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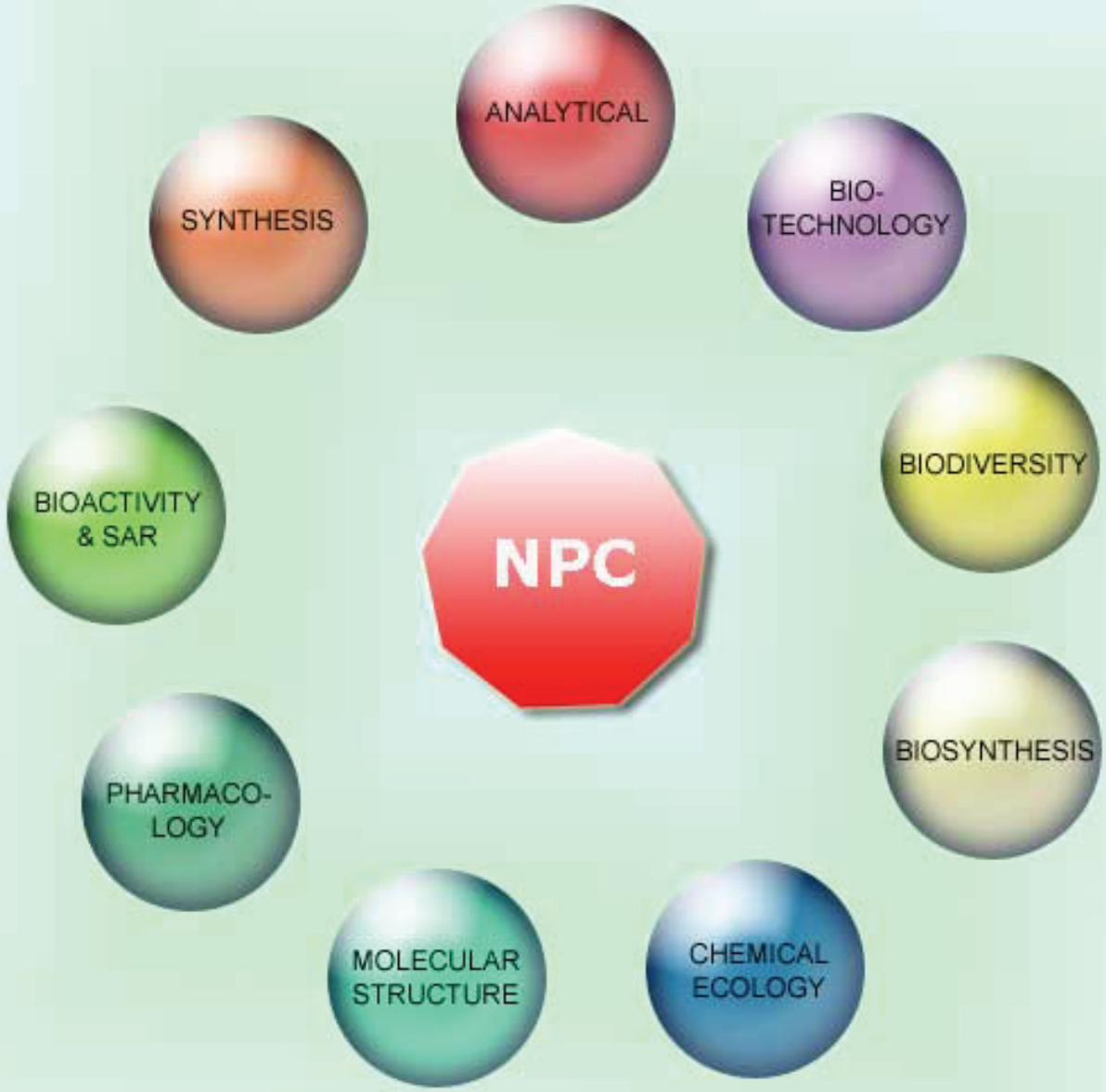

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Phytotoxic Lignans from *Artemisia arborescens*

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A systematic bioassay-guided fractionation of methylene chloride extracts of the aerial part of *Artemisia arborescens* was performed in order to identify its phytotoxic compounds. Two lignans were isolated, sesamin and ashantin, that inhibited growth of *Agrostis stolonifera* (bentgrass), a monocot, and *Lactuca sativa* (lettuce), a dicot, at 1 mg mL^{-1}. In a dose-response screening of these lignans for growth inhibition against *Lemna paucicostata* (duckweed), ashantin was the most active with an IC_{50} of ca. 224 μM. The mode of action of these compounds is still unknown. In mosquito larvicidal bioassays the pure compounds sesamin and ashantin did not cause mortality at the highest dose of 125 mg/L against 1-d-old *Aedes aegypti* larvae. In bioautography bioassays for antifungal activity using *Botrytis cinerea, Fusarium oxysporum, Colletotrichum fragariae, Colletotrichum acutatum,* and *Colletotrichum gloeosporioides*, ashantin and sesamin were inactive at 5 μg and were therefore not subjected to additional screening in secondary antifungal assays.

