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ALLELOCHEMICAL POTENTIAL OF *Callicarpa acuminata*

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Abstract—The allelochemical potential of *Callicarpa acuminata* (Verbenaceae) was investigated by using a biodirected fractionation study as part of a long-term project to search for bioactive compounds among the rich biodiversity of plant communities in the Ecological Reserve El Eden, Quintana Roo, Mexico. Aqueous leachate, chloroform-methanol extract, and chromatographic fractions of the leaves of *C. acuminata* inhibited the root growth of test plants (23–70%). Some of these treatments caused a moderate inhibition of the radial growth of two phytopathogenic fungi, *Helminthosporium longirostratum* and *Alternaria solani* (18–31%). The chloroform-methanol (1:1) extract prepared from the leaves rendered five compounds: isopimaric acid (**1**), a mixture of two diterpenols [sandaracopimaradien-19-ol (**3**) and akhdarenol (**4**)], α-amyrin (**5**), and the flavone salvigenin (**6**)]. The phytotoxicity exhibited by several fractions and the full extract almost disappeared when pure compounds were evaluated on the test plants, suggesting a synergistic or additive effect. Compounds (**4**), (**5**), and the semisynthetic derivative isopimaric acid methyl ether (**2**) had antifeedant effects on *Leptinotarsa decemlineata.* Compound **5** was most toxic to this insect, followed by (**2**), (**4**), and (**6**) with moderate to low toxicity. No correlation was found between antifeedant and toxic effects on this insect, suggesting that different modes of action were involved. All the test compounds were cytotoxic to insect Sf9 cells while (**6**), (**4**), and (**1**) also affected mammalian Chinese Hamster

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Ovary (CHO) cells. Compound **5** showed the strongest selectivity against insect cells. This study contributes to the knowledge of the defensive chemistry and added value of *C. acuminata.*

Key Words—*Callicarpa acuminata,* Verbenaceae, allelochemicals, isopimaric acid, α-amyrin, sandaracopimaradien-19-ol, akhdarenol, salvigenin, bioactivity.

INTRODUCTION

Callicarpa acuminata H. B. K. (Verbenaceae) commonly known as alahualte, elté, chachalaca herb, uvilla, patsumacáshil, and granadilla (Martínez, 1979), is distributed in Mexico from Tamaulipas, Veracruz, Chiapas, Oaxaca, to the Yucatan Peninsula. *C. acuminata* is a shrub or a small tree up to 6 m in height and abundant in secondary plant communities in Quintana Roo (Cabrera Cano et al., 1982). Economically, the family is perhaps most important for its teak (*Tectona grandis*) lumber. A number of genera, such as *Callicarpa* and *Lantana*, are important ornamental species.

This study on *Callicarpa acuminata* is a part of long-term research project on bioactive compounds from the rich biodiversity of different plant communities present in the Ecological Reserve El Eden, Quintana Roo, Mexico. El Eden is located in the Yalahau region in the north of Quintana Roo. This region includes more than 250,000 ha of well-preserved natural environments that represent the main ecosystems of the Yucatan Peninsula and the Caribbean. Chemical diversity studies have been performed on the plant communities in this area as have taxonomic and systematic studies in light of the importance of biological, ecological, and evolutionary information contained therein.

Some chemical studies have already been done on other species of the genus *Callicarpa*. *C. macrophylla* from Calcutta, for example, is used in traditional medicine to relieve stomach disorders and rheumatism. Talapatra et al. (1994) identified L(+)-α-amino-β-(p-metoxyphenyl)-propionic acid, calliterpenone and its monoacetate, β-sitosterol, β-sitosterol-β-D-glucoside, ursolic acid, 2α-hydroxy-ursolic acid, crategolic acid, 7-O-glucuronides of luteolin and apigenin, and 5,4′-dihydroxy-3,7,3′-trimethoxy-flavone from the leaves of *C. macrophylla*. In addition, calliphyllin, betulinic acid, and 5,4′-dihydroxy-3,7-dimethoxy-flavone were isolated and identified from the leaves of this species. Agrawal et al. (1996) described the complete spectral assignment of calliterpenone and related phyllocladane diterpenoids. Xu et al. (2000) isolated four new clerodane-type diterpenoids (pentandranoic acids A–C and pentandralactone) from the leaves of *C. pentandra.* Hu et al. (2002) described the isolation and identification of 14-α-hydroxy-7,15-isopimaradien-18-oic acid, 16-α-17-dihydroxy-3-oxophyllocladane, 8,11,13,15-abietatetraen-18-oic acid, and 6-α-hydroxy nidorellol from *C. pedunculata.* However, the biological activities of these compounds have yet

to be studied in detail, although Kawazu et al. (1967a,b) reported that the tricarbocyclic diterpene callicarpone from *C. candicans* was toxic to fish. In addition, Hayashi et al. (1997) observed that a flavone (5,6,7-trimethoxyflavone) isolated from *C. japonica* exhibited a relatively high inhibitory effect on herpes simplex virus type 1 (HSV-1), human cytomegalovirus, and poliovirus. Tellez et al. (2000) reported some bioactive compounds isolated from the essential oil of *C. americana.* This oil was selectively toxic to *Oscillatoria perornata*, a cyanobacterium, and was only slightly phytotoxic or antifungal.

