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Original Article

Effects of Rainfall on the Antimicrobial Activity and Secondary Metabolites Contents of Leaves and Fruits of *Anadenanthera colubrina* from Caatinga Area.

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ABSTRACT

Background: *Anadenanthera colubrina* (Vell.) Brenan var. cebil (Griseb) is a plant widely used for medicinal proposes in Brazilian Northeast. **Objective:** This study aimed to analyze the influence of rainfall indexes (RI) in antimicrobial activity and phytochemical constituents of extracts from leaves and fruits of *A. colubrina*. **Material and Methods:** Samples were collected in Catimbau National Park (Buíque, Pernambuco, Brazil) at September 2010 (RI: 75 mm) and January (RI: 65 mm), April (RI: 162 mm) and June 2011 (RI: 73 mm). The extracts were prepared by Soxhlet extraction using cyclohexane, chloroform, ethyl acetate and methanol. The antimicrobial activity was determined by MIC and MBC values. **Results:** All extracts showed antimicrobial activity, but ethyl acetate extracts (from all periods) were more active. Strong correlations were found between the RI and the average MIC of MLE (ρ: -0.99), EALE (ρ: -0.81), CHFE (ρ: -0.81), EAFE (ρ: -0.80); while moderate and weak correlations were found for other extracts. Through a HPLC analysis was possible to reveal that the samples collected from dry periods had more chemical diversity (as they presented more peaks). Gallic acid and quercetin (and derivative compounds) were identified. The levels of quercetin were enhanced in extracts from dry months. **Conclusion:** Our results showed that the rainfall has a positive effect on the antimicrobial activity of leaves and fruits of *A. colubrina*, however these extracts showed more chemical diversity during dry months.

Key words: Natural products, Semi arid area, Antimicrobial agents, Medicinal plants.

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INTRODUCTION

The World Health Organization (WHO) has been drawing attention to the increasing problem of microbial resistance to conventional antibiotics for more than a decade.[1] This scenario of antimicrobial resistance has encouraged research using medicinal plants, especially those from under-exploited biomes, such as the Caatinga. This is a unique biome from Northeast of Brazil where traditional communities use hundreds of plant species form medical purposes, such as the control of microbial infections.[2,3] For some of those, scientific evidences confirm these therapeutic aptitudes.[4-7] In recent years, the ethnomedicinal knowledge has stimulated several studies about the pharmaceutical potential of natural products of northeastern Brazil.[8] This is justified by the fact that the intrinsic features of Caatinga area (drought, high solar radiation rates and other environmental stresses) influence the synthesis of secondary metabolites of its plants,[9] making them attractive targets for bioprospecting programs.[10] In traditional medicine, *Anadenanthera colubrina* (Vell.) Brenan var. *cebil* (Griseb) Alstchull (1964), popularly known as Angico (synonyms: *Acacia cebil, Peptadenia macrocarpa, Anadenanthera macrocarpa*),[11] is frequently used by inhabitants of the Caatinga biome to treat anemia, cancer and inflammatory diseases.[2,3] Previous studies demonstrated the antimicrobial potential of leaves and fruits of this plant, especially against *Staphylococcus aureus*.[4-6,12] In this context, the present study aims to analyze the effects of rainfall on the antimicrobial activity and secondary metabolites contents of leaves and fruits of *Anadenanthera colubrina*. With this information, the best collection time to maximize the bioactive compounds extraction for medicinal purposes may be determined.

MATERIAL AND METHODS

Plant material and extract preparation

Leaves and fruits of a single individual of *A. colubrina* were collected at Catimbau National Park (Buíque, Pernambuco, Brazil) at September 2010 and January, April and June 2011. The rainfall index for this months were 75 mm, 65 mm, 162 mm and 73 mm, respectively (as provided by Agronomic Institute of Pernambuco; IPA/PE). The voucher specimen (IPA 84.039) is deposited at IPA/PE. The plant material was dried at 45°C and after 3 days milled to a fine powder in a Macsalab Mill (Model 200 LAB, Eriez®, Bramley), and stored at room temperature in closed containers in the dark until used.

