, 116.07, 115.89, 115.83, 41.02, 38.06, 35.19, 31.08.
*N*-trans-feruloyl 3-phenylpropylamine (17, Figure 5). Yellow liquid; HESIMS m/z 312.1 [M + H] (calcd for C_{19}H_{21}NO_{3}, 311.38); ¹H NMR (500 MHz, DMSO-\textit{d}_6) δ 9.39 (s, 1H), 7.99 (t, \textit{J} = 5.7 Hz, 1H), 7.32 (d, \textit{J} = 15.7 Hz, 1H), 7.28–7.24 (m, 2H), 7.21–7.17 (m, 2H), 7.17–7.13 (m, 1H), 7.11 (d, \textit{J} = 2.0 Hz, 1H), 6.97 (dd, \textit{J} = 8.2, 1.9 Hz, 1H), 6.78 (d, \textit{J} = 8.1 Hz, 1H), 6.44 (d, \textit{J} = 15.7 Hz, 1H), 3.79 (s, 3H), 3.20–3.09 (m, 2H), 2.63 - 2.54 (m, 2H), 1.78 - 1.68 (m, 2H). ¹³C NMR (125 MHz, DMSO-\textit{d}_6) δ 166.26, 149.14, 148.74, 142.63, 139.77, 129.23, 129.20, 129.17, 127.37, 126.65, 122.43, 119.97, 116.57, 111.63, 56.43, 39.21, 33.52, 31.95, 15.01.

Amide structures were confirmed using the triplet with coupling constant of \textit{J} ≈ 5.7 Hz. ¹H NMR spectrum of caffeoyl and feruloyl amides had two vinyl doublets with a coupling constant of \textit{J} ≈ 15.7 Hz, and feruloyl amides also had a methoxyl singlet at 3.80 ppm. 3,4- Dihydroxyhydrocinnamoyl amides lost the vinyl structure, and alternately, characteristic methylene chemical shifts around 2.58 ppm were found. 3,4- Dimethoxylphenethylamine moieties had two singlets with a chemical shift of \textasciitilde 3.70 ppm, which were attributed to two methoxyl groups. An indole singlet above 10 ppm confirmed the tryptamine moieties in compounds 3, 8, and 14.

### 2.3.4 Optimization of UHPLC-MS/MS for HCAA compounds

Although UHPLC-TripleQ provides very limited structural information of analytes, especially those from extracted from plants containing multiple structural isomers and analogs, this drawback could be overcome by targeting synthesis and NMR with structure elucidation and confirmation of each synthetic standard.
0.1% formic acid was added in both water (mobile phase A) and acetonitrile (mobile phase B) as a mobile phase modifier to obtain desirable peak shapes and adequate retention times of analytes. Gradient elution was employed to decrease retention time as well as to further improve peak sharpness. All compounds were eluted within 5 min (Fig. 6), which means that the method is suitable for large-scale application with large number of samples.

Each synthetic analyte was infused into the mass spectrometer, and the precursor ions and at two product ions were preliminarily selected in both positive ion and negative ion modes. Positive ion mode showed better signal response than negative ion mode. Only compound 3 had higher intensity in negative mode. Parameters such as spray voltage, sheath gas, aux gas, ion transfer tube temperature, vaporizer temperature, collision gas pressure and dwell time were evaluated to obtain suitable signal for the precursor and product ions of the analytes. The precursor ion, product ion, collision energy and RF lens voltage of the analytes were individually optimized by direct flow infusion of each standard, and the optimum values are summarized in Table 2.
<table>
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<tr>
<th>Compounds</th>
<th>Polarity</th>
<th>Precursor ion (m/z)</th>
<th>Product ion (m/z)</th>
<th>Collision energy (V)</th>
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<tr>
<td>1</td>
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<td>284.1</td>
<td>163.0</td>
<td>18.0</td>
</tr>
<tr>
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<td>3</td>
<td>Negative</td>
<td>321.1</td>
<td>135.1</td>
<td>23.0</td>
</tr>
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<td>300.1</td>
<td>163.1</td>
<td>16.0</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>316.1</td>
<td>163.1</td>
<td>17.0</td>
</tr>
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<td>6</td>
<td>Positive</td>
<td>298.1</td>
<td>145.0</td>
<td>26.0</td>
</tr>
<tr>
<td>7</td>
<td>Positive</td>
<td>358.2</td>
<td>177.1</td>
<td>15.0</td>
</tr>
<tr>
<td>8</td>
<td>Positive</td>
<td>337.2</td>
<td>177.1</td>
<td>15.0</td>
</tr>
<tr>
<td>9</td>
<td>Positive</td>
<td>314.1</td>
<td>177.1</td>
<td>16.0</td>
</tr>
<tr>
<td>10</td>
<td>Positive</td>
<td>344.2</td>
<td>177.1</td>
<td>15.0</td>
</tr>
<tr>
<td>11</td>
<td>Positive</td>
<td>330.1</td>
<td>177.1</td>
<td>15.0</td>
</tr>
<tr>
<td>12</td>
<td>Positive</td>
<td>286.1</td>
<td>122.1</td>
<td>14.0</td>
</tr>
<tr>
<td>13</td>
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<td>346.2</td>
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<td>14</td>
<td>Positive</td>
<td>325.2</td>
<td>144.1</td>
<td>21.0</td>
</tr>
<tr>
<td>15</td>
<td>Positive</td>
<td>302.1</td>
<td>121.1</td>
<td>21.0</td>
</tr>
<tr>
<td>16</td>
<td>Positive</td>
<td>318.2</td>
<td>137.1</td>
<td>21.0</td>
</tr>
<tr>
<td>17 (internal standard)</td>
<td>Positive</td>
<td>312.1</td>
<td>177.0</td>
<td>18.0</td>
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</tbody>
</table>
2.3.5 Quantification and identification results of HCAAs from fruits, leaves and root barks of *Lycium barbarum*

The LC-MS/MS analytical results showed that the plant had amide
compounds at various concentrations. For example, the content of 9 was greater than 10,000-fold more than that of 7, whereas some compounds only presented in nanogram quantities.

The established LC-MS/MS method was applied to comprehensive analysis and quantitative evaluation of the fruits samples. The analysis was performed in triplicate (n=3). The chromatograms of 16 amide compounds are shown in Figure 6. The compounds were fairly well separated from interferences. The retention times of compounds 1-16 and 17 (internal standard) are 2.93 min, 2.66 min, 2.98 min, 2.32 min, 2.03 min, 3.25 min, 2.97 min, 3.25 min, 2.64 min, 2.36 min, 2.71 min, 2.46 min, 2.81 min, 2.04 min, 1.71 min, 2.68 min and 3.49 min, respectively. Quantification of each analyte in the samples was calculated using the ratio of peak area (analyte peak area versus internal standard peak area) based on the calibration curve of each individual standard. The correlation coefficient values of all models exhibited good linearity ($R^2 > 0.998$, later section). The results indicated successful application of the LC-MS/MS method for the quantification of amide compounds in different quantities. The major constituents in the fruits were found to be 4, 5, 9, 10 and 11. Their contents were between 107.2 and 11109.6 ng/g. The content of 14 was 12.1 ng/g. All compounds contained a tyramine or dopamine moiety, whereas compounds, 6-8, having a phenethylamine, 3,4-dimethoxyphenethylamine or tryptamine moiety, were found to be minor ones (0.7 to 3.1 ng/g). Table 3 lists the mean concentrations of amide compounds detected in the samples.

