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ORIGINAL ARTICLE

Chemical Profiles by Thin-layer Chromatography and High-performance Liquid Chromatography of Plant Species from Northeast Brazil

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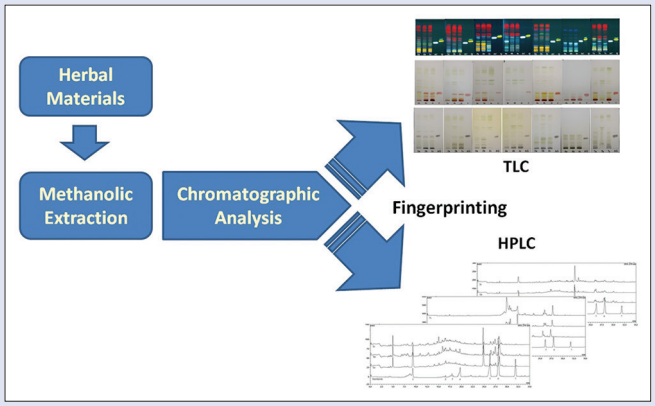
ABSTRACT

Background: Fingerprint analysis plays a key role in quality control of herbal medicines due to its technical capacity to represent the chemical diversity of these complex matrices. Several traditional Brazilian species showed very little data in their chemical profiles. **Objective:** Thus, the purpose of this study was to evaluate the chemical profiles of Brazilian herbal species by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). **Materials and Methods:** The herbal materials of 7 species (*Anacardium occidentale*, *Annona muricata*, *Guazuma ulmifolia*, *Phyllanthus niruri*, *Psidium guajava*, *Punica granatum*, and *Spondias mombin*) were collected from three different locations in Northeast Brazil, botanically authenticated and their chemical profile analyzed by TLC (cinnamics, flavonoids, and tannins) and by HPLC (polyphenols). **Results:** The chromatographic data showed the similarities between chemical profiles of the sample fingerprints, confirming the presence of several classes of secondary metabolites as well as the identification of different chemical standards (catechin, chlorogenic acid, caffeic acid, ellagic acid, gallic acid, quercetin, or rutin). **Conclusion:** The chromatographic profiling of the herbal drugs by TLC and HPLC were successfully characterized and allowed for the identification of promising chemical markers, improving the state of art in the quality control of the herbal species investigated in this study.

Key words: Brazilian medicinal plants, chromatography, fingerprint, quality control

SUMMARY

- Qualitative fingerprints from 7 traditional Brazilian species
- Three collection of each species from different locations in Northeast Brazil
- Development and evaluation of chemical fingerprints by thin-layer chromatography and high-performance liquid chromatography.



Abbreviations used: HPLC: High-performance liquid chromatography; PVDF: Polyvinylidene fluoride; RP-LC: Reverse-phase liquid chromatography; TLC: Thin-layer chromatography; UV-spectrum: Ultraviolet spectrum.

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INTRODUCTION

The use of medicinal plants in the prevention and treatment of several diseases has increased worldwide in the last decades. Phytotherapeutic products are the first choice of treatment for most of the population due to easy access either by self-cultivation and/or by purchasing from popular markets.[1]

Although the phytotherapy is among one of the oldest therapeutic strategies in humanity, there are several problems relating to its safe use, and these are closely linked to the quality of herbal raw materials. The acquisition of high-quality herbal materials is an important challenge and can depend directly on many factors such as cultivation/collection conditions and procedures, as well as the raw material processing (drying, milling, extraction, etc.).[2-4]

Therefore, any significant variation in the execution of any of these procedures may propagate throughout the manufacturing chain resulting in deviations in quality of the finished herbal product. Regarding the chemical control of such products and their manufacturing processes, the concept of chemical markers has been widely used until today.[5]

Although the use of chemical markers is a concept of low complexity, such an approach does not ensure the therapeutic quality of herbal products since the clinical activity of many plants is related to their complex chemical composition. In addition, the active compounds of several medicinal species are still unknown, and the use of one or more makers as an indicator of efficacy or safety can be controversial.[6] Thus, the detailed chemical profile of the plant or its derived products is critical to ensure the reproducibility of efficacy and safety. The chemical