Cruz-Ortega et al. (2002) examined the effect of the aqueous leachate (1%) of *C. acuminata* on protein expression, catalase activity, free radical production, and membrane lipid peroxidation in roots of bean, maize, and tomato. The results showed that the allelochemical stress produced by the mixture of compounds in *C. acuminata* aqueous leachate modified the expression of various proteins in the roots of all treated plants. Catalase activity, free radicals, and peroxidation were increased in some treated roots.

The first biodirected phytochemical study of *Callicarpa acuminata* was aimed at identifying its main bioactive and defensive secondary metabolites against plants, phytopathogenic fungi, *Artemia salina*, insects, and insect and mammalian cell lines.

METHODS AND MATERIALS

Plant Material. Leaves of *Callicarpa acuminata* (Verbenaceae) collected from El Eden Ecological Reserve in 1996 (L.M. Ortega-Torres 2641, Sept. 1996. UCR Herbarium) were air-dried and finely fragmented.

General Experimental Procedures ^{1}H NMR and ^{13}C NMR spectra were recorded on Varian VXR-300 S or Varian VXR-500 S spectrometers. IR spectra were obtained using NaCl discs (oils films) on a Perkin-Elmer 599 B spectrophotometer. Melting points were determined via a Fisher Johns apparatus and are uncorrected. GC-MS analyses were conducted on an Hewlett-Packard Model 5890 gas chromatograph interfaced with a JEOL JMS AX50HA.

HPLC. A Waters binary gradient analytical HPLC consisting of 2 Model 6000A pumps, a Model 680 gradient module, and a Shimadzu SPD-MIOAVP photodiode array detector was used. Column: L-column ODS, 5 micron, 4.6 $\times$ 250 m. Gradient: 65 percent A-95 A, solvent A-acetonitrile/methanol (1:1/v:v); solvent B-water, with flow rate 1.5 ml/min. The effluent was monitored by a UV detector at 214 nm and retention times for compounds **3** and **4** were 21 and 21.7 min, respectively.

A Rainin auto preparative HPLC equipped with 3 Rabbit HP pumps, Erma ERC 3320 degasser, Erma ERC 7510 refractive index detector, Knauer 87 variable wavelength UV/Vis monitor, and MacRabbit controller was used for preparative

HPLC. Conditions for the HPLC were as follows: column Vydac HS-C18, 5 μm, 1×25 cm, solvent 70 percent (1:1 acetonitrile/methanol)/water, flow rate 5 ml/min, detection RI and UV (220 nm). Retention times for compounds **3** and **4** were 62 and 66 min, respectively.

Chemicals and Solvents. Analytical and preparative TLC was performed on precoated silica gel 60 F_{254} plates (Merck). Argentation TLC was carried out with silica gel plates impregnated with a 10% solution of $AgNO_3$. TLC spots were visualized by spraying with a 10% solution of $Ce(SO_4)_2$ in 2N H_2SO_4, followed by heating at 100°C. For open CC, silica gel 60 (70–230 mesh, Merck) was used.

Extraction Procedures. Three kilograms of dried leaves of *C. acuminata* were extracted with a total volume of 15 l of a $CHCl_3$/MeOH (1:1) mixture three times (72 hr each time). The extract was filtered and concentrated *in vacuo* to yield 415 g of dry extract. The extract was fractionated using an open chromatography column on silica gel (2.5 kg) and eluted with a gradient of hexane, chloroform, and methanol. A total of 250 primary fractions were obtained. Similar fractions were pooled using thin layer chromatography (TLC) to obtain 19 fractions (B–T).

Preparation of Aqueous Leachates. Two grams of leaves were soaked for 3 hr in 100 ml of distilled water. The leachates were filtered through Whatman paper No. 4 and a Millipore membrane. The osmotic pressure was measured with a freezing-point osmometer (Osmette A. Precision System, Inc.). The values ranged from 15 to 17 mosm/l.