We also applied it to comprehensively profile HCAA from different plant tissues, which were the root barks and leaves of *Lycium barbarum*, aiming to explore the chemical diversity of the HCAA family as well as systematically quantify them at different locations.
of the plant. The established UHPLC-MS/MS method was applied to quantitative evaluation of target compounds in samples of leaves and root barks. The analysis was performed in triplicate (n=3). HCAA compounds were found in various quantities in the root bark and leaves of Lycium barbarum. Table 3 lists the quantification results of HCAA compounds in the leaves and root barks. Fourteen HCAA compounds were identified from the root barks and 10 from the leaves, which indicated that root barks had more diverse HCAA species compared to the leaves and fruits, compared to our previous work. Compounds 4, 9, 11 and 15 presented at highest concentration in the root bark as 26446.0 ± 154.8, 10600.0 ± 509.2, 5392.1 ± 236.3 and 4864.0 ± 74.9 ng/g respectively. Compounds 9 and 11 were trans-feruloyltyramine and its derivative trans-feruloyl 3-methoxytyramine, which were found to ubiquitously present in plants, serving as major metabolic constituents of cell wall alterations that play a role in plant defense response to pathogen challenge [106]. Compounds 4 and 15 also had tyramine moiety but conjugated with trans-caffeic acid and 3,4-dihydroxyhydrocinnamic acid, which may biosynthesized from similar precursors so that existed at higher concentration. Similar trend was also observed in quantification results of leaves. Following the tyramine conjugated species, compounds 5, 11 and 16 were also found as major HCAA components in both root barks and leaves. They had dopamine extensions and conjugated by trans-caffeic acid, trans-ferulic acid and 3, 4-dihydroxyhydrocinnamic acid, respectively. They may result from the hydroxylation of tyramine, which was the decarboxylated product of tyrosine [107]. Interestingly, compound 6 was found minor in the root barks (3.9 ± 0.1 ng/g) but had much higher quantity in the leaves (182.9 ± 6.8 ng/g).
Table 3 Mean concentrations of amide compounds in different parts of Lycium barbarum (n = 3)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Mean ± SDa (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Root</td>
</tr>
<tr>
<td>1</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>NDb</td>
</tr>
<tr>
<td>3</td>
<td>49.6 ± 2.8</td>
</tr>
<tr>
<td>4</td>
<td>26446.0 ± 154.8</td>
</tr>
<tr>
<td>5</td>
<td>317.6 ± 21.9</td>
</tr>
<tr>
<td>6</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>7</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>8</td>
<td>2.1 ± 0.1</td>
</tr>
<tr>
<td>9</td>
<td>10600.0 ± 509.2</td>
</tr>
<tr>
<td>10</td>
<td>5392.1 ± 236.3</td>
</tr>
<tr>
<td>11</td>
<td>611.3 ± 10.6</td>
</tr>
<tr>
<td>12</td>
<td>5.2 ± 0.2</td>
</tr>
<tr>
<td>13</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>2.7 ± 0.1</td>
</tr>
<tr>
<td>15</td>
<td>4864.0 ± 74.9</td>
</tr>
<tr>
<td>16</td>
<td>324.3 ± 8.0</td>
</tr>
</tbody>
</table>

aStandard deviation  
bNot detected

The quantification results of HCAAs from different part of plant tissues can be possibly attributed to the properties of enzyme THT that catalyze the conjugation between hydroxycinnamic acids and amines. Regardless of the plant sources of this enzyme, THT isolated from different plants exhibit similar substrate affinity patterns, especially at the
level of amine substrates, with the exception of the maize enzyme [108]. For the amine substrates, THT enzymes isolated from potato, tobacco, and tomato show the highest affinity for tyramine and octopamine (β-hydroxytyramine) following by dopamine by enzyme kinetics studies; THT is highest for feruloyl-CoA as the acceptor. In addition, tyramine showed the maximum when feruloyl-CoA was used as the acyl donor [44]. This differential affinity of THT confirmed the quantification results of Lycium barbarum that trans N-feruloyl tyramine is the most abundant species throughout fruits, leaves and roots. Furthermore, when comparing different amine substrate, HCAAs with tyramine moiety all present at relatively larger amount, following by dopamine as amine moiety, which indicates that the affinity THT of amine is more dominant in substrate selection.

In addition to substrate affinity, the expression of THT varies in different plant tissue. The pepper THT mRNAs analysis from different tissues showed a varied expression levels, and stem and roots showed significantly higher expression than leaves, fruits and flower [44]. The differential amount as well as the diversity of HCAAs in different tissues of Lycium barbarum is also attributable to the expression level of THT. HCAAs are recognized as critical molecules of plant defense system such as cell wall cross linker, in which roots are considered play a more important role in this function.

2.5 Conclusions

In summary, there is a large diversity of HCAA species present in different tissues of Lycium barbarum. Due to their similar chemical structures, it is challenging to identify and quantify individual HCAA directly from the plant. The developed method combined organic synthesis and UHPLC-TripleQ specifically targeting at HCAA species identification and quantification largely improved sensitivity. As far as we know, HCAA
compounds were first time reported in leaves of the plant. Among these candidates, nine amide compounds were identified from the fruits. Previous studies only detected several major HCAA compounds in the root bark but not in the leaves [84, 86], and 10 HCAA compounds were newly detected in the root bark. The improvement potentially leads to targeting compartmental simultaneous analysis with fractionation and extraction method.
3. Validation of developed UHPLC-MS/MS method

As of submission of this dissertation, the work in this chapter has been submitted in the title of “Use of UHPLC-TripleQ with synthetic standards to profile anti-inflammatory hydroxycinnamic acid amides in root barks and leaves of Lycium barbarum” to Journal of Food and Drug Analysis for consideration of publication.

3.1 Introduction

Although there are many studies investigating the HCAA species in the plant, the contribution of HCAAs to various plant developmental processes is still not fully clear and under debate that if HCAAs are storage molecules or actual bioactive species [109]. A great deal of effort is still needed in order to gain a detailed understanding of the biosynthesis, function and biotechnological applications of HCAA species in the plants.

In particular, one of the difficulties from an analytical perspective of metabolomics studies is to precisely define the chemical and the physical compartmentation of the HCAAs, aiming to explore the molecular mechanism in relation to developmental and environmental effects. Thus, quantitative procedures with high sensitivity and specificity are required to overcome this challenge to progress the research in this area.

Advances in analytical techniques greatly facilitate biomedical researches by expediting identification and separation of compounds from complex matrices. Directly coupling high performance liquid chromatography with mass spectrometer (LC-MS) provides high sensitivity and enhances quantitative analysis accuracy. Nuclear magnetic resonance (NMR) is the widely used detection tool for structure elucidation of unknown compounds. In this paper, we developed an innovative approach that emerges organic
synthesis and advances in chromatography, mass spectrometry and NMR technologies and to overcome the challenges encountered in both natural product chemistry and plant metabolomics studies, specifically targeting at HCAAs with high detection and quantification sensitivity.

In previous section, an analytical method targeting at analysis of HCAAs has identified a series of HCAA species from the different parts of *Lycium barbarum*. The method successfully identified and quantified those with similar structures and present in trace amount. In order to apply the method to future analysis and targeted applications that require high sensitivity, in this part, the analytical method will be fully validated.

### 3.2 Material and method

LC−MS grade methanol, acetonitrile, water, and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA).

Synthetic standards were obtained using the same method reported in previous section and confirmed by NMR.

Standard and plant extracts preparation and UHPLC-MS/MS operation parameters were followed as previous sections.