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fingerprint of traditional products, such as a spectrum or chromatogram obtained by a defined procedure, is a unique characteristic and allows for the typifying of the chemical profile of this type of product.[7]

Chromatographic methods are excellent tools for monitoring the chemical composition of complex matrices such as herbal drugs. Among the chromatographic techniques, thin-layer chromatography (TLC) is a widely used separation technique, due to its simplicity and ease of execution and interpretation.[8] Another widely used chromatographic method is high-performance liquid chromatography (HPLC). It is a fully automated technique with high resolution, selectivity, and sensitivity.[9,10]

The chemical profile of herbal products obtained by chromatographic techniques (fingerprints) are widely disseminated and accepted by the World Health Organization, the Food and Drug Administration, and the European Medicines Agency as a quality evaluation of herbal raw materials or their derivatives. Thus, the fingerprint may be fundamental in the representation of the integrity of such products.[5,11-13]

Although many of the Brazilian medicinal species have had their activities reported, there is a frequent lack of knowledge surrounding the active chemical composition of these species.Although many of the Brazilian medicinal species have had their activities reported, there is a frequent lack of knowledge surrounding the active chemical composition of these species. Among them we can cite: *Anacardium occidentale* L. (anti inflammatory properties,[14,15] antioxidant and antimicrobial[16] and antidiabetic effects);[17] *Annona muricata* L. (antibacterial, antiviral and antifungal,[18,19] antidiabetic and antioxidant effects);[20] *Guazuma ulmifolia* Lam (renal disorders, alopecia, cough, fever, and skin problems);[19,21,22] *Phyllanthus niruri* L. (anti inflammatory, antinociceptive,[23] and antidiabetic effects);[24] *Psidium guajava* L. (antidiarrheal, anti inflammatory,[25,26] and antifungal effects);[27] *Punica granatum* L. (nematicidal effect in Bursaphelenchus xylophilus[28] and topical anti inflammatory activity);[29] and *Spondias mombin* Jacq. (antioxidant and anti inflammatory activities).[30] In relation to the chemical composition of these species, the presence of polyphenols is widely reported. Cinnamic derivatives, flavonoids, and tannins (hydrolysable and condensates) are profiled in the most common compounds[16,23,26,28,30] which are often attributed to the biological properties of plants.

Therefore, the purpose of this study was the development of standard chromatographic conditions by TLC and HPLC for the execution of reliable fingerprints of the polyphenols present in some medicinal plants popularly used in Brazil.

MATERIALS AND METHODS

Herbal material

The samples of the plant species were collected from three different places within the same month. The voucher specimens were deposited at the Dárdaro de Andrade-Lima Herbarium of the Instituto Agronômico de Pernambuco [Table 1].

Preparation of the herbal materials

The plant parts were dried for 3 days in an air-circulating oven under 40°C (Mod. LUCA-82-480, Lucadema®). Afterwards, the dried materials were powdered using a knife mill (Mod. TE-680, Tecnal®).

Thin-layer chromatography

Samples preparation

The analytical samples were prepared by extraction under reflux at 70°C for 15 min in a water bath (Mod. LUCA-150/24/D, Lucadema®) of 2.0 g from each sample and using 20.0 mL of methanol as solvent. After cooling, the solution was filtered through a piece of cotton.

Thin-layer chromatography analysis

The samples (25 µL) and standards (7 µL) were applied to TLC-plates of Silica gel 60-F_{254} (Macherey-Nagel®, Germany) in the form of bands (10 mm) using a semiautomatic device (Mod. Linomat V, Camag®, Switzerland). The chromatograms were developed in presaturated flasks using as an elution system: toluene: ethyl acetate: methanol: formic acid 85% (75:25:25:6; v: v: v: v). After complete development, the TLC plates were removed and the solvent was evaporated at room temperature. The chromatograms were observed under UV 254 and 365 nm, before and after derivatization with the specific chromogenic agents [Table 2]. The TLC-plates were digitalized using a fotodocumentador MultiDocIt Imaging System® (Mod. 125, USA) with UVP® software and a digital camera (Mod. Rebel T3, EOS 1100 D, Canon®).