Keywords: *Artemisia arborescens*, Allelopathy, Herbicidal, Phytotoxicity, Lactone, Lignans, Ashantin, Sesamin.

Artemisia are the most numerous species within the *Asteraceae* (*Compositae*) family [1, 2]. The genus *Artemisia* includes more than 500 annual, biennial and perennial species which are herbs or small shrubs, mainly distributed in the temperate zones of the Northern hemisphere [3-5]. Known also as silver sage, large wormwood, tree wormwood and other common names, *Artemisia arborescens* (Vaill.) L. is a typical species of the Mediterranean wild flora which usually grows in full sun exposure and is very tolerant of heat and drought conditions. It is a perennial shrub from 1 to 2 m tall, with silver grey-green, deeply divided leaves and clusters of inconspicuous yellow flowers that appear throughout late spring until the summer, depending on the environmental conditions [6].

Secondary compounds of *A. arborescens* have antimicrobial, antiviral, pharmaceutical, insecticidal, and insect repellent activity [*e.g.*, 7-12]. Relatively little research has been done on determination of phytotoxic compounds from *A. arborescens*. *A. arborescens* has been reported to produce phytotoxic compounds [13], but the exact compounds have not been reported. Only the identity of twenty compounds in the most phytotoxic fraction (*n*-hexane) of extracts of the plants shoots was provided. The phytotoxicity of any of the constituents alone was not provided. This was also the case in a study of the phytotoxicity of extracts of *A. arborescens* leaf litter by the same group [14]. No phytotoxicity bioassay-guided isolation of compounds from this species has been done that fractionates the activity to single compounds. This type of study has the potential to lead to the discovery of new compounds, and the genus *Artemisia* is known to have species-specific phytotoxins (*e.g.*, artemisinin [15]) that would not be found by using only GC/MS or LC/MS to identify known compounds. The objective of this study was to find the most potent phytotoxins in *A. arborescens* with bioassay-guided isolation down to the single compound level.

Most of the fractions of the fractionation scheme had little phytotoxicity to lettuce or bentgrass. Fractions 3 and 8 had the strongest inhibition of bentgrass growth, while little effect was observed on lettuce. These fractions were found to be the lignans sesamin and ashantin (Figure 1), respectively. These purified compounds were assayed with a more quantitative bioassay with duckweed (Figures 2 and 3), yielding, IC_{50} values of ca. 401 and 224 μM for sesamin and ashantin, respectively. With this bioassay, these IC_{50} values are in the same range as those for the commercial herbicides naptalam (128 μM), glyphosate (388 μM), and clomazone (126 μM) [16].

Sesamin has been previously reported in *A. arborescens* [13, 14, 17-19] and ashantin has also been found in *A. arborescens* [17, 18]. Both compounds are found in several *Artemisia* species [17]. Araniti *et al*. [13, 14] reported sesamin in a phytotoxic hexane extract, but did not test its phytotoxicity alone. Sesamin has been previously reported as a phytotoxin. Tonelli *et al*. [18] reported sesamin and another lignan, kobusin, to be in a more phytotoxic fraction of *Virola sebifera* against lettuce. This conflicts with our finding of little activity of sesamin against lettuce, but their assay was done with a fraction containing both lignans, and there was no indication of what the concentration of sesamin was in their extract. We find no previous mention of ashantin as a phytotoxin.

Some other lignans are known to be phytotoxic. For example, the aryltetralin lignans podophyllotoxin, α-peltatin, and β-peltatin are active against both dicotyledonous and monocotyledonous plants by interfering with formation of mitotic microtubular organizing centers [20]. Ten lignans from *Helianthus annuus* were reported to be phytotoxic [21]. The results reported in the present paper add to what is known of the phytotoxicity of lignans. Ashantin and sesamin showed good phytotoxic activity against bentgrass; further investigations are necessary about their mode of action, along with additional assays against monocot weeds.

Secondary to our study of phytotoxins, pure compounds ashantin and sesamin were also evaluated for antifungal activity against *Botrytis cinerea, Fusarium oxysporum, Colletotrichum fragariae, Colletotrichum acutatum,* and *Colletotrichum gloeosporioides*. Five days after inoculation, inhibition zones were measured. Ashantin and sesamin were inactive at the threshold value of 5 μg, and were therefore not subjected to additional screening in secondary assays.