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Samples (100g) from each tissue were subjected to Soxhlet extraction using an eluotropic series of solvents in the following order: cyclohexane, chloroform, ethyl acetate and methanol. All samples were subjected to saturation at reflux for 24 hours. After this time, the extracts were filtered (Whatman filter paper No 1). Solvent was completely removed from all extracts from leaves (L) or fruits (F) (cyclohexane: ChLE and ChFE; chloroform: CLE and CFE; ethyl acetate: EALE and EAFE: methanol: MLE and MFE) on a rotating evaporator at 45º under reduced pressure and stored for later antimicrobial and phytochemical analyses.

Antimicrobial assays

Microorganisms

The microorganisms used in this work were provided by Culture Collection UFPEDA (Department of Antibiotics, UFPE), consisting of: *Staphylococcus aureus* (three strains; UFPEDA 02, 733 and 709), *Bacillus subtilis* (UFPEDA 86), *Escherichia coli* (UFPEDA 224), *Klebsiella pneumoniae* (UFPEDA 396) and *Pseudomonas aeruginosa* (UFPEDA 416).

Determination of minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimal inhibitory concentration (MIC) was determined by the microdilution method.[12] Twofold serial dilutions of each extract (initial concentration: 50 mg/mL) were prepared in Müller-Hinton broth (MHB) and 10 µL of bacterial suspension (approximately 1.5×10^8 CFU/mL) were added. The samples were incubated for 24 h at 37°C. Resazurin solution (0.01%) was used as an indicator by color change visualization: any color changes from purple to pink were recorded as bacterial growth. The lowest concentration at which no color change occurred was taken as the MIC. Afterwards, cultures were seeded in MHA and incubated for 24 h at 37°C to determine the minimum bactericidal concentration (MBC), which corresponds to the minimum concentration of the sample that eliminated the bacteria.

Phytochemical Analysis

Thin layer chromatography (TLC) analysis

The qualitative phytochemical analysis was performed by Thin layer chromatography (TLC) using aluminum-precoated plates of silica gel 60 F254 (Merck®) and adequate visualization techniques (Dragendorff, NEU-PEG, KOH-Ethanol, Liebermann-Burchard, vanillin-sulfuric acid and others reagents, according to the respective method).[13]

High-performance liquid chromatography (HPLC) analysis

Investigations of phenolic content were performed by a High-performance liquid chromatography system (HPLC; ProStar, Varian) which is comprised by a quaternary pump, diode array detector, auto-sampler. The reagents used were acetonitrile HPLC grade (Panreac®) and acetic acid (Vetec®). Water was purified through a milli-Q (Merck®) system. Phenolic compounds were analysed on a Phenomenex C18 column (250 x 4.6 mm, 5 µm), applying mobile phase gradient of acidified water (0.3% acetic acid) (solvent A) and acetonitrile (B) as follows: Linear gradient from 10 to 20% (B) from 0 to 10 min; and linear gradient from 20 to 28% (B) from 10 to 60 min. The flow rate was kept constant at 0.8 mL/min and detection was in the range of 190 to 450 nm. Phenolic compounds were identified by comparison of their retention times and their absorption spectra of ultraviolet light (UV). Gallic acid, chlorogenic acid, ellagic acid, rutin and quecertin were used as standard compounds (all purchased from Sigma-Aldrich).

Statistical analyses

Statistical analyses were performed by One-way analysis of variance (ANOVA). All analyses were carried out using software Graph Prism, version 4. The correlation indices were calculated using the Pearson coefficient (ρ). Only for correlation between Rainfall Index and MIC values a negative ρ -value is considered as direct correlation.

RESULTS

Effects of rainfall on the antimicrobial activity of *Anadenanthera colubrina*

All extracts from of *A. colubrina* showed antimicrobial activity, being more active against gram-positive organisms (Table 1). Most tested bacteria were more sensitive to ethyl acetate extracts (from all periods). Taking into account the results for Gram-positive bacteria (all *S. aureus* strains and *B. subtilis*), ethyl acetate extracts from leaves showed the lowest average MIC values (1.06 mg/mL), this value was significantly lower than those observed for other extracts ($p < 0.05$). In the same way, among fruits extracts, the lowest average of MIC values was found to ethyl acetate extracts (17.50 mg/mL), however no significant differences were observed between the results. For this reason, the ethyl acetate extracts were selected for chemical analysis.

