UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Faculdade de Farmácia

Disciplinas de Trabalho de Conclusão de curso I e II

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Matheus Weiler do Amaral

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Trabalho de conclusão
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Antibacterial activity of plant extracts from Caatinga

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Abstract:

The Brazilian semi-arid or Caatinga is a biome located almost exclusively in the Northeast region. The local communities demonstrated numerous uses for the plants, although few studies about the pharmacological properties of these species there have been developed. The present study evaluated the anti-*Pseudomonas aeruginosa* activity of aqueous extracts from *Anadenanthera colubrina* (Fabaceae, Mimosoideae), *Myracrodruon urundeuva* (Anacardiaceae), *Commiphora leptophloeos* (Burseraceae) and *Pityrocarpa moniliformis* (Fabaceae, Mimosoideae) and characterized some aspects of their mechanism of action. The minimal inhibitory concentration and the effect of the extracts on bacterial growth were determined by counting the colony forming units, as well as by antibacterial activity kinectics. Transmission electron microscopy was used to support the previous results. Phytochemical analysis was performed by TLC, bioautography and HPLC. All extracts showed a bacteriostatic effect under the experimental condition. Polyphenols, flavonoids, anthraquinones, anthrones, coumarins, peptides, terpens, steroids and saponins are the supposed class of compounds involved in the antibacterial activity. The four extracts are possible new sources of antibacterial agents against *P. aeruginosa.*

Key Words:

Anadenanthera colubrina (Fabaceae Mimosoideae),

Myracrodruon urundeuva (Anacardiaceae),

Commiphora leptophloeos (Burseraceae),

Pityrocarpa moniliformis (Fabaceae Mimosoideae),

Pseudomonas aeruginosa,

Antibacterial activity

Abbreviations:

MHA: Mueller Hinton Agar

RP-TLC: Reverse phase-thin layer chromatography

MIC: Minimal inhibitory concentration

CFU: Colony forming units

TSB: Triptone Soy Broth

ANVISA: Agência nacional de vigilância sanitária

Introduction:

The Brazilian semi-arid or Caatinga, from the native tupi language-KAA(forest) and-TINGA(white, clear) [1], is a biome located almost exclusively in the Northeast region. This semi-arid region demonstrates different kinds of vegetation due te varying degrees of edaphoclimatic aridity. It is generally associated with the distance from the Atlantic coast, altitude, geomorphology, degree of dissection of the landscape, slope, wind exposure, as well as soil depth and its physical and chemical composition [2].The Caatinga is individualized as a tree and shrub vegetation, in which almost all plant species predominates the expiry from the leaves about the other forms of resistance to the water deficiency; Cactaceae and Bromeliaceae rich, having a huge number of thorny and endemic plants. In most of the Caatinga dominium rains less than 750 mm per year, concentrated in November to June period. The region is characterized by having crystalline, practically impervious and sedimentary lands presenting good water reserves underground. The soils, with rare exceptions, are little developed, mineralogical rich, rocky, little thick and with low capacity for water retention, the limiting factor to primary production in this region[3].

The Caatinga region is known by the low economic development, which reflexes in low access by the population to industrialized drugs, determining that, frequently, the treatment disposable is the use of the medicinal plants. The local communities demonstrated numerous uses for the plants, although few studies about the pharmacological properties of these species have been developed. Taking into account that the ethnopharmacological approach many studies described a wide use of the plants in Caatinga as antiseptic, agents to treat respiratory disorders or to treat general infections [4-8], leading to speculate some possible additional antimicrobial activity.

Pseudomonas aeruginosa is a Gram-negative environmental bacterium and an opportunistic microorganism. It is a leading cause of nosocomial infections, being responsible for 10% of all nosocomial-acquired infections (HAIs)[9] and the second cause of Hospital-acquired pneumonia (10–15% cases)[10]. In addition, it is the most important pathogen in cystic fibrosis patients lung infections (80% of adult patients) [11]. The success of this environmental bacterium at the hospital since the 1950s' mainly relies on its ability to resist the selective pressure exerted on microbial floras by antimicrobials, and to become recalcitrant to any of the commercially available antibiotics through the operation of various intrinsic or transmitted mechanisms of resistance [12]. In Brazil, efflux systems, porin down regulation, AmpC overproduction, and β-lactamases including ESBL and metallo enzymes, particularly SPM-1, interplay a diverse role among Brazilian multidrug-resistant *P. aeruginosa* isolates. The possibility of the *P. aeruginosa* persistence and spread in hospital environments is increased by the colistin-only sensitive Brazilian clone SP (from São Paulo) harborin bla_{SPM-1} that imposes an immense limitation on therapy for treating the infection[13].

According to the WHO, the discovery of antimicrobials is one of the most important advances in health in human history - decreasing suffering from disease and saving lives [14],

but resistance has been barrier to the successful antimicrobial therapy. In this context, the search for new sources is necessary, and natural products seem to be very promising by itself or as prototype models of compounds. Ethnopharmacological data can be considered a valuable pathway in drug discovery. Regarding the antimicrobial problematic, the aim of this study was to evaluate the anti-*P. aeruginosa* activity of four plant extracts from Caatinga and to characterize some aspects of their mechanism of action and chemistry.

Material and methods:

Plant Material:

Plants were collected from various physiognomies in Parque Nacional do Catimbau, Pernambuco, Brazil, between July and August 2009. Botanical identification was performed at the Herbarium of the Instituto Agronômico de Pernambuco (IPA), where voucher of each species have been deposited (Table 1). The parts of plants used were dried using an incubator at 40°C for 2 to 3 days. The dried plant materials were ground into powder form using a grinder followed by a warring blender.

Table 1. Informations about plants investigated.

Scientific name	Family	Popular name	Studied Part	Voucher number	Acronym	Usage forms, preparation and therapeutic indication
Anadenanthera colubrina var. *colubrina* (Griseb.) Altschul	Fabaceae Mimosoideae	Angico, Angico branco	Bark	IPA 84039	Ac26	Drink. A maceration of a handful of stem bark in a liter of wine or “cachaça” is used against coughs, whooping cough and bronchitis [16].
Myracrodruon urundeuva Alemão	Anacardiaceae	Aroeira, Aroeira do Sertão	Bark	IPA 84059	Mu34	Drink or wash the affected site. A decoction or maceration of a handful of stem bark in a liter of water. Used in cases of ovarian inflammation and in ulcerative external afflictions. This species has many other medicinal indications [16].
Commiphora leptophloeos (Mart.) J.B.Gillett	Burseraceae	Umburana, Imburana, Amburana, Imburana de cambão	Bark	IPA 84037	Cl33	Drink or wash/bathe the affected site. A decoction of a handful of roots in a liter of water is prepared with sugar as syrup. Used in the treatment of influenza, coughs, bronchitis, to treat urinary and liver diseases. Also, external use against vaginal ulcers and others [16].
Pityrocarpa moniliformis (Benth.) Luckow & Jobson. = *Piptadenia moniliformis* (Benth.)	Fabaceae Mimosoideae	Catanduva	Leaf	IPA 84048	Pm45	In communication with local people was informed that the stem bark and root are used as healing.

