

# Isolation of active antiphytopathogen compound from extracts of *Anadenanthera colubrina* var. *cebil* (Griseb.) Altschul

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## Abstract

Several microorganisms are responsible for great economic losses in world agriculture. Preventive and treatment methods are applied to avoid contamination of crops by these microorganisms, however, the use of chemical antimicrobials damages health and the environment. Secondary plant metabolites are safe natural sources of antimicrobials for this application. Fabaceae family has its history described in the literature as a potential source for obtaining antimicrobial bioactive. The objective of this work was to isolate bioactive compounds guided by antimicrobial assays against bacteria and fungi *in vitro*. Organic extracts were prepared by elutropic series of leaves of *Anadenanthera colubrina* var. *cebil* and were tested against six bacteria and six fungi phytopathogenic. The antimicrobial assays of minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC) were performed at each purification step that occurred through HPLC-DAD, Flash Chromatography and HPLC-preparative analysis, to confirm the isolation of the bioactive. Through bioguided isolation, the compound p-hydroxybenzoic acid was obtained, which showed activity against the phyto-bacteria *Xanthomonas campestris* pv. *campestris* and *Acidovorax citrulli*.

**Keywords:** Angico. *Xanthomonas campestris*. *Acidovorax citrulli*. HPLC-DAD. Flash Chromatography. HPLC-preparative.

## Introduction

Vegetable diseases, caused by several pathogenic microorganisms, are one of the main problems faced by world agriculture<sup>[1]</sup>. Bacteria and fungi are among the main pathogens that cause a great decrease in productivity and consequently economic losses in this sector<sup>[2]</sup>. Among the methods applied to control diseases in the field are preventive methods, which have low efficiency, and treatment methods, such as the application of synthetic antimicrobials<sup>[3]</sup>. These have caused problems related mainly to damage to human and animal health, in addition to the accumulation of environmental contamination<sup>[4]</sup>.

Secondary plant metabolites are natural sources of substances with antimicrobial properties, which can be used as an alternative in substitution to synthetics<sup>[5]</sup>. This strategy minimizes the negative impacts associated with being considered safe for health and the environment, in addition to being economically advantageous due to the low cost, they are also capable of reducing the impact of microorganisms on agriculture. In addition, plant extracts reduce the possibility of causing microbial resistance, as they are complex mixtures of metabolites<sup>[6]</sup>.

Several studies have demonstrated how the species of the Fabaceae family have potential as sources of antimicrobials for a great diversity of pathogens<sup>[7-9]</sup>. *Anadenanthera colubrina* var. *cebil* (Griseb.) Altschul, popularly known as Angico, belonging to this family, it is widely used in folk medicine because it is related to the antimicrobial properties of its leaves<sup>[10,11]</sup>. This work aimed to conduct a bioguided study on organic extracts of *A. colubrina* var. *cebil*, to obtain compounds with antimicrobial activity against phytopathogenic bacteria and fungi.

## Materials and methods

### Plant material

Leaves of *A. colubrina* were collected in the Catimbau National Park (08°34'30,96" S e 37°14'51,76" W), in the Northeast of Brazil. The collected material was oven dried at 45°C for 72 h, then ground to obtain a thin powder, stored in an airtight container and kept at 4°C until use. One specimen identified and registered by the Herbarium Dárdano de Andrade-Lima of the Instituto Agronômico de Pernambuco (IPA), under voucher IPA - 80350. The plant material was registered in the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen) at number A08E18B.

### Organic Extracts

A hundred grams of the powder of the leaves de *A. colubrina* subjected to eluotropic series of organic solvents: cyclohexane (CHX), chloroform (CHL), ethyl acetate (EtOAc) and methanol (MeOH) in Soxhlet, respecting the boiling temperature of each solvent, and each was kept under reflux for 24 h. The extracts obtained were then filtered (Whatman n°1), and the solvents were entirely removed on a rotary evaporator at 45°C under reduced pressure. The dry extracts were stored at 4°C hermetically sealed until use.