### 3.3 Results and discussion

#### 3.3.1 Method validation

The developed UHPLC-MS/MS method was validated in terms of specificity, linearity, intra- and inter-day precision and accuracy, limit of detection (LOD), limit of quantification (LOQ), recovery, and reproducibility according to the guideline
established by the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Validation of analytical procedures: Text and methodology, 2005 [110].

The specificity was determined by a chromatogram of target compounds (Fig. 7). The retention times of compounds 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16 and 17 (internal standard) are 3.11, 2.80, 3.13, 2.45, 2.19, 3.44, 3.10, 3.42, 2.79, 2.80, 2.50, 2.90, 2.59, 2.97, 2.22, 1.93 and 3.65 minutes, respectively, and the analytes were detected without any apparent interference. Calibration curves were constructed by plotting the ratio of the analyte peak area to the internal standard peak area against the respective analyte concentrations. As presented in Table 4, the calibration curves of analytes were linear over the concentration ranges with high correlation of determination ($r^2$) values above 0.996. The limit of detection (LOD) and limit of quantification (LOQ) were set to three times and ten times the signal to noise ratio by analyzing serial diluted standards, respectively. All LODs and LOQs were estimated at picogram (pg/mL) levels, which means that the method was proven to be highly sensitive to determine trace amount of compounds (Table 4).

The intra- and inter-day precision and accuracy were estimated at three different concentrations (0.05, 4 and 20 ng/mL for compound 1, 0.1, 8 and 40 ng/mL for compounds 6 and 8, 0.25, 20 and 100 ng/mL for compounds 7, 12, 13 and 14, and 0.5, 40 and 200 ng/mL for the others) in the range of the calibration curve. Three replicate analyses were conducted on the same day and on three consecutive days. The precision was defined as the relative standard deviation (RSD, %), and the accuracy was expressed as the observed concentration relative to the nominal concentration. The intra-day
precision was measured from 0.1 to 10.9%, and the inter-day precision was from 0.7 to 11.8%. The intra-day accuracy ranged from 90.5 to 111.6%, and the inter-day accuracy ranged from 93.8 to 112.8% (Table 5).

The recovery was determined by a standard addition method using low and high amounts (40 ng/mL and 100 ng/mL) of analytes. Recoveries from root barks and leaves were evaluated, and each experiment was performed in five times (n = 5). The mean recovery was in the range of 94.8–105.1% and the RSD was less than 15% (Table 6). Lastly, the reproducibility was examined by six replicate analyses (n = 6) of a standard solution (10 ng/mL for compound 1, 20 ng/mL for compounds 6 and 8, 50 ng/mL for compounds 7, 12, 13 and 14, and 100 ng/mL for the others). The resulting RSD was below 5% for all compounds (Table 6).
Figure 7 UHPLC-MS/MS chromatograms of standard mixture. 100 ng/mL N-trans-caffeoyl phenethylamine (1), 200 ng/mL N-trans-caffeoyl 3,4-dimethoxyphenethylamine (2), 200 ng/mL N-trans-caffeoyl tryptamine (3), 200 ng/mL N-trans-caffeoyl tyramine (4), 200 ng/mL N-trans-caffeoyl dopamine (5), 40 ng/mL N-trans-feruloyl phenethylamine (6), 100 ng/mL N-trans-feruloyl 3,4-dimethoxyphenethylamine (7), 40 ng/mL N-trans-feruloyl tryptamine (8), 200 ng/mL N-transferuloyltyramine (9), 200 ng/mL N-trans-feruloyl 3-methoxytyramine (10), 200 ng/mL N-trans-feruloyl dopamine (11), 100 ng/mL N-3,4-Dihydroxyhydrocinnamoyl phenethylamine (12), 100 ng/mL N-3,4-Dihydroxyhydrocinnamoyl 3,4-dimethoxyphenethylamine (13), 100 ng/mL N-3,4-Dihydroxyhydrocinnamoyl tryptamine (14), 200 ng/mL N-3,4-Dihydroxyhydrocinnamoyl tyramine (15), 200 ng/mL N-3,4-Dihydroxyhydrocinnamoyl dopamine (16), and 10 ng/mL Ntrans-feruloyl3-phenylpropylamine (internal standard) (17).
Table 3 Parameters linear range, regression equation, correlation coefficient ($r^2$), retention time ($t_r$), limit of quantification (LOQ) and limit of detection (LOD) for HCAA compounds.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Linear range (ng/mL)</th>
<th>Regression equation</th>
<th>$r^2$</th>
<th>$t_r$ (min)</th>
<th>LOQ (ng/mL)</th>
<th>LOD (ng/mL)</th>
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</thead>
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<tr>
<td>1</td>
<td>0.05-20</td>
<td>$y = 0.0566x - 0.0007$</td>
<td>0.9999</td>
<td>3.1</td>
<td>0.05</td>
<td>0.02</td>
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<tr>
<td>2</td>
<td>0.5-200</td>
<td>$y = 0.0169x + 0.0290$</td>
<td>0.9989</td>
<td>2.8</td>
<td>0.1</td>
<td>0.025</td>
</tr>
<tr>
<td>3</td>
<td>0.5-200</td>
<td>$y = 0.0092x - 0.0097$</td>
<td>0.9996</td>
<td>3.1</td>
<td>0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>4</td>
<td>0.5-200</td>
<td>$y = 0.0243x + 0.0136$</td>
<td>0.9999</td>
<td>2.5</td>
<td>0.05</td>
<td>0.01</td>
</tr>
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<td>0.5-200</td>
<td>$y = 0.0083x - 0.0287$</td>
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<td>0.25</td>
<td>0.1</td>
</tr>
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<td>0.1-40</td>
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<td>0.005</td>
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<td>0.25-100</td>
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<td>0.05</td>
<td>0.01</td>
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<td>0.025</td>
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Table 4 Intra- and inter-day precision, accuracy (n=3) and reproducibility (n=6) of HCAA compounds.

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<th>Precisiona</th>
<th>Accuracya</th>
<th>Reproducibility</th>
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<td>Inter-day (RSD, %)</td>
<td>Intra-day (%)</td>
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<td>96.8-111.6</td>
</tr>
<tr>
<td>2</td>
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<td>4.0-6.3</td>
<td>94.7-104.3</td>
</tr>
<tr>
<td>3</td>
<td>1.4-3.6</td>
<td>1.6-9.1</td>
<td>96.5-106.6</td>
</tr>
<tr>
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<td>1.7-4.7</td>
<td>97.3-102.8</td>
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<tr>
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<td>2.2-5.7</td>
<td>95.0-106.3</td>
</tr>
<tr>
<td>6</td>
<td>1.2-8.5</td>
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<td>101.4-111.0</td>
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Table 5 Recovery of HCAA compounds in root barks and leaves of *Lycium barbarum* (n = 5)

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<td>RSD (%)</td>
<td>Mean (%)</td>
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<td>3.1</td>
</tr>
</tbody>
</table>
3.2.2 HCAAs fragmentation

The HCAAs compounds demonstrate characteristic fragmentation patterns. In the full scan spectra, [M+H]^+ or [M-H]^- (only compound 3) ions were generally the base peak and were accordingly selected as the precursor ion for Q1 for selection reaction monitoring (SRM) analysis. The collision energy-induced fragmentation was always the cleavage of amide bond. Based on the structural characteristics of the 16 compounds, they were divided into two types that showed different daughter ion patterns after fragmentation.

