PHYTOCHEMICAL AND CRYSTALLOGRAPHIC STUDIES OF *Azara dentata* EXTRACTS AND ITS CYTOTOXIC EFFECTS ON HUMAN BREAST CANCER CELL, MCF-7

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ABSTRACT

Azara dentata Ruiz & Pav. also called "Corcolen" is an endemic shrub of Chile. The honey produced in areas with abundance of *Azara dentata* is highly appreciate by its polyphenols. In the present work, we isolated and identified for the first time the phytochemical components of *Azara dentata* and its cytotoxic effects were analyzed on cancer cells together with its antimicrobial activity. The results showed that palmitic acid methyl ester, linolenic acid methyl ester, α-tocopherol and β-sitosterol are the main non-polar constituents of the plant, while the compounds **AD-3** (3-phenylisocoumarin, 0.0001% yield) and **AD-4** (methyl 2-phenacyl-benzoate, 0.00039% yield) were isolated by column chromatography with n-hexane/EtOAc (3:2 v/v) and their structures were determined using NMR analysis. In addition, the structure of **AD-4** was complemented by a single crystal x-ray structural determination. *Azara dentata* showed moderate antibacterial activity against *S. aureus* and *S. epidermidis* with a MIC of 5.0 and 10 mg/mL respectively. The cytotoxic activity of *Azara dentata* against MCF-7 cancer cells showed an IC_{50} of 15.63 µg/mL, this is a promissory value for the deeper study of its metabolites in cancer models.

Keywords: *Azara dentata*, Antimicrobial activity, Cytotoxic activity, Phytochemicals, X-Ray Diffraction, GC-MS.

INTRODUCTION

Azara dentata Ruiz & Pav. Salicaceae, commonly known as "corcolén blanco" or "aromo de Castilla" is one of the species corresponding to the Azara genus, a group of flowering plants which includes *A. alpina, A. integrifolia, A. lanceolata, A. microphylla, A. petiolaris, A. salicifolia, A. serrata, A. uruguayensis*. This endemic specie from Chile, is an evergreen shrub that can reach a height of up to 2.5 m and grows between Santiago and Valdivia, habiting sunny slopes of the Andean pre-cordillera. Phytochemical studies of the honey produced in areas with abundance of *Azara integrifolia* and *Azara petiolaris*, commonly known as corcolén honeys [1], have shown a high content of polyphenols with antioxidant and antibacterial activities [2], in concentrations between 48.79 to 153.30 mg GAE/100 g honey. The main flavonoids identified by UHPLC-MS/MS were pinocembrin, chrysin, and luteolin together with caffeic acid [3]. Here, we report the phytochemical characterization of aerial parts of *Azara dentata* by gas chromatography mass spectrometry, followed by the fractioning and purification of the metabolites together with the crystallographic structural determination of one of the isolated molecules, methyl 2-phenacyl-benzoate (**AD-4**).

The bioactivity of the plant was studied in terms of the cytotoxic activity against MCF-7 cells together with the antimicrobial activity against *S. aureus, S. epidermidis, E. faecalis, E. coli, P. aeruginosa, K. pneumoniae* and a clinical strain of the yeast *Candida albicans.*

EXPERIMENTAL

General Information

Analytical thin-layer chromatography (TLC) was carried out on Merck Silica Gel 60F254 sheets (Darmstadt, Germany). It was employed for monitoring the purification of the compounds, using UV light (254 nm) followed by molybdophosphoric acid and heating for visualization. Preparative chromatography was performed using Merck silica gel 60 and Sephadex LH-20 (25−100 µm; Aldrich, Santiago, Chile). Solvents and fractions were concentrated in a Büchi R100 rotavap. Solvents used in this study were distilled prior to use and dried over appropriate drying agents.

Plant material

Aerial parts of *Azara dentata* (Figure 1) were collected in Padre Las Casas, IX Region of Chile, in December 2018. A voucher specimen has been deposited at the herbal collection of the Laboratory of Natural Products (C. Paz) Universidad de La Frontera, Chile.