### Phytopathogenic microorganisms

The organic extracts tested against 12 phytopathogens. Six bacteria: *Acidovorax citrulli* (*Acc* – strain Ac1.12), *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc* – strain Pcc31), *Ralstonia solanacearum*

(*Rsol* – strain CM10R22), *Xanthomonas campestris* pv. *campestris* (*Xcc* – strain Xcc53), *Xanthomonas campestris* pv. *malvacearum* (*Xcm* – strain Xcv137) and *Xanthomonas campestris* pv. *viticola* (*Xcv* – strain Xcm11.2.1) obtained by the Collection of Cultures of the Phytobacteriology Laboratory of the Department of Agronomy of Universidade Federal Rural de Pernambuco (UFRPE), Brazil. For antimicrobial tests the isolates were grown in a nutrient medium of yeast dextrose - NYDA (5 g.L<sup>-1</sup> Yeast extract; 3 g.L<sup>-1</sup> meat extract; 5 g.L<sup>-1</sup> peptone; 10 g.L<sup>-1</sup> dextrose; 18 g.L<sup>-1</sup> de agar) for 24 h at 30°C. Six fungi: *Aspergillus flavus* (*Af* – strain 6029), *Fusarium moniliforme* (*Fm* – strain URM - 5411), *Fusarium oxysporum* (*Fo* – strain URM - 6185), *F. solani* (*Fs* – strain URM – 6264), *Rhizopus soltonifer* (*Rs* – strain URM – 6525) and *Verticillium lecanii* (*VI* – strain URM – 6171), obtained from the mycological collection of Micoteca-URM of the Department of Mycology of Universidade Federal de Pernambuco (UFPE), Brazil. For antimicrobial tests, the isolates were grown in Potato Dextrose Agar - PDA (4 g.L<sup>-1</sup> potato extract; 15 g.L<sup>-1</sup> dextrose; 18 g.L<sup>-1</sup> agar) for 5 days at 48°C.

### Screening Antimicrobial Activity

To evaluate the antimicrobial activity of *A. colubrina* leaves, the organic extracts, fractions, and isolated compound were solubilized in an aqueous solution at a concentration of 100mg.ml<sup>-1</sup> with 10% dimethyl sulfoxide (DMSO) and were sterilized by filtration through a microfilter 0.22 µm (GV-Millipore).

The minimum inhibitory concentration (MIC) was determined by the microdilution method (CLSI, 2011) with modifications. A serial dilution of the extract/fractions was prepared in NYD or BD and 15 µl (Absorbance 600 nm = 0.150 ± 0.05) of bacteria or fungi suspension was added. The concentration of the samples ranged from 50 mg.ml<sup>-1</sup> to 100 µg.ml<sup>-1</sup> for organic extracts, from 6 mg.ml<sup>-1</sup> to 22 µg.ml<sup>-1</sup> for fractions and 500 to 1 µg.ml<sup>-1</sup> for purified compound. The samples were incubated for 24 h for bacteria and 48 h for fungi, at 30°C for both. As a positive control, chloramphenicol was used for bacteria, and cercobin for tested fungi and sterile water with DMSO (10%) was used as a negative control. All tests were performed in triplicate.

To determine the minimum bactericidal or fungicidal concentration (CMB or CMF) after the microplate incubation period, 5 µL of the solution from each well was transferred to NYDA plates and incubated again for the same period. The complete absence of growth on the agar surface with the lowest concentration of the sample was defined as the MBC or CMF, respectively for bacteria and fungi.

### Flash Chromatography

The active organic extract was fractionated by flash chromatography (Biotage Isolera one, Biotage, Charlotte, NC, EUA). The separation occurred in a 50 g SNAP KP-SIL column (company Biotage, Charlotte, NC, EUA). The mobile phase was a gradient of N-hexane: EtOAc, 30:70 (v / v) with 1 column volume (1 CV); N-hexane: EtOAc, 30:70 to 0: 100, (v / v) with 6 CV; MeOH: EtOAc, 0: 100 to 20:80 (v / v) with 6 CV; MeOH: EtOAc, 20:80 to 80:20 (v / v) with 3 CV. The flow of the mobile phase had a flow rate of 70 ml.min<sup>-1</sup> and scan detection from 200 to 800 nm. The 38 fractions were grouped by software that evaluates the fractions according to the UV absorption spectrum, resulting in 6 fractions.