Regarding the effect of the collection period on antimicrobial activity, the lowest MIC values were observed in the months with the highest rainfall index (RI) for extract from both tissues (Table 1). Strong correlations were found between the RI and the average MIC of MLE (ρ: -0.99), EALE (ρ: -0.81), CHFE (ρ: -0.81), EAFE (ρ: -0.80); moderate correlation was observed for CLE (ρ: -0.62); while weak correlations were found for CFE (ρ: -0.48), MFE (ρ: -0.42), and CHLE (ρ: -0.27).

Effects of rainfall on phytochemical composition of *Anadenanthera colubrina*

It was observed by TLC analysis that the ethyl acetate extracts from both tissues exhibited flavonoids, cinnamic derivatives, terpenes, cyanogenic glycosides and proanthocyanidins. Whereas TLC assays did not show qualitative differences between the extracts obtained from leaves or fruits (Table S1), we were able to detect 15 phenolic compounds with different retention time (Rt) and UV spectra in HPLC chromatograms. The presence and concentration of these compounds varied with tissue and rainfall index.

The extracts from fruits showed more compounds than those from leaves: peaks 2 and 5 were found at all fruits samples, EAFE1 had the highest chemical phenolic diversity as 13 peaks were detected and some compounds were only found in this sample (peaks 3 and 4). EAFE2, EAFE3 and EAFE4 showed 10 peaks. The concentration of some compounds also varied according to collection time. For example, peak 6 had maximum detection on September (EAFE1); while peaks 8, 12 and 15 apparently were more found in April and January (Figure 1). Regarding the qualitative results for leaf extracts, EALE2 showed the highest number of peaks (8). Only two peaks were specific for EALE2 (12 and 13) and peak 14 was only absent for EALE1; the other peaks were present in all extracts. However, quantitative differences for some compounds contents could also be observed, for example, peak 1 was larger in January, and peak 15 in September and January (Figure 1).

The identity of gallic acid (peak 1) and quercetin (peak 15) were confirmed by co-injection of an internal standard for each compound (Figure S1). Some compounds (peak 10 and 11) are presumably quercetin derivatives due to the close similarity in the UV absorption spectra (Figure S2). Gallic acid, quercetin (and its derivatives) had already been

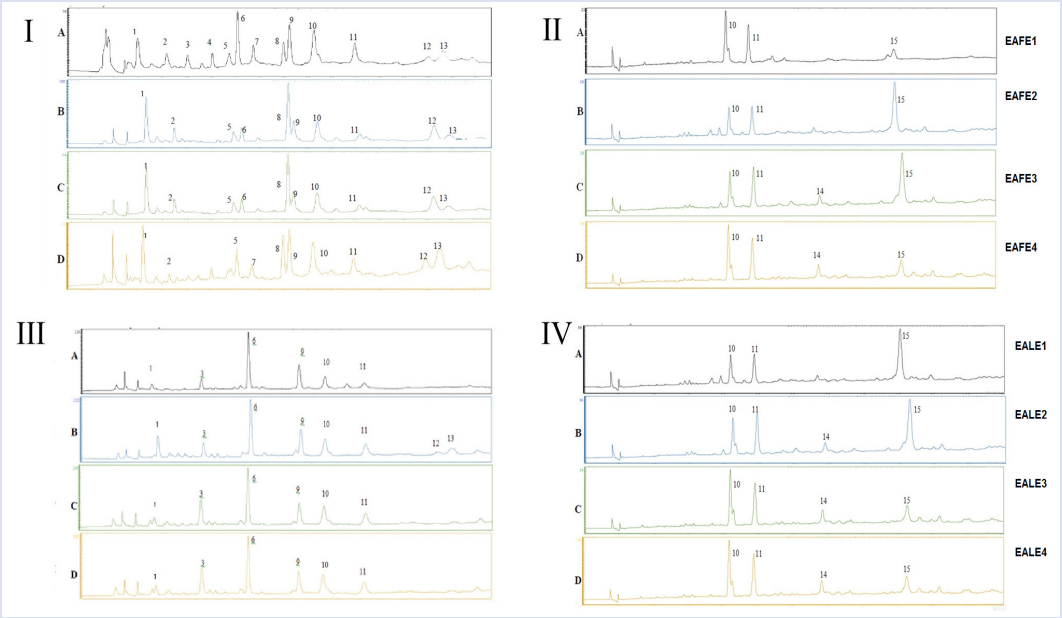

Figure 1: HPLC-UV chromatograms of phenolic compounds from ethyl acetate extracts of leaves and fruits of *Anadenanthera colubrina* collected at September 2010 (A), January 2011 (B), April 2011 (C), June 2011 (D). I: Chromatogram from fruits detected at 280 nm; II: Chromatogram from fruits detected at 340 nm; III: Chromatogram from leaves detected at 280 nm; IV: Chromatogram from leaves detected at 340 nm.