High-performance liquid chromatography

Sample preparation

The extracts were prepared using 0.5 g (of each sample) in a 250 mL round-bottomed flask with ethanolic solution (50%, v/v) and were submitted to a reflux in a water bath for 30 min at 85°C (Mod.

Table 1: Herbal material data

Species	Family	Sample 1		Sample 2		Sample 3		Part of plant
		Local	Voucher	Local	Voucher	Local	Voucher	
Anacardium occidentale L.	*Anacardiaceae*	Fortaleza-CE	90152	Recife-PE	89991	Limoeiro-PE	89990	Leaves
Annona muricata L.	*Annonaceae*	Fortaleza-CE	90153	Caruaru-PE	89992	Bezerros-PE	90450	Leaves
Guazuma ulmifolia Lam	*Malvaceae*	Pau D'alho-PE	90096	Lagoa do Itaenga-PE	91097	Fortaleza-CE	91418	Leaves
Phyllanthus niruri L.	*Euphorbiaceae*	Fortaleza-CE	90149	Recife-PE	89760	Paulista-PE	91098	Areal parts
Psidium guajava L.	*Myrtaceae*	Fortaleza-CE	90151	Caruaru-PE	89761	Bezerros-PE	90449	Leaves
Punica granatum L	*Lythraceae*	Fortaleza-CE	90148	Recife-PE	90095	Paulista-PE	90170	Fruit peels
Spondias mombin Jacq.	*Anacardiaceae*	Caruaru-PE	89762	Limoeiro-PE	*	Paulista-PE	90169	Leaves

*The exsiccate was not possible. CE: Ceará; PE: Pernambuco

Table 2: TLC experimental conditions

Group of compounds	Standards	Eluent System (v/v)	Chromogenic agent
Cinnamics	Caffeic acid	Toluene: Ethyl acetate: Methanol: Formic acid	NEU + PEG 5% (365 nm)
Flavonoids	Quercetin	(75:25:25:6, v:v:v:v)	NEU + PEG 5% (365 nm)
Hydrolyzable tannin	Gallic Acid		Ferric Chloride
Proantocianidins	Catechin		Vanillin-HCl

NEU: Natural product reagent A; PEG: Polyethylene glycol 400, 365 nm - UV under 365 nm; UV: Ultraviolet

LUCA150/24/D; Lucadema®). The extracts were cooled and filtered through a filter paper. An aliquot of 10 mL was transferred to a 25 mL volumetric flask, and the volume was completed with purified water (Mod. Purelab Classic UV, Elga®). Subsequently, this solution was filtered through a 0.45 µm PVDF membrane (MachereyNagel®) and stored in vials.

High-performance liquid chromatography analysis

For the HPLC analysis, the standards were purchased from Sigma Aldrich®: caffeic acid (≥98%), chlorogenic acid (≥95%), catechin hydrate (≥98%), ellagic acid (≥95%), gallic acid (≥98%), quercetin (≥95%), and rutin (≥94%). All solutions were prepared at the concentration of 10.0 µg/mL to obtain a mix of standards, and before use, they were filtered through a 0.45 µm PVDF membrane (Chromafil®).

The analysis of the samples and mix of standards was performed in a HPLC (Mod. Ultimate 3000, Thermo Fisher Scientific®) equipped with a diode array detector, online degasser, and an autosampler with a loop of 20 µL (Mod. ACC3000, Thermo Fisher Scientific®). All HPLC data were processed using the Chromeleon® 6.8 software (Termo Fisher Scientific®). The chromatographic analyzes were performed using a reversed phase column C_{18} (250 mm × 4.6 mm i. d., particle size 5 µm) from Dionex® equipped with a guard column (C_{18}, 4 mm × 3.9 µm, Phenomenex®). Separations were carried out at a column oven temperature of 24°C. The mobile phase consisted of purified water (a) and methanol (b), both acidified with 0.05% trifluoroacetic acid (TFA, Vetec®), at a flow rate constant adjusted to 0.8 mL/min, previously degassed in an ultrasound bath (Ultracleaner®). A gradient program was applied: 0-10 min, 10%–25% (b); 10-15 min, 25%–40% (b); 15-35 min, 40%–75% (b); and, 35-36 min, 75–15% (b).

