JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Tyrosinase Inhibitors from *Calceolaria integrifolia* s.l.: *Calceolaria talcana* Aerial Parts†

Evelyn Muñoz,[‡] Jose G. Avila,[§] Julio Alarcón,[‡] Isao Kubo,[⊥] Enrique Werner,[‡] and Carlos L. Céspedes*[,‡]

[‡]Phytochemical-Ecology Laboratory, Department of Basic Sciences, Faculty of Sciences, University of Bío-Bío, Andres Bello Avenida s/n, Chillán, Chile

[§]Laboratorio de Fitoquímica, Unidad UBIPRO, FES-Iztacala, Universidad Nacional Autónoma de México (UNAM), Mexico DF, Mexico

[⊥]ESPM Department, University of California at Berkeley, Berkeley, California, United States

ABSTRACT: As a defense mechanism of the aerial parts of *Calceolaria talcana* (Calceolariaceae; formerly Scrophulariaceae) against herbivore offenses and insect pest attack, diterpenoids, triterpenoids, phenylethanoids, flavonoids, and iridoids are rapidly accumulated along the aerial parts, resulting in a unique natural biopesticide complex from this plant. In addition to verbascoside a series of known compounds were screened for their inhibitory activity against mushroom tyrosinase and protease enzymes. Ethyl acetate and *n*-hexane extracts, together with cyclopropyl-7,15-ent-pimaradiene (**1**), abietatriene (**2**), ursolic acid (**3**), α-lupeol (**4**), β-sitosterol (**5**), 2-hydroxy-3-(1,1-dimethylallyl)-1,4-naphthoquinone (**6**), α-dunnione (**7**), verbascoside (**8**), martynoside (**9**), and some known model compounds proved to be inhibitors of oxidation of L-3,4-dihydroxyphenylalanine (L-DOPA) catalyzed by tyrosinase (EC 1.14.18.1) with an IC_{50} between 10.0 and 200 ppm or μM, respectively, suggesting that phenolic moieties in the molecules assayed are important for the activity.

KEYWORDS: *Calceolaria talcana, verbascoside, iridoid monoterpenes, triterpenes, diterpenes, phenylethanoids, naphthoquinones, tyrosinase inhibitory activity, protease inhibition, insect growth inhibitory activity, defense mechanism, plant−insect interaction*

INTRODUCTION

As a defense mechanism of the aerial parts of *Calceolaria talcana* (Calceolariaceae; formerly Scrophulariaceae) against herbivore offenses and insect pest attack, diterpenoids, triterpenoids, phenylethanoids, flavonoids, and iridoids are rapidly accumulated along the aerial parts, resulting in a unique natural biopesticide complex from this plant.[1−4] In addition to verbascoside a series of known compounds were screened for their inhibitory activity against mushroom tyrosinase and protease enzymes.

In a previous report some extracts together with diterpenes, triterpenes, naphthoquinones, and phenylethanoids such as verbascoside were found to exhibit growth inhibitory activity against the fall armyworm, *Spodoptera frugiperda*, and fruit fly, *Drosophila melanogaster*, in an artificial diet feeding assay.[13,14] In previous works we have reported the insect growth regulatory activity of extract and natural compounds.[9,12,24−26] This work deals with our continuing search for alternative insect control agents; here we report that the *n*-hexane and ethyl acetate extracts as well as several other secondary metabolites previously isolated from *Calceolaria talcana* inhibited oxidation of L-DOPA catalyzed by mushroom tyrosinase.

Tyrosinase (EC 1.14.18.1), also known as polyphenol oxidase (PPO),[5] is a copper-containing enzyme widely distributed in microorganisms, animals, and plants. This mixed function oxidase catalyzes two distinct reactions of melanin synthesis,[6] the hydroxylation of a monophenol (monophenolase activity) and the conversion of an *o*-diphenol to the corresponding *o*-quinone (diphenolase activity). In our continuing search for alternative insect control agents from plants,[7−14] tyrosinase inhibitors have recently been targeted[15−19] because tyrosinase is one of the key enzymes in the insect molting process.[20] Hence, tyrosinase inhibitors might ultimately provide clues to control insect pests by inhibiting tyrosinase, resulting in incomplete cuticle hardening and darkening.[21] For example, this enzyme was previously reported to be strongly correlated with aphid resistance of the *Solanum berthaultii* plants,[22] and phenylpropanoids and their glycosides remarkably inhibit tyrosinase and consequently have a pronounced effect on insect growth.[2,23]

Our initial attempt to clarify the mechanism of defense of this plant against insect attack on a molecular level was limited by availability of sufficient plant material of *C. talcana*. Therefore, on the basis of previous results obtained from the effects of extracts as growth inhibitors,[13] we examined these substances as tyrosinase and protease inhibitors

MATERIALS AND METHODS

Chemicals. Many authentic samples of natural compounds were provided by Dr. J. G. Avila, FES-Iztacala, Universidad Nacional Autónoma de Mexico, Mexico D.F., Mexico. Kojic acid, gallic acid, quercetin, tannic acid, 1,1-diphenyl-2-*p*-picryhydrazyl (DPPH), and L-3,4-dihydroxyphenylalanine (L-DOPA) were purchased from Sigma-Aldrich Chemical Co. (Santiago, Chile; Milwaukee, WI, USA). L-Tyrosine and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich Chemical Co. (Santiago, Chile; St. Louis, MO, USA). Silica gel 60 F_{254} precoated aluminum sheets (0.2 mm layer thickness) were purchased from Merck (Darmstadt, Germany).

Received: February 4, 2013
Revised: April 18, 2013
Accepted: April 22, 2013
Published: April 22, 2013

dx.doi.org/10.1021/jf400531h | J. Agric. Food Chem. 2013, 61, 4336−4343

Plant Materials. Samples of aerial parts of *Calceolaria talcana* Grau & C. Ehrhart were collected along the roadside 4.7 km NW of Confluencia on the road to Trehuaco on the north shore of the Itata River (36°37′21″ S, 72°28′16″ W, elev. 172 ft), Ñuble Province, VIII Región, Chile, in November 2010. Voucher specimens have been deposited in the Herbarium of the Basic Science Department, University of Bío-Bío (Voucher DS-2010/05-16243/44), and in the Herbarium of the University of Illinois, at Urbana−Champaign, Illinois, USA (ILL, Voucher DS-16243/44). Samples of aerial parts were dried and milled.

Extracts and Compounds from *C. talcana* Aerial Parts. Plant material was air-dried at room temperature, milled, extracted with methanol overnight, and filtered, and the process was repeated five times. The resulting methanol extract was concentrated at reduced pressure in a rotatory evaporator at 40 °C and 200 mb to yield a syrupy methanol extract (645 g).

A portion of the methanol extract (410 g) was dissolved in distilled water, diluted with methanol to a ratio 60/40 methanol−water, placed in a separatory funnel, and washed with *n*-hexane (150 mL, 20 times). The *n*-hexane phases were then combined and concentrated under reduced pressure. An identical process was repeated with ethyl acetate, leaving a residual mixture of methanol−water.