Table 1: Antimicrobial activity of ethyl acetate extracts from leaves and fruits of *Anadenanthera colubrina*.

Tissues	Strain	September		January		April		June	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Leaves	*S. aureus* 02	0.390	6.25	0.781	12.5	0.195	0.781	0.390	1.562
	S. aureus 733	0.781	3.125	0.781	25	0.390	0.781	6.25	12.5
	S. aureus 709	1.562	50	1.562	>50	0.781	3.125	0.390	12.5
	E. coli	6.25	6.25	6.25	50	0.390	>50	6.25	25
	P. aeruginosa	3.125	6.25	6.25	50	12.5	12.5	0.781	6.25
	B. subtilis	0.781	1.5625	0.781	50	0.390	0.390	0.781	>50
	K. pneumoniae	25	>50	25	>50	1.562	6.25	25	>50
Fruits	*S. aureus* 02	0.390	3.125	0.390	12.5	0.097	0.390	0.781	>50
	S. aureus 733	0.781	3.125	1.562	6.25	0.390	0.781	1.562	3.125
	S. aureus 709	0.193	6.25	1.562	6.25	0.195	0.781	0.019	0.039
	E. coli	6.25	6.25	6.25	6.25	0.195	>50	6.25	6.25
	P. aeruginosa	1.562	1.5625	1.562	3.125	6.25	12.5	6.25	6.25
	B. subtilis	0.781	3.125	0.781	50	0.195	0.390	0.390	6.25
	K. pneumoniae	25	>50	25	>50	0.781	>50	6.25	6.25

Results are expressed in mg/mL

Table S1: Phytochemical profile of ethyl acetates from leaves and fruits of *Anadenanthera colubrina*.

Class of secondary metabolite	*Anandenanthera colubrina* var. Cebil							
	Leaves				Fruits			
Flavonoids	++	++	++	++	++	++	++	++
Cinnamic Acid Derivatives	traces	traces	traces	traces	++	++	++	++
Triterpenes and Steroids	+	+	+	+	+	traces	traces	traces
Mono and Sesquiterpenes	-	-	-	-	-	-	-	-
Alkaloids	-	-	-	-	-	-	-	-
Proanthocyanidins	++	++	++	++	+++	+++	+++	+++