Figure 1. Photography of *Azara dentata*.

Extraction and purification of Azara dentata

3.5 kg of aerial parts of *Azara dentata* (humid weight) were dried, powdered and extracted by maceration with ethyl acetate/methanol 1:1 (EtOAc/MeOH) for 3 days (5 L x 2-folds). The organic solvent was evaporated *in vacuo* giving a total extract (860 g, gummy oil). Total extract was further fractionated by silica gel column chromatography, giving a primary fractioning of eight fractions (AD-F1 to AD-F8) by using increasing polarity of solvents from n-hexane to EtOAc, scheme 1.

Fractions AD-F1 and AD-F2 were eluted with hexane 100% giving fatty acids. AD-F3 (9 g) gave a red oil as a concentrate of alpha tocopherol, this was not further purified. AD-F4 eluted with n-hexane/EtOAc (9:1 v/v) gave β-sitosterol (250 mg, white solid, 0.0066% yield) followed by a concentrate of chlorophyll in the AD-F5 eluted with n-hexane/EtOAc (4:1 v/v). AD-F6 was further purified by exclusion chromatography using Lh-20 and isopropanol giving compound AD-3 (25 mg, colorless crystals, 0.0001% yield). AD-F7 was eluted in silica gel column chromatography with n-hexane/EtOAc (3:2 v/v) giving a gummy residue which was further eluted using Lh-20 and isopropanol giving compound AD-4 (15 mg, colorless crystals, 0.00039% yield). AD-F8 was eluted with EtOAc 100% giving a dark gummy residue, formed by a mixture of sugars and lignin. The AD-4 was recrystallized by slow evaporation at 4°C from (1:1) ethyl acetate/hexane mixture, to obtain crystals over 99% purity by NMR. Pure compounds and fractions were air-dried and frozen at -20 °C until use.

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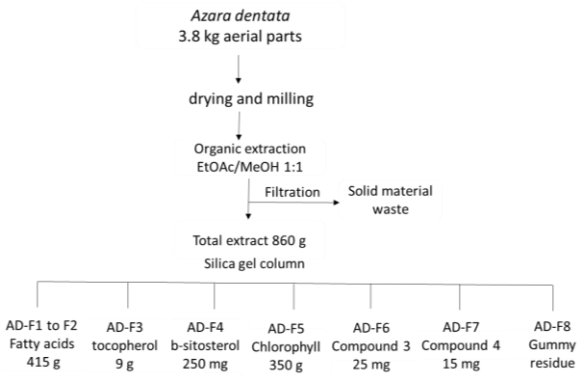

Scheme 1. Method of obtaining extracts, fractions, and compounds from aerial parts of *Azara dentata*.

Identification of natural compounds from Azara dentata

The compounds of *Azara dentata* were identified by gas chromatography mass spectrometry (CG-MS; Agilent 7890 and Agilent 5975 mass detector, California, USA), using a column capillary of silica HP5-MS of 30m x 0.25mm and a film of 0.25µm in thickness. Mass spectra were obtained from total ion current (TIC) and compared to standard mass spectra in addition to the NIST05a database (NIST 2018, National Institute of Standards and Technology). The structure of compound AD-4 was unambiguously elucidated by 1D and 2D NMR together with X-Ray Structure Analysis. The ^{1}H- and ^{13}C NMR spectra were recorded in $CDCl_3$ solution in 5 mm tubes at RT on a Bruker Avance III 500 MHz spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany), with the deuterium signal of the solvent as the lock and TMS (for ^{1}H) or the solvent (for ^{13}C) as internal standard. All spectra (^{1}H, ^{13}C, gs-H,H−COSY, edited HSQC, and gs-HMBC) were acquired and processed with the standard Bruker software.

X-Ray Structure Analysis of AD-4

Colourless crystals were obtained from a (1:1) ethyl acetate/hexane mixture, at 277 K. The X-ray diffraction data were collected at 150 K on a Bruker *APEX*-II CCD diffractometer with Mo Kα radiation (λ= 0.71075 Å).