Extraction:

Aqueous extracts were prepared with sterile Milli-Q water (1:10, [w/v]). The extraction occurred during 24 hours without shaking at room temperature and protected from light. The extracts were filtered under vacuum, lyophilized and stored at 4°C.

Inoculum preparation:

Stock bacterial cultures of *P. aeruginosa* ATCC 27853 were streaked on MHA plate and incubated at 37°C for 18 h. Inocula were prepared by colony suspension in sterile saline solution (0,9% NaCl [w/v]) up to reach 0,150 ±0,003 optical density at 600 nm (10^8 CFU/mL corresponding to 1 in MCFarland scale).

Bioassays:

MIC and effect on bacterial growth:

The work solutions of the four extracts and the reference antimicrobials, gentamicin and erythromycin, were made with sterile Milli-Q water and then filtered by a 0,22 µm membrane filter. In sterile polystyrene 96 well microplates were added 80 µL of the work solutions (concentration of the sample Ac26: 6 - 0.5 mg/mL, sample Cl33: 4 - 0,25 mg/mL, sample Mu34: 7 - 2 mg/mL, sample Pm45: 16 - 2 mg/mL), 40 µL of TSB and 80 µL of the inocula. Bacterial growth and sterility controls were performed. Microplates were incubated for 6 h at 37°C. The experiment was performed in eightfold and three times. MIC was defined as the lowest concentration of the plant extract that inhibits growth compared with the untreated bacteria, in experimental conditions. After, a serial dilution of the MIC well (100 µL) was performed in sterile saline solution. Aliquots of 50 µL of the 10^{-5} and 10^{-6} dilutions were placed in MHA and incubated for 18 h at 37 °C. Bactericidal or bacteriostatic activity was taken by counting the CFU per plate. The extract can be considered bactericide if there was no significant bacterial growth.

Antibacterial activity kinectics:

In sterile polystyrene 96 well plates, 80 µL of samples at 0.25xMIC, 0.5xMIC, MIC, 2xMIC concentration and erythromycin at MIC concentration were added, 40 µL of TSB and 80 µL of the inoculums were added and incubated in 37 °C. The OD_{600nm} was read in microplate reader at 0, 3, 6, 9, 24, 30, 48, 54 and 72 h of incubation time. Three controls were performed: (i) samples were replaced by sterile water in growth control, (ii) bacterial inoculums were replaced by saline solution in samples colour wells (this avoided the interference of the samples colour in the results), and (iii) sterility controls only with TSB. Experiments were conducted four times for each sample concentration.

Transmission electron microscopy

Inocula (400 µL) were incubated (6h, 37 °C) in sterile tubes containing 400 µL of four extracts at MIC concentration and the final volume was completed to 1 mL with TSB medium. Untreated cells replaced the extract to sterile Milli-Q water. Samples were centrifuged (10000 rpm, 60 min) and the pellets were washed twice with 900 µL of PBS (pH 7.0). Cells were fixed in glutaraldehyde 2,5% and p-formadehyde 2%, at 4 °C for 12 h, and were postfixed with osmium tetroxide 1% at room temperature for 2h 30 min. Cells were dehydrated with acetone solutions (30%, 50%, 70%, 90% and 100%) and then embedded gradually in resin solutions (25%, 50%, 75% and 100%). Series of ultrathin sections were cut and collected on copper grids. Following staining with uranyl acetate (30 min) and lead citrate (20 min), the sections were examined using a JEOL-1200 EX transmission electron microscope.

Chemical analysis:

Thin Layer Chromatography and bioautography:

Aqueous solutions of the four extracts (10 mg/mL) were analyzed by TLC on silica gel 60 F_{254} and on silica gel RPC-18 plates. The solvent system was ethyl acetate:water:acetic acid:formic acid (9:2.3:1:1), and for RPC-18 plates was methanol:water (3:1). Visualization was performed only to silica gel 60 F_{254} under visible and UV light (254 and 365 nm) and after staining with colorimetric specific groups reagents: Dragendorff reagent, Eckert's reagent, Neu-reagent following with plate dipping in polyethylene glycol, Ninhydrin reagent, Iodine vapors, Potassium hydroxide reagent and Iron(III) chloride [17, 18]. The plates used to visualize under UV light and the RPC-18 were used to the bio-autography assays. They were covered with a misture of 12 mL of MHA and 8 mL of the inocula prepared as previously described. The plates incubated at 4°C for 2 h 40 min, and at 37°C for 18 h. Afterwards, the plates were covered with triphenyltetrazolium 5% solution and incubated at 37 °C for 2,5 h. Cream-white spots appear against a purple background on the TLC plate surface, pointing the presence of antibacterial agents [19].

Liquid Chromatography:

The HPLC qualitative analyses were carried out on a Shimadzu LC-20AT coupled to a diode array detector Shimadzu SPD M20A, and a reversed-phase column Shim-pack VP-ODS (250 x 4.6 mmI.D., Shimadzu) was used. Gradient elution was performed with solution A, acetonitrile: water (5:95, [v/v]), and solution B comprising 100 % acetonitrile, delivered at a flow rate of 0.6 mL min^{-1} as follows: initially 95 % of solution A increasing up to 100 % of solution B during 60 min and finally 100 % of B for 15 min. The injection volume was 20 µL from a solution with concentration of 2.5 mg mL^{-1}. Maximums of absorption of the compounds were evaluated at 210, 254, 273 and 365 nm.

Results:

MIC and effect on bacterial growth:

Aqueous extracts of the four plants showed inhibitory activity against *P. aeruginosa* under the experimental conditions, presenting a bacteriostatic effect (table 2). The MICs of references antimicrobials are under expected with literature [20] being a control test conditions. Erythromycin MIC concentration was used to determine the kinetics of antibacterial activity.

Table 2 MIC, counting CFU/mL and effect on bacterial growth from plant extracts and reference antimicrobials.

Aqueous solution	MIC µg/mL	CFU 10^8/mL	Effect on bacterial Growth
Ac26	2500	4,98±0.72	Bacteriostatic
Cl33	1000	3.45±0.12	Bacteriostatic
Mu34	4000	6,03±0.66	Bacteriostatic
Pm45	10000	2.02	Bacteriostatic
Gentamicin	2	-	Bactericide [21]
Erythromycin	512	-	Bacteriostatic [22]

Antibacterial activity kinectics:

A significant decreased in the bacterial growth was observed when *P. aeruginosa* was exposed to the samples, characterizing the bacteriostatic effect of them (Fig.1). It is possible to visualize that bacterial growth is inhibited until 9 h. When the kinects inhibition of erythromycin is compared with the extracts, is possible to see that they are similar, confirming the bacteriostatic action in the experimental conditions against *P. aeruginosa*.