3.2.2.1 Caffeoyl and feruloyl amide fragmentation

Compounds 1, 6 and 7 were selected as the representatives in the structural elucidation. The full scan mass spectrum of 1 and 6, 7 gave a [M+H]^+ ion at m/z 284.1, 298.1 and 358.2 respectively. Collision-induced dissociation of [M+ H]^+ was preferential to cleave the amide bond to eliminate the amine moiety as neutral loss and produce a base ion at m/z 163.04 and 177.1 which ascribed to a caffeoyl and feruloyl group, respectively (Fig. 8a and c). Further fragmentation might consecutively cause loss of a molecule of CH₃OH (Fig. 8b), giving base ion of 145.03.
Figure 8 LC-MS/MS spectrum of caffeoyl and feruloyl amide. a) fragmentation of compound 1. b) loss of CH$_3$OH from m/z at 177.04, which is feruloyl group. c) fragmentation of compound 7.
3.2.2.2 3,4-hydroxyhydrocinnamoyl amide fragmentation

Compared to structure of caffeoyl and feruloyl amine family, the double bond in the caffeoyl moiety was reduced in 3,4-hydroxyhydrocinnamoyl amide, which resulted in different fragmentation behaviors in the MS/MS experiment. Here, compound 12 is used as representative to elucidate fragmentation details of this family. Two predominant ions at m/z 122.15 and 105.13 were observed in the MS/MS spectrum (Fig. 9). It was speculated that after the cleavage of amide bond the corresponding electrons were transferred to the carbon atom to generate the amine ion, in this case, phenethylamine (m/z 122.15), which subsequently lost a molecule of NH$_3$ to produce the ion of m/z 105.13.

![Figure 9 LC-MS/MS spectrum of compound 12](image-url)
3.3 Conclusion

In this study, we developed and validated a highly sensitive method UHPLC-MS/MS combined with organic synthesis and NMR, targeting at comprehensively profiling HCAA species from plant tissues. The method was fully validated with respect to specificity, linearity, intra- and inter-day precision and accuracy, limit of detection (LOD), limit of quantification (LOQ), recovery, and reproducibility.

The method significant improvement in terms of quantification and detection sensitivity. The developed methodology also facilitates natural product studies that can be expanded to identification of other bioactive components and metabolomics studies of plants.
4. *In vitro* and *in vivo* anti-inflammatory studies of HCAA compounds

4.1 Introduction

Due to the intimidated relationship between inflammation and many human disease conditions, an increasing number of research focuses on identification of anti-inflammatory natural products because these molecules are able to interact with inflammatory molecules and modulate inflammatory pathways. As mentioned in previous section, one of the key inflammatory transcription regulator is NF-κB that controls expression of a series of pro-inflammatory cytokines and enzymes that generate inflammatory mediators. For instance, activated macrophages transcriptionally express inducible nitric oxide synthase (iNOS), which catalyzes the oxidative deamination of L-arginine to produce nitric oxide. Excessive generation of NO can trigger deleterious consequences such as cytotoxicity, tissue damage, and inflammatory diseases [111]. In addition to the central transcription factor, many studies imply involvement of upstream signaling transduction in activation of inflammation, such as extracellular signal-regulated 1/2 (ERK)1/2 (p42/44), p38 MAPK, and PI3K/AKT pathways [112].

Lipopolysaccharide-activated RAW264.7 murine macrophage cells are widely used as an *in vitro* inflammatory model for the screen of effective anti-inflammatory molecules. In inflammation, macrophages exert three major functions, including antigen presentation, phagocytosis, and immunomodulation through production of various cytokines and growth factors. Macrophages play a critical role in the initiation, maintenance, and resolution of inflammation [113]. The induction of edema in the mouse
ear has been established as a reliable \textit{in vivo} model for anti-inflammatory agent studies [114].

The anti-inflammatory properties demonstrated by some of HCAAs motivate a screening of those HCAAs identified from the plant as well as a detail molecular mechanism of their actions, specifically targeting at NF-κB activities. Furthermore, mouse ear edema model was used as \textit{in vivo} model to further explore and confirm the mechanism.

\textbf{4.2 Materials and method}

\textbf{4.1.1 Materials}

Sulfanilamide, naphthylethlenediamine dihydrochloride, molecular biology grade dimethyl sulfoxide (DMSO), lipopolysaccharides (LPS) (\textit{Escherichia coli} O127: E8), \(N^G\)-methyl-L-arginine acetate (L-NMMA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Gibco Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Halt Protease inhibitor cocktail 100X and streptomycin/penicillin solution were purchased from ThermoFisher Scientific (Hagerstown, MD, USA).

DC Protein Assay, 2X Laemmli Sample Buffer, 10\% pre-casting SDS-PAGE gel, 10X running buffer, 10X transfer buffer, Tris-HCl pH 6.8, Tris-HCl pH 8.8 solutions, PVDF membrane, SDS, and blocker were purchased from Bio-rad (Hercules, CA). TBS and TBST were purchased from Sigma-Aldrich (St. Louis, MO). The iNOS, COX-2, NF-κB antibody sampler, Lamin-B1, Akt and phosphor-Akt (Thr202/Tyr204) antibodies, anti-rabbit and anti-mouse IgG antibody conjugated to horseradish peroxidase were purchased from Cell Signaling Technology (Beverly, MA). β-actin was purchased from Santa Cruz
Biotechnology (Dallas, Texas). ProteoExtract ®Subcellular Proteome Extraction Kit was purchased from Merk Millipore (Billerica, MA).

Hematoxylin solution modified acc. to Gill II, Eosin Y solution, ethanol, Xylene were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.2 Cell culture

RAW264.7 murine macrophages were purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in high-glucose Dulbecco’s Modified Eagle’s medium, supplemented with 100 IU/mL penicillin/streptomycin, 1 mM sodium pyruvate and 10% fetal bovine serum. Cells were incubated in 10 cm culture Petri dishes in 5% CO$_2$ with 70% humidity at 37 °C.

4.2.3 Cell viability Assay

4×10$^5$ cells/mL were seeded into 96-well plates and incubated for 12 hours before treatment. Compounds of interest were first dissolved in molecular biology grade DMSO at a concentration of 100 μM and were further diluted with growth media to reach the final assay concentration. Growth media with 0.01% v/v DMSO served as the control. 75 μg/mL of L-NMMA was used as a positive control. Either the compound of interest or a vehicle was added to the medium and then incubated for 24 hr. After treatment, cells were washed twice with phosphate buffered saline. Phenol red free medium containing 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to cells and then incubated at 37 °C for 4 hours. After removing the supernatant, formazan crystals were dissolved in 150 μL of DMSO. Optical densities were measured at 570 nm.
4.2.4 Nitrite Assay

100 μM DMSO stock solutions of test compounds were diluted with growth media to achieve assay concentrations. 75 μg/mL L-NMMA was used as positive control. Cells (4×10⁵ cells/mL) were treated with E. coli LPS (100 ng/mL) in either the presence of the compound of interest or 0.01% dimethyl sulfoxide (DMSO) as a vehicle in phenol red free medium for 24 hours. After a 12-hour incubation period, 50 μL of conditional supernatant was removed, mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethlenediamine dihydrochloride in water), and incubated at room temperature for 10 minutes. Production of nitrite was measured at an absorbance of 550 nm.