The crystal structure was solved by direct methods and difference Fourier techniques, with non-hydrogen atoms refined on F**2 using full-matrix least squares. After being identified in difference electron density maps, hydrogen atoms were placed at idealized positions and refined as riding onto their hosts, with isotropic U_{iso}(H) = x.U_{eq}(host), x (1.2-1.5). Crystallographic data for AD-4 have been deposited at the Cambridge Crystallographic Data Centre (CCDC) under the CCDC Deposition Number 2001020. Copy of the data can be obtained, free of charge, on application to the retrieval service of the CCDC, (https://www.ccdc.cam.ac.uk/structures/).

Crystal data for AD-4

$C_{16}H_{14}O_3$, M_r = 254.27, Monoclinic, space group *P*21/c, a = 9.7290 (16) Å, b = 9.2274 (12) Å, c = 14.355 (2) Å, β = 99.856 (7) °, V = 1269.7 (3) Å^3, Z = 4, ρ_{Calcd} = 1.330 g.cm^{-3}, μ= 0.09 mm^{-1}, and *F* (0 0 0) = 536. Crystal size: 0.24 × 0.16 × 0.12 mm^3. No. of measured, independent and observed [*I*> 2σ (*I*)] reflections: 15933, 2416, 1594 [*R*int = 0.115]. Final *R and S* indexes: *R*1 [F^2> 2 σ(F^2)]= 0.066, *wR*2 = 0.152, *S* = 1.14.

Computer programs used: data collection: Bruker APEX3 [4]; data reduction: Bruker SAINT [4]; absorption correction: Bruker SADABS [4]; structure resolution: SHELXS97 [5]; structure refinement: SHELXL2018/1 [6]; graphic material: Bruker SHELXTL [5].

Microbial Strains and Culture

In the antimicrobial tests, strains of three Gram-positive bacteria were investigated (*Staphylococcus aureus* ATCC 25930, *Staphylococcus epidermidis* ATCC 14990, *Enterococcus faecalis* ATCC 51299), three Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* Boston 41501, *Klebsiella pneumoniae* NCTC 13438) and clinical strain of fungus *Candida albicans*. The species were grown at 35°C for 24 h, bacteria in tryptone soy agar (Graso, Poland), while fungus in Sabouraud dextrose agar (Graso, Poland).

Studies of minimal inhibitory concentrations (MICs)

The minimal inhibitory concentrations (MICs) of samples were determined by the micro-dilution method using the 96-well plates (Nest Scientific Biotechnology). Studies were conducted according to the CLSI, European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations, and as described in our previous publications. In study of MIC the following concentrations of samples were used: 50 mg/mL (50,000 µg/mL), 20 mg/mL, 10 mg/mL, 5 mg/mL, 2.5 mg/mL, 1.25 mg/mL, 625 µg/mL, 312 µg/mL, 156 µg/mL, 78 µg/mL and 39 µg/mL. The plates with Mueller-Hinton broth (Graso, Poland) were incubated at 35°C for 24 h. Next, 20 µL of 1% MTT water solution (Sigma-Aldrich) was added and plates were additionally incubated 2-4 hours at 35°C. The MIC value was taken as the lowest concentration of the sample that inhibited any visible microbial growth.

Clonogenic assays to evaluate cytotoxic effect of Azara dentata extract in MCF-7 cells

Azara dentata total extract was dissolved in DMSO and stored at −20°C. The solution was diluted with DMSO to obtain working concentrations. The IC_{50} determination of the *Azara dentata* extract was analyzed by clonogenic assay, the cells were grown at a density of 1.000 per well in a six wells plate and then the cells were incubated with increased concentration of the *Azara dentata* extract (0-1000 µg/mL) in RPMI media with a DMSO concentration minor to 1% (v/v). After 24 hours, the extract was removed, and the cells were grown for 14 days with refresh RPMI media. The colonies were stained with crystal violet, images were photo-documented and the total colonies number were counted for each condition.