### High-performance liquid chromatography

The active extract and fraction, and the isolated compound were analyzed on HPLC-DAD (1260 infinity LC System-DAD, Agilent OpenLAB CDS EZChrom Edition software, version 04.05 of Agilent Technologies,

Santa Clara, CA, USA) equipped with the Zorbax, SB-C18, 5  $\mu\text{m}$  and 4.6 x 250 mm column and Zorbax SB-C18 pre-column of 5  $\mu\text{m}$  and 4.6 x 12.5 mm. For this, samples at a concentration of 5  $\text{mg}\cdot\text{ml}^{-1}$  were solubilized in methanol and filtered through 0.22  $\mu\text{m}$  polytetrafluoroethylene (PTFE) filters. The mobile phase was composed of the following solutions: (A) 0.3% acetic acid with Milli-Q water (Millipore) and (B) 100% acetonitrile (Merck).

The active extract was analyzed under exploratory chromatographic conditions using the linear-gradient method of 95 - 40% (A) between 0-30min, with a flow rate of 2.4  $\text{ml}\cdot\text{min}^{-1}$ , the initial pressure of 202 Bar and ultraviolet detection (UV) from 196 to 400nm. The chromatographic conditions for analysis of the active fraction and the purified compound followed a linear gradient of 92 - 65% (A) 0-15 min, with a flow rate of 2.4  $\text{ml}\cdot\text{min}^{-1}$ , the initial pressure of 202 Bar and detection at 256 nm. In both analyzes, the identified peaks had retention time and UV spectrum compared to the commercial reference standards: gallic acid, p-coumaric acid, caffeic acid, catechin, trans-ferulic acid, quercetin 3 $\beta$  D-glucoside, chlorogenic acid, quercetin, rutin, and ellagic acid.

### Preparative High-performance liquid chromatography

To isolate the active compound, the active fraction obtained by the flash chromatography system had its compounds separated by AutoPurification HPLC System <sup>TM</sup> (model: 2767 Sample Manager, 2545 Binary Gradient Module, System Fluidics Organizer, System Fluidics Organizer, 2489 UV / Vis Detector, MassLynx Software with FractionLynx Application Manager and ACQUITY QDa Detector - Waters). The fraction solubilized in methanol at a concentration of 15  $\text{mg}\cdot\text{ml}^{-1}$  and filtered through 0.22  $\mu\text{m}$  PTFE filters. The separation and isolation of the compounds occurred through the preparative column XBridge Prep C18 (5  $\mu\text{m}$  and 10 x 100 mm), through the following mobile phase; 0.1% formic acid in Milli-Q water (A) and 100% acetonitrile (B) (Merck) with a linear gradient from 94 to 65% (A) from 0 to 9 min; 9-10 min (65%-0% A), 10-14 min (0% A), 16-20 min (0% -94% A), at room temperature, flow rate 9  $\text{ml}\cdot\text{min}^{-1}$  and detection at 256 nm.

## Results and Discussion

**TABLE 1** shows the results obtained about the antimicrobial power of organic extracts from *A. colubrina* leaves against six phytopathogenic bacteria, according to the extraction solvent used. It is observed that all extracts showed reduced growth in the tested phyto-bacteria when compared to the control. However, it is observed that MIC  $\leq 1.56 \text{ mg}\cdot\text{ml}^{-1}$  is registered in 66.6% of the phyto-bacteria in the ethyl acetate extract, followed by 33.3% in the chloroform and methanol extracts, and the cyclohexane extract showed no activity for any of the species. When evaluating the MBC, it is observed that only the ethyl acetate extract has bactericidal activity in concentrations  $\leq 1.56 \text{ mg}\cdot\text{ml}^{-1}$  for *A. citrulli* and *X. campestris* pv. *campestris*.

The antifungal activity of the extracts against phytopathogenic fungi shown in **TABLE 2**. It observed that there was a reduction in growth in the tested concentrations when compared to the control, however, no extract was considered to have relevant inhibitory or antifungal activity due to all results being higher than 6.25  $\text{mg}\cdot\text{ml}^{-1}$ . In both microbiological tests, it was observed that the DMSO used to solubilize the organic extracts in an aqueous medium, did not affect the bacterial or fungal growth in the negative controls in the concentration used.

**TABLE 1:** Antibacterial activity, Minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC), in mg.ml<sup>-1</sup>, of organics extracts of *Anadenanthera colubrina* var. *cebil* against plant pathogenic bacterial.