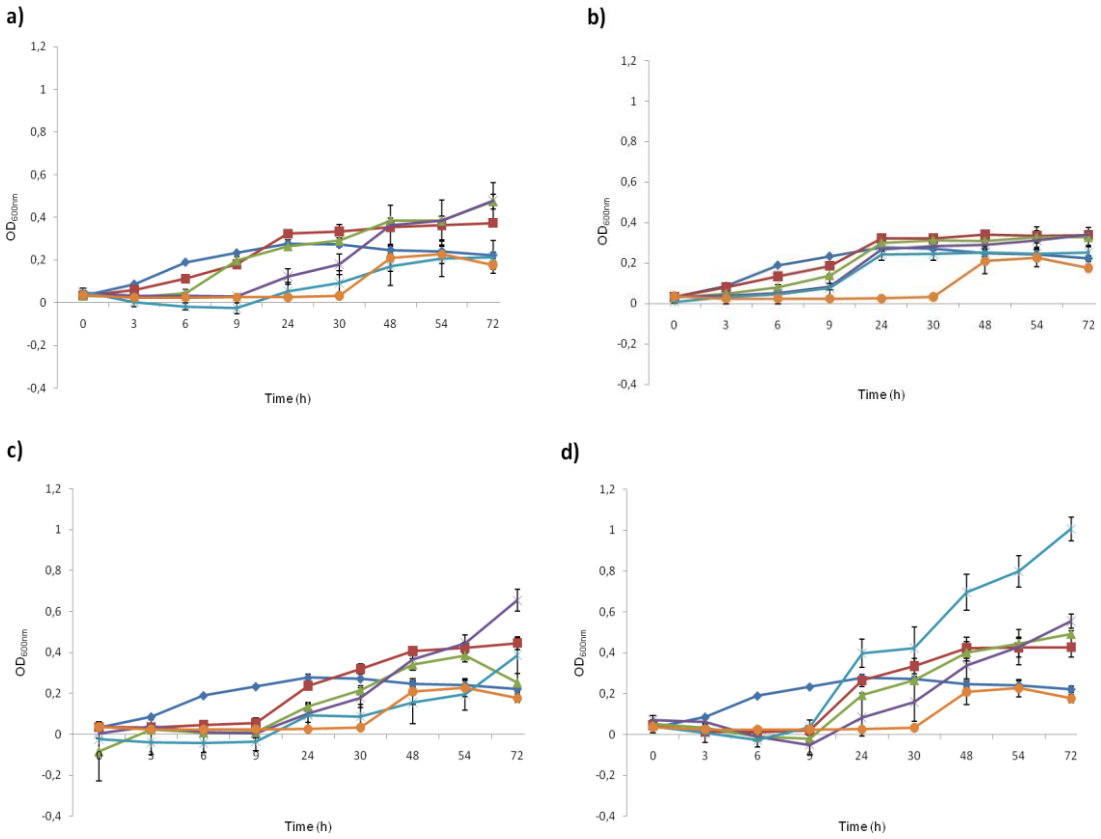

Fig. 1 The antibacterial activity kinectics of plant extracts from Caatinga: a) *Anadenathera colubrina*; b) *Commihora leptophlocos*; c) *Myracrodruoum urundeuva*; d) *Pityrocarpa moniliformis*.♦ Bacterial Growth control,● Erythromycin, ■ Extracts at 0.25xMIC concentration, ▲ Extracts at 0.5xMIC concentration, X Extracts at MIC concentration, X Extracts at 2xMIC concentration.

Transmission electron microscopy:

Bacterial cells treated with plant extracts at MIC concentration, during 6 h at 37 °C and were examined by TEM. In MIC, at magnification 15000 x all extracts appear to promote extra- and intracellular modifications in some cells (Fig. 2d,g,j,m) when compared with untreated cells (Fig.2a). Furthermore the Ac26 extract showed a visible decrease in the number of cells (Fig.2d). Also, some cells seem to be not affected by extracts. Higher magnification (120000 x) showed that all extracts coagulated cytosolic components and Pm45 causes empty spaces in the extremity of the cell (Fig.2n). Disrupted cytoplasm membrane and cell wall are seen in all treatment when compared with untreated cells (Fig. 2b) and the Mu34 causes separation of cytoplasm membrane from the cell wall (Fig.2i).