Bioassays with Seeds. Aqueous leachates were mixed (1:1) with 2% pure agar to obtain a 1% w/v test solution. Pure agar (1%) and 2,4-dichlorophenylacetic acid (2,4-D, 100 μg/ml) were used as controls. Germination and seedling root growth bioassays for the aqueous leachates were carried out according to procedures published previously (Anaya et al., 1990) using *Amaranthus hypochondriacus* L. (Amaranthaceae), *Echinochloa crus-galli* (L.) Beauv. (Poaceae), and *Lycopersicon esculentum* Mill. (Solanaceae) as test species. *A. hypochondriacus* seeds were purchased from a local market in Tulyehualco, Mexico, D. F. Seeds of *E. crus-galli* were from Valley Seed Service, Fresno, California, USA, and *L. esculentum* seeds from Semillas Berentsen, Celaya, Guanajuato, Mexico. Bioassays were set up in 6-cm Petri dishes. Ten seeds of each test plant were sown directly on to the agar of each Petri dish following a completely random design with four replicates. Petri dishes were kept in darkness at 27°C. Root lengths were measured 24 hr after treatment for *A. hypochondriacus*; 48 hr for *E. crus-galli*, and 72 hr for *L. esculentum*. Data were analyzed by ANOVA and Tukey's tests (Mead et al., 2002). Bioassays with organic extracts and compounds were set up using the same conditions as for aqueous leachates, but with filter paper as a substrate. The filter paper (Whatman 42) was impregnated with 1.5 ml of the solutions at 100 μg/ml (or 2,4-D) and, after total evaporation of the solvents, it was moistened with 1.5 ml of distilled water. Test seeds and time of harvesting for these bioassays were the same.

Bioassays with Phytopathogenic Fungi. Organic extract and isolated compounds were tested on the growth of some phytopathogenic fungi with economic importance, such as *Alternaria solani* and *Helminthosporium longirostratum.* The bioassays were performed by incubating, in 6-cm Petri dishes, a fungal bud on potato-dextrose-agar (PDA) sterile medium. The medium contained the respective treatment. Extracts and fractions were evaluated at 100 μg/ml. Isopimaric acid and isopimaric acid methyl ether were evaluated at 25, 50, 150, 250, and 500 μg/ml, and Captan (50 *N*-(trimetil)tiol-4 ciclohexen, 1,2 dicarboximide), a commercial fungicide, was used at the same concentrations as a control. The bioassay was performed by following a completely random design with four replicates. Fungi were incubated in darkness at 27°C. The effects of treatments were determined by measuring the radial growth of the mycelium after 72 hr of incubation. Two perpendicular measurements of colony diameter were taken and the mean value was calculated. Results were analyzed by ANOVA and Tukey's statistical tests (Mead et al., 2002).

Brine Shrimp Lethality Test. Organic extracts and pure compounds were evaluated for lethality to brine shrimp (*Artemia salina*) larvae (BST). This crustacean is a good indicator to test for bioactive compounds that can be used as insecticides or cytotoxic substances (Meyer et al., 1982). Eggs of *A. salina* were hatched by incubating them in saline water during 48 hr at 30°C with light.

Twenty mg of each treatment were dissolved in 2 ml of an appropriate solvent. From each solution, aliquots of 500, 50, and 5 μl were added to vials in triplicate. Solvent was evaporated to dryness at room temperature or by vacuum. Five ml of saline water and 10 larvae were added to each vial. Vials were maintained at room temperature and light for 24 hr and lethality was subsequently measured. LC_{50} values of <1000 μg/ml were the activity standard for extracts or chromatographic fractions. LC_{50} values of <200 μg/ml were the activity standard for pure compounds (Anderson et al., 1991). In all bioassays, control treatments contained only saline water.

Insect Bioassays. Leptinotarsa decemlineata and *Spodoptera littoralis* colonies were reared on potato foliage, artificial diet (PoitOut and Bues, 1970) and bell pepper (*Capsicum annuum*) plants, respectively, and maintained in a growth chamber at 22 ± 1°C, >70% RH with a photoperiod of 16:8 (L:D).

Feeding Preference Assays. These experiments were conducted with adult *L. decemlineata*, newly emerged fifth-instar, and *S. littoralis* larvae. Percent feeding inhibition (% FI $= [1-(T/C)] \times 100$, where $T =$ surface of treated leaf disks and $C =$ surface of control leaf disks) was calculated as previously described (Reina et al., 2001). Compounds with FI > 70% were tested in a dose–response experiment to calculate their relative potency (EC_{50} values, the effective dose for 50% feeding reduction), which was determined from linear regression analysis (%FI on log dose).

Oral Cannulation. This experiment was performed with preweighed newly molted *S. littoralis* L6-larvae as previously described (Reina et al., 2001). At the end of the experiments (72 hr), the relative consumption rate (RCR) and the relative growth rate (RGR) were calculated on a dry weight basis according to Farrar et al. (1989). All dry larval weight measures were log-transformed prior to ANOVA to test treatment effects.