4.2.5 Western blot

RAW 264.7 cells were washed with ice-cold PBS and lysed in an ice-cold RIPA buffer (25 mM Tris–HCl, pH 7.2; 0.1%, SDS; 1%, Triton X-100; 1%, sodium deoxycholate; 0.15 M, NaCl; EDTA (ethylene- diaminetetra acetic acid) 1 mM) with 1X Halt Protease inhibitor cocktail, 1 mM of phenylmethylsulphonyl fluoride (PMSF) and 1mM sodium metavanadate. After centrifugation, the protein concentration in supernatant was measured by DC Protein Assay. 50 μg of protein sample was mixed with 2X Laemmli Sample Buffer with 5% β-mercapitoethanol and heated at 100 C° for 5min. The sample was run on 10% pre-casting SDS-PAGE gel with 150V and transferred to PVDF membrane with 100V for 1hr. The membrane was blocked with blocking solution (5% blocking agent in TBST solution) at room temperature for 1hr. The blocked membrane was incubated with primary antibody (3% blocking agent in TBST, antibody dilution 1:1000) at 4 C° overnight. The membrane was subsequently probed with anti-mouse or anti-rabbit IgG antibody
conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence. The densities of the bands were quantitated with a computer densitometer. All the membranes were normalized with β-actin (or lamin-B1).

For fractionation of subcellular compartments, ProteoExtract ®Subcellular Proteome Extraction Kit was used and downstream western blotting was applied according to the method described previously.

All Western blotting experiments were triplicated independently.

4.2.6 Animals

Male ICR mice aged 6 weeks were purchased from BioLASCO Experimental Animal Center (Taiwan Co., Ltd). All animals were randomly housed in a controlled atmosphere (25 ± 1 °C at 50% relative humidity) at National Taiwan University animal facility with a 12 h light−12 h dark cycle. Animals were able to free access to food and water at all times. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the National Taiwan University (IACUC, NTU).

4.2.7 Mouse ear edema assay

After 1 week of acclimation, the animals would be ready for the experiment. Both ears of male ICR mice (n =3) will be topically treated with 20 μL of acetone (vehicle control) or two HCAA compounds at different concentration (1 or 5 μmol) in 20 μL of acetone 30 min prior to application of acetone or 2 nmol of TPA in acetone. The mice were sacrificed 2 and 4 hr after TPA treatment. Ear punches (6 mm in diameter) will be taken immediately for analysis. The ear pouches will be measured by its thickness and weight. Furthermore, whole cell lysate of the ear punches will be subjected to western blotting according to the
protocol discussed in previous section.

4.2.8 H&E Staining

The mouse ear samples were cut into 3 mm sections and deparaffined at 65 °C for 30 min. Then the slides were immersed into following solvent: xylene for 30 min, xylene for 30 min, 100% ethanol for 10 min, 100% ethanol for 10 min, 90% ethanol for 5 min, 70% ethanol for 1 min, 50% ethanol for 1 min, 30% ethanol for 1 min. The slides were rinsed in PBS for 5 min. Ear tissues were firstly stained in 200 μL hematoxylin solution and incubated at room temperature for 5 min, following by washing in running tap water and rinse in PBS for 5 min. The counterstain was performed by staining in 400 μl Eosine solution for 30 seconds, followed by washing in running tap water and rinse in PBS for 5 min. The slides were dehydrated in absolute alcohol for two changes of 2 min each and then cleared in xylene for two changes of 10 min each. The slides were mounted and observed under microscope.

4.2.9 Statistical analysis

Statistical analysis for IC\textsubscript{50} was performed using Prism 7 by nonlinear regression. IC50 values are shown as mean ± standard error. All experimental data were obtained independently and replicated a total of three times. Significant differences were determined as \( p < 0.05 \).

4.3 Results and discussion

4.3.1 Cytotoxicity of HCAAs

In order to select proper concentrations of the compounds of interest for the anti-inflammatory studies, murine macrophage RAW264.7 cells were treated with the
compounds of interest at various concentrations or 0.01% DMSO vehicle for 24 hours, and cell viability was determined by MTT assay (Fig. 10). Among the IC₅₀ values shown in Table 7, 10 of the 16 compounds possessed IC₅₀ values larger than 100 μM, indicating low cytotoxicity of these compounds. The compounds exhibiting higher cytotoxicity contain large amounts of caffeic acid or 3,4-dihydroxyhydrocinnamic acid moiety, and were compounds 1, 3 and 12-14, which each contain a catechol structure. These results led to the hypothesis that the ortho bis-hydroxylation structure is crucial for cytotoxicity. Similar findings were observed by studying the anti-cancer properties of caffeic acid derivatives due to hydroxyl groups in the molecule that enhances the affinity for proteins and nucleic acids through potential hydrogen donating and accepting [115-117]. We confirmed that methylation of the hydroxyl group did reduce cytotoxicity by comparing compounds 1, 3 and 12-14 with compound 5-10 that had lost the catechol structure of ferulic acid moiety. High cytotoxicity narrows the concentration range for anti-inflammatory studies. However, compounds with high cytotoxicity could be candidates for the treatment of certain cancers. In addition, the structural characteristics of compounds with high cytotoxicity provide insights into the design of anti-cancer drugs.

For further studies, concentrations with 90% cell viability will be selected in order to eliminate any background effects caused by compounds.
Table 6 Cytotoxicity IC50 value and NO production inhibition IC50 value of HCAA compound 1-16 to RAW 264.7.

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>Cytotoxicity IC50, Mean ± STD&lt;sub&gt;a&lt;/sub&gt; μM</th>
<th>NO production IC50 , Mean ± STD μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>35.25±3.148</td>
<td>2.381±0.1497</td>
</tr>
<tr>
<td>2</td>
<td>&gt;100 μM</td>
<td>5.575±0.3469</td>
</tr>
<tr>
<td>3</td>
<td>72.71±7.811</td>
<td>4.227±0.3854</td>
</tr>
<tr>
<td>4</td>
<td>&gt;100 μM</td>
<td>12.760±1.611</td>
</tr>
<tr>
<td>5</td>
<td>&gt;100 μM</td>
<td>39.050±3.527</td>
</tr>
<tr>
<td>6</td>
<td>&gt;100 μM</td>
<td>14.38±2.099</td>
</tr>
<tr>
<td>7</td>
<td>&gt;100 μM</td>
<td>&gt;10 μM</td>
</tr>
<tr>
<td>8</td>
<td>&gt;100 μM</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>9</td>
<td>&gt;100 μM</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>10</td>
<td>&gt;100 μM</td>
<td>&gt;50 μM</td>
</tr>
<tr>
<td>11</td>
<td>&gt;100 μM</td>
<td>15.08±0.8049</td>
</tr>
<tr>
<td>12</td>
<td>35.53±4.066</td>
<td>&gt;5 μM</td>
</tr>
<tr>
<td>13</td>
<td>52.00±4.849</td>
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<tr>
<td>14</td>
<td>40.34±6.697</td>
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<td>15</td>
<td>&gt;100 μM</td>
<td>40.36±4.648</td>
</tr>
<tr>
<td>16</td>
<td>&gt;100 μM</td>
<td>&gt;20 μM</td>
</tr>
</tbody>
</table>

<sup>a</sup>Standard error

<sup>b</sup>IC50 value was above final assay concentrations
Figure 10 RAW 264.7 cell viability of 16 HCAAs compounds examined by MTT assay.

RAW 264.7 cells were incubated with compounds of interest for 24hr. 5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide was added to cells and then incubated at 37 °C for 4 hours. After removing the supernatant, formazan crystals were dissolved in 150 µL of DMSO. Optical densities were measured at 570 nm.