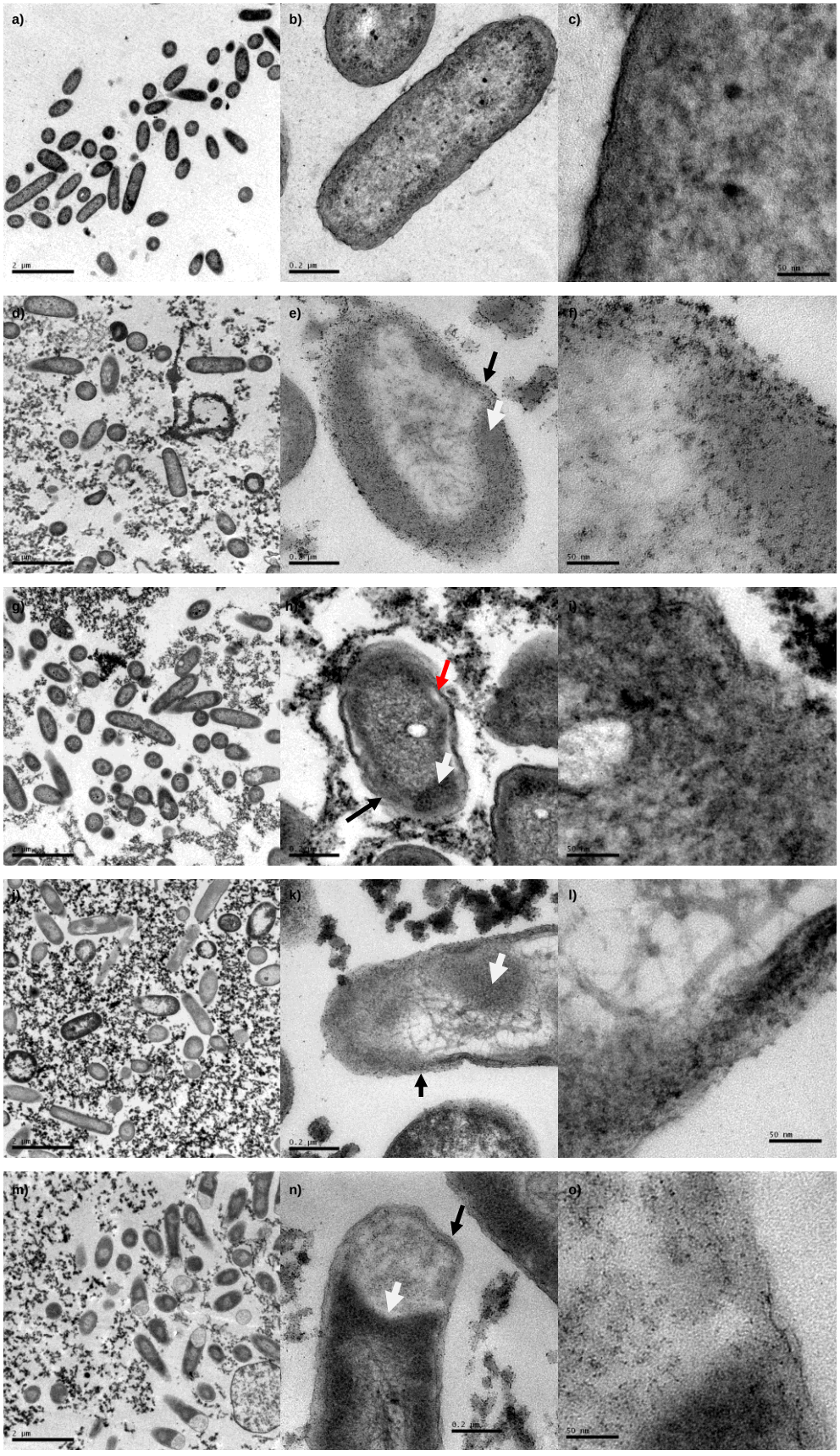

Fig 2: Transmission electron micrographs of *P. aeruginosa* ATCC 27853. Fig. 2a-c: Untreated cells at 15000x, 120000x and 500000x magnification, respectively. Fig.2d-f: Bacterial cells treated with Ac26 at 15000x, 120000x and 500000x magnification, respectively. Fig.2g-i: Bacterial cells treated with Cl33 at 15000x, 120000x and 500000x magnification, respectively. Fig.2j-l: Bacterial cells treated with Mu34 at 15000x, 120000x and 500000x magnification, respectively. Fig.2m-o: Bacterial cells treated with Pm45 at 15000x, 120000x and 500000x magnification, respectively. The white arrows shows the coagulated cytosolic components, the black arrows shows the damage to the cell envelope and the red arrow shows the separation of cytoplasm membrane of cell wall.

Thin Layer Chromatography and bio-autography:

Analysis of the TLCs (Fig. 3) shows the complexity of the extracts. Only presence or absence of positive reaction were evaluated, indicating phytochemistry composition of the extracts preliminary (Table 3). Bioautography of the extracts showed that hydrophilic compounds are involved with antibacterial activity, but Cl33 and Mu34 also presented hydrophobic compounds with activity (Fig.4). These data were confirmed in the RP-TLC, where the actives appear in the top of the plate, a zone of hydrophilic compounds. While Cl33 and Mu34 showed antibacterial activity of compounds in the application point a zone of hydrophilic compounds in the RP-TLC.

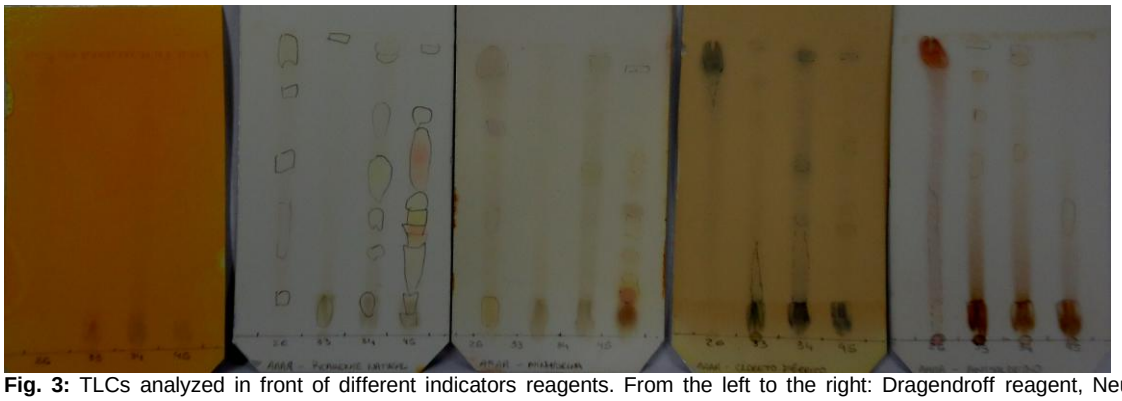

Fig. 3: TLCs analyzed in front of different indicators reagents. From the left to the right: Dragendroff reagent, Neu-reagent, Ninhydrin reagent, Iron(III) chloride and Eckert's reagent. The order of application of extracts in the TLCs was, from the left to the right: Ac26, Cl33, Mu34 and Pm45. TLC analysis with potassium hydroxide not shown.

Table 3. Analysis of TLC plates with indicators reagents for specific groups. (+) indicates positive reaction, (-) indicates negative reaction.

Extract	**Reagents**						
	Eckert's	**Dragendorff**	**Iodine**	**Ninhydrin**	**KOH**	**$FeCl_3$**	**Neu-reagent**
Possible detected compounds	Terpens, steroids, saponins	Alkaloids	Conjugated double bounds	Amino acids, biogenic amines	Anthraquinones, coumarins	Polyphenols	Flavonoids
Ac26	+	-	-	+	-	+	+
Mu34	+	-	-	+	-	+	+
Cl33	+	-	+	+	-	+	+
Pm45	+	-	-	+	-	+	+

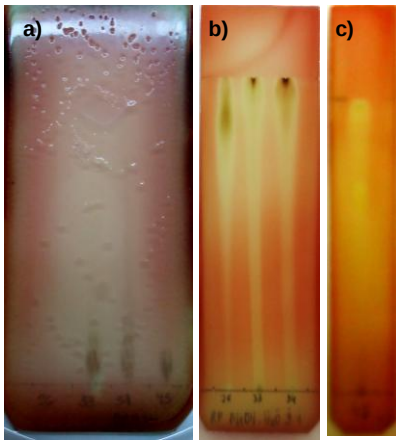

Fig. 4: Bioautography of the extracts. (a) TLC silica gel 60 F_{254}. The order of application of the extracts in the TLCs was, from the left to the right: Ac26, Cl33, Mu34 and Pm45. (b) RP-TLC of extracts Ac 26, Cl33 and Mu 34, (c) RP-TLC of Pm45 extract. The cream-white zones show the antibacterial activity from compounds of the extracts.

Liquid Chromatography:

HPLC analysis showed a great number of absorption peaks, leading us to confirm the complexity of the samples (Fig. 5). The most representative wavelength was 210 nm (Fig 5.a1-d1) due to the major number and definition of the absorption peaks observed. Although the Pm45 extract is originated from leaves (Table 1), surprisingly all extracts have a similar HPLC profile, in agreement with TLCs analysis (Table 3) and showed a dropped peak, which is indicative of compounds with high molecular weight. Among Ac26, Cl33 and Mu34, in which the used part was bark, the Cl33 (Fig. 5b) appear to had less complexity due the lowest number of absorption peaks.