Isolation of Diterpenes, Triterpenes, Phenylethanoids, and Naphthoquinones. To extract verbascoside, the ethyl acetate extract (60.1 g) was fractionated by open CC using silica gel 60 F_{254} (0.063−0.200 mm particle size, 70−230 mesh ASTM, 60 Å pore diameter). Elution was carried out with addition of methanol to hexane−ethyl acetate mixtures in different ratios of increasing polarity until 100% methanol was reached. All fractions were analyzed by TLC using ceric sulfate as the visualization system. Verbascoside was obtained as the major compound by successive washes with CH_2Cl_2−MeOH (8:2) (4.345 g, 7.23% of the ethyl acetate extract). This compound was purified further by silica gel TLC using CH_2Cl_2−MeOH (7:3) as the developing solvent.[13,27]

The *n*-hexane extract was submitted to an extensive chromatographic purification. On the basis of an insect pest model, the most bioactive fractions were **F-3**, **F-4**, and **F-5**. Two new diterpenes, **1** and **2**, were obtained from fraction **F-3**, and compounds **3**, **4**, and **5** were obtained from **F-4**. Finally a mixture of naphthoquinones was obtained from **F-5**. These were identified as 2-hydroxy-3-(1,1-dimethylallyl)-1,4-naphthoquinone (**6**) and α-dunnione (**7**), both isolated previously from *Calceolaria sessilis* Ruiz & Pavón and *C. integrifolia* L.[28,29] Other quinones from this fraction remain unidentified. The chemical structures of diterpenes, triterpenes, and naphthoquinones isolated from *C. talcana* were determined by comparison of spectroscopic and chromatographic data with those of authentic samples and were reported previously.[14]

Radical Scavenging Assay against DPPH. The assay was performed as previously described.[30−32] The reaction mixture consisted of 1 mL of 100 mM acetate buffer (pH 5.5), 1 mL of ethanol, and 0.5 mL of an ethanolic solution of DPPH. After allowing the mixture to stand at room temperature for 20 min, the absorbance of the remaining DPPH was determined colorimetrically at 517 nm. The scavenging activity was measured as the decrease in absorbance of the DPPH expressed as a percent of the absorbance of a control DPPH solution.[31] Inhibitory activity was expressed as the mean 50% inhibitory concentration of triplicate determinations, obtained by interpolation of concentration−inhibition curves.

Determination of the Total Phenolic Contents. The content of total phenolic compounds was measured using a method described previously by Céspedes et al.[32] A 1 mL aliquot of the appropriate diluted extract was added to 1 mL of Folin−Ciocalteu reagent (Sigma-Aldrich, Santiago, Chile) (diluted with distilled water 1:1), and after 10 min 2 mL of 20% Na_2CO_3 was added. After an additional 5 min, the absorbance was measured at 730 nm with a UV2310 Techcomp spectrophotometer with multichannel detection. Additionally, 10 μL of sample or standard (10−100 μM catechin) plus 150 μL of diluted Folin−Ciocalteu reagent (1:4 reagent/water) was placed in each well of a 96-well plate and incubated at room temperature for 3 min. Following the addition of 50 μL of sodium carbonate (2:3 saturated sodium carbonate/water) and a further incubation of 2 h at room temperature, absorbance was read at 725 nm with a BIOTEK Epoch microplate spectrophotometer equipped with Gen5 microplate data analysis software. Results are expressed as micromoles of Cat E per gram using catechin as the standard.[30] All tests were conducted in triplicate.

Yield of verbascoside and total phenolic compounds were determined and expressed as the amount of each component extracted per gram of dried extract of *C. talcana*.

Tyrosinase Assay. In order to obtain a high specific activity of mushroom tyrosinase (EC 1.14.18.1) used for this bioassay, the tyrosinase (Sigma Chemical Co. Santiago de Chile, Chile) was repurified according to a previously reported procedure.[33] Although mushroom tyrosinase differs somewhat from that from other sources,[34] this fungal source was used for all experiments because of its ready availablility. The preliminary assay was tested at 167 μg/mL, unless otherwise specified. All samples were first dissolved in DMSO and diluted 30 times for use in the experiments. Tyrosinase catalyzes a reaction between two substrates, a phenolic compound and oxygen, but the assay was carried out in air-saturated aqueous solutions. The enzyme activity was monitored by dopachrome formation at 475 nm up to the appropriate time (not exceeding 10 min, unless otherwise specified.). The extent of inhibition by the addition of samples is expressed as the percentage necessary for 50% inhibition (IC_{50}).

This assay was performed as previously described.[16−18] First, 1 mL of a 2.5 mM L-DOPA solution was mixed with 1.8 mL of 0.1 M phosphate buffer (pH 6.8) and incubated at 25 °C for 10 min. Then, 0.1 mL of the sample solution and 0.1 mL of the aqueous solution of the mushroom tyrosinase (138 units) were added in this order to the mixture. This solution was immediately monitored for the formation of dopachrome by measuring the linear increase in optical density at 475 nm.

The preincubation mixture consisted of 1.8 mL of 0.1 M phosphate buffer (pH 6.8), 0.6 mL of water, 0.1 mL of the sample solution (equivalent amount of IC_{50}), and 0.1 mL of the aqueous solution of mushroom tyrosinase (138 units). The mixture was preincubated at 25 °C for 5 min. Then, 0.4 mL of 6.3 mM L-DOPA solution was added, and the reaction monitored at 475 nm for 2 min.

The percentage of inhibition of tyrosinase reaction was calculated as follows:

$$\%\ \text{inhibition} = \{[(A - B) - (C - D)]/(A - B)\} \times 100$$

where A is optical density at 475 nm without test sample, B is optical density at 475 nm without test sample and enzyme, C is optical density at 475 nm with test sample, and D is optical density at 475 nm with test sample, but without enzyme.

Gallic acid (**10**), kojic acid (**11**), quercetin (**12**), and tannic acid (**13**) were used as positive controls. All measurements were carried out in triplicate. The bathochromic shift of phenolic samples was monitored by adding 0.05 mM $CuSO_4$.

Protease Assay. Type II fungal protease of *Aspergillus oryzae* and casein powder used for the assay were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bacto-agar was obtained from Difco Laboratories (Detroit, MI, USA). The assay was performed using the previous literature as a reference.[35] Agar plates were prepared as follows: 10 mL of a solution of M/15 phosphate buffer (pH 7.4), 1% agar, and 4 mg/mL casein as substrate were placed in Petri dishes (100 × 15 mm) and allowed to harden. The samples were dissolved in DMSO and used for the experiment at 10 times dilution. Protease (2.5 mg) was dissolved in 1 mL of 10% DMSO−M/15 phosphate buffer (pH 7.4) or 1 mL of 10% sample solution−M/15 phosphate buffer (pH 7.4) as control and test solutions, respectively. Filter paper disks were dipped in the control or test solution and placed on the agar plates. The plates were covered and incubated at 37 °C overnight and then flooded with 5% trichloroacetic acid solution. The casein, which had not been hydrolyzed, precipitated in situ, gave a milky-white background. Clear zones were measured and percent inhibitions were calculated from a standard curve made from different concentrations of enzyme solutions.

