

Cytotoxic Constituents from Bark and Leaves of *Amyris pinnata* Kunth.

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Abstract: From leaves and bark of *Amyris pinnata* Kunth twelve compounds were isolated, corresponding to six lignans **1-6**, three coumarins **7-9**, a sesquiterpene **10**, an oxazole alkaloid **11**, and a prenylated flavonoid **12**. Metabolites were identified by spectroscopic techniques (¹H and ¹³C NMR, EIMS) and by comparison with published data in the literature. Cytotoxicity against leukemia, solid tumors, and normal cells was evaluated for all isolated compounds. Lignans were found to be the most cytotoxic compounds occurring in *A. pinnata*.

Keywords: *Amyris pinnata*; lignans; coumarins; alkaloid; flavonoid; cytotoxicity. © 2015 ACG Publications. All rights reserved.

1. Plant Source

An important neotropical genus from Rutaceae family is *Amyris* genus, comprising 126 species as trees and shrubs [1]. Its distribution is in the Antilles and from Florida and Texas in the United States through Central America to Peru and Venezuela [2]. Genus *Amyris* are usually very aromatic plants and some species have been used for timber as well as a source of incense and oil, attributing antiseptic, emollient, and sedative properties as well as for the treatment of skin diseases [3-5]. An important number of metabolites have been isolated from *Amyris* plants such as nicotinamides [6], chromene amides [7,8], prenylated furo and pyranocoumarins [5, 9-13], prenylated flavonoids [14], lignans [4], and alkaloids [15,16]. Additionally, several biological activities have been described for *Amyris* species such as cytotoxic [7], antifungal [15], larvicidal [17], and cytochrome inhibition [7].

2. Previous Studies

So far, only a work had described the isolation and the cytotoxic activity of five coumarins and three butyrolactone lignans from leaves and twigs of *A. pinnata* [18].

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3. Present Study

Plant material (leaves and bark) of *Amyris pinnata* Kunth was collected in reserve forest at Tuluá Botanical Garden, Valle del Cauca Department, Colombia, in September 2009, by Botanist Wilson Devia. A voucher specimen (number 384414) has been deposited at “Juan María Cespedes” Herbarium at Tuluá Botanical Garden.

Dried, powdered leaves (3459 g) of *A. pinnata* were extracted by percolation at room temperature using 96% ethanol. 244.3 g of ethanolic extract were obtained. 25 g crude extract were fractionated by CC on silica gel using a mixture of petroleum ether (PE)/ethyl acetate (EtOAc) in increasing polarity, thereby producing 18 fractions in total. Fraction 4 was subjected to CC on silica gel using a mixture of CHCl₃/MeOH as eluent in increasing polarity. Fraction 4.2 was purified by preparative TLC and eluted with CHCl₃/MeOH 9:1 to obtain the compounds **5** (22 mg) and **6** (18 mg). Fraction 5 was also subjected to CC on silica gel with PE/EtOAc as mobile phase obtaining the fraction 5.3, which was subsequently purified by CC eluted with CHCl₃/MeOH 9.5/0.5 affording compounds **8** (36 mg) and **9** (12 mg). Fraction 9 was purified by CC on silica gel using a mixture of toluene/EtOAc as mobile phase in increasing polarity. From the collected fractions 9.3 and 9.7 were independently isolated two solids recrystallized in *n*-hexane, obtaining compounds **3** (8 mg) and **10** (130 mg), respectively.