Hemolymph Injection. Twenty adult *L. decemlineata* beetles were injected with DMSO solutions of the test compounds (5 μg/insect) as described (Reina et al., 2001). Beetle mortality was recorded up to 3 days after injection. Percent mortality was analyzed with contingency tables and the data (Table 6) were corrected for the mortality of the control (Abbott, 1925).

Cytotoxicity Studies. Sf9 cells derived from *S. frugiperda* pupal ovarian tissue (European Collection of Cell Cultures, ECCC) were maintained in TC-100 insect cell medium supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin at 26°C. Mammalian Chinese hamster ovary cells (CHO) contributed by Dr Pajares, I.C. Biomédicas, CSIC, were grown in RPMI 1640 medium supplemented as above at 37°C under a humidified atmosphere of 5% CO_2/95% air. Cytotoxicity was measured by the MTT method (Mossman, 1983) as described in González-Coloma et al. (2000a). The active compounds were tested in a dose–response experiment to calculate their relative potency (LD_{50}values, the effective dose to give 50% cell viability), which was determined from linear regression analysis (% cell viability on log dose). A cytotoxic selectivity index (CSI = Sf9-LD_{50}/CHO-LD_{50}) was used to compare the value of the selective cytotoxic action between insect and mammal cells of a given compound.

Isolation and Identification of Compounds

Isopimaric Acid. From fractions H and K, isopimaric acid (**1**) spontaneously crystallized (1.011 g) as clear, plane square crystals, m.p. of 155–158°C. The spectral properties (IR, NMR, and MS) were identical to those previously described in the literature (Talapatra et al., 1994) (Figure 1).

Isopimaric Acid Methyl Ether. One-hundred-milligram isopimaric acid was dissolved in 20-ml methanol and treated with an excess of ethereal solution of diazomethane. After 24 hr, the excess diazomethane was eliminated and the solution evaporated to dryness. Clear crystals of isopimaric acid methyl ether (**2**) precipitated from the residue and were identified by IR, NMR, and MS (Figure 1).

α-Amyrin, Sandaracopimaradien-19-ol, and Akhdarenol. Fraction K (22 g) that was toxic against *A. salina*, *A. hypochondriacus*, and *E. crus-galli* was subjected to open column chromatography on silica gel (220 g). The initial elution system was hexane/$CHCl_3$ (1:1). A total of 239 fractions were obtained and similar fractions were pooled using TLC to yield 21 fractions (K1–K21). Further column chromatography (50 g of silica gel impregnated with 5 ml of 10% silver nitrate

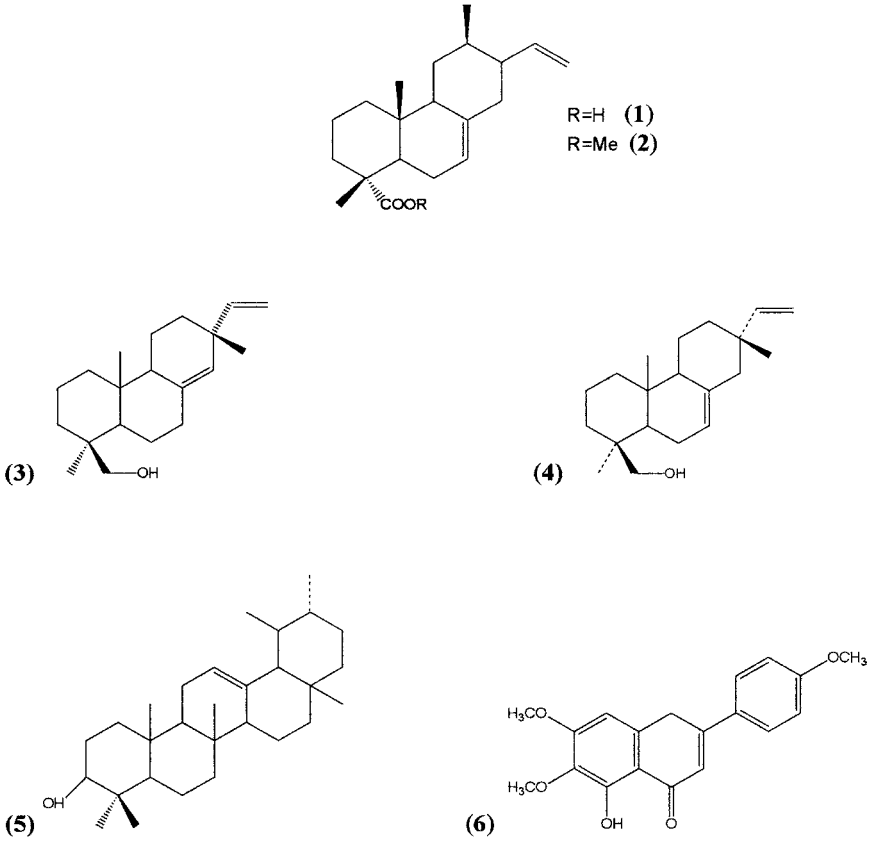

FIG. 1. Structures of compounds from *Callicarpa acuminata:* isopimaric acid (**1**), sandaracopimaradien-19-ol (**3**), akhdarenol (**4**), α-amyrin (**5**), salvigenin (**6**), and isopimaric acid methyl ether (**2**).