Statistical Analyses. Data shown in figures and tables are average results obtained by means of three or 10 replicates and are presented as average ± standard errors of the mean. Data were subjected to analysis of variance (ANOVA) with significant differences between means identified by General Linear Procedures (GLM). Results are given in the text as probability values, with $p < 0.05$ adopted as the criterion of significance. Differences between treatment means were established with a Student−Newman−Keuls test. The IC_{50} values for each activity were calculated by probit analysis based on percentage of inhibition obtained at each concentration of the samples. IC_{50} is the concentration producing 50% inhibitory activity. Complete statistical analysis was performed by means of the MicroCal Origin 6.0 statistical and graphs PC program.

RESULTS AND DISCUSSION

C. talcana, an ethnomedicinal species, in addition to being an attractive ornamental plant, is used for protection of some crops and as an anti-inflammatory material in ethnomedicine.[36] Plants belonging to the Calceolariaceae have aerial parts with medicinal activity that are used for their digestive properties and as diuretics and antimicrobial medicines.[37,38] Only 15% of the approximately 180 species of *Calceolaria* in Chile have been studied chemically; these studies indicate the presence of flavonoids, glucophenylpropanoids, and diterpenes.[36,39,61]

Additionally, studies of other *Calceolaria* species from other places in the Americas show that this genus is characterized by the presence of substances with important agrochemical and pharmacological potential such as insecticides,[40] antimicrobials,[38] and antioxidants[41] and in similar form in other species such as *Stachys* spp., a mint in the Labiatae family.[42]

The chemistry and biological activity of *C. talcana* have been little studied. In previous reports,[13,14] this plant was found to contain mainly three types of phytochemicals: terpenes (di- and triterpenes), phenylethanoids, and phenolic compounds. Terpenoid compounds were found to consist mainly of diterpenes (pimaradienes and abietatriene), triterpenes (ursolic acid, α-lupeol, β-sitosterol), phenylethanoids (verbascoside, martynoside, among others), and naphthoquinones (dunnione). These compounds were analyzed by UV, IR, NMR, GC-MS, and HPLC analysis. In addition, the *n*-hexane and ethyl acetate extracts of this plant species were found to exhibit various biological activities.[13,14,36,38]

Verbascoside, Martynoside, and Other Secondary Metabolites. The ethyl acetate extract was first found to exhibit growth inhibitory activity against the fall armyworm *Spodoptera frugiperda* and *Drosophila melanogaster* in an artificial diet feeding assay.[13] Lastly, a mixture of naphthoquinones showed more potent insecticidal and growth inhibitory activities.[14]

Fractionation was first guided by tyrosinase inhibitory activity using L-DOPA. Successive partitioning of the crude extract with *n*-hexane, CH_2Cl_2, EtOAc, and H_2O revealed that the strongest enzymatic activity was retained in the EtOAc layer. Further fractionation and purification with Sephadex LH-20 column chromatography gave two purified phenolic compounds. The ^{1}H NMR spectrum of **8** proved to be almost identical to an authentic sample of verbascoside, with aromatic protons near 6.9 ppm corresponding to a cinnamoyl group and other peaks due to anomeric signals of the sugars. The ^{13}C NMR data confirm that compound **8** is verbascoside and compound **9** is martynoside. Confirmation was also obtained by comparing **8** and **9** with authentic samples (chromatographic and spectral data) (Figures 1 and 2).[14,27]

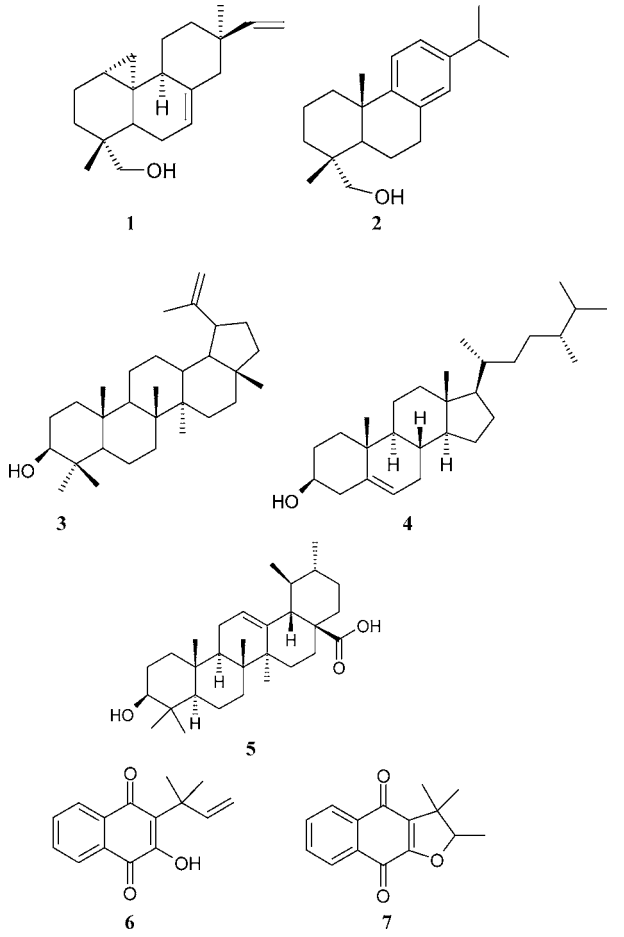

Figure 1. Chemical structures of secondary metabolites **1**−**7** isolated from an *n*-hexane extract of *Calceolaria talcana*.

As a result, the polar part of the methanol extractable fraction of the EtOAc extract consists almost exclusively of verbascoside **8** and martynoside **9** and a mixture of other compounds such as flavonoids and iridoids, whose structures remains without identity until now. In addition, when the fractionation was also guided by the artificial diet feeding assay against the fruit fly, the same compounds were characterized as active principles.[13]

On the other hand, diterpenes **1** and **2**, triterpenes **3**, **4**, and **5**, 2-hydroxy-3-(1,1-dimethylallyl)-1,4-naphthoquinone (**6**), and α-dunnione (**7**) are abundant in the *n*-hexane extract part of leaves and stems (Figure 1).[14]

Interestingly, it is possible to observe that some phenolic compounds are rapidly accumulated in the trichomes during the first growing steps of the plant after initial attack by some coleoptera during spring season, as in an additional experiment with an extract obtained by soaking of air-dried leaves with *n*-hexane−CH_2Cl_2. This extract showed a very similar TLC profile of secondary metabolites to those obtained from conventional extraction procedures (see section 2.3). The chemicals involved in trichome formation are undoubtedly numerous. However, as an initial step to understanding this process, we focused the current study on the biological activity of secondary compounds of the aerial parts, especially their effects on insects[14] and tyrosinase inhibitory activity. In order to facilitate it, gallic acid (**10**), kojic acid (**11**), quercetin (**12**), and tannic acid (**13**) were used as model compounds for comparison (Figure 3) since these compounds are known tyrosinase inhibitors.