Extracts		Acc	Pcc	Rsol	Xcc	Xcm	Xcv
CHX	MIC	6.25	12.5	6.25	3.12	3.12	3.12
	MBC	6.25	25	12.5	6.25	6.25	6.25
CHL	MIC	3.12	6.25	1.56	1.56	3.12	3.12
	MBC	6.25	12.5	3.12	1.56	3.12	6.25
EtOAc	MIC	1.56	3.12	1.56	0.78	3.12	1.56
	MBC	1.56	3.12	3.12	1.56	3.12	3.12
MeOH	MIC	1.56	6.25	3.12	1.56	3.12	12.5
	MBC	3.12	6.25	3.12	3.12	6.25	12.5
Chloramphenicol	MIC	0.009	0.039	0.078	0.019	0.039	0.004
	MBC	0.009	0.078	0.156	0.019	0.039	0.004

Legend: *Acidovorax citrulli* (Acc), *Pectobacterium carotovorum* subsp. *carotovorum* (Pcc), *Ralstonia solanacearum* (Rsol), *Xanthomonas campestris* pv. *campestris* (Xcc), *X. campestris* pv. *malvacearum* (Xcm) e *X. campestris* pv. *viticola* (Xcv); Cyclohexane extract (CHX); Chloroform extract (CHL); Ethyl acetate extract (EtOAc); Methanolic extract (MeOH).

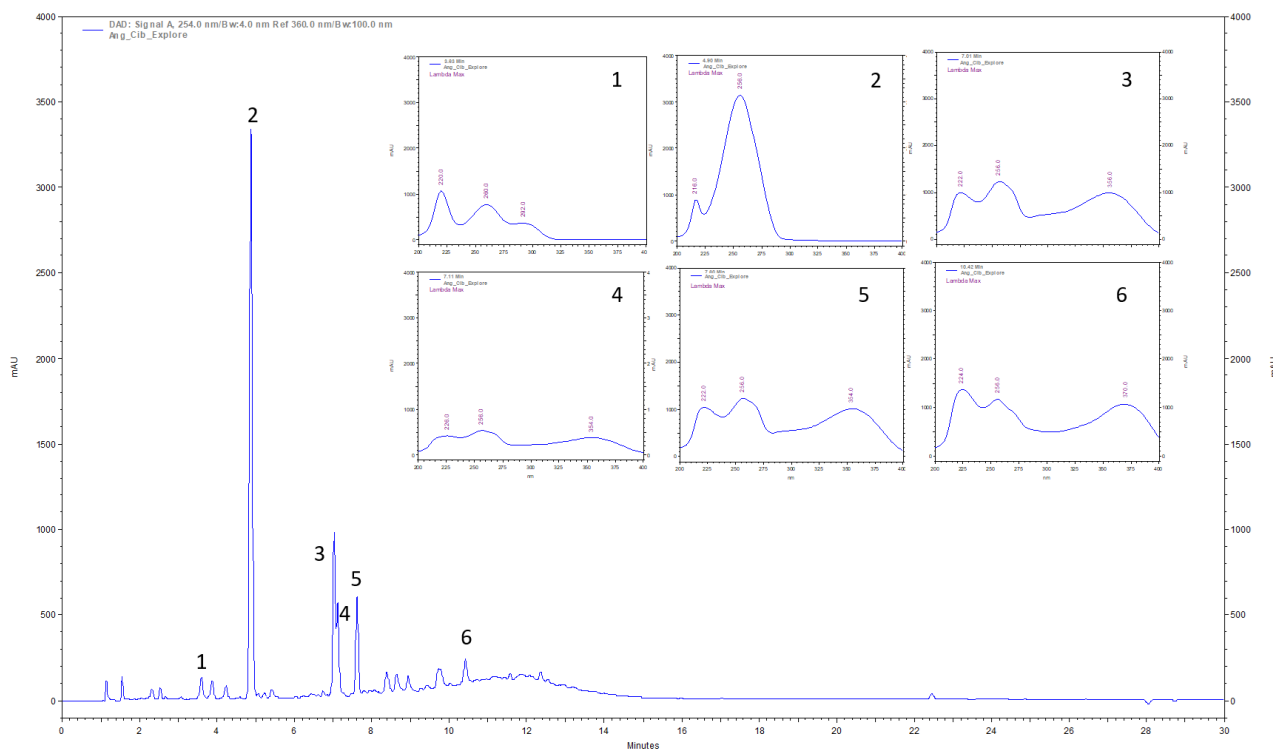
**TABLE 2:** Antifungal activity, Minimum inhibitory concentrations (MIC) and minimum fungicidal concentration (MFC) of organics extracts from *Anadenanthera colubrina* var. *cebil* against plant pathogenic fungal. Concentrations in mg.ml<sup>-1</sup>.

