INHIBITION OF CRUZAIN BY OCOTEA LEAF ESSENTIAL OILS FROM MONTEVERDE, COSTA RICA

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Summary

Introduction: Over 18 million people in tropical and subtropical America are afflicted by American trypanosomiasis or Chagas disease. Symptoms of the disease include fever, swelling, and heart and brain damage, usually leading to death. There is currently no effective treatment for this disease. Cruzain is a cysteine protease from *Trypanosoma cruzi* that is key to replication and differentiation of the parasite and has been identified as an important biochemical target for treatment of this parasitic infection. **Materials and Methods:** The leaf essential oils of ten species of *Ocotea* from Monteverde, Costa Rica, were obtained by hydrodistillation and analyzed by GC-MS. The enzyme inhibitory activities of the essential oils against cruzain have been examined using a fluorometric assay. **Results:** *O. meziana* leaf oil was the most active (IC_{50} = 14.9 μg/mL) followed by *O. whitei* (15.8 μg/mL), *Ocotea* sp. nov. "los llanos" (17.1 μg/mL), *Ocotea* sp. nov. "small leaf" (19.2 μg/mL) and *O. holdridgeana* (76.9 μg/mL). The leaf oils of *O. floribunda*, *O. tonduzii*, and *O. valeriana* were somewhat active (IC_{50} 100-200 μg/mL), but *O. sinuata* and *O. veraguensis* essential oils were inactive (IC_{50} > 500 μg/mL). The *Ocotea* leaf essential oils are generally dominated by the monoterpenes α- and β-pinene and the sesquiterpenes β-caryophyllene and germacrene D. α-Pinene and β-pinene show slight cruzain inhibitory activity (IC_{50} = 160 and 155 μg/mL, respectively), but neither β-caryophyllene nor germacrene D are active (IC_{50} > 500 μg/mL). **Conclusions:** The cruzain inhibitory activity of *Ocotea* leaf essential oils cannot be explained by the activities of the major components alone. There may be synergistic effects of the components or the activities may be due to minor components.

Key Words: *Ocotea*, Monteverde, Costa Rica, leaf essential oil, composition, *Trypanosoma cruzi*, cruzain inhibition.

Introduction

Parasitic protozoal infections are of increasing importance, not only in developing nations where they are endemic, but also to people of industrialized countries due to world travel. Over 18 million people in tropical and subtropical America are afflicted by American trypanosomiasis or Chagas disease, caused by *Trypanosoma cruzi* (1). There is a pressing need for new anti-trypanosomals. An effective treatment for Chagas Disease has not been found. Both nifurtimox and benznidazole have been used in treatment of the acute phase of the disease, however they suffer from severe side effects and require prolonged use (2,3). There are currently no vaccines available for treatment of trypanosomiasis (4), and there is little optimism for development of vaccines for these parasitic infections in the near future (5,6). Proteases play critical functions in the metabolism, replication, survival, and pathology of parasitic protozoa. Cysteine proteases of the papain family have been found in most parasitic protozoans, including *T. cruzi* (7). The cysteine protease cruzain (from *T. cruzi*) has been suggested to be a valid biomolecular target for antiparasitic drug discovery (8). For thousands of years, plant products and their modified derivatives have been rich sources for clinically useful drugs. In this work, we investigate leaf essential oils from ten species of *Ocotea* from Monteverde, Costa Rica, as inhibitors of cruzain.

Methods

Plant Collection. The plants were collected from Monteverde, Costa Rica, and identified by William A. Haber. Essential oils were obtained by hydrodistillation of freshly chopped leaves and dichloromethane extraction of the distillate (Table 1).