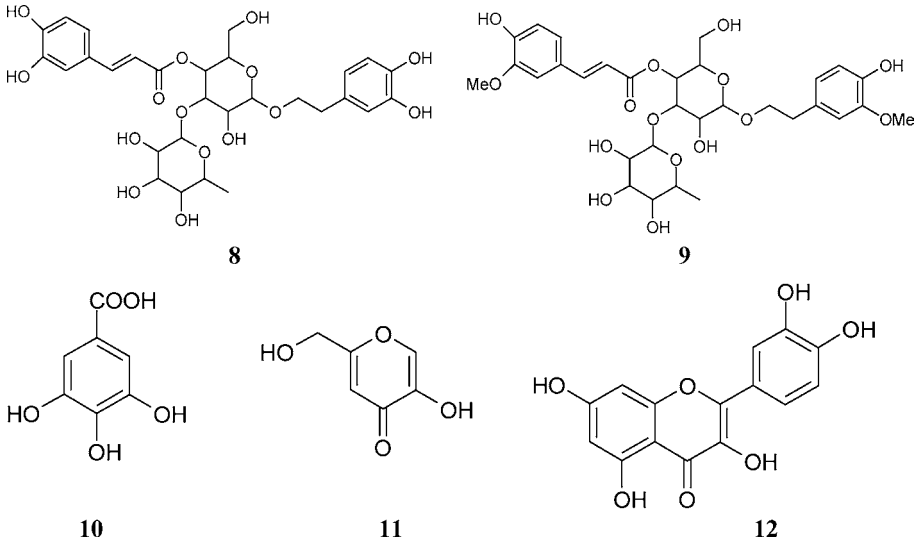

Figure 2. Chemical structures of secondary metabolites verbascoside (**8**) and martynoside (**9**) isolated from the ethyl acetate extract of *Calceolaria talcana* and pattern compounds gallic acid (**10**), kojic acid (**11**), and quercetin (**12**).

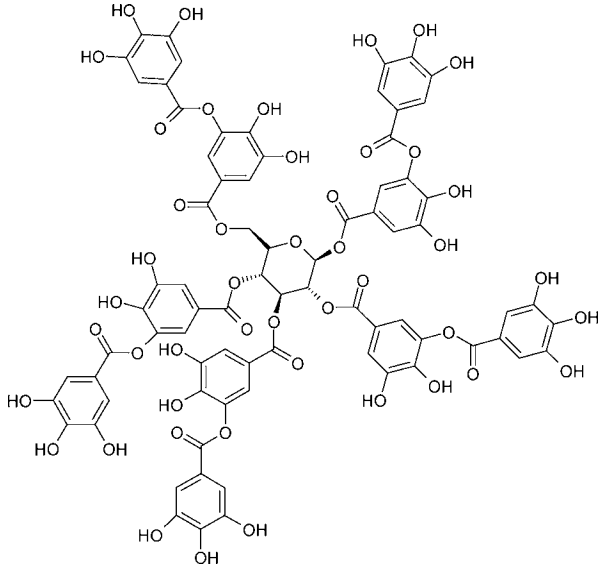

Figure 3. Representative structure of tannic acid (**13**).

Tyrosinase Inhibition. Bioassays with purified phenolics showed a concentration-dependent inhibitory effect on the oxidation of L-DOPA by mushroom tyrosinase. The IC_{50} values for **8**, **9**, and **6** were determined as 108.4, 177.7, and 91.2 μM, which is about 5-fold less potent in activity than those of tannic acid (Table 1), a well-documented tyrosinase inhibitor.[17] It seems possible that verbascoside (**8**) and naphthoquinone **6** could be responsible for the original tyrosinase inhibitory activity of the ethyl acetate (IC_{50} = 97.7 μg/mL) and *n*-hexane extracts observed in a preliminary screening (the most active fraction is **F-5** (IC_{50} = 87.3 μg/mL) from the *n*-hexane extract).

Thus, in the ethyl acetate (EtOAc) extract, verbascoside and martynoside are the main products, whereas iridoid monoterpenes and flavonoids were also encountered. Then, we can infer that the combined compounds contribute to the inhibitory activity of the ethyl acetate extract. However in the *n*-hexane extract ($IC_{50} \geq 100$ ppm), the activity is attributable only to the presence of naphthoquinones that were isolated from the most active fraction, **F-5**.

Table 1. Tyrosinase Inhibitory Activity of Compounds 6, 8, and 9, Extracts, and Reference Compounds[a]

sample	IC_{50} [μM]	K_I [μM]	inhibition type
6	91.2	0.0397	competitive
8	108.4	0.0465	competitive
9	177.7	0.0808	competitive
F-5[b]	87.3 [μg/mL]	N.D.[c]	N.D.
ethyl acetate	97.7 [μg/mL]	N.D.	N.D.
gallic acid	4.5	N.D.	N.D.
quercetin	70	0.031	competitive
benzoic acid	640	0.29	mixed
tannic acid	22	0.007	competitive
kojic acid	14	0.00135	competitive

[a]With respect to L-DOPA. [b]Values correspond to most active fraction (**F-5**) from the *n*-hexane extract; units of concentrations are expressed as [μg/mL]. [c]N.D., not determined.

Interestingly, verbascoside (**8**) and naphthoquinone **6** both can be oxidized as substrates by the tyrosinase enzyme.[43] Oxidation of verbascoside (**8**) was characterized by a new peak with a maximum at 383 nm (shift of Cu^{2+}, from 329.5 to 383 nm) in accord with data reported previously[44] and a new peak at 520 nm for naphthoquinone **6** (shift of Cu^{2+}, from 460 to 520 nm). The shift of verbascoside (**8**) was not observed in the case of martynoside (**9**) (a methylated phenylethanoid); thus the inhibitory activity of verbascoside could be attributed to the presence of ortho-hydroxyls on the phenolic rings, which give them the chelating property of metals. A similar case is between naphthoquinone **6** (IC_{50} = 24.7 μg/mL), which has a hydroxyl group vicinal to carbonyl, and quercetin (**12**), where the carbonyl group has vicinal hydroxyls (one at the A ring and other at the B ring) and two catechol hydroxyls in the C ring, thus increasing the inhibitory potency with an IC_{50} of 19.9 μg/mL.

It should be noted that this oxidation was at a much slower rate but did not have the lag period. Oxidation of L-DOPA is not subject to the lag period because the recruitment step takes place as soon as the substrate is presented with rapid conversion of met-enzyme to the oxygen-binding deoxy form.[62] In contrast, there was a progressive increase in the

observed rate of this oxidation as soon as catalytic amounts (0.01 mM) of L-DOPA became available as a cofactor. Thus, upon addition of both tyrosinase and L-DOPA, a yellow color was immediately detected optically for verbascoside (**8**) and an orange color for the naphthoquinone **6**.

