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Three constituents with biological activity from *Coccoloba uvifera* seeds

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Abstract

"Uvero de playa" (*Coccoloba uvifera* Jacq.) seeds were extracted with methanol and the antibacterial, antifungal, toxic and phototoxic activities were determined together with their phytochemical analysis. The extract was active against *Salmonella typhimurium* and *Staphylococcus aureus*, showing the presence of flavonoids, saponins, polyphenols and tannins. The partition with EtOAc of the MeOH extract gave a brown precipitate that inhibited the growth of *Escherichia coli* and *Pseudomonas aeruginosa*, and showed antifungal activity against *Candida albicans*, *Fusarium oxysporum* and *F. decencellulare*. This solid was fractionated by means of vacuum columns (low pressure columns) chromatographies, molecular exclusion and bidimensional paper chromatographies obtaining a tannic compound, an organic acid and a benzopyran from the bioactive fractions. These compounds were identified by means of IR, GC/MS and NMR (^{1}H and ^{13}C) as: gallic acid, hexenedioic acid and 1,3,4,6,7,8–hexahydro-4,6,6,8,8,8-hexamethylcyclopenta-2-benzopyran.

Key words: Benzopyran; *Coccoloba uvifera*; gallic acid; hexenedioic acid; Polygonaceae.

Tres constituyentes con actividad biológica de las semillas de *Coccoloba uvifera*

Resumen

Las semillas del "uvero de playa" (*Coccoloba uvifera* Jacq.) fueron extraídas con MeOH y se les determinó su actividad antibacteriana, antifúngica, tóxica y fototóxica; además se les realizó un análisis fitoquímico. El extracto fue activo contra *Salmonella typhimurium* y *Staphylococcus aureus*; también mostró reacción positiva en las pruebas de flavonoides, saponinas, polifenoles y taninos. Se particionó el extracto metanólico con AcOEt, obteniéndose un precipitado que inhibe el crecimiento de *Escherichia coli* y *Pseudomonas aeruginosa*, y mostró actividad antifúngica contra *Candida albicans*, *Fusarium oxysporum* y *F. decencellulare*. Este sólido fue fraccionado por columnas cromatográficas de: vacío, exclusión molecular y bidimensional de papel, obteniendo un compuesto tánico, un ácido orgánico y un benzopirano de las fracciones bioactivas. Estos compuestos fueron identificados por medio de IR, CG/EM y RMN (^{1}H y ^{13}C) como: ácido gálico, ácido hexenedioico y 1,3,4,6,7,8-hexahidro-4,6,6,8,8,8-hexametilciclopenta-2-benzopirano.

Palabras clave: Ácido gálico; ácido hexenedioico; benzopirano; *Coccoloba uvifera*; Polygonaceae.

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Introduction

In the Polygonaceae family we can find species of the *Coccoloba*'s gender, many of them are used to treat some diseases. Some of these plants have been analyzed for their biological activity and their chemical constitution. The *Coccoloba barbadensis* dry leaves are used in Mexico for the treatment of kidneys illness in adults (1), while in India the *Coccoloba diversifolia* bark have been boiled in order to stop the diarrhea (2). In 1996 Nicaraguan scientists evaluated the biological activity of some *coccoloba* extracts, the aqueous extract of the *C. cozumelensis* had antibacterial activity against *Staphylococcus aureus*, *Bacillus punullus* y *Pseudomonas aeruginosa*, the *C. pubescens* ethanol extract exhibit an antimalarian activity (3). Concerning to the chemical therapeutic agents isolation, it has been obtained a successful substance like gallic acid and benzoic acid from *Coccoloba dagandicena* (4), also it has been isolated betulinic acid, lupeol and sitosterol obtained from *Coccoloba excoriata* (5).

The *Coccoloba uvifera* Jacq. is another vegetable species that belongs to this family of plants. This species is original from the Antilles, the Bahamas and South American tropics; it could be found frequently in Venezuelan coasts by the common name "sea grape". This plant has been used whit pharmaceutic purposes, the roots and barks boiled in hot water are used in popular medicine against diarrhea and dysentery. Some studies show that the bark contain tannins used to dye (6), and other investigations show that it is the source of "quine", resin of medical interest that appears in the wound of the trunk. *Coccoloba uvifera* Jacq. (Polygonaceae) is a very common medicinal plant on Venezuelan coasts. Previous studies have reported the presence of some secondary metabolites in its leaves and bark (7), but neither the chemical composition of its seeds or the biological activity of its extracts has been studied.