Extracts		Af	Fm	Fo	Fs	Rs	VI
CHX	MIC	6.25	12.5	25	6.25	6.25	6.25
	MFC	6.25	12.5	50	12.5	6.25	6.25
CHL	MIC	12.5	12.5	50	6.25	12.5	6.25
	MFC	12.5	25	50	12.5	12.5	12.5
EtOAc	MIC	12.5	12.5	25	6.25	12.5	6.25
	MFC	12.5	12.5	25	12.5	12.5	12.5
MeOH	MIC	12.5	25	50	6.25	12.5	12.5
	MFC	12.5	25	50	12.5	12.5	12.5
Cercobin	MIC	0.12	0.12	0.5	0.25	0.12	0.12
	MFC	0.12	0.25	-	0.25	0.25	0.25

Legend: Fm: *Fusarium moniliforme*; Fo: *Fusarium oxysporum*; Fs: *Fusarium solani*; Rs: *Rhizopus soltonifer*; VI: *Verticillium lecanii*; CHX: Cyclohexane extract; CHL: Chloroform extract; EtOAc: Ethyl acetate extract; MeOH: Methanolic extract.

From the results obtained in the screening of antimicrobial activity, bioguided purification of the bioactive compound was continued. Given the observed, the EtOAc extract was selected to be analyzed on HPLC-DAD (**FIGURE 1**). This analysis allowed the detection of six main peaks ( $\geq 500$  mAU), between the retention time (Rt) 3 and 4 min, peak 1 ( $\lambda_{\max}$  228, 260 and 294), between Rt 4 and 5 min, peak 2 ( $\lambda_{\max}$  256), between 7 and 8 min, peak 3 ( $\lambda_{\max}$ , 265, 354) peak 4 ( $\lambda_{\max}$  256, 352) and peak 5 ( $\lambda_{\max}$  256, 356), and between 10 and 11 min peak 6 ( $\lambda_{\max}$  256 and 370). Peak 2 is the major compound of the extract. Peak 6, when compared to retention time (Rt) and UV absorption spectrum, was identified as quercetin, in addition to traces of catechin and gallic acid were also identified. Except for peak 6, it was not possible to identify the other peaks obtained in the HPLC-DAD analysis by the reference standards used.

**FIGURE 1:** HPLC-DAD analysis of the ethyl acetate (EtOAc) extract of *Anadenanthera colubrina*. In the image we can see the six main compounds: peak 1 ( $\lambda$  max 228, 260 and 294), peak 2 ( $\lambda$ max 256), peak 3 ( $\lambda$ max, 265, 354), peak 4 ( $\lambda$ max 256, 352), peak 5 ( $\lambda$ max 256, 356) and peak 6  $\lambda$ max 256, 370).