The oxidation products of these two compounds are a complex mixture of polar compounds, and the unstable nature of the intermediates makes their characterization difficult. Despite our efforts, an attempt to characterize them failed. Enzymes are known to catalyze the oxidation of naphthoquinones.[45−47] Thus, the hydroxylquinone moieties of the naphthoquinone **6** may condense with one another through a Michael-type addition, yielding a relatively stable quinol−quinone intermediate.[47,48] However, the possibility that the naphthoquinone may form adducts with other nucleophilic groups in the enzyme and inactivate it cannot be entirely ruled out. The former case seems to be more likely since the remaining L-DOPA in the cuvette was oxidized when oxygen was supplied by mixing, as shown in Figure 4. This result

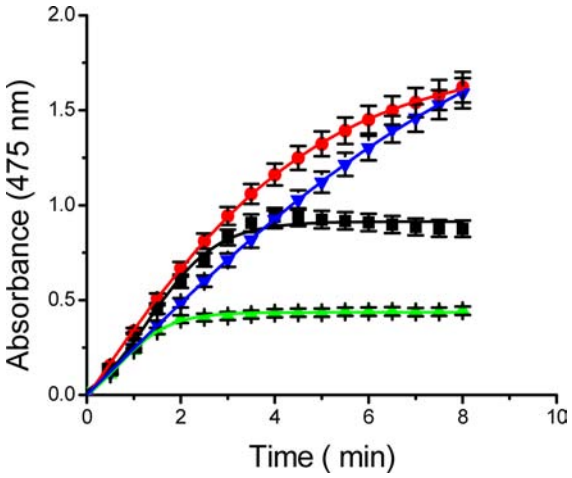

Figure 4. Mixing effect of **6** (110.0 μg/mL): (■) control without compound **6** and without mixing; (●) control without compound **6** and with mixing; (▲) with compound **6** and without mixing; (▼) with compound **6** and with mixing.

indicates that the enzyme was not inactivated by a K_{cat}-type inhibition (inactivation of the enzyme by products of the reaction)[49] as long as the current experiment was carried out.

On the other hand, tannic acid exhibits tyrosinase inhibitory activity, with an IC_{50} of 22 μM, and benzoic acid has an IC_{50} of 640 μM. The assayed compounds in this work are in the middle of this range. The inhibition kinetics of naphthoquinone **6** for the oxidation of L-DOPA by mushroom tyrosinase were analyzed by Eadie−Hofstee plots and found to be those characteristic for a competitive inhibitor, as shown in Figure 5. Because phenolics are known to react with proteins by cross-links, they definitely react of course in a process known as tanning.[50] Thus the naphthoquinone **6** should irreversibly inactivate the tyrosinase (protein); however, this does not occur with this naphthoquinone **6**, which competitively inhibits tyrosinase. It appears that tannic acid exhibits much more potent tyrosinase inhibitory activity compared to that of compounds **6** and **8**.[51]

Antioxidant and Protease Inhibitory Activity. The insect growth inhibitory activity shown by the extracts and compounds from *C. talcana*[14] seems to correlate with the

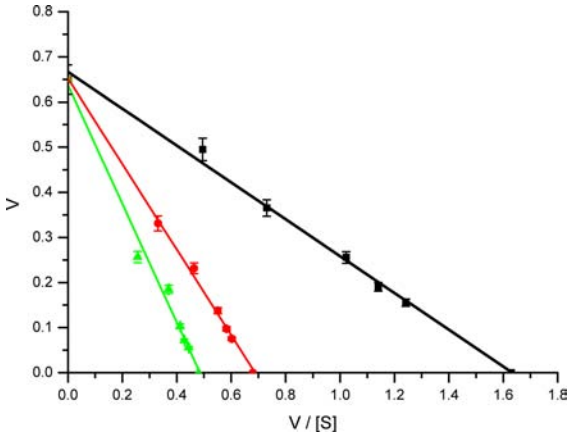

Figure 5. Eadie−Hofstee plots of tyrosinase and L-DOPA without (■) and with compound **6** [(●) 80.0 μg/mL and (▲) 150.0 μg/mL]. 1/V: 1/Δ475 nm/min.

tyrosinase inhibitory activity, but it should be borne in mind that plant secondary metabolites can act by a variety of different mechanisms in insects such as proteinase inhibitors.[52,53] The sensitivity of insect pest models to ingestion of compounds **6** and **8** may be a consequence of the extensive chemical modification in the midgut by oxidation. If so, naphthoquinones should be the first oxidized structure. Quinones are usually toxic to insects as well as to many other organisms.[40,54−56] However, it should be noted that benzoic acid inhibited tyrosinase (Table 1), but did not inhibit insect growth. This result may support that the oxidized products are responsible for the activity.

Radical scavenging activity, which can be measured as decolorizing activity following trapping of the unpaired electron of DPPH, was examined. In fact, compounds **6** (IC_{50} = 24.7 μg/mL) and **8** (IC_{50} = 58.13 μg/mL) exhibited almost equal radical scavenging activity, as shown in Table 2. The possibility that their adverse effects are a consequence of their potential to act as a prooxidant should also be considered. In fact, gallic acid, under certain conditions, can produce superoxide anion.[57] Furthermore, compound **6** may irreversibly inactivate enzymes (proteins) in the midgut, a process known as tanning, prior to being oxidized. This process also needs to be taken into consideration, as compound **6** may bind with proteins in the gut and, as a result, inhibit digestive enzymes as well as protein digestion.[58] This can be supported by the observation that compound **6** showed significant inhibitory activity against fungal protease. At a concentration of 10 μg/mL, compound **6** inhibited enzymatic activity 68%, while dunnione (**7**) did not exhibit any inhibitory activity up to 75 μg/mL. In addition, compound **6** inhibited the two assayed digestive enzymes (Table 2, Figure 6). Dunnione (**7**) also inhibited these enzymes but much more weakly compared to compound **6**. This fact shows that the addition of a hydroxyl group resulted in a significant increase in the toxicity.[56] On the other hand, dunnione (**7**) did not show insecticidal activity, although it has an unusually high activity on fungal proteinase at concentrations higher than 75 μg/mL (>90% at 250 μg/mL) (data not shown), similar to findings of Khambay et al.[54]

Digestive proteases catalyze the release of peptides and amino acids from dietary protein, and they are found most abundantly in the midgut region of the insect digestive tract.[52] Different proteases can be inhibited by many secondary metabolites from the plants; this process can be termed

Table 2. Scavenging Activity of 6, 8, and 9, Reference Compounds 10, 11, 12, and 13, and Amounts of Phenolics Content (μg/mL ± standard error) from Extracts from *Calceolaria talcana* Needed To Inhibit 50% of Activity[a]

sample tested	IC_{50}[b]	IC_{50} type II fungal protease	IC_{50} casein
ethyl acetate extract	61.3 ± 3.4 a	55.6 ± 2.80 a	58.4 ± 3.46 a
F-5	39.7 ± 2.5 b	4.79 ± 0.89 b	6.36 ± 0.21 b
naphthoquinone **6**	24.7 ± 1.77 b	6.01 ± 1.29 c	5.75 ± 0.35 c
verbascoside, **8**	58.13 ± 1.24 a	9.38 ± 2.18 d	8.28 ± 0.44 d
martynoside, **9**	87.35 ± 1.34 c	20.75 ± 1.79 e	37.50 ± 2.12 e
gallic acid, **10**	10.8 ± 0.21 d	13.29 ± 0.55 f	29.61 ± 1.78 f
quercetin **12**	19.9 ± 0.89 e	6.38 ± 0.91 c	6.89 ± 0.07 b
kojic acid, **11**	2.19 ± 0.09 f	9.75 ± 0.09 d	7.26 ± 0.04 b
tannic acid, **13**	1.06 ± 0.01 f	6.20 ± 0.08 c	5.71 ± 0.05 c

[a]IC_{50} for inhibition of DPPH radical formation. [b]Values expressed as μg/mL (ppm), mean ± SD, $n = 3$. Different letters show significant differences ($p < 0.05$), using Duncan's multiple range test.