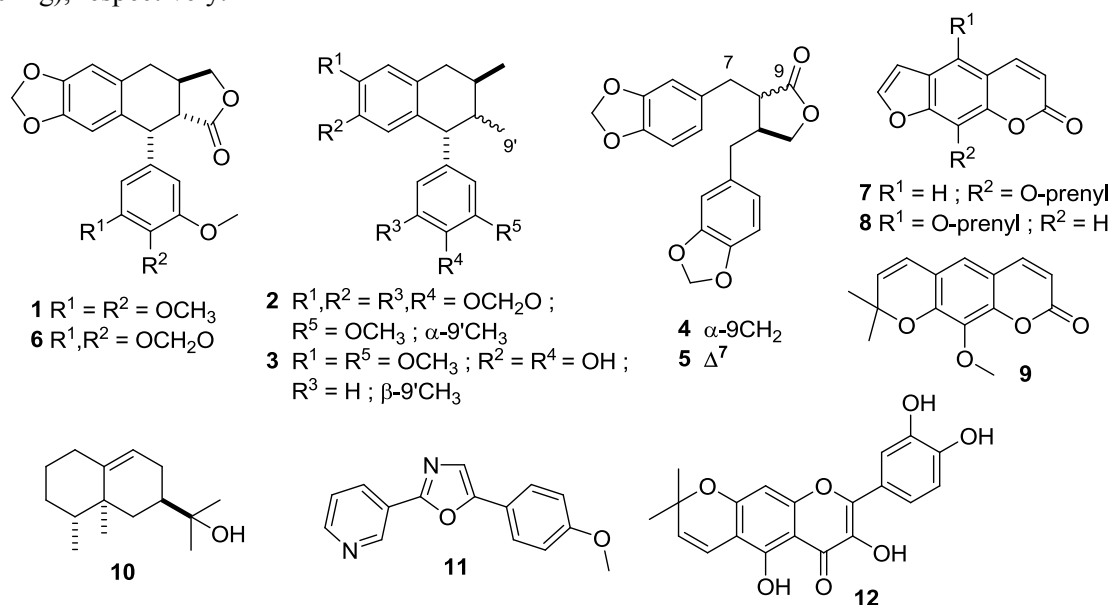


Figure 1. Structures of secondary metabolites isolated from *A. pinnata*

Dried, powdered bark (1200 g) was extracted with 96% ethanol by percolation at room temperature. 88 g of ethanolic extract were obtained. Crude extract (20 g) was subjected to CC on silica gel using a mixture of Tol/EtOAc as eluent in increasing polarity, thereby obtaining 11 fractions. Fraction 5 was subjected to CC on silica gel and eluted with PE/EtOAc 7:3, yielding compounds **1** (10 mg), **2** (15 mg), and **7** (22 mg). Fraction 7 was subjected to CC on silica gel eluted with a mixture of Tol/EtOAc 6:4 and then a subsequent preparative TLC eluted with CHCl₃/MeOH 9:1 yielded compound **4** (33mg). Fraction 9 was purified by CC eluted with Tol/EtOAc 1:1 afforded the compounds **11** (15 mg) and **12** (7 mg).

The compounds were identified as deoxypodophyllotoxin **1**, austrobailignan-3 **2**, (-)-isoguaiacin **3**, hinokinin **4**, hibalactone **5**, austrobailignan-1 **6**, imperatonin **7**, isoimperatonin **8**, luvangetin **9**, jinkoh-eremol **10**, methylhalfordinol **11**, sarothranol **12**, whose structures were elucidated by spectroscopic techniques (¹H and ¹³C NMR, IR, EIMS) and by comparison with published data in the literature [4-18], (Figure 1).

All compounds are reported for the first time in *A. pinnata* species, excepting compounds **6** and **7** [18]. The occurrence of lignans and coumarins in the genus *Amyris* has been revealed in previous studies, but the report of oxazole alkaloids and prenylated flavonoids are less indicated in *Amyris* [4,14,15]. A detailed literature review shows that this is the second report for the isolation of a

halfordinol-derivative, hinokinin **4** and prenylated flavonoids from this genus, since the prenylated O-(3-3-dimethylalil)-halfordinol and compound **4** have been previously isolated from *A. brenesii* [4] as well as O-prenylated flavonoids (Amyrisins A–C) from *Amyris madrensis* [14]. However, so far there are no reports on C-prenylated flavonoids in *Amyris* genus.

The ethanolic extracts from the leaves and the bark of *A. pinnata* and all isolated compounds were tested in a disk diffusion soft agar colony formation assay (disk assay) [19]. The disk assay is designed to compare the relative cytotoxicity of a chemical agent against leukemia cells, solid tumor cells (including multidrug resistant solid tumors), and normal cells. The inhibition is expressed in zone units, with 200 units = 6.5 mm. On average, a 10-fold dilution of a cytotoxic agent produces a 330 zone unit change. Activity against a drug sensitive leukemia (L1210 or P388) provides the reference point. The leukemic cell can represent antiproliferative leukemic active agents of past discoveries. The agent needs greater activity against the drug insensitive solid tumors than against the leukemia cells. Normal fibroblasts were also used in such studies. The tumor cells are isolated from a tumor growing in a mouse. The cells are then seeded in the soft agar. The drug is placed on a filter paper disk (standard hole punch of Whatman no 1) (500 µg per disk), which is then placed on top of the soft agar (60 mm plate). The drug diffuses off the disk as the tumor cells are replicating, creating a zone of inhibition of colony formation. Sufficient cytotoxicity is considered when the inhibition exhibited >400 units.

Table 1. Cytotoxicity of isolated compounds in the disk diffusion soft agar colony formation assay^a.

Cmpd	Leukemia		Mouse		Human	Normal
	L-1210	P-03	C-38	M-17	H-116	Fibroblast
ApL^b	>950	>950	>950	>950	>950	>950
ApB^c	>950	>950	>950	>950	>950	>950
1	>950	>950	>950	>950	>950	>950
2	900	270	440	540	100	280
3	650	600	600	640	800	600
4	900	430	470	500	640	500
5	630	600	610	650	560	600
6	>950	>950	>950	>950	>950	>950
7	70	0	40	70	0	60
8	80	80	70	40	100	30
9	400	420	400	520	300	220
10	220	330	260	340	200	150
11	200	150	150	300	150	0
12	30	60	60	30	30	0
XK469^d	600	400	800	850	150	500

^aZone units recorded: 200 units = 6.5 mm; ^bLeaves-derived extract; ^cBark-derived extract; ^dXK469 as positive reference.