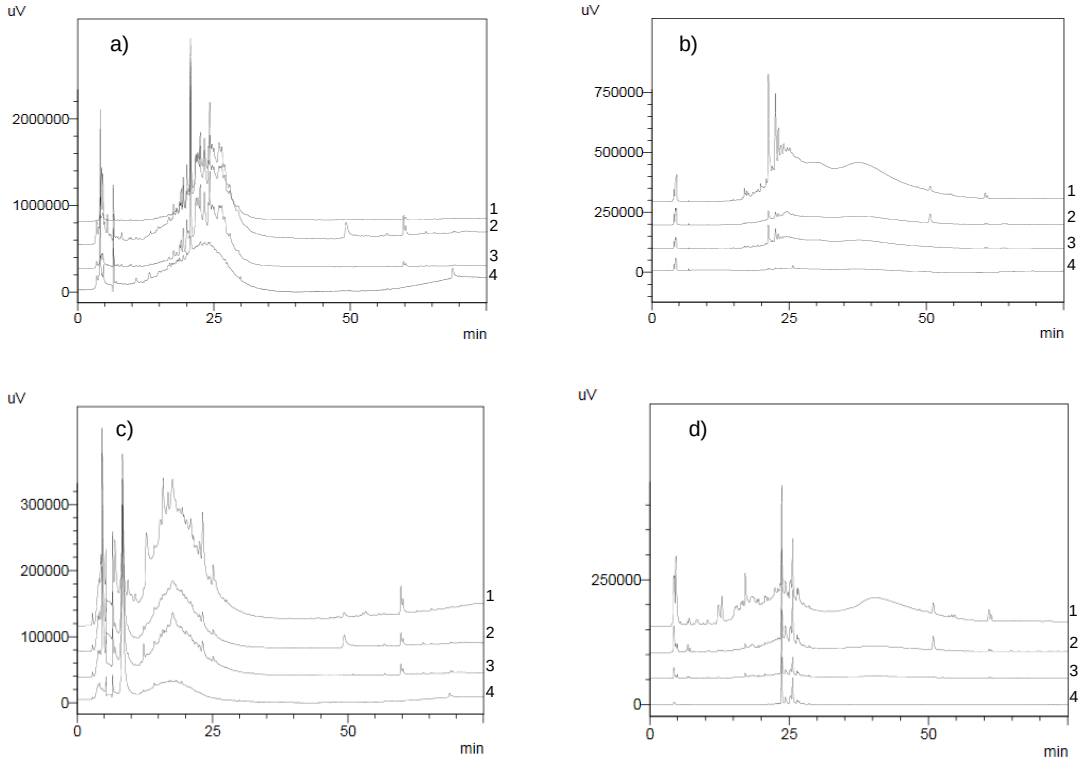

Fig. 5: Qualitative HPLC-PDA chromatogram of the extracts: a) *Anadenathera colubrina*(Ac26); b) *Commiphora leptophloeos* (Cl33); c) *Myracrodruoum urundeuva* (Mu 34); d) *Pityrocarpa moniliformis* (Pm45). 1- 210 nm; 2- 254 nm; 3- 273 nm; 4- 365 nm.

Discussion:

In spite of many conventional drugs have arisen from natural products, including plants, few antimicrobial agents have come from this source, with the vast majority in clinical use derived from products naturally produced by microorganisms [23]. Nonetheless, the use of medicinal and herbal plant remedies to treat infectious diseases is common in many countries [24]. Plant-derived antimicrobials are promising to be effective against resistant microorganism since compounds with new mechanism of action could be discovery. Increasing levels of antimicrobial resistance and declining numbers of pharmaceutical companies involved with the development of anti-infective agents create a significant public health threat to the effective management of bacterial infections [14, 25]. Trying to control bacterial resistance in Brazil, the ANVISA has restricted the sale of antimicrobials [26]. Prevalence of multidrug resistance strains of *P. aeruginosa* and the fact that no newer antipseudomonal agents are available [27] reinforce the need of new drugs to treatments.

The present work showed a study of the antibacterial activity of *A. colubrina, M. urundeuva, C. leptophloeos and P. moniliformis* against *P. aeruginosa*. All aqueous extracts showed bacteriostatic activity under the experimental conditions, although results of TEM showed that there is damage to the cell structure. This observation contradicts the classical concept of action of bacteriostatic antimicrobials, which causes some metabolic injury which is reversible when the antimicrobial agent is removed or neutralized [29]. Bactericidal action results from irreparable and irreversible damage to a vital cellular structure or function [30]. However, as it is possible to see that there were no damage cells between whole populations. The *in vitro* microbiological determination of whether an antibacterial agent is bactericidal or bacteriostatic may be influenced by many factors, as time of experiment and bacterial cell density, among others. This definition of the mechanism of action is even more arbitrary, since most antibacterials are better described as potentially being both bactericidal and bacteriostatic. Most so-called “bacteriostatic” agents kill some bacteria after the time of expirement- often more than 90%-99% of the inoculum, but not enough (>99.9%) to be called “bactericidal.” [31]. Some cells seemed not be affected by extracts or recover the growth ability and restart to growth. The possibility of there are no sufficient amount of active molecules to ensure that all cells are unable to growth due their low amount of active compounds in the crud extract cannot be excluded. Comparing the kinects of antibacterial activity of the extracts with erythromicyn, the reference antimicrobial, the same profile is obtained, corroborating with the hypothesis of bacteriostatic effect, in this experimental conditions involved.

In order to assume the class of compounds that are involved to the activity the TLCs showed the great complexity of the extracts, although the chemical revealing can react with more than one class due to their mechanism of action. TLC is a rapid qualitative valuable tool of detection of compounds, and when coupled with bioautography gives the positive or negative response of the antibacterial activity to different compounds separated by TLC [32]. All extracts appear to have similar constitution, with exception of the extract from *C. leptophloeos*, which

reacts in the presence of iodine vapors. The HPLC analysis supported the results from the TLC, confirming the complexity of samples. The absence of alkaloids and the dropped peak in HPLC analyses can be explained by singularities of the Caatinga environment. Plants that develop in rigorous environments have slower growth rates, and tend to invest in high molecular weight compounds. There is an important metabolic cost in the production of bioactive toxic compounds, such alkaloids [33]. Phytochemical classes observed in the extracts by TLC, polyphenols, flavonoids, anthraquinones, anthrones, coumarins, peptides, terpens, steroids and saponins have antibacterial activity widely described [34-37] and could be the responsible to the antibacterial activity of the extracts.

Among the plants analysed, *A. colubrina* and *M. urundeuva* have a great importance to the Caatinga communities [5-8,38,39]. Moreover, the part used is the bark, putting our study in accordance with the popular use. The use of this part is explained due to the seasonality of the region, when it remains as medicinal source unlike others parts of plants, such as leaves [38]. *C. leptophloeos* is less cited in ethnopharmacological studies and *P. moniliformis* is cited in only one report, but with both species showing antibacterial and antibiofilm activity, respectively [15]. The *A. colubrina* tannins, which are at higher concentration in bark [38], are supposed to have antimicrobial activity [40]. In the bark of *M. urundeuva* it is described the presence of phenols, tannins, flavonoids, terpenes, triterpenes, and anthraquinones [41]. However, the antibacterial and antifungal activities of its heartwood are reported to lectins, a class of proteins that recognizes carbohydrates and have been considered as participants in plant defense mechanisms [28]. The result of TLCs corroborated the reported activity, due the positive reaction of *M. urundeuva* extracts with ninhydrin, which is indicative of the presence of proteins.

The study presented here shows the value of plants from the Caatinga as possible sources of new antibacterial agents against *P. aeruginosa* and also validate the popular use of plants. The prospects of the work are the isolation and elucidation of the active molecules from the extracts.