There was an injection of 20 µL of each solution, and each one was analyzed in triplicate. Scans from 190 nm to 400 nm were obtained and the chromatograms were recorded at 270 nm.

RESULTS AND DISCUSSION

Thin-layer chromatography

Regarding the natural origin of herbal materials, the quality analysis becomes more critical and requires several strategies of identification/authentication. Along with the botanical approach, the chemical evaluation, being either qualitative or quantitative, plays a key role as a complementary tool in the identification of herbal drugs (notably for powdered materials) and is also closely linked to the efficacy, safety, and pharmaceutical quality of herbal products.[3] The TLC profiles reflect the phytochemical integrity of herbal medicines and can be easily used to provide a higher amount of data about the complex composition of such biological matrices.[31-33]

Thus, the development and evaluation of TLC systems with the required reproducibility is not a simple task and requires time-consuming effort. In addition, the chemical variability within and between drug batches is a complex and extensive challenge.[34]

In the case of most Brazilian medicinal species, the active compounds are not yet known, making the task of determining a chemical profile that represents the active composition of such herbal products even more difficult. On the other hand, the development of a reliable TLC profile is the first step to standardizing the chemical quality of such products, and together with the use of high-quality raw material, it can be used as a parameter or reference to typify the biological activity through *in vitro* and *in vivo* assays.

In our study, TLC chromatographic systems were applied to the analysis of different samples from several plant species of recognized importance in traditional Brazilian therapeutics, in order to identify bands or sets of bands that are typical in all samples and that could work as an initial step in further understanding their chemical and pharmacological relationships.

Anacardium occidentale

If we take into consideration the TLC data from leaves of *A. occidentale* (cashew) samples, the plates revealed NEU and observed under UV 365 presented bands indicative of cinnamic derivatives and flavonoids [Figure 1A: 1a-c]. The three samples showed very similar profiles indicating the suitability of the experimental conditions for the analysis of this herbal drug. The literature reports the presence of heterosides derived from quercetin (quercetin 3-*O*-rutinoside and quercetin 3-*O*-rhamnoside) in addition to the respective aglycone observed in this study.[16] The bands for caffeic acid (R_f = 0.30) and quercetin (R_f = 0.42) were observed in all samples and these can be used as references for the control of this herbal material. The analysis of condensed tannins allowed for the detection of catechin (R_f = 0.21) in only two samples [Figure 1B: 1a and c]. On the other hand, gallic acid was used as a reference for hydrolysable tannins and was detected (R_f = 0.33) in all samples [Figure 1C: 1a-c].

Annona muricata

The TLC analysis of leaves from *A. muricata* showed the absence of caffeic acid or quercetin [Figure 1A: 2a-c], but the chromatogram shows clear orange-colored bands that indicate the presence of flavonoids. The presence of catechin (R_f = 0.16) and gallic acid was detected only in two samples [Figure 1B and 1C: 2b and c]. Since the herbal samples were authenticated and the sample 1a has another geographical origin, the TLC data suggest that the analysis of these drug materials could be focused on the tannins, by using either catechin and/or gallic acid. Previous studies[35,36] performed with butanolic, methanolic, and aqueous extracts of *A. muricata* leaves reported the presence of flavonoids and tannins by TLC, corroborating with our results.

Guazuma ulmifolia

The chemical profile observed in samples of *G. ulmifolia* showed caffeic acid (R_f = 0.44) and some typical bands for flavonoids [Figure 1A: 3a and b]. The presence of catechin (R_f = 0.20) and some catechin derivatives were observed [Figure 1B: 3a and b]. The presence of gallic acid was not observed in the samples [Figure 1C: 3a-c]. Despite there being few studies in the literature for *G. ulmifolia*, the presence of flavonoids, tannins, and other polyphenols were detected by TLC in several extracts from leaves of this species.[37]

Phyllanthus niruri

The presence of caffeic acid [Figure 1A: 4a and b] and cinnamic derivatives, as well as the detection of gallic acid [Figure 1C: 4a and b], plays a key role in the chemical characterization of aerial parts from *P. niruri*. The complete absence of condensed tannins [Figure 1B: 4a and 4b] corroborates with the results of phytochemical prospecting performed by Nascimento *et al.*[38] Therefore, our data suggest that these metabolites cannot be used as reference substances for this herbal drug.