So far, only a work had described the isolation and the cytotoxic activity of five coumarins and three butyrolactone lignans from *A. pinnata*, leading to the identification of the cytotoxic lignan austrobailignan-1 **6** [18], which was found to be active in the 9KB cell culture system (ED₅₀ 0.027 µg/mL). In the present study, extracts from bark and leaves exhibited good activity in the disk diffusion soft agar colony formation assay (>950 units). This result directed our aim towards the isolation and evaluation of the cytotoxicity of the constituents from the leaves and bark of *A. pinnata*. Major number of test compounds exhibited antitumor activity at different levels (Table 1). Aryltetralin lignan lactones (**1** and **6**) exhibited the best cytotoxic activity (>950 units) against cell lines without selectivity and high toxicity, which is recognized in several works for these compounds [4,20]. The activity of these compounds was higher to that than positive reference used, XK469, a known antitumor agent [19], which exhibited different pharmacological profile to that of the most active compounds.

On the other hand, butyrolactone (**4-5**) and aryltetralin (**2-3**) lignans showed lower activity to that of **1** but with sufficient cytotoxicity (>400 units against fibroblast), excepting lignan **2**, which has no free hydroxyl groups like in **3**. In addition, lignan **4** demonstrated good selectivity towards Leukemia cell line (900 units) and lignan **3** against solid tumor cells (800 units).

The prenylated coumarins **7** and **8**, prenylated flavonoid **12**, and oxazole **11** exhibited practically no activity against test cell lines. This result is in agreement with the previous study on *A. pinnata* where imperatorin **7** showed lower activity (KB ED₅₀ = 33.0 µg/mL). However, luvangetin **9**, a non-prenylated coumarin, demonstrated moderate activity (300-520 units) against all cell lines but no higher to that of **1**. The cytotoxicity of compound **9** has already been reported against human lung A-549 tumor cells, resulting in a remarkable activity (IC₅₀ = 4.28 µg/mL) [21]. Sesquiterpene **10** exhibited some selectivity on mouse solid tumor cells but lower cytotoxic activity than that of **9**.

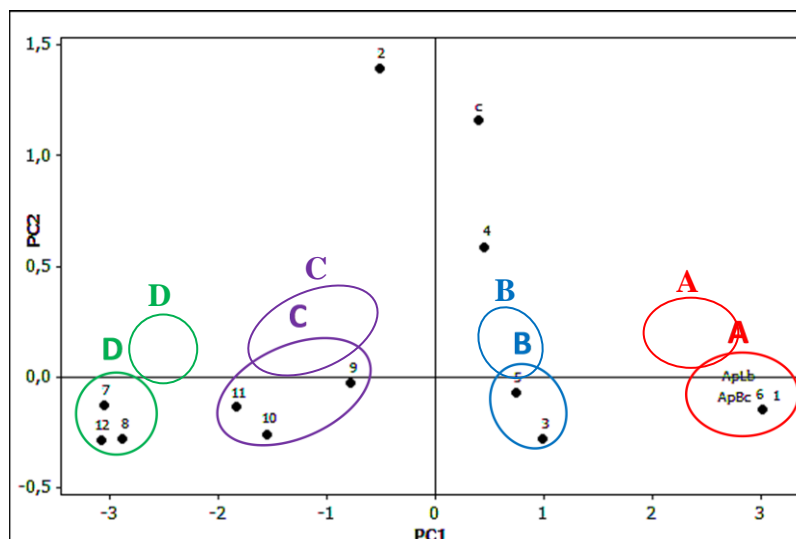


Figure 2. PC1 and PC2 Score Plot for the isolated compounds from *A. pinnata*.

In order to discriminate observed the activity by test compounds **1-12**, a Principal Component Analysis (PCA) was performed using the cytotoxic activity dataset per compound. The resulting Score Plot PC1 vs PC2 (Figure 2) separated the data in four principal groups. The group A consisted in the most active substances (bark and leaves-derived extracts and compounds **1,6**) while group D comprised the non-active compounds (**7, 8, 12**). Groups B and C clustered the compounds with intermediate activity, containing group B those compound more active than those grouped in C. Curiously, PCA algorithm separated compound **4** which exhibited selectivity against Leukemia cells, on the one hand, and compound **2** and the reference showing no significant activity against human H-116 cell line (100 units), on the other hand.

Present study depicted the cytotoxic activity of twelve isolated constituents of *A. pinnata* for the first time, excepting for compounds **6** and **7**. These results indicate that deoxypodophyllotoxin **1** and austrobailignan-1 **6** are the active principles responsible for the observed activity of extracts from the leaves and bark, respectively. The findings of this biological activity denote this neotropical endemic medicinal plant as good source of lignan-related cytotoxic agents.

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