RESULTS AND DISCUSSION

Phytochemical characterization of Azara dentata

The purification of the methanol/ethyl acetate extract from the aerial parts of *Azara dentata* were performed by silica gel column chromatography and exclusion chromatography. Thus, two fatty acids esters were obtained in the less polar fraction of the chromatography, identified as methyl palmitate and (Z,Z,Z) 9,12,15-octadecatrienoic acid methyl ester or linolenic acid methyl ester, in addition to β-sitosterol (250 mg, white solid, 0.0066% yield) and α-tocopherol. Then, two phenolic compounds were isolated, **AD-3** and **AD-4** (Figure 2). All compounds were determined by CG-MS (Figure 3) and contrasted with the chemical library NIST05a database and their available standards in the laboratory of C.P. (Table 1).

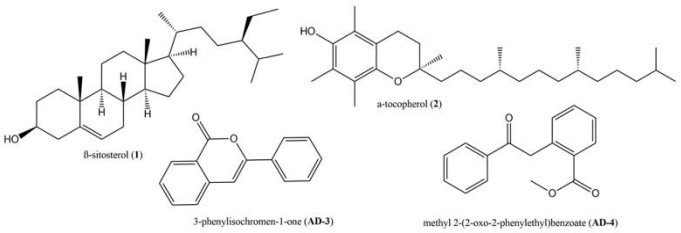

Figure 2. Molecular structures of compounds identified in *Azara dentata*.

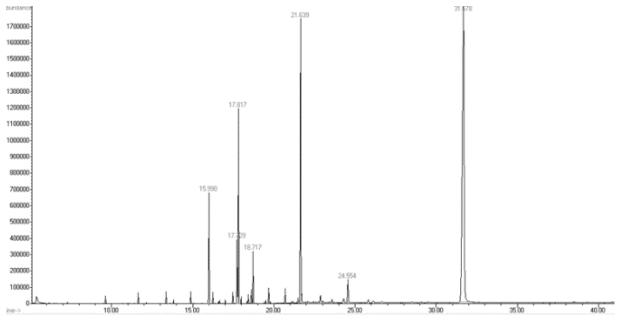

Figure 3. CG-MS chromatography of total extract of *Azara dentata*.

Table 1. CG-MS profile of total extract of *Azara dentata*.

Retention time (min)	Compound	Molecular weight	Five main ions peaks (m/z)
15.990	methyl palmitate	270	74 / 87 / 55 / 75 / 143
17.817	linolenic acid methyl ester	292	79 / 67 / 93 / 95 / 55
21.639	β-sitosterol	414	43 / 55 / 41 / 57 / 107
31.678	α-tocopherol	430	165 / 430 / 164 / 431 / 166
24.554	AD-3	222	222 / 194 / 165 / 77 / 89
18.717	AD-4	254	105 / 77 / 222 / 51 / 106

Methyl palmitate or methyl hexadecanoate is a saturated fatty acid ester with acaricidal activity to *T. viennensi*, causing disruption of membranous structures in the mitochondria [7]. While (Z,Z,Z) 9,12,15-octadecatrienoic acid methyl ester or linolenic acid methyl ester is an unsaturated fatty acid often found in vegetable oils, for instance, as part of lipid profile of *Plumbago zeylanica*, a pharmaceutically important plant of India [8], also this is a precursor in the biosynthesis of many ω3 family fatty acids in the brain [9]. Together with these nonpolar compounds, it was isolated 250 mg of β-sitosterol as a white solid in 0.0066% yield. β-sitosterol is a phytosterol, potential anti-Alzheimer's agent with inhibitory activity against AChE and BChE showing an IC_{50} value of 55 and 50 μg/ml respectively. In transgenic animals models, β-sitosterol showed gradual improvement in working memory, spontaneous alternation behavior and motor coordination [10]. α-tocopherol or vitamin E is an antioxidant and a singlet-oxygen trap, that prevents the cyclic progression of lipid peroxidation [11].