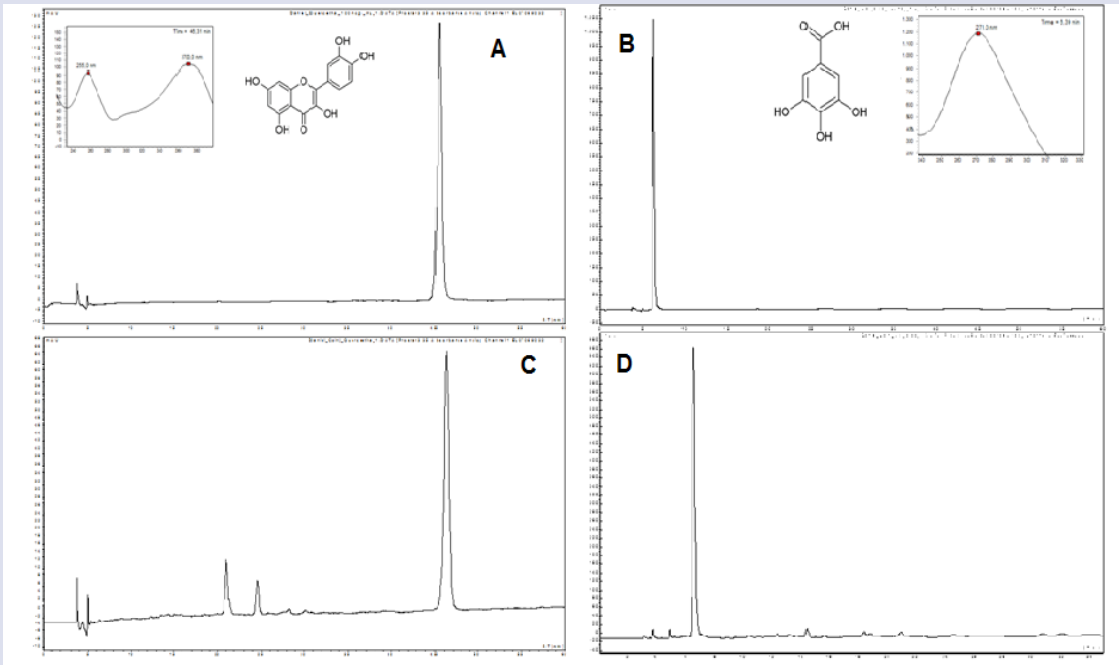

Figure S1: HPLC-UV chromatograms of quercetin (A), gallic acid (B), quercetin + ethyl acetate extracts of *A. colubrina* leaves (C) and gallic acid + ethyl acetate extracts of *A. colubrina* leaves (D). Detection at 340 nm.

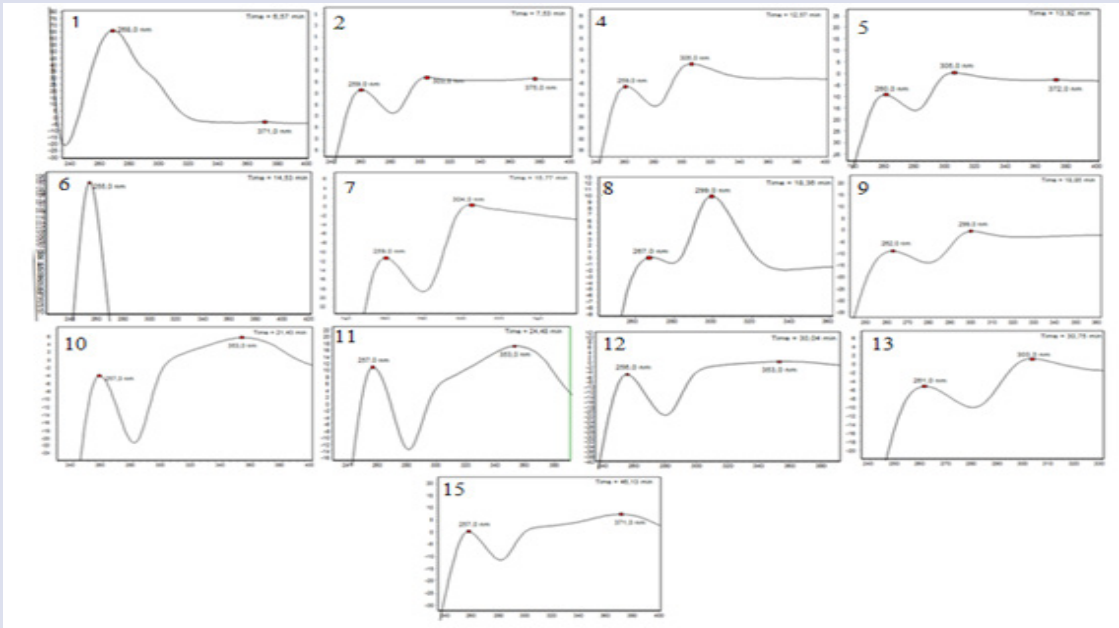

Figure S2: UV spectra analysis of the main peaks. Peak 1:gallic acid (UV 268nm); peaks 10 e 11: quercetin derivatives (UV 257 353nm) and peak 15: quercetin (UV 257 371nm).

detected in the extracts from *A. colubrina*.[6,21] Antimicrobial activity of plants is commonly attributed to these compounds.[14-16]

DISCUSSION

This work analyzed the effects of rainfall on the antimicrobial action and phytochemical constituents of extracts from leaves and fruits of *A. colubrina*. This plant has been subject of several studies about its pharmaceutical potential.[4-7,12,17] In our previous studies the antimicrobial action of extracts and fractions from leaves and fruits of *A. colubrina* was reported against Gram-positive bacteria, and no activity was observed against Gram negative bacteria.[4-6,12] In the present work we employed a different extraction method, by which we obtained extracts with activity against tested Gram negative bacteria. Anyway, the best activity was found against *S. aureus* and *B. subtilis,* and ethyl acetate extracts had the best activity. It is also important to highlight that some variation on antimicrobial activity of products derived from *A. colubrina* has been found according with area of cultivation. For example, extracts derived of samples from Cerrado biome did not show antagonist activity,[18] while others from Caatinga area were able to inhibit microbial grow.[19]