solution) of fraction K-9 (2 g) was performed. Altogether 110 fractions were obtained and pooled on the basis of their TLC profiles into four fractions (A–D). TLC from fraction C showed two spots (C1 and C2). The proton and carbon NMR spectra of C1 were very close matches for literature values for α-amyrin (Ahmad and Ur-Rahman, 1994). Compound **5** was positively identified as α-amyrin (Figure 1). Mixture of Fraction C2 was resolved via HPLC into two predominant components **3** and **4**, present in about a 1:2 ratio. The chromatography system utilized was 70% (1:1 methanol-acetonitrile) in water on a 25 $\times$ 1 cm Vydac HS-C18 column. At a flow rate of 5 ml/min, retention times were 62 min for **3** and 66 min for **4**. They were about 85% resolved, but apparently pure samples were obtained by peak shaving techniques.

Salvigenin, 5-Hydroxy-6,7,4'-Trimethoxyflavone. Fraction L (23 g) was further fractioned by CC using an elution system of $CHCl_3$ and $CHCl_3$:MeOH (100, 95:5, and 90:10). A total of 206 fractions were obtained and pooled using TLC to obtain 22 fractions (L1–L22). A total of 173 mg of a yellow compound crystallized from fraction L9 and was further purified by TLC using $CHCl_3$:MeOH (98:2). The pure compound, m.p. 193–195°C, MW 328.32 was identified as the known flavone 5-hydroxy-4',6,7-trimethoxyflavone (salvigenin) (**6**) by spectral means (Xaasan et al., 1980) (Figure 1).

RESULTS AND DISCUSSION

Activity of Aqueous Leachates and Organic Extracts. One percent aqueous leachate of *C. acuminata* leaves significantly inhibited the radical growth of *A. hypochondriacus* (48%), *E. crus-galli* (72%), and *L. esculentum* (42%). The effects of the chloroform:methanol extract (1:1) of the leaves of *Callicarpa,* and the most active primary fractions following column chromatography of the full extract (named by letters) on the root growth of the same three test species are shown in Table 1.

The chloroform-methanol (1:1) extract significantly inhibited the root growth of *A. hypochondriacus* (23.5%), *E. crus-galli* (59%), and *L. esculentum* (53.5%). The root growth of the latter species was strongly inhibited by fraction D (56.6%). Fractions B, C, K, L, M, and N also inhibited the root growth of the test plants. *A. hypochondriacus* and *E. crus-galli* were strongly inhibited by fractions O and P.

TABLE 1. EFFECT OF *Callicarpa acuminata* LEAF EXTRACT AND ACTIVE PRIMARY FRACTIONS FOLLOWING COLUMN CHROMATOGRAPHY ON ROOT GROWTH

	A. hypochondriacus	*E. crus-galli*	*L. esculentum*
Extract/Fraction (100 μ/ml)	% of root growth vs. control[a]		
A (Full extract)	76.5*	41*	46.5*
Active column fractions			
B	64*	67.8*	52*
C	89	57.6*	62.4*
D	62.6*	82.2	43.4*
I	114	89.7	74*
K	53.4*	61.8*	104
L	76.7*	59.4*	106
M	63.4*	61*	90
N	64.8*	59.7*	96.5
O	44*	30.4*	61*
P	46.6*	40.8*	77*

[a] Distilled water.
* $p < 0.05$.

TABLE 2. EFFECT OF *Callicarpa acuminata*, LEAF EXTRACT AND PRIMARY FRACTIONS ON RADIAL GROWTH OF PHYTOPATHOGENIC FUNGI

Treatments (100 $\mu g/ml$)	*H. longirostratum*	*A. solani*
	% of radial growth vs. control[a]	
A (Full extract)	109	106.04
Active column fractions		
B	68.7*	95.5
J	70.7*	111.6
K	76.7*	93.5
M	80*	105
O	111	82*

[a] PDA (potato–dextrose–agar).
* $P < 0.05$.

Biological tests during fractionation showed that both *Artemia salina* and the three test plants were negatively affected by the allelochemicals from *C. acuminata* indicating the presence of bioactive compounds. Fractions I (BST $LC_{50} = 213.79$ μg/ml), J (BST $LC_{50} = 311.17$ μg/mL), and K (BST $LC_{50} = 500$ μg/ml) were toxic to brine shrimp. In addition, biological tests with *A. salina* showed that fraction (K-9) was highly toxic to this crustacean (BST $LC_{50} = 2\mu$g/ml).