Compound **AD-3** and **AD-4** are related to the isocoumarin. Isocumarins are a family of bioactive compounds isolated from fungal and plants; for instance they have been isolated from the tubers of *Sparganium stoloniferum* [12], also from the endophytic fungus *Xylomelasma sp.* showing antibacterial and antioxidant activities [13]. The compound **AD-3** is a natural 3-phenylisocoumarin, chemically called 3-phenylisochromen-1-one was purified as colorless crystals with 0.0001% yield. **AD-3**, is structurally related to thunberginol A but without the hydroxylated rings, previously isolated from leaves of Hydrangea macrophylla, which promoted adipogenesis of 3T3-L1 cells [14]. The structure of **AD-4** was determined using NMR and single crystal x-ray structure analysis. This compound had not been reported so far as a natural product, though it appears to have been already synthesized and used as an intermediate for the obtainment of different isocoumarins [15, 16]. Furthermore, a search in the CSD database [17], for structures with a similar phen-CH_2CO-phen nucleus showed just a handful of entries (FUHVEK, JULJEF, SELZUG, UJAWIN), all of them from a synthetic origin.

AD-4 was identified as methyl 2-phenacyl-benzoate. This compound was crystallized as colorless crystals in a 0.00039% yield.^{1}H-NMR (500 MHz, in $CDCl_3$.δ in ppm. *J* in Hz): 3.68 (3H, s); 4.66 (2H, s); 7.18 (1H, m); 7.30 (1H, dt, J= 1.6, 0.9); 7.42 (3H, m); 7.51 (1H, m); 7.99 (3H, m). ^{13}C-NMR (500 MHz, in $CDCl_3$, δ in ppm): 45.1 (q); 52.1 (t); 127.4 (d); 128.3 (d); 128.8 (d); 129.8 (s); 131.3 (d); 132.5 (d); 132.7 (d); 131.1 (d); 137.1 (s); 137.3 (s); 167.6 (s); 197.4 (s).

X-Ray Structure Analysis of AD-4

Figure 4 shows an ellipsoid plot (40% probability level) of **AD-4**. The molecule can be basically described as consisting of three planar sections of (A, B and C in Figure 4). Deviation from the overall planarity (H atoms not considered) is due to rotations around two Cp3—Cp3 bonds, C07—C08 (torsion angle C06 C07 C08 C09 = -162.1°), and C14—C15 (torsion angle C09—C14—C15—O1 = -15.5°) ending up with the aromatic ring planes A—B at 86.0° from each other. Non covalent interactions are few and weak, as expected from the absence of good H-bonding donors. All the H-bonds are of the C—H···O type. Two of them are intra-molecular, and serve to clamp the eventual rotation around the C07—C08 and C14—C15 single bonds (Figure 4). The remaining one links molecules in a herringbone pattern defining strips along [010].

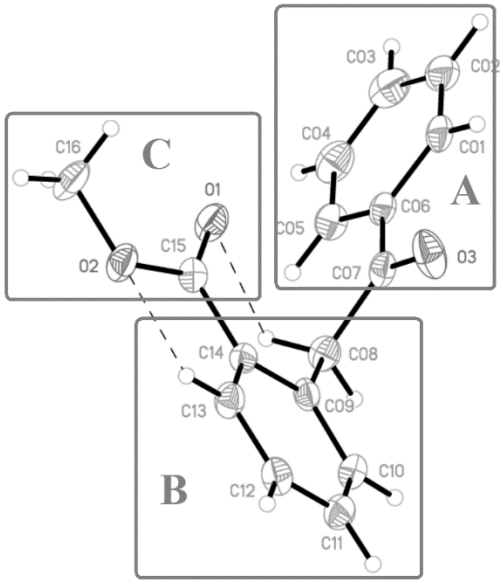

Figure 4. Molecular structure of AD-4, as determined by x-ray diffraction. Broken lines stand for intramolecular H-bonds.