Coccoloba uvifera Jacq. effectivity in the treatments indicated above what is a potential source of secondary metabolism with significant biological activity, which could be present either in the leaves, the bark and fruit seeds. This species have not been studied yet, in spite of their medical uses. In addition we don't have any information or reports about secondary metabolites in this part of the plant, making of great interest that is why it has been selected the *Coccoloba uvifera* in order to isolate the responsible components of the biological activity.

Materials and methods

Plant material

Seeds from *C. uvifera* were collected in July 2001 in the gardens of the Universidad de Oriente in Cumaná, State of Sucre, Venezuela. The plant was identified by Prof. Luis Jose Cumana. A voucher specimen (No. 01072001) was kept in the IRBR Herbarium at the same university.

Extract preparation

Plant material (93.5 g) was dehydrated in the shade at room temperature. It was then grinded in an electric mill, and the resulting powder was weighed and extracted successively with MeOH until exhaustion. The MeOH seed extract (yield: 21.6%) was dissolved in water and kept in the refrigerator for 24 h at 4°C, and a resulting brown solid was separated by filtration. The remaining aqueous solution was continuously and exhaustively extracted with AcOEt and the organic layer was rotaevaporated after drying over $MgSO_4$ (the brown extract was named ASE). Chemical tests performed on the former extract gave positive results for polyphenols and catechinic and gallic tannins (8).

Polyphenols and tannis test

Phenolic compounds are detected by the brown coloration that take place in presence from a solution of $FeCl_3$ to 1%. For that the total extract is evaporated to dryness,

dissolved in H_2O and filtrated before the reaction with ferric chloride. If the phenols are present, they produce a brown coloration. If the raw contains tannis, a precipitate is produce with 1% Jello in 1% NaCl sln.

Catechinic and gallic tannins test

The sample is dissolved in MeOH-H_2O 1:1, 1mL of this solution is added to 8mL of Stiasny reagent (twice formaldehyde volumens to 30% + a volume of concentrated HCl) it warms to boil for 30min, the presence of catechinic tannis is evidenced by appearance of a brown precipitate, it is filtrated and the solution is saturated with sodium acetate, them are added some drops of 2% ferric chloride. A blue coloration indicates the presence of gallic tannis.

Biological activity

All extracts and fractions were evaluated by means of antibacterial (9), antifungal (10), toxic (11) and phototoxic (12) bioassays. Antibacterial and phototoxic tests were realized using the Gram-positive bacteria strains *Staphylococcus aureus* Rosenbach (ATCC 6538P) and *Bacillus cereus* Frank. Frank. (ATCC 9634); while the Gram-negative ones were: *Escherichia coli* T. Esch. (ATCC 0389), *Pseudomonas aeruginosa* Schroeter (ATCC 25416) and *Salmonella typhimurium* D. E. Salmon (ATCC 14028). In the toxicity test brine shrimp *Artemia salina* Linn., Crustacea, Anostraca was used. Two opportunistic fungi (*Candida albicans* Langenbeck and *Trichosporum* sp. Elmer) and two phytopathogenic fungi (*Fusarium oxysporum* Killiam Maire and *F. decencellulare* Bender) were used for the antifungal assay.

The biological activity was used to biodirection the whole isolation process.

Isolation

Column chromatography was done on a silica gel Aldrich 20–70 mesh 60Å (123.5g) starting with hexane followed by dichloromethane, acetone, methanol, methanol/water (9:1) and methanol/acetic acid (9:1). Molecular exclusion chromatography was performed on Sephadex LH 20, 25-100 m starting with AcOEt (100%), followed by AcOEt-MeOH (1:1), MeOH (100%) and MeOH-H_2O (8:2). For vacuum chromatography Sigmacell cellulose type 50 S-5504 was employed with dichloromethane, acetone and methanol using a Cole-Parmer pump, model 7049-00.

IR spectra were recorded on a Perkin Elmer FTIR 16 PC spectrophotometer using KBr tablets, 24 scans and a 2 cm^{-1} resolution. The GC-MS was performed with a Hewlett Packard model 5890, series II, gas chromatograph coupled to a EI (70eV) mass spectrometer, Hewlett-Packard, model 5971 A. A methyl silicone column DB-5 (25 m) was used, with a 280°C injector temperature**.** The initial and final temperatures were 70°C and 310°C, respectively (rate of heating 6°C/min) with helium as carrier gas. The ^{1}H, ^{13}C NMR spectra were recorded on Jeol 400 MHz spectrometer using deuterated methanol, acetone and water like reference standard.