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Anexo 1. Normas para publicação de artigo científico na Revista Planta Medica.

Guidelines for Authors

1. Editorial Policy

PLANTA MEDICA – Journal of Medicinal Plant and Natural Product Research is published in 18 issues a year. The following areas of medicinal plant and natural product research are covered:
1. Biological and pharmacological activity
2. Pharmacokinetic investigations and clinical studies (Pharmacokinetic investigations examining the kinetics of drug disposition and bioavailability including the use of *in vitro, in vivo* and human studies)
3. Natural product chemistry
4. Analytical studies

Only papers of highest scientific quality, concisely written and complying with these Guidelines for Authors can be considered for publication. All contributions are peer-reviewed by independent referees.

Submission of a manuscript to *Planta Medica* implies that it represents **original research not previously published and that it is not being considered for publication elsewhere.**
The corresponding author must declare that the manuscript is submitted on behalf of all authors. Copyright belongs to the publisher upon acceptance of the manuscript. There are no page charges.

The language of publication is **English**. Manuscripts written by authors whose mother tongue is not English should be checked by a native speaker or a professional language editing service before submission. **Manuscripts which do not meet acceptable standards will be returned to the authors.**

Authors investigating the chemistry of a single species should aim to publish their results in a single manuscript rather than in a series of papers. Manuscripts should not report fragmentary parts of a larger study. **Pharmacological investigations of extracts require detailed extract characterisation** (see below).

Submission of a manuscript signifies acceptance of the journal's Guidelines for Authors. Submissions which are not in line with these principles may be returned directly to the authors by the Editorial Office.

A statement clarifying the **conflicts of interests** of **all** authors must be included at the end of the manuscript (before the references); this will be published. Conflicts of interest also need to be declared during the submission process. Declaration of conflicts of interest is mandatory; if none, this also needs to be stated.

2. Submission of Manuscripts

Manuscripts can be submitted exclusively online at http://mc.manuscriptcentral.com/plamed or using the link at http://www.thieme.de/plantamedica. Submissions of hardcopy manuscripts or by e-mail will not be accepted.

A **sample manuscript** (for Original Papers) and a **sample letter** are available at http://mc.manuscriptcentral.com/plamed → Instructions and Forms, and at www.thieme.de/plantamedica. In addition to the Guidelines, authors are urged to follow these formats when preparing a manuscript.

10 Basic Rules for a Publication in *Planta Medica*

Manuscripts will not be considered for publication in *Planta Medica* unless the following conditions, if applicable, are fulfilled:

1. Ethical considerations: Submission of a manuscript to *Planta Medica* implies that it represents **original research** not previously published and that it is **not being considered for publication elsewhere**. Authors investigating the chemistry of a single species should aim to publish their results in a single manuscript rather than in a series of papers. **Manuscripts should not report fragmentary parts of a larger study.**
2. Language of publication is **English.** Manuscripts written by authors whose mother tongue is not English should be checked by a native speaker or a professional language editing service before submission.
3. Plant material (as well as other organisms) must be properly identified. The scientific name (in *italics*), the author of this name and the family must be given. It should be mentioned who identified the material. The manuscript must include references to **voucher specimens** of the plants (deposited in a major regional herbarium) or the material examined.
4. Isolation of compounds: Extraction and isolation should be described in detail. The kind and amount of material, solvents and extraction methods must be indicated. The description of chromatographic systems should contain the quantitative information that allows the reader to repeat the work. Column dimensions, elution volumes, fraction sizes, etc. should be reported.
5. Analytical studies: Key data on method validation must be provided and should typically include information on specificity, linearity, limit of detection, limit of quantification, accuracy, precision, intermediate precision, and some robustness studies. Information on the purity of reference compounds, and on the methods used for the determination of purity must be given. Recoveries of extraction and sample pre-purification steps have to be indicated. Adequate statistical treatment of data is required. Analytical studies of a routine nature will not be considered for publication.
6. Pharmacological investigations of extracts require detailed extract characterisation. Chromatographic profiling (e.g. HPLC profile with at least the major peaks identified) should be carried out, or qualitative and quantitative information on active or typical constituents should be provided.
7. Pharmacological investigations: *Planta Medica* will only consider manuscripts in which conclusions are based on adequate statistics. In each case **positive controls** (reference compounds) should be used and the dose/activity dependence should be shown.
8. Pharmacological investigations: When working with **experimental animals**, reference must be made to principles of laboratory animal care or similar regulations, and to approval by the local ethical committee. The approval number and the corresponding date must be provided.
9. Clinical studies must be designed, implemented and analyzed in a manner to meet current standards of randomised controlled trials. Reference must be made to approval of the study by the local ethical committee. The approval number and the corresponding date must be provided.
10. Biological screening: Papers dealing with the biological screening of a meaningful number of extracts of plants or other organisms can be considered for publication in *Planta Medica*. Identification of the material must be properly documented, and preparation of the extracts must be clearly described. Biological activities should be reported by listing IC_{50} values, or at least a dose-response relationship should be shown by using at least two test concentrations. Positive controls (reference compounds) must be included. Results should be presented in a concise format, and the discussion should be kept to a minimum.

Commonly used text processors should be used for preparation of the manuscripts. No pdf files must be submitted. The manuscript has to be accompanied by a **cover letter**, in which the authors briefly explain the significance of their findings and the interest to the readership of *Planta Medica*.

The **manuscript** (main text, tables, structural formulas and figures) should be submitted as **one file**. Colour reproduction of figures is available without any charge, if necessary for scientific reasons. If not, colour prints can only be reproduced without charge when submitted as Supporting Information. Authors are strongly encouraged to provide non-essential but useful data, figures and tables as **Supporting Information** (see below).

3. Format of Manuscripts

3.1. Original Papers. Original papers are research articles describing original experimental results. The material should be arranged in the order: Title Page / Abstract / Keywords / Abbreviations / Introduction / Materials and Methods / Results / Discussion / Acknowledgements / References / Figure Legends / Tables / Structural Formulas / Figures. Results and Discussion sections may appear as two separate parts or as a combined "Results and Discussion" section. No subheadings are allowed within this section. The normal length of the **main text** of an Original Paper, **excluding** references, tables, figures and figure legends, is about **3,000 words**. In exceptional and well justified cases longer manuscripts may be accepted. When submitting such manuscripts, authors should provide a justification statement, giving compelling reasons for the length of the paper.

3.2. Letters should be concise reports on new specific results of general interest. The Letter should be arranged in the order: Title Page / short Abstract / Keywords / Abbreviations / Introductory Remarks, Results and Discussion as one body of text without headlines / Materials and Methods with brief experimental details without subheadings / Acknowledgements / References / Figure Legends / Tables / Structural Formulas/Figures. The normal length of the **main text** of a Letter, **excluding** references, tables, figures and figure legends, is about **1,000 words**. The number of references should normally not exceed 30.

3.3. RapidCommunications are intended for the publication of exceptionally significant new and original results, such as unusual structures, bioactivities and innovative analytical techniques that deserve rapid publication, in the format of an Original Paper or a Letter.
If authors want their submission to be considered as a Rapid Communication, they should provide a justification statement for this with their manuscript. However, also regular submissions can be selected by the Editors for rapid communication after the review process.