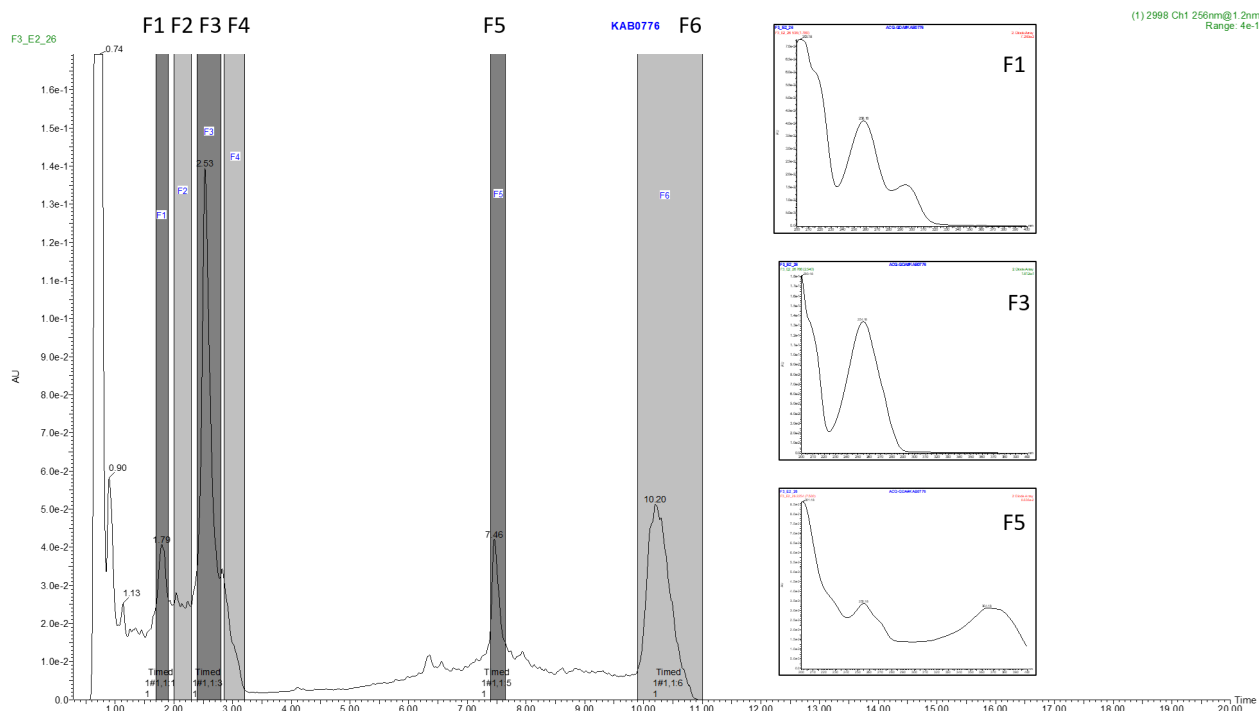
The EtOAc extract was subjected to Flash Chromatography (Biotage™), this semi purification generated six fractions grouped according to the UV absorption spectrum (**FIGURE 2**). These were tested against the microorganisms *Acc* and *Xcc* according to the activity recorded for the crude EtOAc extract (**TABLE 3**). Fraction 3 for the *Xcc* bacteria showed activity, as it had a MIC less than 1 mg.ml<sup>-1</sup>, so it was selected for the purification of the active compound.

**TABLE 3:** Antibacterial activity, Minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC), in mg.ml<sup>-1</sup>, of Flash Chromatography fractions of ethyl acetate (EtOAc) extract against selected plant pathogenic bacterial.

Fractions		<i>Acc</i>	<i>Xcc</i>
Flash Fraction 1	MIC	>5.8	>5.8
	MBC	>5.8	>5.8
Flash Fraction 2	MIC	>5.8	>5.8
	MBC	>5.8	>5.8
Flash Fraction 3	MIC	1.45	0.72
	MBC	1.45	1.45
Flash Fraction 4	MIC	5.8	5.8
	MBC	5.8	5.8
Flash Fraction 5	MIC	>5.8	>5.8
	MBC	>5.8	>5.8
Flash Fraction 6	MIC	>5.8	>5.8
	MBC	>5.8	>5.8

Legend: *Acidovorax citrulli* (*Acc*), *Xanthomonas campestris* pv. *campestris* (*Xcc*).

**FIGURE 2:** Purification of Fraction F3 (flash chromatography) by preparative HPLC. The fraction components were tracked using their respective UV spectra and purified. Compound with  $\lambda$  max 228, 260 and 294 (F1), compound with  $\lambda$ max 256 (F3), compound with  $\lambda$ max 256 and 370 (F5) and wash with 100% CAN (F6).