4.3.2 NO inhibitive effects of HCAAs
Chronic inflammation is a complex process mediated by activation of inflammatory or immune cells and has been shown to trigger chronic disorders. During the inflammatory response, macrophages play a critical role in managing inflammatory phenomena such as the overproduction of pro-inflammatory cytokines and inflammatory mediators, including nitric oxide (NO). In order to investigate the inhibitory effects of these HCAA compounds on NO production, RAW264.7 murine macrophages were co-incubated with HCAA compounds and LPS (100 ng/mL) for 24 hours. The NO accumulation in cell medium was measured by Griess reagent (Fig. 11). After LPS stimulation, the NO production significantly increased compared to the negative control groups and could be observed in all experimental groups. These results are shown in Table 7. A positive control compound, 50 μM of L-NMMA was also included in order to compare NO inhibitive effects of HCAAs. A total of 8 HCAA compounds exhibited NO inhibitory properties, and these compounds all showed higher potency than L-NMMA to suppress NO production. Furthermore, compound 1 (2.49±0.30 μM) and compound 3 (4.30±0.22 μM) showed potent NO inhibitory activity with a low NO IC50 value, and compound 4, which was abundantly present also effectively inhibited NO production with a 50% inhibition concentration of 12.760±1.611 μM.

According to the results, the compounds with a caffeic acid moiety exhibited the most significant inhibition of NO accumulation. All five caffeic acid derivatives (compounds 1-5) have a 50% inhibition concentration (< 50 μM) of NO production from macrophages. Among ferulic acid derivatives, two compounds, 6 and 11, exhibited inhibitory effects of NO production, which implies a reduced NO suppression effect. Moreover, only compound 15 in the 3,4-dihydroxyhydrocinnamic acid derivatives group
was found to significantly decrease NO levels with a 50% inhibition concentration of 40.71±0.95 μM. The reduction of effectiveness on NO production by macrophages suggests that catechol and tethered conjugated double bond structures are essential for inhibitory potency, and the conjugated double bond structure may be more important for inhibitory effects. The inhibitory activity of HCAAs containing the caffeic acid moiety decreased in following order: 5 < 4 < 2 < 3 < 1. Furthermore, the inhibitory properties of NO accumulation in the cell medium are not fully related to the free radical scavenging effects of these compounds. A free radical scavenging study of caffeoyl amide compounds shows effectiveness decreases in the order: 1 < 4 < 5 [118], which is not consistent with our results, and indicates these compounds exhibited NO inhibitory activities through cellular mechanisms. This sequence suggested that the effectiveness of NO inhibition is proportional to hydrophobicity resulting from a decrease in the number of hydroxyl and methoxyl groups. The difference in NO inhibition between 3 and 1 may be due to the size of the side chain of the amine moiety. Thus, the effectiveness to inhibit NO production may also require an optimal bulkiness of the side chain of amine moieties. Similar trends were observed in studies of anti-inflammatory properties of caffeic acid alkyl ester derivatives to suppress NO production [119, 120]. However, this trend was only observed in the caffeic acid derivative group, not the other two phenolic acid derivatives. The detailed mechanism underlying the structure-activity relationship remains unclear.
Figure 11 Inhibitive effects of 16 HCAAs compounds on LPS-induced RAW264.7. RAW 264.7 cells were co-incubated with compounds of interest and 100 ng/mL LPS for 24hr. NO production was examined by Griess reagents and measured at 450 nm.

4.3.3 The inhibition of LPS-induced iNOS and COX-2 expression by two representative HCAA compounds

Based on the inhibitive and abundance of the HCAAs in the plant, there were two
HCAA species were chosen as representatives in order to investigate in more detail of their potential anti-inflammatory mechanism, compound 3 (trans-caffeoyltryptamine, tryp) and 4 (trans-caffeoyltyramine, tyr). trans-caffeoyltyramine was reported as NF-κB inhibitor identified from root bark of *Lycium barbarum* with an IC$_{50}$ value of 18.4 μM by luciferase assay [101]. Thus, the purpose to include trans-caffeoyltyramine fall into twofold; firstly, the detail mechanism of its inhibitive effects on NF-κB activation as well as its prevalence to inflammation are missing; secondly, trans-caffeoyltyramine may serve as positive control compound to compare anti-inflammatory efficacy.

iNOS, which is regulated by NF-κB, is the pro-inflammatory enzyme that generates NO as mediators. Exposure to lipopolysaccharide induce PGE$_2$ production of COX-2, resulting in inflammatory symptoms, such as swelling and pain. The protein expression levels of iNOS and COX-2 were investigated. As shown in Fig. 12, LPS significantly induced expression of both iNOS and COX-2, compared to the control. Co-incubation with two HCAA compounds suppressed iNOS and COX-2 expression in a dose-dependent manner. The reduction of protein expression indicated that both iNOS and COX-2 expression is regulated mainly at the transcriptional level. When we compared the efficacy of the two compounds at the same concentration. Tryp was more effective to inhibit iNOS expression. Similar trends were observed in both HCAA compounds treatment that when concentration doubled from 5 to 10 μM (10 to 20 μM in Tyr), the COX-2 inhibitive effect did not show significant decrease, which indicated that the IC$_{50}$ values of both HCAA compounds to COX-2 were between the low and middle concentration treatments.
Figure 12 Effects of two HCAA compounds (3, Tryp and 4, Tyr) on LPS-induced iNOS and COX-2 protein levels in RAW 264.7 cells. The cells were treated with HBA different concentrations and LPS (100 ng/mL) for 24 h. Equal amounts of total proteins (50 μg) were subjected to 10% SDS–PAGE. The expression of iNOS and COX-2 and β-actin protein was detected by Western blot using specific antibodies. These experiments were repeated three times with similar results. Quantification of iNOS and COX-2 expressions were normalized to actin using a densitometer.
NO is a free radical synthesized from L-arginine by iNOS and it is centrally involved in inflammatory response as signaling molecules as well as reactive nitrogen species (RNS). Specifically, NO can react with superoxide leading to formation of the highly reactive peroxynitrite which cause cellular damage and activate cell death pathway [121]. Thus, the expression of iNOS enhance ROS damage in the inflammatory sites. It induces various harmful responses including tissue injury which are considered to be pathogenic in many diseases, such as septic shock and apoptosis [122, 123]. As mentioned before, Inhibition of the excessive NO and PGE$_2$ by reducing iNOS and COX-2 might be a useful strategy for the treatment of inflammatory disease.

From the Western blotting results of COX-2, the two HCAA compounds do not only act as inhibitor that reduce production pro-inflammatory mediators; instead, they inhibit COX-2 at translational level. This is critical advantage of using HCAA molecules as anti-inflammatory agent. Most of non-steroid anti-inflammatory drugs serve as selective COX-2 inhibitor, minimizing their effects on COX-1 actions. This idea is based on the notion that he COX-1 predominates in the stomach, yielding protective prostaglandins, while COX-2 is induced majorly at inflammatory sites giving rise to pain, swelling, and stiffness [101]. However, COX-2 inhibitors may have negative impact on protective prostaglandins when they are used to alleviate intestinal inflammation [101]. From this perspective, HCAAs may have advantages to use as anti-inflammatory agents by reducing protein expression level of COX-2.
4.3.4 Inhibitory effect of HCAA on LPS-induced activation of IKK complex and phosphorylation and degradation of IκBα

IκB kinase (IKK) complex contains essentially two catalytic units IKKα and IKKβ that serves as a core element of NF-κB. IKK complex is responsible for phosphorylation and activation of IκBα at Ser 32, a natural inhibitor of nuclear transcription factor NF-κB, which inhibits certain nuclear DNA transcription. In order become active, IKK complex need to be phosphorylated on two serine residues, Ser 177 and 181 for IKKβ, and Ser 176 and 180 for IKKα, located in an activation loop, which results in a conformational change and to kinase activation [124, 125]. In addition, IKKα shows a putative nuclear localization signal, possibly linked to its nuclear activity on the activation of NF-κB [126]. After IκBα is phosphorylated, the protein unit will be labeled with poly-ubiquitin and degraded afterwards. LPS-mediated activation and translocation of NF-κB to the nucleus is preceded by proteolytic degradation of IκBα.