Anti-bacterial Activity Assay

The antibacterial activity was evaluated *in vitro* against the Gram-positive bacteria *Staphylococcus aureus* ATCC 25930 and *Staphylococcus epidermidis* ATCC 14990 showing a moderate activity with minimum inhibitory concentration values of 5.0 and 10 mg/mL respectively. No activity up to 10 mg/mL was shown against three Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* Boston 41501, *Klebsiella pneumoniae* NCTC 13438) and clinical strain of fungus *Candida albicans*, indicating that the isolated compounds do not display promissory antibacterial activity.

Cytotoxic effect of Azara dentata extract

The cytotoxic effect of *Azara dentata* extract was evaluated on breast cancer cells MCF-7 by clonogenic assay. The results showed that the MD extract reduced significantly the cell viability of MCF7 cells by 11, 33, 81, 98, 99 and 100 %, at concentrations of 5, 10, 50, 100, 500, 1000 μg/mL respectively, (Figure 5).

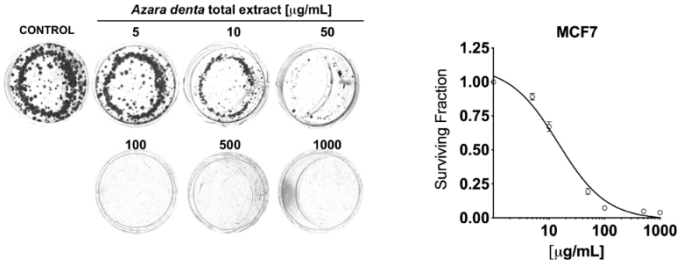

Figure 5. *Azara dentata* extract has cytotoxic effects in human breast cancer cell MCF-7. The cytotoxicity was evaluated by clonogenic assay in MCF-7 cells treated with 5 – 100 μg/mL of *Azara dentata* extract by 24 hours. The IC_{50} was calculated using GraphPad prism software.

The cytotoxic activity of *Azara dentata* in MCF-7 cancer cells, showed an IC_{50} of 15.63 μg/mL. This activity values, just for a total extract, turns to *Azara dentata* and compounds **AD-3** and **AD-4** as a promissory source of anti-cancer agents, which deserve more attention to their activities on breast cancer.

CONCLUSION

Azara dentata Ruiz & Pav. is a native Chilean shrub appreciate for the production of honey. The variety of honey types depend of the plant species where nectar is collected by bees, in Chile, honeys of species *Azara* commonly known as corcolén honeys, showed a high content of total phenol with beneficial

properties for human health, but there is no information about phytochemicals produced by the plant. In this study, we determinate by first time the phytochemical and bioactive study of *Azara dentata*, results showed that leaves of the plant have high content of vitamin E together with isocoumarins as secondary metabolites in minor proportions. Isocoumarins and stilbenoids derivatives have shown a broad spectrum of bioactivities, such as, antibacterial and antioxidant [13], anti hyperglycemic [18] as well as, anti-cancer and antifungal activities, reviewed by Hussain and Green 2017 [19] and Saeed 2016 [20]. Interestingly, we found a precursor of isocoumarin, **AD-4,** its structure was determined using NMR and single crystal x-ray structure analysis. This compound had not been reported so far as a natural product, though it appears to have been already synthesized and used as an intermediate for the obtainment of different isocoumarins [15]. Moreover, *A. dentata* displays moderate antibacterial activity against *S. aureus* and *S. epidermidis*, while the cytotoxic activity against MCF-7 cancer cells is promissory, with an IC_{50} acceptable for a total extract, suggesting that *Azara dentata* is a source of anticancer agents, which deserve deeper pharmacological studies.

ACKNOWLEDGMENTS

This research was funded by ANID of Chilean Government by the grant number 11181076 to CP. The authors thank to Professor Bernd Schmidt and Dr. Matthias Heydenreich from Universität Potsdam, Institut für Chemie, Germany, for their valuable assistance.

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