Because of the scarce quantities of fractions O and P, fractionation was continued with fraction K, which was more abudant.

Phytopathogenic fungi were less sensitive to the full extract and primary chromatographic fractions than the test plants or *Artemia salina* (Table 2). In general, *H. longirostratum* was more sensitive than *A. solani.* The chloroform extract had no effect on the radial growth of either fungal species, but fractions B, J, K, and M had a moderate inhibitory effect on *Helminthosporium*. Fraction M was the only fraction to inhibit *Alternaria* growth.

Isolation and Characterization of Sandaracopimaradien-19-ol and Akhdarenol. NMR data (1D and 2D proton and carbon correlations) indicated that **3** and **4** were isomers with the formula $C_{20}H_{32}0$. The NMR spectra of both compounds were too complex and too overlapped to completely assign, even with advanced 2D methods. Therefore, we employed the lanthanide shift reagent (LSR), europium (III) tris (1,1,1,2,2,3,3,)-heptafluoro-7,7,-dimethyl-d_6-octane-4,6-dione-d_3 ($Eu(fod)_3$), to resolve the resonances in the NMR spectra and deduce the complete stereochemistry and structure of these diterpenols. We used a combination of ^{1}H-^{1}H-COSY, CHCOR, LRCOR, INAPT, and NOESY 2D-experiments in combination with shift reagent to verify these structures (König and Wright, 1995).

Finally, the full relative stereochemistry of C2a was determined by NOESY experiments. The top face of the molecule could be defined by NOe's observed between CH_3-20 and H-1e, H-2a, H-6a, H-11a, CH_3-17, and H-19a and between CH_3-17 and H-11a. The bottom face was shown by NOe's between H-1a and H-5 and H-9, and between CH_3-18 and H-5 and H-6e. In conclusion, C2a was assigned the structure 4*S**, 5*R**, 9*S**, 10*R**, 13*R**-isopimara-8(14),15-dien-19-ol, also known as sandaracopimaradien-19-ol (**3**) (Grant et al., 1969) (Figure 1). The complete relative stereochemistry of C2b was determined by lanthanide-shifted NOESY experiments. The top face of the molecule was defined by NOe's between CH_3-20 and H-1e, H-11a, CH_3-17, and CH_2-19, and by NOe's between CH_3-17 and H-14e. The bottom face showed NOe's between H-9 and H-1a, H-5, and H-14a and between H-5 and CH_3-18. Thus, all stereocenters of the C2b molecule are defined. The CH_2OH must be axial as are the ring junction protons, H-5 and H-9, and the methyl groups at C-10 and C-13. In conclusion, C2b was identified as 4*S**, 5*R**, 9*S**, 10*R**, 13*S** isopimara-7,15-dien-19-ol. This compound is also known as akhdarenol (**4**) (Kitajima et al., 1982) (Figure 1).

Activity of Compounds. The phytotoxicity exhibited by several fractions and the full extract almost disappears when pure compounds were evaluated (Table 3). Only compounds **5** and **6** caused a significant inhibition on two of them, α-amyrin (**5**) inhibited the root growth of *Amaranthus* and *Echinochloa* (26.8 and 34%, respectively). In addition, **5** was toxic to *Artemia salina* (BST $LC_{50} = 200\ \mu$g/ml). α-Amyrin is an ursane type of triterpenoid widely prevalent in nature. It also occurs in the latex of many plants: *Ficus variegata* (Moraceae), rubber trees, *Erythroxylon coca* (Erythroxylaceae), and *Balanophora elongata* (Balanophoraceae), among others (Harborne and Baxter, 1993). Salvigenin (**6**) inhibited the root growth of *Amaranthus* by 26.2%. Miura et al. (2001) isolated salvigenin from the leaves of

TABLE 3. EFFECTS OF ISOPIMARIC ACID (**1**), METHYL ISOPIMARIC ACID (**2**), THE MIXTURE OF TWO ISOMERIC DITERPENOLS (**3, 4**), α-AMYRIN (**5**), AND SALVIGENIN (**6**) ON PLANT RADICLE GROWTH

Treatments (100 μg/ml)	*A. hypochondriacus*	*E. crus-galli*	*L. esculentum*
	% of radicle growth vs. control[a]		
Control	100	100	100
2,4-D	0	0	0
1	85.5	82.6	100
2	86	72.4	113.8
3+4	103.7	78.3	94.0
5	73.2*	66.0*	105.4
6	73.8*	100	110

[a] Distilled water.
* $P < 0.05$

Salvia officinalis together with some apianane terpenoids and other known compounds. These findings may suggest a defensive role played by both compounds (**5** and **6**) in all these plants, as in *C. acuminata*. Further studies on the allelochemical potential of *C. acuminata* could allow us to determine the compounds responsible for the phytotoxic effects. *L. esculentum* was not affected by any of the isolates, but 2,4-D, which served as control. This suggests a probable synergistic or additive effect of compounds in the mixtures, a common phenomenon observed in phytotoxic studies. In most cases, chemical inhibition is the result of the simultaneous action of several compounds, and often these include compounds whose chemistry is divergent (Einhellig, 2002).