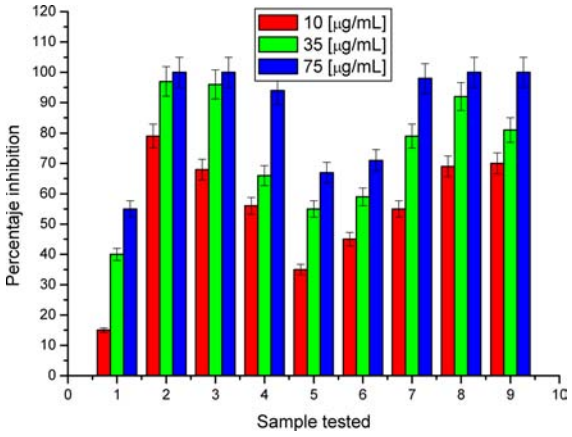

Figure 6. Percentaje inhibition of fungal protease type II by 1 = ethyl acetate extract, 2 = fraction **F-5**, 3 = hydroxylnaphthoquinone **6**, 4 = verbascoside (**8**), 5 = martynoside (**9**), compounds isolated from *Calceolaria talcana*, and model compounds 6 = gallic acid (**10**), 7 = kojic acid (**11**), 8 = quercetin (**12**), and 9 = tannic acid (**13**).

proteinase inhibitor inducing factor (PIIF).[59,60] The more polar fractions of the aerial parts (dried stems and leaves) of the plant species *C. talcana* yielded the most active compounds (verbascoside (**8**) and 2-hydroxy-3-(1,1-dimethylallyl)-1,4-naphthoquinone (**6**)), which inhibited tyrosinase as well as two digestive proteinases tested that were used as a protease enzyme model, than those of medium or low polarity.

Then, it may be logical to assume that phenolics such as verbascoside (**8**) and the hydroxynaphthoquinone **6** for example are synthesized during the preliminary attacks on *C. talcana* by an unidentified coleopterous observed on this plant, which after several hours searches for another plant. It appears therefore that the releasing mechanism of phenolics may be one of the key processes to understanding plant defense.[47]

CONCLUDING REMARKS

Finding alternate insect control agents by searching for plant compounds that inhibit tyrosinase is one of the goals of our continuing research. However, in vitro results using fungal tyrosinase as described are still far from our goal. Mushroom tyrosinase used for the initial screening differs somewhat from that of insects.[20] More importantly, it has not been intentionally overlooked, but, as a result, the dynamic function of tyrosinase in insect cuticle formation has not been thoughtfully taken into account. Thus, tyrosinase does not always exist as the active form in insects, and tyrosinase inhibitors cannot always reach the cuticle in sufficient concentrations to be effective. In addition, the reaction time and amount of available oxygen need to be considered from a practical point of view, as insect tyrosinase is an aerobic oxidase. As far as the artificial diet feeding assay against *S. frugiperda* and *D. melanogaster* larvae is concerned, some tyrosinase inhibitors characterized inhibit insect growth but some do not. The fact that plant secondary metabolites function by a variety of different mechanisms in insects needs to be kept in mind. The relevance of the results of in vitro experiments in simplified systems to the in vivo situation should be carefully considered. We are doing further studies using more appropriate bioassay methods.

AUTHOR INFORMATION

Corresponding Author

*Phone: +56-42-463277. Fax: +56-42-463046. E-mail: cespedes.leonardo@gmail.com.

Author Contributions

[†]Taken as part of the M.Sc. thesis of E.M. conducted in the laboratory of Dr. Carlos L. Céspedes.

Funding

This paper is based on work supported by a grant from the Comision Nacional de Investigacion Cientifica y Tecnologica de Chile (CONICYT), through FONDECYT Program grants 1101003 and 1130242.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are indebted to M.Sc. C. Lamilla for technical assistance with the antioxidant assay and for his help in performing insect feeding assays, to Prof. Ana M. García-Bores (Laboratorio de Fitoquimica, Unidad UBIPRO, FES-Iztacala, UNAM, Mexico DF, Mexico) for proteinase assays, and to Prof. David S. Seigler, Ph.D. (Emeritus Professor, Department of Plant Biology, and Curator, Herbarium of University of Illinois at Urbana–Champaign) for identification of the plant samples.

REFERENCES

(1) Bowers, M. D. Unpalatability as a defense strategy of western checkerspot butterflies (*Euphydrias* Scudder, Nymphalidae). *Evolution* **1981**, *35* (2), 367–375.

(2) Nieminen, M.; Suomi, J.; Van Nouhuys, S.; Sauri, P.; Riekkola, M.-L. Effect of iridoid glycoside content on oviposition host plant choice and parasitism in a specialist herbivore. *J. Chem. Ecol.* **2003**, *29* (4), 823–844.