Gas chromatographic-mass spectral analysis. The leaf oils of the *Ocotea* species were subjected to gas chromatographic-mass spectral analysis using an Agilent 6890 GC with Agilent 5973 mass selective detector, fused silica capillary column (HP-5ms, 30 m x 0.25 mm), helium carrier gas, 1.0 mL/min flow rate; inj temp 200^{o}C, oven temp prog: 40^{o}C initial temperature, hold for 10 min; increased at 3^{o}/min to 200^{o}C; increased 2^{o}/min to 220^{o}C, and interface temp 280^{o}C; EIMS, electron energy, 70 eV. The sample was dissolved in $CHCl_3$ to give a 1% w/v solution; 1-μL injections using a splitless injection technique were used. Identification of oil components was achieved based on their retention indices (determined with reference to a homologous series of normal alkanes), and by comparison of their mass spectral fragmentation patterns with those reported in the literature (9) and stored on the MS library [NIST database (G1036A, revision D.01.00)/ChemStation data system (G1701CA, version C.00.01.08)].

Table 1. Leaf essential oils of *Ocotea* species from Monteverde, Costa Rica.

Plant	Voucher number	Collection Site (Date)	Mass of leaves	Mass of leaf oil
O. floribunda	Haber 9571	Los Llanos Field Station (May 19, 2006)	65.0 g	89.7 mg (0.138%)
O. holdridgeana	Haber 12771	Monteverde Cloud Forest Preserve (May 23, 2006)	50.9 g	13.2 mg (0.026%)
O. meziana	Haber 238	Monteverde Cloud Forest Preserve (May 23, 2006)	71.5 g	21.8 gm (0.030%)
O. sinuata	Haber 7201	Los Llanos Field Station (May 19, 2006)	61.6 g	38.3 mg (0.062%)
O. tonduzii	Haber 10041	Monteverde Cloud Forest Preserve (May 23, 2006)	110.5 g	189.2 mg (0.171%)
O. valeriana	Haber 6643	Upper Monteverde (May 23, 2005)	49.5 g	23.9 mg (0.048%)
O. veraguensis	Haber 10647	San Luis Reserve (May 28, 2006)	66.2 g	189.8 mg (0.287%)
O. whitei	Haber 11068	Monteverde Cloud Forest Preserve (May 23, 2006)	67.3 g	163.0 mg (0.242%)
Ocotea "los llanos"	Haber 11063	Los Llanos Field Station (May 19, 2006)	100.0 g	23.9 mg (0.024%)
Ocotea "small leaf"	Haber 11515	Monteverde Cloud Forest Preserve (May 23, 2006)	106.5 g	167.1 mg (0.157%)

Cruzain inhibition assay. The activity of essential oils and essential oil components against recombinant cruzain (prepared as described in Eakin et al., 1992) was measured by a fluorescence assay using Z-Phe-Arg-AMC·HCl as the fluorescent enzyme substrate. The cruzain solution (4 nM) was prepared with 20 μL of cruzain per liter of 100 mM sodium acetate buffer with 5 mM DTT and a pH of 5.5. The substrate solution (40 μM) was prepared with 26 mg Z-Phe-Arg-AMC·HCl first dissolved in DMSO per liter of 100mM sodium acetate buffer with 5 mM DTT and a pH of 5.5. The essential oils and components were prepared as 1% solutions in DMSO. For each well of a 96 well plate 475 μL of cruzain was mixed with 25 μL of the sample solution to be tested. 100 μL of this mixture was pipetted into each well. Each sample was tested in quadruplicate with DMSO negative controls and TLCK positive controls. After approximately 10 minutes incubation at room temperature, 100 μL of the substrate solution was pipetted into each well (the final sample concentration is 500 μg/mL). The plate was then immediately read using a SpectraMax M2 fluorescence plate reader. After an initial mixing period of 5 seconds the fluorescence was measured 9

times over a period of 5 minutes with an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The slope given by the change in fluorescence was then exported into an Excel spreadsheet for the calculations of percent inhibition and standard deviation. Samples that showed >50% inhibition at 250 μg/mL were retested at 50 μg/mL and 5 μg/mL. IC_{50} Values were determined using the Reed-Muench method (10). The cruzain inhibitory activities of the *Ocotea* leaf oils and components are summarized in Table 2.