Since the production of secondary metabolites is influenced by environmental conditions (such as temperature, soil composition, solar irradiation, water availability), we attempted to evaluate the influence of rainfall on the antimicrobial action and chemical composition of extracts from both leaves and fruits. First we analyzed the variation on antimicrobial

activity of those extracts. We correlated the RI for each month where the collection was performed with the MIC average obtained. Strong correlations were found for most of extracts. There is no consensus on the effect of rainfall index in the biological activity of plants, both positive and negative correlations are reported.[20,21] Our results corroborate with several reports which showed positive effects of rainfall on the biological activity of plant extracts.[20,21]

The influence of rainfall on the phytochemical composition of *A. colubrina* extracts was first analyzed by TLC based assay. In general, we observed the same composition reported in our previous work.[12] It was not noted qualitative differences between the extracts prepared in each period. Thus, we attempted to perform a HLPC analysis which revealed some quantitative differences: the samples collected from dry periods had more diversity (as they presented more peaks). Some compounds were identified: gallic acid (peak 1), quercetin (peak 15) and two quercetin derivatives (peaks 10 and 11). In addition, quercetin levels increased in those extracts from dry months. Enhanced levels of quercetin have been reported to other plants during dry seasons.[22] The production of quercetin and other flavonoids was shown to be up-regulated during stressful conditions such as excessive ultra-violet radiation and high salinity.[23] These findings could explain the well-known photoprotective effect of quercetin.[24]

CONCLUSION

Our results confirm that the rainfall levels have influence on the antimicrobial activity and chemical diversity of leaves and fruits of *A. colubrina*. Specifically, the antimicrobial activity was enhanced during months with higher rainfall levels, while the most chemical diversity was found in dry months. The increased levels of quercetin found in samples from dry months suggest metabolic alterations in order to produce compounds related to plant resistance during stressful conditions. These analyses may direct for the best collection time for obtain antimicrobial products derived from *A. colubrina* which can be applied for biomedical purposes.

CONFLICT OF INTEREST

Nil

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ABBREVIATIONS USED

A. colubrina: Anadenanthera colubrina; **B. subtillis:** Bacillus subitilis; **CFE:** Chloroform Fruits Extract; **CHFE:** Cyclohexane Fruits Extract; **CHLE:** Cyclohexane Leaves Extrac; **CLE:** Chloroform Leaves Extract; **E. coli:** Escherichia coli; **EAFE:** Ethyl Acetate Fruits Extract; **EAFE1:** Ethyl Acetate Fruits Extract September; **EAFE2:** Ethyl Acetate Fruits Extract January; **EAFE3:** Ethyl Acetate Fruits Extract April; **EAFE:** Ethyl Acetate Fruits Extract June; **EALE:** Ethyl Acetate Leaves Extract; **EALE1:** Ethyl Acetate Leaves Extract September; **EALE2:** Ethyl Acetate Leaves Extract January; **EALE3:** Ethyl Acetate Leaves Extract April; **EALE4:** Ethyl Acetate Leaves Extract June; **HPLC:** High Performance Liquid Chromatography; **IPA:** Agronomic Institute of Pernambuco; **K. pneumoniae:** Klebsiella pneumoniae; **MBC:** Minimum Bactericidal Concentration; **MFE:** Methanol Fruits Extract; **MIC:** Minimal Inhibitory Concentration; **MLE:** Methanol Leaves Extract; **P. aeruginosa:** Pseudomonas aeruginosa; **PE:** Pernambuco; **RI:** Rainfall Indexes; **Rt:** Retention Time; **S. aureus:** Staphylococcus aureus; **TLC:** Thin Layer Chromatography; **UFPEDA:** Culture Collection of Department of Antibiotics; **UFPE:** UV: Ultraviolet; **WHO:** World Health Organization.

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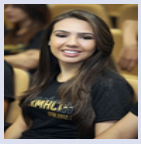

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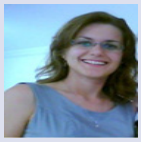

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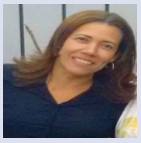

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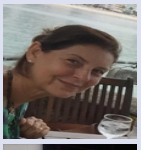

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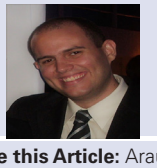

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