Upon LPS induction, the signaling transduction is relatively fast. Time course study of phosphorylation of IKK as well as IκBα (data is not shown) indicated 20 min LPS treatment was sufficient to reach highest phosphorylation level of IKK and IκBα.

From the Western blotting results, catalytic unit with LPS treatment was significantly increased compared to the control (Fig. 13). As a result, the phosphorylation of IκB was also increased due to activation by IKK; meanwhile, the total IκB level was significantly reduced which caused by ubiquitin-labeled degradation of IκB. With both of two HCAA compounds, the phosphorylation of IKK was decreased in a dose dependent manner. In Fig. 13, treatment with two HCAA compounds effectively attenuated the phosphorylation of IκBα and sustained the IκBα protein content. Furthermore, the pattern
of inhibition on phosphorylation and degradation of \( I\kappa B\alpha \) by HCAA was paralleled to each other. These results suggest that inhibition the phosphorylation and the degradation of \( I\kappa B\alpha \) protein can prevent the activation and nuclear translocation of NF-\( \kappa B \) and further transcriptional activity.
Figure 13 Effects of HCAA on LPS-induced IKK complex phosphorylation, phosphorylation and degradation of IκBα. RAW 264.7 cells were treated with LPS (100 ng/mL) for 20 min after 4 hr pre-treatment of two HCAA compounds, and the cellular lysates were prepared and analyzed for the content of IKKα, p-IKKα/β, IκBα, p-IκBα and β-actin by Western blot. These experiments were repeated three times with similar results. Quantification of protein expression was normalized to actin using a densitometer.
4.3.5 Inhibitory effect of HCAA on LPS-induced nuclear translocation and phosphorylation of NF-κB

The activation of NF-κB is critical for expression of pro-inflammatory enzymes and cytokines upon LPS stimulation. The activation of NF-κB involves two steps: nuclear translocation and nuclear activation. IKKα was shown to regulate the additional step of NF-κB-dependent gene transcription. IKKα phosphorylates chromatin-bound p65 subunit on Ser 536, leading to acetylation of p300 to p65 at Lys310, an event necessary for full transcription [127].

Nuclear accumulation was used to test if two HCAAs would perturb the distribution and activation of NF-κB subunits. Nucleus and cytosolic extracts were prepared and subjected to immunoblot analysis. Lamin B₁ and β-actin were used as internal references for quantification, respectively. As shown in Fig. 14, after 4 hr pre-treatment of two HCAA compounds, the accumulation of p65 unit in the nucleus was significantly decreased, indicating nuclear retention of NF-κB, potentially leading less downstream DNA transcription. Furthermore, phosphorylation of p65 inside the nucleus was also reduced which resulted in attenuated transcriptional activities of NF-κB transcription factor.
**Figure 14** Effects of HCAA on LPS-induced p65 translocation and NF-jB activation in RAW 264.7 cells. Cells were pre-treated two HCAA compounds for 4hr. Then the cells were induced with LPS (100 ng/mL) for 30min. Cytosolic and nuclear fractions were prepared and analyzed by Western blotting. These experiments were repeated three times with similar results. Quantification of protein expression was normalized to either β-actin or Lamin B1 using a densitometer.
4.3.6 Involvement of PI3K/Akt pathway in anti-inflammatory effects of HCAAs in vitro

To further understand the molecular mechanism of anti-inflammatory effect by HCAAs, MAPKs (ERK, JNK, and p38) and phosphoinositide-3 kinase (PI3K)/Akt which have been suggested to be involved in pro-inflammatory signaling cascades [112, 128]. HCAAs did not show any inhibitive effects on MAPKs (data is not shown).

Active Akt stimulates IKK activity by phosphorylation on the IKKα subunit, which consequently activates the NF-κB cascade. PI3K/Akt pathway serves as an important element in upstream regulation of NF-κB-mediated inflammatory response. From Fig. 15, two HCAA compounds decreased LPS-stimulated activation of PI3K/Akt. The results of our immuno-blot analyses suggest that HCAA’s inhibitory effect on LPS-induced NF-κB activation by inhibiting PI3K/Akt/IKK pathway, which interrupts downstream signaling pathway.

Activation of PI3K/Akt plays an important role in the expression of iNOS and COX-2 in vascular smooth muscle cells, peritoneal macrophages, and mesangial cells, which involve in inflammatory conditions of diseases [129]. The inhibitive effects of HCAAs on PI3K/Akt not only reveal how HCAAs modulate NF-κB signaling and inflammation, but also lead their additional health potential on metabolism and inflammation-related metabolic disorders. PI3K/Akt-dependent signaling is increased in the diet-induced insulin resistance and hyperinsulinemia [130]. The anti-inflammatory actions of HCAAs may also alleviate inflammation-associated insulin resistance and hyperinsulinemia in diabetes and other metabolic disorders.

Now, many natural product and molecule drugs have been identified to
demonstrate inhibitive effects on activation NF-κB via various action mechanisms, including inhibiting IKK, IκB ubiquitination and degradation, NF-κB nuclear translocation, p65 modification and DNA binding of NF-κB [131]. Our results indicated that HCAAs were able to interact with NF-κB activation at multiple points as well as upstream PI3K/Akt signaling pathway. It was not clear that the suppressive action of HCAAs on transcription factor activities was due to directly inhibition of NF-κB or upstream signaling and therefore disrupting corresponding downstream effectors. To elevate the complexity of the question, if HCAAs can directly act on NF-κB, in addition to PI3K/Akt, they may show differential inhibiting efficacy on each step of NF-κB activation (i.e. IKK complex, IκB ubiquitination and degradation etc.). Furthermore, structure modifications of HCAA family may have specificity towards each step and result in different efficacy, depending on the therapeutic targets. Further investigations are needed to answer these questions and address clear mechanism of HCAAs as novel anti-inflammatory agents.
4.3.6 Effect of HCAAs on TPA-induced ear edema
The *in vitro* study using RAW264.7 cell model demonstrated anti-inflammatory mechanism mediated through inhibition of NF-κB and PI3K-Akt pathways, further anti-inflammatory mechanisms were also confirmed *in vivo* by mice ear edema model. In this *in vivo* study, HCAAs were observed to clearly inhibit the ear swelling induced by TPA (Fig. 16B). Topical application of 1 nmol TPA to the ear of mice increased the average ear thickness and weight of ear punch (6 mm diameter) 10 h after exposure to TPA. However, the topical application of 1 and 5 μmol HCAAs before TPA treatment to the ears of mice inhibited the TPA-induced ear thickness (Fig. 16B) and weight (Fig. 16A).
Figure 16 Inhibitory effect of two HCAAs on TPA-induced ear edema in mice. Both ears of male ICR mice (n = 3) were topically treated with 20 μL of acetone (vehicle control) or Tryp/Tyr (1 or 5 μmol) in 20 μL of acetone 30 min prior to application of acetone or 2 nmol of TPA in acetone. The mice were sacrificed 10 h after TPA treatment. Ear punches (6 mm in diameter) were taken immediately and weighed. Data are shown as the mean ± standard deviation. Different notations in the bar charts indicate statistical significance (P < 0.05, n = 3) by One Way ANOVA. #, p < 0.05, ###, p < 0.001, Control VS TPA. **, p < 0.01 and ***, p < 0.001. (A) Morphological changes. The weight (B) or thickness.
Furthermore, the expressions of pro-inflammatory enzymes, iNOS and COX-2 were also investigated in each ear punch biopsy. Consistent with the inhibitory effect on the *in vitro* model, two HCAAs compounds inhibited expression of iNOS and COX-2 by 30 min topical preventive application (Fig. 17). It was noteworthy that COX-1 expression in the ear punch was not affected by the two HCAAs compounds which may eliminate negative effects caused by anti-inflammatory agents on the positive prostaglandins expression as well as functions. Therefore, these findings for novel anti-inflammatory compound, HCAAs, could provide therapeutic opportunities against various pathological conditions with an inflammatory component.
Figure 17 Inhibitory effects of topical application Tryp and Tyr on TPA-induced iNOS and COX-2 expression. Ears of male ICR mice were treated either with acetone, Tryp and Tyr (1 or 5 mmol) 30 min prior to TPA (2 nmol) except control animals, which were treated with acetone only. Total cell lysates were analyzed for iNOS, COX-2 and COX-1 expression by immunoblotting. The values below the figure represent change in protein expression of the bands normalized to β-actin.
The anti-inflammatory effect of HCAAs was further confirmed by histological examination of the inflamed ear tissue by H&E staining. The histopathological results of