3.4. Reviews will generally be **invited** by the Editor-in-Chief or the Review Editor. They should be as concise as possible and do not need to include experimental details. The main purpose of reviews is to provide a concise, accurate introduction to the subject matter and inform the reader critically of the latest developments in this area. Reviews should contain an abstract, and 4–6 keywords should be listed.

3.5. Minireviews and **Perspectives** will generally be **invited** by the Editor-in-Chief or the Review Editor. Minireviews provide concise and critical updates on a subject of high interest. Perspectives are written by leading experts in an emerging field and provide a concise assessment of the current state-of-the-art and an outlook on future developments. The normal length of the **main text** of Minireviews and Perspectives, **excluding** references, tables, figures and figure legends, is about **1,500 words**.

3.6. Editorials addressing topical issues of general interest to the readership of *Planta Medica* will be published on an irregular basis. They are written by the Editor-in-Chief, other Editors, or by experts on a specific issue in the form of an Invited Editorial.

4. Preparation of Manuscripts

In addition to the Guidelines, authors should consult the **sample manuscript** (for Original Papers) or the **sample letter** at http://mc.manuscriptcentral.com/plamed → Instructions and Forms, or at www.thieme.de/fz/plantamedica prior to preparing their contribution. Commonly used text processors should be used for preparation of the manuscripts.

For submission of all manuscripts, follow the instructions of the online submission system. Before submission, prepare the cover letter, and keep ready all information on the manuscript (title, full name and affiliation of all authors, abstract, name of all files to be submitted). **The author submitting the manuscript will be corresponding author.**

4.1. The **Title Page** must contain the title of the manuscript, the full names referenced by numerical superscripts with affiliation and addresses of all authors, and the full address of the corresponding author.

4.2. The **Abstract** should contain brief information on purpose, methods, results and conclusion (without subheadings).

4.3 The **Keywords** should include the scientific name and family of the plant(s) or other organism(s) investigated. 4–6 keywords should be listed.

4.4. Abbreviations should generally be used sparingly. Standard abbreviations such as m. p., b. p., K, s, min, h, µL, mL, µg, mg, g, kg, nm, mm, cm, ppm, mmol, HPLC, TLC, GC, UV, CD, IR, MS, NMR can be used throughout the manuscript. Non-standard abbreviations must be defined in the text following their first use. Provide a list of all non-standard abbreviations after the keywords. Define all symbols used in equations and formulas. If symbols are used extensively, provide a list of all symbols together with the list of abbreviations.

4.5. The **Introduction** should state the purpose of the investigation and relate to current knowledge in the specific topic addressed.

4.6. Materials and Methods. Specific details about test materials and test compounds, instrumentation and experimental protocols should be given here. This section should contain sufficient details so that others are able to reproduce the experiment(s). Purity (%) of all reference and standard compounds should be mentioned, as well as the method how it was determined. Previously reported methods should be referenced only. Suppliers for major equipment, cell lines, chemicals, biochemical reagents and major disposables should be indicated.

4.6.1. Documentation of plants and other organisms or starting materials. Use the correct scientific nomenclature. For plants, the Index Kewensis (electronic Plant Information Centre ePIC, Royal Botanic Gardens, Kew, UK: http://www.kew.org/epic), and/or the Inter-

national Code of Botanical Nomenclature (www.bgbm.fu-berlin.de/iapt/nomenclature/code/tokyo-e/default.htm) should be followed. Give the scientific name (in *italics*), the author of this name and the family. Indicate who identified the material. The manuscript must include references to voucher specimens of the plants (deposited in a major regional herbarium) or the material examined including their registration number(s). It should be mentioned which plant parts have been used.

4.6.2. Description of the preparation of extracts and isolation of compounds. The kind and amount of starting material, solvents and extraction methods must be indicated. The description of chromatographic systems should contain the quantitative information that allows the reader to repeat the work. Column dimensions, stationary phase, particle size, mobile phase composition, flow rate, sample amount, and elution volumes (or retention times, k' values) of fractions should be given. E.g.: "MPLC on silica gel (40–63 µm; 2 × 50 cm), MeOH/EtOAc 8 : 2, 3 mL/min; t_R of **1**: 60–70 mL, **2**: 120–140 mL, **3**: 145–175 mL; detection of eluates by TLC (SiO_2, MeOH/H_2O 9 : 1; Dragendorff reagent), Rf **1**: 0.35, **2**: 0.55, **3**: 0.73)." When using gradients the volumes of solvents should be presented; fractions should be defined by their elution volume. Similar information is necessary for HPLC, GLC, DCCC, MLCC and all other methods of purification. Figures of chromatograms will only be accepted if they are essential for understanding the methods or the results described. GC identifications of constituents of essential oils must be supported by retention indices on a polar and an apolar column. Identification by GC-MS is preferred.

4.6.3. Physico-chemical characterisation of compounds. Data provided for new compounds should enable an unambiguous identification of the substance and have to appear in the following order, if available: visual appearance, chromatographic mobility in TLC, GC, or HPLC, mp, UV-vis, specific optical rotation, CD, IR, ^{1}H-NMR, ^{13}C-NMR, low resolution MS, high resolution MS, elemental analysis. Note that for specific optical rotation $[\alpha]_D^{temp}$, the symbol *c* is defined as mass of substance (in g) in 100 mL of solution. For specific optical rotation no unit should be specified; the "degree" symbol "°" should not be used. In case of spectroscopic work on known substances refer, if possible, to published data; the manuscript should then contain the following indication: *Copies of the original spectra are obtainable from the corresponding author.* Such original spectra and/or spectral assignments can be provided as Supporting Information (see below), as well as structural formula outlining NMR spectral correlations, MS fragmentations, etc. IR, NMR, mass, and UV spectra should normally not be given in the manuscript as figures, but only if the listing of characteristic signals is not sufficient.

4.6.4. Chemical nomenclature used should be based on the systematic rules adopted by Chemical Abstracts and IUPAC. Trivial names should be avoided unless they are definitely advantageous over the corresponding systematic names. Trivial names are not accepted for close analogues and derivatives of known compounds. For reference drug substances the INN names should be used.

4.6.5. X-Ray crystallographic data must include a line drawing of the structure, a perspective drawing, and a discussion of bond lengths and angles. A supplement describing full details of the structure and methods and means of its determination in a form suitable for deposition must be submitted to the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 (0)1223 33 60 33 or e-mail: deposit@ccdc.cam.ac.uk). Deposition of the data has to be prior to submission of the manuscript, and appropriate reference has to be made in the Materials and Methods section, including the deposition number.

4.6.6. Analytical studies. Key data on method validation must be provided and should typically include information on specificity, linearity, limit of detection, limit of quantification, accuracy, precision, intermediate precision, and some robustness studies. Information on the purity of reference compounds, and on the methods used for the determination of purity must be given. Recoveries of extraction and sample pre-purification steps have to be indicated. Adequate statistical treatment of data is required. For more information regarding validation issues, prospective authors should also refer to ICH guidelines. Analytical studies of a routine nature will not be considered for publication.