Table 2. Cruzain inhibitory activity of *Ocotea* leaf essential oils and some components.

Essential Oil	IC_{50} (μg/mL)	Compound	IC_{50} (μg/mL)	Compound	IC_{50} (μg/mL)
O. floribunda	119 (±12)	Borneol	197 (±14)	Limonene	>500
O. holdridgeana	76.9 (±1.6)	Camphene	48.0 (±1.6)	Linalool	>500
O. meziana	14.9 (±0.9)	Camphor	>500	Myrtenal	>500
O. sinuata	>500	β-Caryophyllene	>500	α-Pinene	160 (±9)
O. tonduzii	153 (±5)	Caryophyllene oxide	112 (±14)	β-Pinene	155 (±10)
O. valeriana	177 (±4)	1,8-Cineole	>500	4-Terpineol	>500
O. veraguensis	>500	α-Copaene	363 (±20)	α-Terpineol	>500
O. whitei	15.8 (±0.2)	*p*-Cymene	>500		
Ocotea "los llanos"	17.1 (±0.3)	Germacrene D	>500		
Ocotea "small leaf"	19.2 (±0.1)	α-Humulene	>500		

Results

The leaf essential oil of *O. floribunda* was made up largely of monoterpene hydrocarbons and diterpenes (48.9% and 34.7%, respectively), with smaller amounts of oxygenated sesquiterpenoids (10.6%), sesquiterpene hydrocarbons (5.1%), and oxygenated monoterpenoids (0.8%). The key chemical characteristic of *O. floribunda* was the abundant quantity of diterpenes, which were not observed in the other nine *Ocotea* species. The most abundant essential oil components of *O. floribunda* were kaurene (34.0%), α-pinene (22.5%), and β-pinene (21.3%). Although a large amount of the diterpene kaurene was found in *O. floribunda*, none of the other nine species contained the compound. The most abundant components of the essential oil of *O. holdridgeana* were α-pinene (29.9%), germacrene D (19.9%), and β-pinene (9.1%). Sesquiterpene hydrocarbons (46.9%) made up the bulk of the leaf oil with monoterpene hydrocarbons (40.5%), fatty-acid-derived compounds (alcohols, ketones, and aldehydes), oxygenated sesquiterpenoids, and oxygenated monoterpenoids (8.4%, 3.5% and 0.7%, respectively) making up the remainder. *O. meziana* leaf oil was composed primarily of sesquiterpene hydrocarbons (86.2%), with lesser amounts of oxygenated sesquiterpenoids (6.8%), monoterpene hydrocarbons

(4.1%), and fatty-acid-derived compounds (2.9%). The large proportion of sesquiterpene hydrocarbons was due to high concentrations of germacrene-D (50.6%), and β-caryophyllene (13.2%).

The leaf essential oil of *O. sinuata* was rich in monoterpene and sesquiterpene hydrocarbons (44.0% and 31.6%, respectively) with low amounts of fatty-acid-derived compounds (17.0%), oxygenated monoterpenoids (5.9 %), and oxygenated sesquiterpenoids (1.5%). *trans*-2-Hexenal (17.0%), camphene (16.2%), germacrene-D (15.4%), α-pinene (15.0%), and β-caryophyllene (9.4%) were the most abundant essential oil components of *O. sinuata*. *O. tonduzii* leaf oil was composed primarily of monoterpene hydrocarbons (70.5%) and contained α-pinene (41.2%) and β-pinene (25.0%) as its main components. The concentrations of sesquiterpene hydrocarbons (22.6%), oxygenated sesquiterpenoids (4.5%), oxygenated monoterpenoids (1.9%), and fatty-acid-derived compounds (0.5%) were significantly lower than monoterpene hydrocarbons. The leaf essential oil of *O. valeriana* was dominated by germacrene-D (69.7%), and therefore sesquiterpene hydrocarbons (84.6%) made up a large part of the leaf oil of *O. valeriana*. α-Cadinol (4.5%), τ-cadinol (3.4%), δ-cadinene, and *cis*-3-hexenol (2.5 %) were also found in notable quantities. The amounts of oxygenated sesquiterpenoids (12.7%), monoterpene hydrocarbons (1.4%), and fatty-acid-derived compounds (1.2%) were small when compared to the percentage composition of sesquiterpene hydrocarbons.