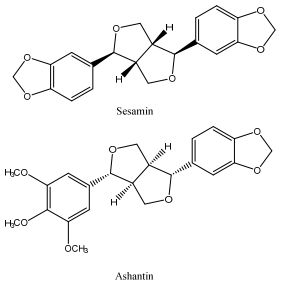

Figure 1: Structures of sesamin and ashantin.

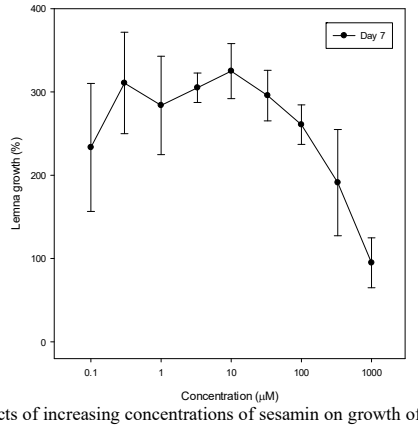

Figure 2: Effects of increasing concentrations of sesamin on growth of *L. minor* after 7 days of exposure. Bars represent the ± standard error of each mean.

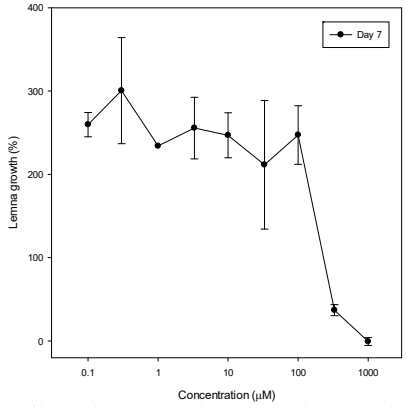

Figure 3: Effects of increasing concentrations of ashantin on growth of *L. minor* after 7 days of exposure. Bars represent the ± standard error of each mean.

Lastly, pure compounds ashantin and sesamin were also evaluated for larvicidal activity against *Aedes aegypti*. Sesamin and ashantin did not show any larvicidal activity at the highest dose of 125 mg/L against 1-d-old *Aedes aegypti* larvae.

Experimental

Instrumentation: NMR spectra (H and ^{13}C) were recorded in $CDCl_3$ on a Varian ANOVA 400 MHz spectrometer. Column chromatography was carried out using a Biotage, Inc. Isolera One pump equipped with a flash collector and a photo-diode array detector. Fractions and purified compounds were analyzed by GC/MSD on an Agilent Technologies 7890A GC system coupled to a 5975C Inert XL MSD. The GC was equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm, film thickness of 0.25 μm) operated using the following conditions: injector temperature, 240°C; column temperature, 60–240°C at 3°C/min then held at 240°C for 5 min; carrier gas, He; injection volume, 1 μL (splitless). The MS mass range was from 40 to 650 m/z, a filament delay of 3 min, target TIC of 20,000, a prescan ionization of 100 μs, an ion-trap temperature of 150°C, a manifold temperature of 60°C, and a transfer line temperature of 170°C. High-resolution mass (ESI-MS) spectra of isolated compounds in MeOH were acquired by direct injection of 20 μL of sample (approximately 0.1 mg mL-1) in a JEOL USA, Inc. (Peabody, MA) AccuTOF (JMS-T100LC).

Plant material: Shoots of *A. arborescens*, were collected from near Altofonte (N 38°2'27.182'' E 13°18'25.555''), Palermo, Italy, on June, 2016 and dried at room temperature for one week. A voucher specimen (number PAL109315) was deposited at the Herbarium Mediterraneum Panormitanum.

Plant extraction: A 200 g sample of dried material was ground to a powder and soaked in 2.5 L of dichloromethane (DCM) for 60 h. It was then filtered and dried in two steps, first by rotary evaporator and then under nitrogen. Product yield was 13.1 g of crude extract, which was stored at 0°C until use.

Phytotoxicity-guided fractionation: Five grams of DCM extract were dissolved in 200 mL of MeOH/H_2O, 90:10 (v/v) and subjected to a modified Kupchan [22] liquid/liquid partitioning with 200 mL of *n*-Hexane, for three times, obtaining 2.13 g of hexane extract. Hence, to the remaining partition 57 mL of distilled de-ionized (DDI) water was added to make the solution MeOH/H_2O, 70:30 (v/v). Then it was partitioned with 200 mL of $CHCl_3$, for 3 times, obtaining 2.63 g of $CHCl_3$ extractables. The remaining MeOH was removed by rotary evaporator. The water was removed using a freeze dryer.