4.6.7. Pharmacological investigations. *Planta Medica* will only consider manuscripts in which conclusions are based on adequate statistics that incorporate the appropriate tests of significance, account for the type of data distribution and are based on the number of experimental observations required for the application of the respective statistical method. In each case **positive controls** (reference compounds) should be used and the dose/activity dependence should be shown. When working with experimental animals, reference must be made to principles of laboratory animal care or similar regulations, and to approval by the local ethical committee. The approval number and the corresponding date must be provided.

Pharmacological investigations of extracts require **detailed extract characterisation.** This includes botanical characterisation of plant material, solvent(s), duration and temperature of extraction, plus other method(s) used for preparation(s). The drug to extract ratio (DER) must be given. Chromatographic profiling (e.g. HPLC profile with a reference compound recorded at different wavelengths) should be carried out, with at least the major peaks identified, or qualitative and quantitative information on active or typical constituents should be provided. Altogether the phytochemical standardisation of an extract and/or fraction(s) require state-of-the-art methods.

4.6.8. Clinical studies. Studies reporting on plant preparations tested in humans will be accepted for review and publication. Clinical studies must be designed, implemented and analyzed in a manner to meet current standards of randomised controlled trials. For guidelines see the following reviews: Begg C et al. JAMA 1996; 276: 637–639 and Altmann DG. BMJ 1996; 313: 570–571. Reference must be made to approval of the study by the local ethical committee. The approval number and the corresponding date must be provided. All methods and variables used in a trial should be described; the data must be based on adequate statistics. Herbal medicinal products used must be characterised as described above for pharmacological investigations.

4.6.9. Biological screening. Papers dealing with the biological screening of a meaningful number of extracts of plants or other organisms can be considered for publication in *Planta Medica.* Identification of the material should properly be documented, and preparation of the extracts should clearly be described (see above, sections 4.6.1 and 4.6.2). Biological activities should be reported by listing IC_{50} values, or a dose-response relationship should be shown by using at least two test concentrations. Positive controls (reference compounds) should be included. Results should be presented in a concise format, and the discussion should be kept to a minimum.

4.7. Results should be presented in a concise manner. Tables and figures should be presented in a manner which maximises clarity and comprehension. The **Discussion** should provide an interpretation of the data and relate them to existing knowledge. Subtitles are only admitted in exceptional cases.

4.8. Acknowledgements should list persons who made minor contributions to the investigation and organisations providing support.

4.9. References should be numbered in the order in which they are cited in the text, using arabic numbers between square brackets, e.g. [1]; for multiple references, e.g. [1 – 3] or [1, 2, 5].The list of references should be arranged consecutively according to the numbers in the text. Use Index Medicus abbreviations for journal titles. Authors bear complete responsibility for the accuracy of the references. The following examples illustrate the format for references:

a) Journals

Trute A, Nahrstedt A. Separation of rosmarinic acid enantiomers by three different chromatographic methods and the determination of rosmarinic acid in *Hedera helix*. Phytochem Anal 1996; 7: 204 – 208

Article in press without doi:
Lim EK, Ashford DA, Hou B, Jackson RG, Bowles DJ. Arabidopsis glycosyltransferases as biocatalysts in fermentation for regioselective synthesis of diverse quercetin glucosides. Biotech Bioeng, in press

Article in press with doi:
Lim EK, Bowles DJ. A class of plant glycosyltransferases involved in cellular homeostasis. EMBO J, advance online publication 8 July 2004; doi: 10.1038/sj.emboj.7600295

b) Books

Citation to complete book:
Mabberley DJ. The plant book, 2nd edition. Cambridge: Cambridge University Press; 1997: 520 – 521

Citation to article within a book:
Lechtenberg M, Nahrstedt A. Cyanogenic glycosides. In: Ikan R, editor. Naturally occurring glycosides. Chichester: Wiley & Sons; 1999: 147 – 191

Lorberg A, Hall MN. TOR: the first ten years. In: Thomas G, Sabatini DM, Hall MN, editors. TOR – target of rapamycin. Heidelberg: Springer Verlag; 2004: 1 – 18

Multi-volume books and encyclopedias:
Warren SA. Mental retardation and environment. In: International encyclopedia of psychiatry, psychology, psychoanalysis and neurology, Vol. 7. New York: Aesculapius Publishers; 1977: 202 – 207

Pharmacopoeia of China, Part 1. Beijing: People's Health Press; 1977: 531 – 534

c) PhD and Diploma Theses
Dettmers JM. Assessing the trophic cascade in reservoirs: the role of an introduced predator [dissertation]. Columbus: Ohio State University; 1995

d) Patents
Cookson AH. Particle trap for compressed gas insulated transmission system. US Patent 4554399; 1985

e) Conference Paper
Okada K, Kamiya Y, Saito T, Nakagawa T, Kaawamukai M. Localization and expression of geranylgeranyldiphosphate synthases in *Arabidopsis thaliana*. Annual Meeting of the American Society of Plant Physiologists, Baltimore, MD; 1999

f) Electronic Sources
Agatep R, Kirkpatrick RD, Parchaliuk DL, Woods RA, Gietz RD. Transformation of *S. cerevisiae* by the lithium acetate/single-stranded carrier DNA/polyethylene glycol protocol. Technical tips online. Available at http://research.bmn.com/tto. Accessed September 22, 2005.

If no author is given, the title is used as the first element of the citation.

If reference is made to papers submitted or in press, authors are requested to add a file of the manuscript or galley proof to the online submission. Avoid references to unpublished personal communications.

4.10. Structural formulas should be prepared with ChemDraw® or a similar program using the following settings: bond lengths 0.508 cm, bond width 0.021 cm, bold bond width 0.071 cm, bond spacing 18 % of length, hash spacing 0.088 cm, atom labels Helvetica 10, compound numbers Helvetica 10 bold. These settings correspond to American Chemical Society document settings preset in ChemDraw®. The configuration of all stereocenters present should be indicated; use of bold and dashed lines rather than solid and dashed wedges is recommended. The formulas should be integrated into the manuscript file (see above: 2. Submission of Manuscripts). They will be reproduced without reduction and the charts should be prepared with maximum widths of up to 8.0 cm for single column print and up to 17 cm for double column print.

4.11. Supporting Information: To keep articles as concise and at the same time as informative as possible, authors are strongly encouraged to submit part of their tables and figures as Supporting Information. The following type of data will be preferentially published as Supporting Information rather than in the print article: High-resolution halftone and colour illustrations, spectra, chromatograms, structural drawings outlining NMR correlations, experimental procedures of secondary importance, tables summarising data that are non-quintessential but useful to the understanding of an article. Tables, figures and text provided as Supporting Information must be referred to in the manuscript as follows: (Table 1S, Supporting Information, etc.).
The cover page for Supporting Information must contain the title of the manuscript, names and affiliations of all authors, and the full address of the corresponding author. Legends for Figures and Tables must appear directly on the respective figure pages. Pages have to be numbered consecutively. **Supporting Information has to be submitted as a separate file.**
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January, 2011

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