Psidium guajava

The TLC profiles observed for leaves from *P. guajava* showed several intense bands for flavonoid-like compounds, including the presence of quercetin [Figure 1A: 5a-c]. Mild bands of caffeic acid (R_f = 0.34) were also observed in all samples. The presence of catechin (R_f = 0.18) was confirmed in all samples through intensive bands [Figure 1B: 5a-c]. On the other hand, the data from literature reporting the presence of gallic acid in leaves from *P. guajava*,[27,39] could not be confirmed in this study [Figure 1C: 5a-c].

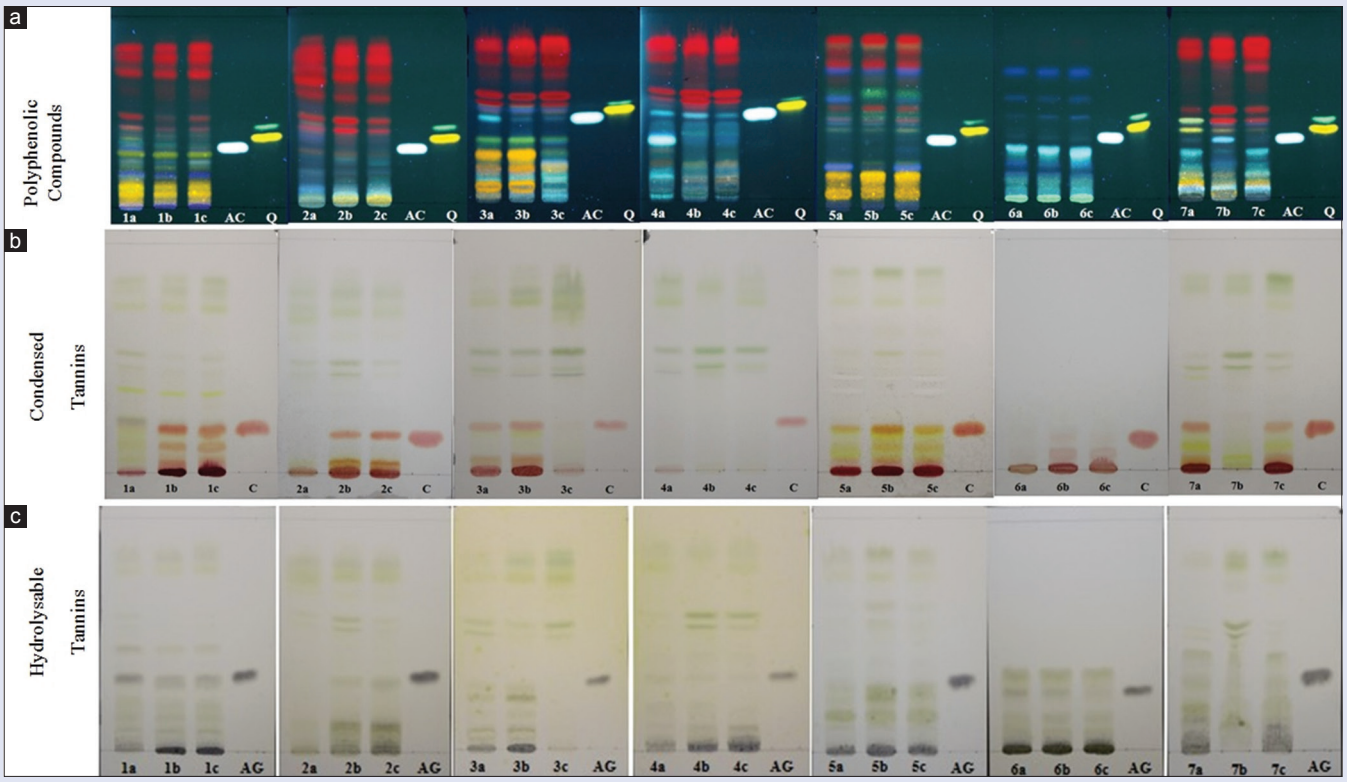

Figure 1: Chromatographic profile by TLC of samples of *A. occidentale* (1a-1c), *A. muricata* (2a-2c), *G. ulmifolia* (3a-3c), *P. niruri* (4a-4c), *P. guajava* (5a-5c), *P. granatum* (6a-6c), *S. mombin* (7a-7c), and standards caffeic acid (CA), quercetin (Q), catechin (C), gallic acid (GA)

Punica granatum

The analysis of cinnamic derivatives and flavonoids in fruit peel from *P. granatum* [Figure 1A: 6a-c] showed a higher similarity among the TLC profiles, with intensive bands for cinnamic derivatives but showing no evidence of flavonoids. Alper *et al*.[40] reported some phenolic compounds in the juice of the pomegranate fruit including caffeic and chlorogenic acids, which agree with our data. The cinnamic derivatives are compounds that present important biological activities, such as antimicrobial, antioxidant, antifungal, and anti-inflammatory effects among others.[41] The standard of caffeic acid (R_f = 0.33) was detected in the samples. In regard to the analysis of tannins, the TLC chromatograms indicated the presence of catechin [Figure 1B: 6b and c] and gallic acid [Figure 1C: 6a-c].

Spondias mombin

The leaves of *S. mombin* presented thin-layer chromatograms containing cinnamic derivatives (caffeic acid, R_f = 0.31), flavonoid (quercetin, R_f = 0.43), catechin (R_f = 0.17), and gallic acid (R_f = 0.31). The profiles were similar for samples 7a and 7c, while the sample 7b showed bands of higher intensity for caffeic acid, traces of gallic acid, and absence of catechin.

High-performance liquid chromatography