(3) Dobler, S.; Petschenka, G.; Pankoke, H. Coping with toxic plant compounds—the insect's perspective on iridoid glycosides and cardenolides. *Phytochemistry* **2011**, *72*, 1593−1604.
(4) Nawrot, J.; Harmatha, J. Phytochemical feeding deterrents for stored product insect pests. *Phytochem. Rev.* **2012**, *11*, 543−566.
(5) Mayer, A. M. Polyphenol oxidases in plants−recent progress. *Phytochemistry* **1987**, *26*, 11−20.
(6) Robb, D. A. Tyrosinase. In *Copper Proteins and Copper Enzymes*; Lontie, R., Ed.; CRC Press: Boca Raton, FL, 1984; Vol. II, pp 207−240.
(7) Calderón, J. S.; Céspedes, C. L.; Rosas, R.; Gómez-Garibay, F.; Salazar, J. R.; Lina, L.; Aranda, E.; Kubo, I. Acetylcholinesterase and insect growth inhibitory activities of *Gutierrezia microcephala* on fall armyworm *Spodoptera frugiperda* J. E. Smith. *Z. Naturforsch. C* **2001**, *56c*, 382−394.
(8) Céspedes, C. L.; Alarcón, J. Biopesticides of botanical origin, phytochemicals and extracts from Celastraceae, Rhamnaceae and Scrophulariaceae. *Bol. Latinoam. Caribe Plant Med. Aromat.* **2011**, *10*, 175−181.
(9) Céspedes, C. L.; Calderón, J. S.; Lina, L.; Aranda, E. Growth inhibitory effects on fall armyworm *Spodoptera frugiperda* of some limonoids isolated from *Cedrela* spp. (Meliaceae). *J. Agric. Food Chem.* **2000**, *48*, 1903−1908.
(10) Céspedes, C. L.; Alarcón, J.; Aranda, E.; Becerra, J.; Silva, M. Insect growth regulator and insecticidal activity of β-dihydroagarofurans from *Maytenus* spp. (Celastraceae). *Z. Naturforsch. C* **2001**, *56c*, 603−613.
(11) Céspedes, C. L.; Martínez-Vázquez, M.; Calderón, J. S.; Salazar, J. R.; Aranda, E. Insect growth reguladory activity of some extracts and compounds from *Parthenium argentatum* on fall army worm *Spodoptera frugiperda*. *Z. Naturforsch. C* **2001**, *56c*, 95−105.
(12) Céspedes, C. L.; Avila, J. G.; Marin, J. C.; Dominguez, M.; Torres, P.; Aranda, E. Natural compounds as antioxidant and molting inhibitors can play a role as a model for search of new botanical pesticides. In *Naturally Occurring Bioactive Compounds*; Rai, M., Carpinella, M. C., Eds.; Advances in Phytomedicine Series, Vol. 3; Elsevier: The Netherlands, 2006; pp 1−27.
(13) Muñoz, E.; Lamilla, C.; Marín, J. C.; Alarcón, J.; Céspedes, C. L. Antifeedant, insect growth regulatory and insecticidal effects of *Calceolaria talcana* (Calceolariaceae) on *Drosophila melanogaster* and *Spodoptera frugiperda*. *Ind. Crops Prod.* **2013**, *42*, 137−144.
(14) Muñoz, E.; Escalona, D.; Salazar, J. R.; Alarcón, J.; Céspedes, C. L. Insect growth regulatory effects by diterpenes from *Calceolaria talcana* Grau & Ehrhart (Calceolariaceae: Scrophulariaceae) against *Spodoptera frugiperda* and *Drosophila melanogaster*. *Ind. Crops Prod.* **2013**, *45*, 283−292.
(15) Kubo, I. Tyrosinase inhibitors from plants. In *Phytochemicals for Pest Control*; Hedin, P., Hollingworth, R., Masler, E., Miyamoto, J., Thompson, D., Eds.; ACS Symposium Series 658; American Chemical Society: Washington, D.C., 1997; pp 310−326.
(16) Kubo, I.; Kinst-Hori, I. Tyrosinase inhibitors from cumin. *J. Agric. Food Chem.* **1998**, *46*, 5338−5341.
(17) Kubo, I.; Chen, Q.-X.; Nihei, K.-I.; Calderón, J. S.; Céspedes, C. L. Tyrosinase inhibition kinetics of anisic acid. *Z. Naturforsch. C* **2003**, *58c* (9/10), 713−718.
(18) Kubo, I.; Kinst-Hori, I.; Soria, F.; Takasaki, M.; Calderón, J. S.; Céspedes, C. L. Tyrosinase inhibitors from galls of *Rhus javanica* leaves and their effects on insects. *Z. Naturforsch. C* **2003**, *58c* (9/10), 719−725.
(19) Satooka, H.; Kubo, I. Resveratrol as a Kcat type inhibitor for tyrosinase: potentiated melanogenesis inhibitor. *Bioorg. Med. Chem.* **2012**, *20* (2), 1090−1099.
(20) Andersen, S. O. Sclerotization of insect cuticle. In *Molting and Metamorphosis*; Ohnishi, E., Ishizaki, H., Eds.; Japan Sci. Soc. Press: Tokyo, 1990; pp 133−155.
(21) Kramer, K. J.; Hopkins, T. L. Tyrosine metabolism for insect cuticle tanning. *Arch. Insect Biochem. Physiol.* **1987**, *6*, 279−301.
(22) Ryan, J. D.; Gregory, P.; Tingey, W. M. Phenolic oxidase activities in glandular trichomes of *Solanum berthaultii*. *Phytochemistry* **1982**, *21*, 1885−1887.
(23) Korkina, L. G. Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. *Cell Mol. Biol.* **2007**, *53* (1), 15−25.
(24) Alarcón, J.; Molina, S. C.; Villalobos, N.; Lillo, L.; Lamilla, C.; Céspedes, C. L.; Seigler, D. S. Insecticidal activity of Chilean Rhamnaceae: *Talguenea quinquenervis* (Gill et Hook). *Bol. Latinoam. Caribe Plant Med. Aromat.* **2011**, *10* (4), 383−388.
(25) Céspedes, C. L.; Molina, S. C.; Muñoz, E.; Lamilla, C.; Alarcón, J.; Palacios, S. M.; Carpinella, M. C.; Avila, J. G. The insecticidal, molting disruption and insect growth inhibitory activity of extracts from *Condalia microphylla* Cav. (Rhamnaceae). *Ind. Crops Prod.* **2013**, *42*, 78−86.
(26) Torres, P.; Ávila, J. G.; Romo de Vivar, A.; García, A. M.; Marin, J. C.; Aranda, E.; Céspedes, C. L. Antioxidant and insect growth regulatory activities of stilbenes and extracts from *Yucca periculosa*. *Phytochemistry* **2003**, *64*, 463−473.
(27) Domínguez, M.; Marín, J. C.; Esquivel, B.; Céspedes, C. L. Pensteminoside, an unusual catalpol-type iridoid from *Penstemon gentianoides* HBK (Plantaginaceae). *Phytochemistry* **2007**, *68*, 1762−1766.
(28) Chamy, M. C.; Jimenez, I.; Piovano, M.; Garbarino, J. A.; Didyk, B. Secondary metabolites from *Calceolaria sessilis*. *Bol. Soc. Chil. Quim.* **1993**, *38*, 187−190.
(29) Rüedi, P.; Eugster, C. H. Isolierung von (-)-Dunnion aus Calceolaria integrifolia Murr. (Scrophulariaceae). *Helv. Chim. Acta* **1977**, *60* (3), 945−947.
(30) Domínguez, M.; Nieto, A.; Marin, J. C.; Keck, A.-S.; Jeffery, E.; Céspedes, C. L. Antioxidant activities of extracts from *Barkleyanthus salicifolius* (Asteraceae) and *Penstemon gentianoides* (Scrophulariaceae). *J. Agric. Food Chem.* **2005**, *53*, 5889−5895.
(31) Blois, M. S. Antioxidant determination by the use of a stable free radical. *Nature* **1958**, *181*, 1199−1200.
(32) Céspedes, C. L.; Valdez-Morales, M.; Avila, J. G.; El-Hafidi, M.; Alarcón, J.; Paredes-López, O. Phytochemical profile and the antioxidant activity of Chilean wild black-berry fruits, *Aristotelia chilensis* (Mol) Stuntz (Elaeocarpaceae). *Food Chem.* **2010**, *119*, 886−895.
(33) Espín, J. C.; Wichers, H. J. Slow-binding inhibition of mushroom (*Agaricus bisporus*) tyrosinase isoforms by tropolone. *J. Agric. Food Chem.* **1999**, *47*, 2638−2644.
(34) van Gelder, C. W. G.; Flurkey, W. H.; Wichers, H. J. Sequence and structural features of plant and fungal tyrosinases. *Phytochemistry* **1997**, *45*, 1309−1323.
(35) Stauffer, C. E. Measurement methods. In *Enzyme Assays for Food Scientists*; Van Nostrand Reinhold: New York, 1989; pp 128−130.
(36) Garbarino, J. A.; Chamy, M. C.; Piovano, M. Chemistry of the *Calceolaria* genus. Structural and biological aspects. *Molecules* **2000**, *5*, 302−303.
(37) Harborne, J. B.; Baxter, H. *Chemical Dictionary of Economic Plants*; J. Wiley & Sons: Chichester, England, 2001; p 753.
(38) Woldemichael, G. M.; Wächter, G.; Singh, M. P.; Maisse, W. M.; Timmermann, B. N. Antibacterial diterpenes from *Calceolaria pinifolia*. *J. Nat. Prod.* **2003**, *66*, 242−246.
(39) Di Fabio, A.; Bruni, A.; Poli, F.; Garbarino, J. A.; Chamy, M. C.; Piovano, M.; Nicoletti, M. The distribution of phenylpropanoid glycosides in Chilean *Calceolaria* spp. *Biochem. Syst. Ecol.* **1995**, *23*, 179−182.
(40) Khambay, B. P. S.; Batty, D.; Matthew, C.; Denholm, I.; Mead-Briggs, M.; Vinall, S.; Niemeyer, H.; Simmonds, M. S. J. Isolation, characterization, and biological activity of naphthoquinones from *Calceolaria andina* L. *J. Agric. Food Chem.* **1999**, *47*, 770−775.
(41) Falcao, D. Q.; Costa, E. R.; Alviano, D. S.; Alviano, C. S.; Kuster, R. M.; Menezes, F. S. Atividade antioxidante e antimicrobiana de *Calceolaria chelidonioides* Humb. Bonpl. & Kunth. *Braz. J. Pharmacogn.* **2006**, *16*, 73−76.