The three extracts were dried, as above, and re-tested. The chloroform extract had the highest phytotoxic effects. Hence, 1 g of $CHCl_3$ extract was separated by a flash chromatography system: Biotage (Isolera One), through Cartridge SNAP 100 g running at a 40 mL/min of flow rate, using a hexane: EtOAc step gradient beginning with 100:0 to 70:30 over 2400 mL, followed by 50:50 over 600 mL, then 0:100 over 400 mL.

One hundred and seventy-two fractions (in racks of 22 mL each), plus column wash by MeOH, were collected and combined in thirteen fractions which were assayed: 1 (1.52 mg), 2 (0.42 mg), 3 (1.73 mg), 4 (1.93 mg), 5 (2.62 mg), 6 (2.78 mg), 7 (3.84 mg), 8 (4.07 mg), 9 (21.13 mg), 10 (8.19 mg), 11 (10.10 mg), 12 (7.60 mg), 13 (29.94 mg). Fraction #3 and #8 had the most significant inhibition of bentgrass growth. Little effect was observed on lettuce.

Phytotoxicity Bioassays: The initial bioassay was done with both a monocot and dicot species and slightly modified from Dayan *et al*. [23]. Lettuce (*Lactuca sativa* L.) Iceberg A Crisphead cv. seeds from Burpee Seeds (W.Atlee Burpee & Co., Warmister, PA) were used. Creeping bentgrass (*Agrostis stolonifera* L. cv. Penncross) seeds were obtained from Turf Seed, Inc. of Hubbard, OR. All seeds were surface-sterilized prior to use by mixing in a 5 to 10 % sodium hypochlorite solution for approximately 10 min. Seeds were thoroughly rinsed with deionized water from a Millipore System, and air-dried in a sterile environment. All bioassays were done in duplicate in sterile non-pyrogenic polystyrene 24-well cell culture plates (CoStar 3524, Corning Incorporated). One filter paper disk

(Whatmann Grade 1, 1.5 cm) was placed in each well. The control wells contained 200 µL of DDI water. The control + solvent well contained 180 µL of water and 20 µL of the solvent. All sample wells contained 180 µL of water and 20 µL of the appropriate dilution of the test solution. Water was always pipetted into the well before the sample or solvent. All plate preparation was done in a sterile environment to reduce chances of any possible contamination. When preparing lettuce plates, five seeds were placed in each well. Lids were sealed with parafilm. The plates were incubated in a Percival Scientific CU-36L5 incubator under continuous light conditions at 26°C and 120 µmol $s^{-1}m^{-2}$ average photosynthetically active photon flux.

Plates were incubated for at least 7 days. A subjective ranking of plant growth was used, based on a scale from 0 to 5, where 0 indicated no apparent inhibition (sample well plants looked identical to the control + solvent well plants), and 5 indicated no growth or complete inhibition. A ranking of 5 was given only when no seed germinated.

For a more quantitative assay of isolated, pure compounds, the duckweed bioassay of Michel et al. [16] was slightly modified. *Lemna paucicostata* Hegelm. (duckweed) was grown in Hoagland's No. 2 Basal Salt Mixture (Sigma H2395) (1.6 g/L) with added iron (1 ml of 1000X FeEDTA solution to 1 L of Hoagland media). The 1000X iron solution contained 18.355 g/L of Fe-EDTA. The pH of the media was adjusted to 5.5 with 1 N NaOH. The media was filter sterilized using a 0.2 µm filter and stored in sterile 1 L bottles. The duckweed stocks were grown in approximately 100 mL of media in sterile baby food jars with vented lids. Duckweed stocks were started from one or two three-frond plants and grown in a Percival Scientific CU-36L5 incubator under continuous light conditions at 26°C and 120.1 µmol $s^{-1}m^{-2}$ average light intensity. Media was changed every 2 to 3 days or new stocks were prepared in fresh media. Plant doubling time was approximately 24 to 36 hours.

Screening and Replicate Series Tests: both screening and replicate series test were conducted using non-pyrogenic polystyrene sterile 6-well plates (CoStar 3506, Corning Incorporated). Each well contained 4950 µL of the Hoagland's media plus 50 µL of water, or the solvent, or the compound dissolved in the appropriate solvent (at a concentration of 100x). Final concentration of the solvent was therefore approximately 1 % by volume.