Gallic acid (A). IR bands (KBr): 3500 – 3000, 1700, 1600, 760 cm^{-1}; EIMS m/z: 170 (M^+), 152, 125 (base peak), 110; ^{1}H – NMR (400 MHz, MeOH – d_4): δ 6.9 – 7.4 (H aromatic and phenolic); ^{13}C - NMR (400 MHz, MeOH – d_4): δ 170.4 (C = O), 146.4 (C – 3 and C – 5), 139.6 (C – 4), 121.9 (C – 1), 109.9 (C – 2 and C – 6).

Hexenedioic acid (B). EIMS m/z: 144 (M^+), 126, 108, 57 (base peak).

1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-2-benzopyran (C). EIMS m/z: 258 (M^+), 243 (base peak), 227, 213, 198, 185, 171, 158, 143, 128, 113, 86. ^{1}H–NMR (400 MHz, DMSO–d_6): δ 7.2 (H_5), 4.6 (H_4), 4,4 (H_7), 3.9 (H_1), 3.6 (H_3), 1.6 (H_4'), 1.2 (H_6), 0.8 (H_7'); ^{13}C - NMR (400 MHz, DMSO–d_6): δ 128.9 and 131.0 (C aromatic), 66.9 (C–7), 63.1 (C–3), 30 (C–6 and C-8), ~ 23 (C–6', C–8' and C–4'), 14.1 (C–7').

Results and Discussion

The MeOH extract from the seeds (MSE) of *Coccoloba uvifera* gave positive results for the catechinic tannins test and an antibacterial effect against *Salmonella typhimurium* and *Staphylococcus aureus* was observed with halos inhibition diameters of 15 mm and 17 mm respectively, this test was carried out at 40mg/mL of concentration. However, the extract did not show any antifungal, toxic or phototoxic activities. MSE was partitioned with AcOEt giving a brownish organic extract (ASE) that gave positive results for the tests for polyphenols and tannins and showed an antibiotic effect against *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, *Fusarium oxysporum* and *F. decencellulare*. ASE (5.70g) was chromatographed by gravity giving 25 fractions. Fractions 7 to 14 showed positive results for polyphenols and catechinic tannins, except for fraction 10 that showed the presence of gallic tannins. Fractions 6 to 14 showed antibacterial activity (Table 1). The solvents also used like blanks, didn't show biological activity.

Fractions 9, 10 and 11 were rechromatographed by column vacuum and molecular exclusion chromatographies. All subfractions were studied by IR, GC/MS and ^{1}H and ^{13}C NMR identifying gallic acid (A) from $F_{10\text{-}5\text{-}3}$; hexenedioic acid (B) from $F_{11\text{-}4\text{-}5\text{-}2}$ and 1,3,4,6,7,8 – hexahydro-4,6,6,8,8,8-hexamethylcyclopenta-2-benzopyran (C) from the same subfraction (Figure 1).

Gallic Acid. The IR spectrum showed absorption bands at 3000 – 3500 (vR – OH), 1700 (vC= O), ~ 1600 (vC= C aromatic), ~ 760 (δC – H aromatic) cm^{-1}. The EIMS showed an ion peak at m/z 152 that corresponds to the loss of H_2O. The intense peak at m/z 125 represents the loss of the carboxylic group and formation the $C_6H_5O_3]^{\bullet+}$.The ^{1}H – NMR spectrum showed resonances between δ 6.9 and 7.4 ppm, that were attributed to aromatic and phenolic protons. The ^{13}C – NMR spectra of commercial gallic acid and the obtained acid in our work, showed identical absorptions (Table 2). This acid has previously been isolated from *Coccoloba dugandiana* Fernandez (Polygonaceae) (4).

Hexenedioic Acid. Its EIMS showed a peak at m/z 144 corresponding to the molecular mass of the acid, and a peak at m/z 126 formed by the loss of H_2O producing $[C_6H_6O_3]^{\bullet+}$.