Oxygenated sesquiterpenoids (58.8%) comprised a large part of the leaf oil of *O. veraguensis*, which demonstrates unusual chemical characteristics compared to the other nine *Ocotea* species. The remainder of the oil was composed of smaller amounts of monoterpene and sesquiterpene hydrocarbons (27.5% and 10.1%, respectively) with a very small amount of oxygenated monoterpenoids (2.3%), fatty-acid-derived compounds (1.1%), and others (0.1%). The leaf essential oil of *O. veraguensis* was dominated by bulnesol (29.5%) and *p*-cymene (19.8%). Although *O. veraguensis* had abundant bulnesol, it was not observed in any of the other nine species. Based on the number of chemical components, *O. veraguensis*, with 55 compounds, had the most complex oil out of the ten *Ocotea* species examined. The leaf essential oil of *O. whitei* was composed largely of monoterpene and sesquiterpene hydrocarbons (22.0% and 31.6%, respectively) as well as oxygenated sesquiterpenoids (33.8%) with smaller amounts of fatty-acid-derived compounds (0.8%), oxygenated monoterpenoids (3.1%), oxygenated sesquiterpenoids (1.5%), and aromatics (0.5%). The most abundant essential oil components of *O. whitei* were spathulenol (15.3%), β-caryophyllene (15.2%), α-pinene (12.7%), and farnesyl acetate (10.1%).

Monoterpene hydrocarbons (76.5%) were the main constituents of the essential oil of *Ocotea* "los llanos" and contain α-pinene (27.5%), *trans*-β-ocimene (24.1%), and β-pinene (17.2%). The remainder of the essential oil was comprised of oxygenated sesquiterpenoids (10%), sesquiterpene hydrocarbons (9.8%), fatty-acid-derived compounds (2.1%), oxygenated monoterpenoids (1.2%), and others (0.1). In addition, *Ocotea* "los llanos" had the second most complex oil out of the ten *Ocotea* species with 51 compounds. Similar to *O. valeriana*, the leaf essential oil of *Ocotea* "small leaf" was dominated by germacrene-D (60.4%). Germacrene-B (5.3%), α-pinene (4.3%), and δ-cadinene (4.3%) were also found in notable quantities, similar to *O. valeriana*. Sesquiterpene hydrocarbons (88.0%) made up a large part of the leaf oil of *Ocotea* "small leaf" with smaller amounts of monoterpene hydrocarbons (7.3%), oxygenated sesquiterpenoids (3.8%), and fatty-acid-derived compounds (0.9%).

Discussion

Out of the ninety-one compounds identified in this study, there were only nine compounds that were common to all ten *Ocotea* species: α-pinene (abundant in *O. floribunda*, *O. holdridgeana*, *O. sinuata*, *O. tonduzii*, *O. whitei*, and *Ocotea* "los llanos"), β-pinene (abundant in *O. floribunda*, *O. holdridgeana*, *O. tonduzii*, and *Ocotea* "los llanos"), β-elemene (only small amounts in any of the *Ocotea* species), β-caryophyllene (abundant in *O. meziana*, *O. sinuata*, and *O. whitei*), α-humulene (only small amounts in any of the *Ocotea* species), germacrene-D (abundant in *O. holdridgeana*, *O. meziana*, *O. sinuata*, *O. valeriana*, and *Ocotea* "small leaf"), γ-cadinene (only small amounts in any of the *Ocotea* species), δ-cadinene (only small amounts in any of the *Ocotea* species), and α-cadinol (only small amounts in any of the *Ocotea* species). The nine common compounds (α-pinene, β-pinene, β-elemene, β-caryophyllene, α-humulene, germacrene D, γ-cadinene, δ-cadinene, and α-cadinol) had been previously reported in *O. angustifolia* (11) and *O. brenesii* (12) leaf essential oils. Furthermore, *O. comoriensis* bark essential oil (13) contained eight of the nine common compounds found in the *Ocotea* species of this study; β-elemene was not reported. However, *O. quixos* had very different chemical characteristics with only five of the common nine compounds (β-pinene, α-humulene, γ-cadinene, and α-cadinol were not found). These nine compounds are apparently common components of other genera of the Lauraceae, including *Beilschmiedia*, *Cinnamomum*, *Laurus*, *Lindera*, *Nectandra*, and *Persea*. For instance, *Beilschmiedia alloiophylla* (14), *Beilschmiedia tilaranensis* (14), *Laurus azorica* (15), and *Nectandra membranacea* (16) leaf essential oils had all the nine compounds. Additionally, *Cinnamomum tamala* (17), *Laurus nobilis* (18), and