Isopimaric acid (**1**) was toxic to *Artemia salina* (BST $LC_{50} = 10\,\mu$g/ml). Table 4 shows that (**1**) also produced a significant inhibition of the radial growth of *Helminthosporium longirostratum* at 150, 250, and 500 μg/ml (41, 50, and 47%, respectively) ($IC_{50} = 403\,\mu$g/ml). The effect of this compound on *H. longirostratum* was similar to the commercial fungicide captan ($IC_{50} = 355\,\mu$g/ml).

TABLE 4. RADIAL GROWTH OF TWO PHYTOPATHOGENIC FUNGI IN RESPONSE TO VARIOUS CONCENTRATIONS OF ISOPIMARIC ACID (**1**), ISOPIMARIC ACID METHYL ETHER (**2**), AND A COMMERCIAL FUNGICIDE (CAPTAN)

	H. longirostratum	*A. solani*
Treatments (μg/ml)	% of radial growth vs. control[a]	
Control	100	100
Isopimaric acid (1)		
25	94	84.5
50	88.2	80.2*
150	58.8*	77*
250	50.4*	80.8*
500	53*	78*
Isopimaric acid methyl ether (2)		
25	104.7	95.0
50	98.6	95.3
150	95.2	96.5
250	105.6	89.6
500	ND	ND
Captan		
25	60	82.3
50	57.3*	75.2*
150	55.3*	75.6*
250	54.2*	56.6*
500	46*	49.1*

[a] Potato-dextrose-agar (PDA).
* $P < 0.05$. ND = not determined.

TABLE 5. EFFECTIVE ANTIFEEDANT DOSES (EC_{50}) AND 95% CONFIDENCE LIMITS (LOWER, UPPER) OF THE TEST COMPOUNDS

Compounds	$EC_{50}(\mu g/cm^2)$	
	S. littoralis	*L. decemlineata*
1	>100	>100
2	>100	29.7 (21.2, 41.6)
3	>100	>60
4	>100	4.7 (1.7, 18.5)
5	>100	14.6 (4.8, 44.4)
6	>100	>60

Isopimaric acid (**1**) caused moderate, but significant, growth inhibition of *A. solani* from 50 to 500 μg/ml. Captan also inhibited the growth of *A. solani* from 50 to 500 μg/ml (25–51%) ($IC_{50} = 426.36\,\mu$g/ml). Isopimaric acid methyl ether (**2**) had no significant effect on the growth of either fungus, but was toxic to *Artemia salina* (BST $LC_{50} = 50\,\mu$g/ml). Isopimaric acid and other related resin acids such as pimaric, abietic, dehydroabietic, neoabietic, dehydroisopimaric, and sandaracopimaric acids are common compounds in oleoresins of Pinaceae (Harborne and Baxter, 1993). Their presence in *C. acuminata* suggests that they probably play an important role in defense against herbivores in plants from different taxa.

Table 5 shows the antifeedant effects of the test compounds on *S. littoralis* and *L. decemlineata*. None of them had any effect on the polyphagous *S. littoralis*, while the oliphagous *L. decemlineata* was sensitive to akhdarenol (**4**), followed by α-amyrin (**5**), and isopimaric acid methyl ether (**2**). These results are consistent with the model suggesting that differences in taste sensitivity to deterrent compounds could account for the difference in host range (Bernays and Chapman, 2000).

Previous results have shown that some diterpene acids and alcohols have antifeedant, repellant, and acaricidal effects against several insect species (Morita, 1989; Yamaaki et al., 1998; Yamada, 1998; Powell and Raffa, 1999), but this is the first report of the antifeedant properties for akhdarenol, α-amyrin, and isopimaric methyl ether acid.

Comparison of the activity of the diterpenes indicated that the unsaturation of the B ring (>12-fold increase in activity of **4** with respect to **3**) with a hydroxy (**4**) or methyl ester (**2**) in C-19 (>20 and >3-fold increase in activity of **4** and **2** in relation with **1**) play an important role in the antifeedant effect of this class of diterpenes.