1, 3, 4, 6, 7, 8–Hexahydro-4, 6, 6, 7, 8, 8–Hexamethylcyclopenta–2–Benzopyran. Its EIMS showed a peak at m/z 258 corresponding to $C_{18}H_{26}O$, m/z 243 corresponding to the ion formed by the loss of a methyl group. The ^{1}H – NMR spectrum exhibited signals assigned to protons in the methyl terminal groups [δ 0.8 (H_7), 1.2 (H_6) and 1.6 (H_4) ppm]. At δ ~3.6 and ~3.9 ppm two signals appeared which were attributed to the two methylene groups adjacent to the oxy-

Table 1
Antibacterial activities from ASE fractions expressed in mm.

Microorganism	F_6	F_7	F_8	F_9	F_{10}	F_{11}	F_{12}	F_{13}	F_{14}
Escherichia coli	–	12	12	–	14	13	12	–	–
Pseudomonas aeruginosa	–	–	16	–	20	16	12	–	–
Salmonella typhimurium	–	–	15	12	20	20	12	–	–
Staphylococcus aureus	–	12	17	13	24	22	12	11	11
Bacillus cereus	11	11	12	12	20	17	11	–	–

F: Fraction.

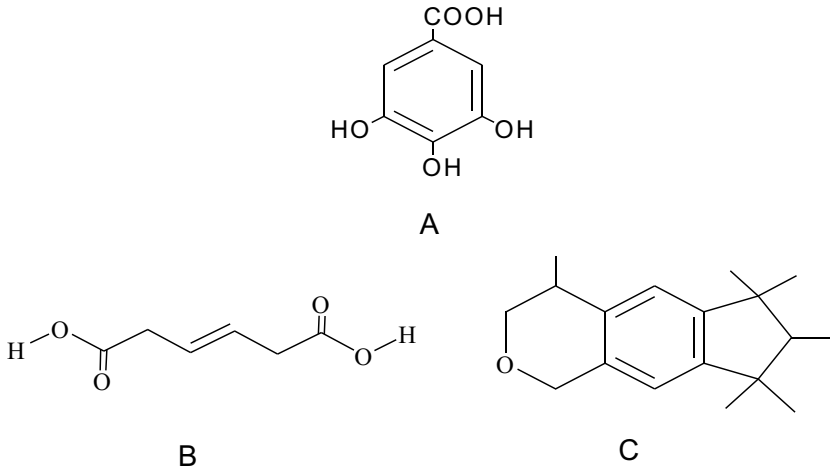

Figure 1. A: Gallic acid; B: Hexenedioic acid; C: 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-2-benzopyran.

Table 2
Chemical shifts (ppm) for the different carbons of F_{10-5-3} and commercial gallic acid.

Carbon	d [F_{10-5-3}]	d [Gallic acid]
C_1	121.9	121
C_2 and C_6	109.9	110
C_3 and C_5	146.4	146
C_4	139. 6	140
C = O	170.4	168 - 170

gen atom, whilst two multiplets at 4.4 and 4.6 ppm correspond to the methyne protons (H_7) and ~4,6 (H_4) ppm. Aromatic protons H_5 and H_9 appear as a singlet at 7.2 ppm. The ^{13}C – NMR spectrum showed the methyl group C_7' at 14.1 ppm, whilst the methyl groups at C_6, C_8 and C_4 appear around 23 ppm. Signals around 30 ppm correspond to the quaternary carbons, aromatic carbons appear at 128.9 and 131.0 ppm whilst two signals at 63.1 and 66.9 ppm correspond to C_3 and C_7, respectively. In a previous study (13) benzopyranic compounds with organic acids and tannins were isolated from propolis.

Conclusions

The presence of tannis in *Coccoloba uvifera* seeds was demonstrated.

The seed's methanolic extract inhibit the growth of *S. aureus* and *S. typhimurium* the fractions and sub fractions of *Coccoloba uvifera* seeds also show antibacterial activity, these may have been considerate potential fountain of useful compounds for the combat of pathogens effect of these bacteria.

The seed's methanolic extract also contain compounds with antifungal activity against *C. albicans*, *F. oxysporum* and *F. decencellulare*.

In the sub fractions of *Coccoloba uvifera* seeds were identified: Gallic acid, hexenedioic acid and 1,3,4,6,7,8-hexahydro -4,6,6,8,8,8-hexamethylcyclopenta-2-benzopyran. These have antibacterial and antifungal activity.

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