Figure 18 Histological morphology of the ear biopsies evaluated from H&E-stained sections and ear thickness. Shown in mm, 100× magnification.
ear edema are shown in Fig. 18. According to Fig. 18, no cellular infiltration and edema were observed in the control group that was treated with acetone. In contrast, immune cells infiltration and swelling followed the TPA stimulation. H&E staining showed that the extent of cell population as well as tissue swelling were significantly reduced in mice treated with HCAAs compared to TPA alone.

The result suggested that HCAAs could act by inhibiting the neutrophil infiltration into the inflammatory site, which was in accordance with the results of histological examination. The histological morphology provided direct observation of anti-inflammatory actions of the HCAAs, including edema caused by inflammatory response as well as immune cell infiltration. In general, innate immune cells respond to inflammation-induced traffic cues, but majorly leukocytes, including granulocytes, monocytes, and Mast cells, which can respond to stimulating signals by altering the composition, expression and/or functional activity of their trafficking molecules [132]. After being activated, different leukocyte subsets localize together in affected tissues, communicating through short-range cytokines and/or direct cell-cell contact. Among these leukocytes subset, neutrophils are the most abundant bloodborne leukocytes in healthy humans. The host response to acute infection or injury, in our case TPA treatment, consists of a vigorous neutrophilic inflammatory response. These short-lived cells accumulate in the same target tissue, thus necessitating combination therapy for maximal elimination of infection and injuries. Shown in Fig. 18, TPA 2 nmol, large number of neutrophils migrated to the inflamed ear tissue. As is often the case in inflammation, this is a ‘double-edge sword’, and this robust protective response can also be deleterious to host tissue; for instance, neutrophils play an essential role in pathogenesis of ischemia-reperfusion injury,
which later contributes to stroke, shock and acute respiratory distress syndrome [133]. In our experiment, HCAAs significantly reduced neutrophils flux into the inflamed tissues. Therapies aimed at hindering neutrophil influx into tissue are being evaluated as likely new strategies. Furthermore, this process has been closely associated with progression of various human diseases. Infiltrating immune cells leads dysfunction of adipose tissue by producing cytokines, metalloproteinases, reactive oxygen species, and chemokines that participate in tissue and vessel remodeling and cell signaling, which aggravates insulin resistance and metabolic dysfunction [134]. In spontaneously diabetic animal model, apoptotic pancreatic β-cell destruction was also characterized with IL-1β and TNF-α expression in the immune cells and the induction of iNOS expression in the β-cells, revealing the cytotoxic effects caused by immune cell infiltration [135]. The inhibitive effects of HCAAs on immune cell infiltration caused by inflammation could potentially be applied to target other inflammation-related conditions.

4.3.7 Conclusion

Based on abundance and potency of the HCAA species found from the plant, there were two HCAA compounds (3, trans-caffeoyltryptamine and 4, trans-caffeoyltyramine) were chosen and investigate their cellular an-inflammatory properties by using RAW 264.7 murine macrophage in vitro model. Both HCAA compounds inhibited NF-κB signaling pathway in murine macrophage, accompanied by inhibition of PI3K/Akt/IKK pathway. Moreover, the anti-inflammatory action of HCAAs was also evaluated in vivo mouse ear edema model. With treatment of two representative HCAAs compounds, TPA-induced ear edema was significant reduced by showing as reduction of ear weight and thickness. Histological examination and immunoblotting found inhibition
of immune cells infiltration and expression of pro-inflammatory enzymes the at inflamed tissue. From the results, HCAAs showed great potential to be a novel anti-inflammatory agent, which could be used to treat chronic inflammation and inflammation-related diseases.
4. Significances and future work

Discovery of bioactive components from food sources have attracted more popularity in past few decades because of simple and diverse sources and safe consumption methods, ultimately in order to promote human health. However, traditional separation and purification technology does have certain drawbacks, for instance, which misses trace active compounds from complex matrices and fails to identify novel compounds or the actually functional ones through bioactivity-guided studies. These drawbacks not only largely limit functional food research but also impede drug discovery and plant breeding program.

The current project successfully developed novel identification and quantification method that combined analytical instrument and organic synthesis for natural products with high sensitivity and selectivity. Focusing on hydroxycinnamic acid amides family, minor and analogous chemical constituents were identified and differentiated from each other. The novel method emerges various analytical techniques, based on plant biosynthesis research, which largely compensated the drawback of traditional purification and isolation process. By using this method, chemical diversity of different parts of Lycium barbarum was expanded with wider applications in functional foods and medicines.

Furthermore, the developed method was fully validated in regarding to limit of detection, limit of quantification, linear range, inter- and intra- precision and accuracy, and recovery test. The method could be potentially used for quality control.

The identified hydroxycinnamic acid amide species exhibited promising anti-inflammatory activities. Some of them demonstrated improved potency, compared to analogs that were reported before, which further confirm developed method with better
sensitivity and selectivity. Among 16 compounds, eight of them showed *in vitro* anti-inflammatory activities by inhibiting nitric oxide production in RAW264.7 cell stimulated with Lipopolysaccharide. Two compounds were chosen to further investigate their underlying mechanism both *in vitro* and *in vivo* that they were effectively inhibited NF-κB pathway as well as downstream iNOS and COX-2 through attenuation of PI3K/Akt pathway. The identification and investigation of anti-inflammatory mechanisms of hydroxycinammmic acid amides allow them to be potentially used as prevention and treatment for chronic inflammation and inflammation-related diseases.

Although 16 HCAAs compounds potential exert anti-inflammatory action via similar mechanism, targeting at suppression of NF-κB signaling pathway, they demonstrated differential efficacy in terms of suppressing inflammatory mediator production and transcription factor activation. The current findings showed potential structure-activity relationships among HCAA family. They could be candidates of drug intermediates for better efficacy against inflammation and other functions with chemical modifications.

For further application as novel anti-inflammatory agents, investigation of oral toxicity and pharmacokinetic properties of HCAAs are necessary. These include both in vitro and in vivo assessment of ADME (absorption, distribution, metabolism and elimination) and pharmacokinetic properties. By doing so, the information will provide comprehensive picture of HCAAs for future applications as functional ingredients to prevent chronic inflammation and inflammation-related diseases.
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