Persea caerulea (unpublished results from our laboratory) leaf essential oils had eight compounds except α-cadinol. *Cinnamomum sintoc* (19) leaf essential oil had eight compounds except α-pinene, but α-pinene was found in bark essential oil. Also, *Persea tolimanensis* (20) and *Licaria excelsa* (unpublished results from our laboratory) leaf essential oil also had eight compounds except β-elemene, but δ-elemene was found instead in *Persea tolimanensis* leaf essential oil. Furthermore, *Lindera benzoin* (unpublished results from our laboratory) leaf essential oil had seven compounds except α- and β-pinene.

Twelve compounds were found to be common to seven or more of the *Ocotea* species: *trans*-2-Hexenal (not found in *O. floribunda* and *O. valeriana*), camphene (except *O. meziana* and *Ocotea* "small leaf"), myrcene (except *O. meziana* and *O. valeriana*), limonene (except *O. holdridgeana*, *O. valeriana*, and *O. veraguensis*), 1,8-cineole (except *O. meziana* and *O. veraguensis*), α-copaene (except *O. sinuata*), β-bourbonene (except *O. holdridgeana* and *Ocotea* "small leaf"), bicyclogermacrene (except *O. floribunda*, *O. veraguensis*, and *Ocotea* "small leaf"), Germacrene-A (except *Ocotea* "los llanos" and *Ocotea* "small leaf"), cadina-1,4-diene (except *O. floribunda* and *O. sinuata*), 1-*epi*-cubenol (*O. holdridgeana*, *O. sinuata*, and *O. whitei*), and τ-cadinol (except *O. whitei*).

Other investigators have examined the essential oils of other *Ocotea* species and have discovered similar chemical compositions among them. For instance, *O. brenesii* (12) had similar chemical characteristics to the *Ocotea* species in this present study. In the *O. brenesii* leaf oil, sesquiterpene hydrocarbons were the main constituents (53.4%) of the oil and contained α-copaene (21.1%), δ-cadinene (9.2%) and β-caryophyllene (5.2%) as the major compounds. The second highest group in terms of quantity was oxygenated sesquiterpenes (29.9%) with spathulenol (7.3%) and globulol (5.6%) predominating. Also, *O. comoriensis* bark essential oil (13) had compounds that were identified in the leaf oils of this study. There are some *Ocotea* species, however, that have different chemical characteristics. Thus, for example, cinnamyl acetate and cinnamaldehyde, which have been attributed to the cinnamon-like taste of *O. quixos* leaf essential oil (21), were not detected in any of the ten species of *Ocotea* in our study. In addition, the major component of the chemical composition of the leaf essential oil of *O. foetens* was ethyl *p*-coumarate (69.6 %) (22), but none of the ten *Ocotea* species in this present study had the compound. Furthermore, most of the *Ocotea* species from previous reports contained phenylpropanoids such as safrole, *O*-methyleugenol, cinnamaldehydes and benzenoids (12). However, none of the ten *Ocotea* species of this study exhibited these chemical characteristics.

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