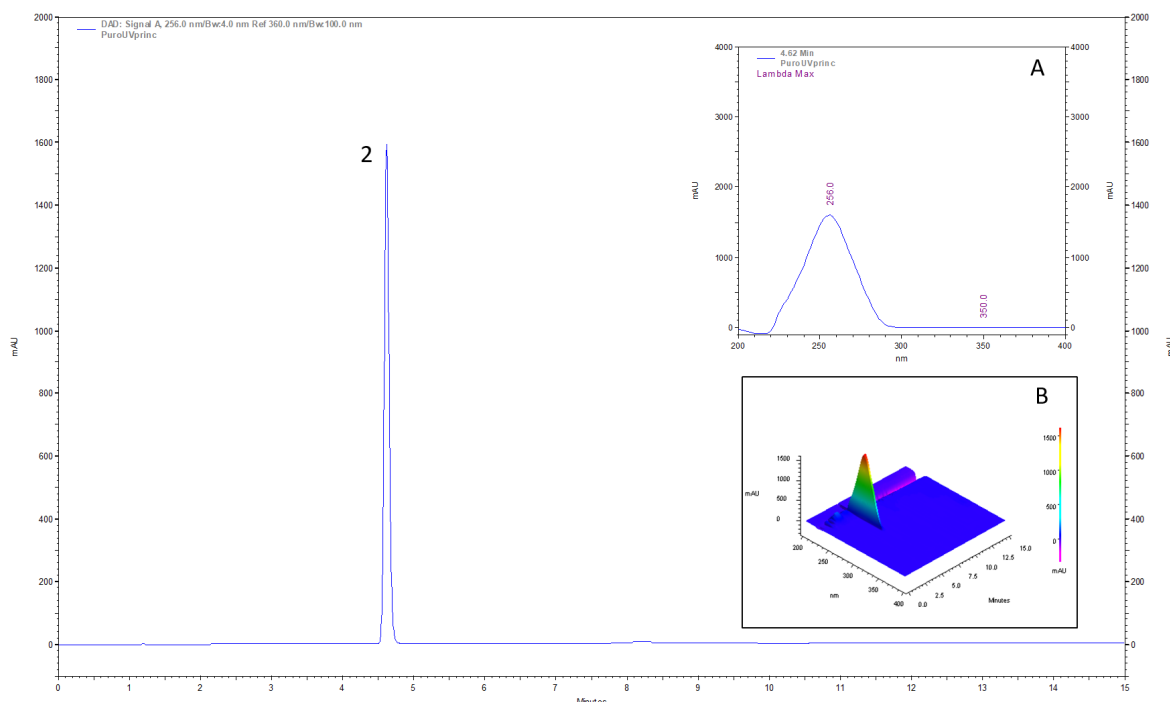


Thus, flash fraction 3 was analyzed and was subfractionated by HPLC-Preparative, giving rise to six subfractions that were again evaluated for their antimicrobial activity (**TABLE 4**). Subfraction 3 showed activity with MIC and MBC of 0.5 mg.ml<sup>-1</sup> for *Xcc*. In view of its antibacterial potential, subfraction 3 was analyzed by HPLC-DAD, and a pure compound was detected. The UV spectrum associated with data available in the literature indicates that this compound is p-hydroxybenzoic acid (**FIGURE 3**), tracked through its UV absorption spectrum using flash chromatography (Biotage™) and HPLC-Preparative (Autopurification System™).

**TABLE 4:** Antibacterial activity, Minimum inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC), in mg.ml<sup>-1</sup>, of preparative fractions against selected plant pathogenic bacterial.

Subfractions		<i>Xcc</i>
Preparatory Subfractions 1	MIC	>0.5
	MBC	>0.5
Preparatory Subfractions 2	MIC	>0.5
	MBC	>0.5
Preparatory Subfractions 3	MIC	0.5
	MBC	0.5
Preparatory Subfractions 4	MIC	>0.5
	MBC	>0.5
Preparatory Subfractions 5	MIC	>0.5
	MBC	>0.5
Preparatory Subfractions 6	MIC	>0.5
	MBC	>0.5

Legend: *Xanthomonas campestris* pv. *campestris* (*Xcc*).

**FIGURE 3:** F3 subfraction analyzed by HPLC-DAD. In the image, we can see that compound 2 was isolated. Peak UV spectrum 2  $\lambda_{max}$  256 (A). 190-400nm 3D chromatogram (B).

Given the importance of developing a biopesticide, several studies have been carried out, in search of this objective, thus, a screening carried out by Silva *et al.*<sup>[11]</sup> with several plants of medicinal importance against several phytopathogens pointed to *A. colubrina* as an important source of antimicrobial compounds. From this result, a bioguided purification approach was carried out, starting with an extraction following the elutropic order of solvents and using an *in vitro* anti-phytopathogenic bioassay. According to Santos *et al.*<sup>[12]</sup> to fight bacteria, an extract with MIC > 2.0 mg.ml<sup>-1</sup> is considered inactive, therefore, the active and promising extract for the isolation of bioactive compound was the one with the lowest MIC and MBC values. According to this criterion, the EtOAc extract was the most active, with MIC and MBC ≤ 1.56 mg.ml<sup>-1</sup> against the bacteria *A. citrulli* and *X. campestris* pv. *campestris*.