(42) Asnaashari, S.; Delazar, A.; Seyed-Alipour, S.; Nahar, L.; Williams, A. S.; Pasdaran, A.; Mojarab, M.; Fathi-Azad, F.; Sarker, S. D. Chemical composition, free-radical-scavenging and insecticidal activities of the aerial parts of *Stachys byzantine*. *Arch. Biol. Sci. Belgrade* **2010**, *62*, 653−662.
(43) Duckworth, H. W.; Coleman, J. E. Physicochemical and kinetic properties of mushroom tyrosinase. *J. Biol. Chem.* **1970**, *245* (7), 1613−1625.
(44) Karioti, A.; Protopappa, A.; Megoulas, N.; Skaltsa, H. Identification of tyrosinase inhibitors from *Marrubium velutinum* and *Marrubium cylleneum*. *Bioorg. Med. Chem.* **2007**, *15* (7), 2708−2714.
(45) Klapper, M. H.; Hackett, D. P. The oxidatic activity of horseradish peroxidase I. Oxidation of hydro- and naphthohydroquinones. *J. Biol. Chem.* **1963**, *238* (11), 3736−3742.
(46) Handerson, G. B.; Ulrich, P.; Fairlamb, A. H.; Rosenberg, I.; Pereira, M.; Sela, M.; Cerami, A. "Subversive" substrates for the enzyme trypanothione disulfide reductase: alternative approach to chemotherapy of Chagas disease. *Proc. Natl. Acad. Sci.* **1988**, *85*, 5374−5378.
(47) Singh, Sh. B.; Graham, P. L.; Reamer, R. A.; Cordingley, M. G. Discovery, total synthesis, HRV 3C-protease inhibitory activity, and structure-activity relationships of 2-methoxystypandrone and its analogues. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 3143−3146.
(48) Sayre, L. M.; Nadkarni, D. V. Direct conversion of phenols to *o*-quinones by copper(I) dioxygen. Questions regarding the monophenolase activity of tyrosinase mimics. *J. Am. Chem. Soc.* **1994**, *116*, 3157−3158.
(49) Golan-Goldhirsh, A.; Whitaker, J. R. Kcat inactivation of mushroom polyphenol oxidase. *J. Mol. Catal.* **1985**, *32*, 141−147.
(50) Ryan, C. A. Proteinase inhibitors in plants: genes for improving defenses against insects and pathogens. *Annu. Rev. Phytopathol.* **1990**, *28*, 425−449.
(51) Passi, S.; Nazzaro-Porro, M. Molecular basis of substrate and inhibitory specificity of tyrosinase: phenolic compounds. *Br. J. Dermatol.* **1981**, *104*, 659−665.
(52) Jongsma, M. A.; Bolter, C. The adaptation of insects to plant protease inhibitors. *J. Insect Physiol.* **1997**, *43* (10), 885−895.
(53) Farmer, E. E.; Ryan, C. A. Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proc. Natl. Acad. Sci.* **1990**, *87*, 7713−7716.
(54) Khambay, B. P. S.; Batty, D.; Jewess, Ph. J.; Bateman, G. L.; Hollomon, D. W. Mode of action and pesticidal activity of the natural product dunnione and of some analogues. *Pest Manage. Sci.* **2003**, *59* (2), 174−182.
(55) Akhtar, Y.; Isman, M. B.; Lee, Ch.-H.; Lee, S.-G.; Lee, H.-S. Toxicity of quinones against two-spotted spider mite and three species of aphids in laboratory and green house conditions. *Ind. Crops Prod.* **2012**, *37* (1), 536−541.
(56) Akhtar, Y.; Isman, M. B.; Niehaus, L. A.; Lee, Ch.-H.; Lee, H.-S. Antifeedant toxic effects of naturally occurring and synthetic quinones on the cabbage looper, *T. ni*. *Ind. Crops Prod.* **2012**, *37* (1), 8−14.
(57) Serrano, A.; Palacios, C.; Roy, G.; Cespon, C.; Villar, M. L.; Nocito, M.; Gonzalez-Porque, P. Derivatives of gallic acid induce apoptosis in tumoral cell lines and inhibit lymphocyte proliferation. *Arch. Biochem. Biophys.* **1998**, *350*, 49−54.
(58) Feeny, P. Plant apparency and chemical defense. In *Biochemical Interactions between Plants and Insects*. Wallace, J. W., Masell, R. L., Eds.; Plenum Press: New York, 1976; pp 1−40.
(59) Ryan, J. D. Proteinase inhibitors. In *Herbivores: Their Interaction with Secondary Plant Metabolites*; Rosenthal, G. A., Janzen, D. H., Eds.; Academic Press: New York, 1979; pp 599−618.
(60) Guerrero, A.; Rosell, G. Biorational approaches for insect control by enzymatic inhibition. *Curr. Med. Chem.* **2005**, *12* (4), 461−469.
(61) Cespedes, C. L.; Salazar, J. R.; Alarcon, J. Chemistry and biological activities of *Calceolaria* spp. (Calceolariaceae: Scrophulariaceae). *Phytochem. Rev.* **2013**, DOI: 10.1007/s11101-013-9276-6.
(62) Naish-Byfield, S.; Riley, P. A. Tyrosinase autoactivation and the problem of the lag period. *Pigment Cell Res.* **1998**, *11*, 127−133.