Some pentacyclic triterpene derivatives have been described as insect antifeedants against *S. littoralis* and *L. decemlineata* (Lugemwa et al., 1990; Huang et al., 1995; Jagadeesh et al., 1998). Here, we have found that triterpene **5** (α-amyrin) had moderate antifeedant activity against *L. decemlineata*. Previous results

TABLE 6. EFFECT OF TEST COMPOUNDS ON INSECT LARVAL PERFORMANCE (20 μG INSECT), ADULT MORTALITY (5 μG/INSECT) FOLLOWING ORAL AND HEMOLYMPH INJECTION, AND EFFECTIVE CYTOTOXIC DOSES (IC_{50}) AND 95% CONFIDENCE LIMITS ON INSECT AND MAMMALIAN CHO CELLS

	S. littoralis		*L. decemlineata*	LD_{50} (μg/ml)		CSI[d]
Compounds	RCR[a] (% of control)	RGR[b] (% of control)	% Mortality (72 hr)[c]	Sf9	CHO	
1	144.4	108.9	0	4.1 (6.3,2.6)	16.31 (4.9,53.9)	0.25
2	87.8	78.1	27[e]	na	na	—
3	102.5	87.0	23[e]	14.3 (9.0,22.8)	>100	<0.14
4	103.3	104.7	11	4.3 (2.9,6.6)	6.9 (4.3,11.0)	0.60
5	91.9	80.7	60[e]	8.66 (6.8,11.09)	>100	<0.05
6	77.3	75.7	20[e]	5.74 (3.5,9.3)	5.8 (3.2,10.6)	0.9

[a] RCR = $I/(\mathrm{BI})\times T$, I = mg consumed diet, T = length (days), BI = initial insect weigth (mg).
[b] RGR = $\Delta B/(BI) \times T$, ΔB = change in insect body weight (mg).
[c] Corrected according to Abbot (1925).
[d] Cytotoxic Selective Index (CSI = IC_{50}Sf9: IC_{50}CHO).
[e] Significantly different from the control. Contingency table analysis ($p < 0.05$).

with triterpenes structurally-related to **5** have shown that the presence of a hydroxy (as in **5**) or a ketone substituent in C-3 is needed for the antifeedant effect against *L. decemlineata* in the absence of an unsaturation in the E ring (as in **5**) (Santana, 2000).

None of the compounds had detrimental, postingestive effects on *S. littoralis* (Table 6). However, on abdominal injection, compound **5** was toxic to *L. decemlineata,* followed by compounds **2**, **3**, and **6** with moderate-to-low toxicity. We did not find any correlation between antifeedant and toxic effects on this insect as previously shown for other terpenoid antifeedants (see González-Coloma et al., 2002b), suggesting different modes of action for the antifeedant and toxic effects.

Authors have also tested the cytotoxicity of the natural products on insect- and mammalian-derived cells (Sf9 and CHO, respectively) (Table 6). Overall, the compounds were cytotoxic to Sf9 cells, suggesting that the lack of postingestive effects is probably due to metabolic detoxification. Among the diterpene alcohols, **1** and **4** were similarly cytotoxic to Sf9 cells. CHO cells were sensitive to compounds **6**, **4**, and **1**. Compound **5** showed the strongest selectivity against insect cells (with the lowest CSI value), followed by compounds **3**, **1**, and **4**, with **6** being equally toxic to both cell lines.

Some diterpene acids, including isopimaric acid (**1**) and triterpenes structurally related to **5** have been described as being cytotoxic to mammalian and tumor cells (Montagnac et al., 1997; Chang et al., 2000). Furthermore, there is indirect evidence of the antitumor action of α-amyrin (**5**) (Akihisa et al., 1996)

and salvigenin (**6**) (Tezuka et al., 2000). However, this is the first report on the cytotoxic effects of these compounds on insect-derived cells. The selective cytotoxic action of the diterpene alcohol **3** and the triterpene **5** against Sf9 cells is probably due to membrane-related factors. The antibacterial and cytotoxic effects of some isopimaranes have been attributed to membrane-damaging effects (Woldemichael et al., 2003).

In the present study, we found diverse and selective bioactivity of an organic extract, chromatographic fractions, and pure compounds from *C. acuminata* on a variety of test organisms. In addition, we confirmed the ecological importance of terpenoids as infochemicals (compounds that carry some information in chemical interactions between two organisms), particularly showing the toxicity of some of them on fungi, insects, and mammalian and insect cells. The terpenoid pathways generate a great structural diversity and complexity of compounds, which have an enormous potential for mediating ecological interactions (Langenheim, 1994).

Current studies on plants and fungi of El Eden, Quintana Roo, Mexico will contribute to the knowledge of their allelochemical potential, and as a consequence, to the discovery of the added value of these organisms from the endangered dry tropical forests.

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