HPLC-DAD analysis of the crude EtOAc extract revealed a phenolic acid as the major (peak 2), with  $\lambda_{max}$  256 nm, its ultraviolet (UV) absorption spectrum being very similar to p-hydroxybenzoic acid<sup>[13-17]</sup>. This acid was previously isolated and identified in *Anadenanthera colubrina* in the works of Gutierrez-Lugo *et al.*<sup>[18]</sup> and Weber *et al.*<sup>[19]</sup>. It was also possible to verify the presence of four flavonoids (FIGURE 1) with characteristic UV spectra. The UV spectrum of flavonoids shows two main peaks in the region of 240-400 nm, these two peaks are referred to as the band I of the molecule (usually 300-380nm) and band II (usually 240-280 nm). Thus the peaks 3 ( $\lambda_{max}$ , 265, 354), 4 ( $\lambda_{max}$  256, 352) and 5 ( $\lambda_{max}$  256, 356) are referred to as quercetin derivatives and maybe quercetin 3,7-O-diglucoside ( $\lambda_{max}$  256, 355), quercetin 3-O-glucoside 7-O-rhamnoside ( $\lambda_{max}$  257,358), quercetin 3-methyl ether ( $\lambda_{max}$  257,358), quercetin 3-O-glucoside 7-O-rutinoside ( $\lambda_{max}$  257,358) among others<sup>[20]</sup>. Peak 6  $\lambda_{max}$  256 and 370 identified through the standard, as already mentioned, is quercetin.

The purification of the bioguided EtOAc extract by the assay against phytopathogens *in vitro* resulted in the isolation (FIGURE 2) of the major component (peak 2  $\lambda_{max}$  256) which when tested separately presented MIC and MBC of 0.5 mg.ml<sup>-1</sup> *X. campestris* pv. *campestris*. The difference between the MIC and MBC values of the extract and the isolated substance may be due to the presence of nutritional components, common in extracts,



such as proteins and sugars, which can contribute to the development of the microorganism<sup>[21]</sup>. Our data are in agreement with the findings of Araújo *et al.*<sup>[22]</sup> which isolated and identified by nuclear magnetic resonance (NMR) p-hydroxybenzoic acid the major compound of the ethyl acetate extract of aerial parts of *A. colubrina*.

Phenolic acids are part of the group of phenolic compounds, rarely occur as free acids, are divided into benzoic, cinnamic acids and their derivatives, p-hydroxybenzoic acid is the simplest form found in nature<sup>[23]</sup>. Despite data on the antimicrobial effects of phenolic acids<sup>[24,25]</sup>, studies dealing with the anti-phytopathogenic properties of its metabolites or derivatives are still scarce. Literature data indicate that p-hydroxybenzoic acid inhibits the growth of plant pathogens. Cho *et al.*<sup>[26]</sup> found that the acid inhibits the growth of *X. campestris* with an IC<sub>50</sub> of 0.136 mg.ml<sup>-1</sup>. However research indicates that the phytopathogen *Xcc* may have developed a functional degradation pathway of 4-HBA (4-hydroxybenzoate) that plays a role in detoxifying phenolic metabolites in the host during infection, however, the mechanistic details and the biological significance of this phenomenon have yet to be elucidated<sup>[27]</sup>.

In fungi assays, all extracts were  $\geq 6.25$  mg.ml<sup>-1</sup>, however, the CHX extract was the most active with MIC and MBC of 6.25 mg.ml<sup>-1</sup> for *Aspergillus flavus*, *Rhizopus sotolonifer*, and *Verticillium lecanii*, the rest of the extracts presented MIC and MFC  $\geq 12.5$  mg.ml<sup>-1</sup>. Campos *et al.*<sup>[28]</sup> perform a bioguided assay with *A. colubrina* against fungi and identify or extract hexane as the most active, assign an antimicrobial activity to three substances, among them:  $\beta$ -sitosterol and  $\beta$ -sitosterol linoleate, both in fruits and leaves and with MICs of 0.25 and 0.5 mg.ml<sup>-1</sup> in front of *Alternaria alternata* respectively. Due to the similarity with ergosterol, steroidal substances can compete with fungal proteins involved in the synthesis of this metabolite and can be lethal to the fungus<sup>[28]</sup>.

## Conclusion

The bioguided approach carried out with the extracts of the leaves of *A. colubrina* through *in vitro* antimicrobial tests led to p-hydroxybenzoic acid which, in addition to being the major component of the active extract, showed antimicrobial activity against the phytopathogens *Xanthomonas campestris* pv. *campestris* with MIC and MBC of 0.5 mg.ml<sup>-1</sup>. The data highlight the potential of the species as an alternative source of this compound to fight diseases of economic importance in agriculture.

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