Frond growth was determined with a Lemnatec Scanalyzer PL (Aachen, Germany) image analysis instrument which made a photograph of each well daily. The software is an image analysis system and detects the fronds as they lay on the surface of the media. It can assess frond numbers and quantify colors as well as frond areas. A color scheme was set up initially so that the software would categorize different colors. The software then measures the area that it sees: plant areas at day 0 and 7 and varied days in between. The LemnaTec results are saved as .csv files, showing frond number and total frond area. The averages of the triplicates were plotted along with the standard deviation using the SigmaPlot software program. Results are shown as duckweed growth (% increased frond size) versus log concentration (µM). I_{50} values were determined with R software.

Mosquito larvicidal bioassays: Bioassays were conducted using the system described by Ali *et al*. [24] to determine the larvicidal activity of pure compounds, sesamin and ashantin against *Ae. aegypti*. Five 1-d-old larvae were transferred to individual wells of a 24-well tissue culture plates in a 30-40 µL droplet of water. Fifty µL of larval diet of 2% slurry of 3:2 beef liver powder (Now Foods, Bloomingdale, Illinois) and Brewer's yeast (Lewis Laboratories Ltd., Westport, CT) and 1 mL of deionized water were added to each well by using a Finnpipette stepper (Thermo Fisher, Vantaa, Finland). Sesamin and ashantin were diluted in DMSO. Eleven microliters of the test chemical was added to the labeled wells, while 11 µL of DMSO was added to the control treatments. After treatment application, the plates were swirled in clock-wise and counter clockwise motions and front and back and side to side five times to ensure even mixing of the tested compounds. Permethrin (positive standard, 46.1% *cis* – 53.2% *trans*, Chemical Service, West Chester, PA, USA) at 0.025 ppm gave 100% mortality in the screening bioassays. Larval mortality was recorded 24 h post treatment.

Fungal Pathogen production and inoculum preparation: Isolates of *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. & Sacc. in Penz. were obtained from B. J. Smith, USDA- ARS, Small Fruit Research Station, Poplarville, MS. The three *Colletotrichum* species were isolated from strawberry (*Fragaria x ananassa* Duchesne). *Botrytis cinerea* Pers. Fr was isolated from commercial strawberry. *Fusarium oxysporum* Schlechtend. Fr was isolated from orchid (*Cynoches* sp.). Fungal cultures were initiated on ½ strength potato dextrose agar (PDA, Difco, Detroit MI) from spores stored in sterile 10% glycerol RPMI (Roswell Park Memorial Institute, Gibco) buffer with MOPS (3-(N-morpholino) propane sulfonic acid) at -80°C. Fifty µL suspended spore solution was inoculated on PDA plate using crossed inoculation method. Inoculated plates were incubated at 24 ± 2°C under cool-white fluorescent lights (55 ± 5 µmols/m^2/s) with a 12h photoperiod. *Colletotrichum* cultures were subcultured or harvested from PDA every 7-10 days. Conidia were harvested by flooding plates with 3-5 mL of sterile distilled water and dislodging conidia by softly brushing the colonies with an L-shaped glass rod. Conidial suspensions were filtered through sterile Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA) spread on a sterile funnel to remove mycelia. Conidia suspensions were adjusted with sterile DDI water to a concentration of 1.0×10^6 CFU (colony forming units)/mL. Conidia concentrations were determined photometrically from a standard curve based on the percent of transmittance (%T) at 625 nm. Conidial suspensions of each fungal species were then adjusted to a concentration of 3.0×10^5 conidia/mL with liquid potato-dextrose broth (PDB, Difco).

Direct Bioautography: This method provided a simple technique to visually follow antifungal components through the separation process. Matrix, one-dimensional, and two-dimensional bioautography protocols on silica gel TLC plates with fungi as the test organisms are used to identify the antifungal activity according to published bioautography methods [25-27]. Direct bioautography is a successful technique to pre-screen large numbers of crude extracts [28], or pure compounds [29]. Bioautography is especially important in evaluating lipophilic extracts and determining the number of active compounds in an extract [30-31].

After sample application and solvent evaporation to provide a solid amount of compound or pure compound applied, each TLC plate was subsequently sprayed with a spore suspension (3.0×10^5 spores/mL) of the fungus of interest and incubated in a moisture chamber for 4 days at 26°C with a 12 h photoperiod. Clear zones of fungal growth inhibition on the TLC plate indicated the presence of antifungal constituents in each extract or pure compound. Bioautography experiments using fungi were used to drive the bioassay guided fractionation and isolation of pure compounds, sesamin [32] and ashantin [33]. Means of inhibitory zone size and

standard deviations, when available were used to evaluate antifungal activity of extracts and pure compounds. Commercial technical grade azoxystrobin, captan, benomyl and cyprodinil (without formulation) were used as controls.

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