The chemical analysis of herbal materials and herbal products by the HPLC technique is the most widely used procedure. The LC data can provide qualitative and quantitative information, which can be improved in accordance to the attached detector. However, even using the most common detector (UV-Vis), chromatographic profiles have played an important role either in identification or quality assays of herbal medicines.[42-43] Sensitivity, selectivity, and reproducibility are some of the advantages of HPLC analysis compared to TLC; which can often overcome its higher operating costs.[44]

Therefore, in thus study, the samples were also submitted to RP-LC analysis to perform their fingerprints. The qualitative profiles were acquired by using the same chromatographic conditions and then compared with the LC data from standard substances.

The analytical conditions allow for the separation and identification of the standard substances as well as their respective peaks in the samples. The chromatograms for each herbal sample and standard substances are presented in Figure 2a-g.

In addition to the standards, several peaks or chromatographic regions were assigned as "typical peaks" and are represented in the boxes.

Anacardium occidentale

The analysis of leaves from *A. occidentale* by RP-LC confirmed the presence of tannins in all samples (gallic acid and catechin) as was observed in the TLC plates (including catechin in the sample 1a). Despite the several bands for flavonoid-like compounds detected by TLC, they could not be identified in the LC-Chromatogram [Figure 2a]. However, some reproducible peaks observed in all the samples at 21 and 27 min showed the typical spectrum of flavonoid with a maximum at 216.0 and 280.7 nm and 201.7, 267.5, 349.0 nm, respectively.

Annona muricata

The chromatographic data of samples from *A. muricata* allowed for the separation and identification of catechin [Figure 2b]. These results corroborate with previous reports in which the presence of catechin in the aqueous extract[36] and its isolation from an ethanol-water extract (3:1, v/v) was observed.[45] On the other hand, presence of quercetin or gallic acid were not detected in our study.[45] In addition, the profiles showed several zones of similarities. One of them (between 25 and 30 min) contained peaks with UV-spectrums of flavonoids (at 26.3 and 28.15 min, and maximum of 203.0, 257.0, and 356.3 nm;

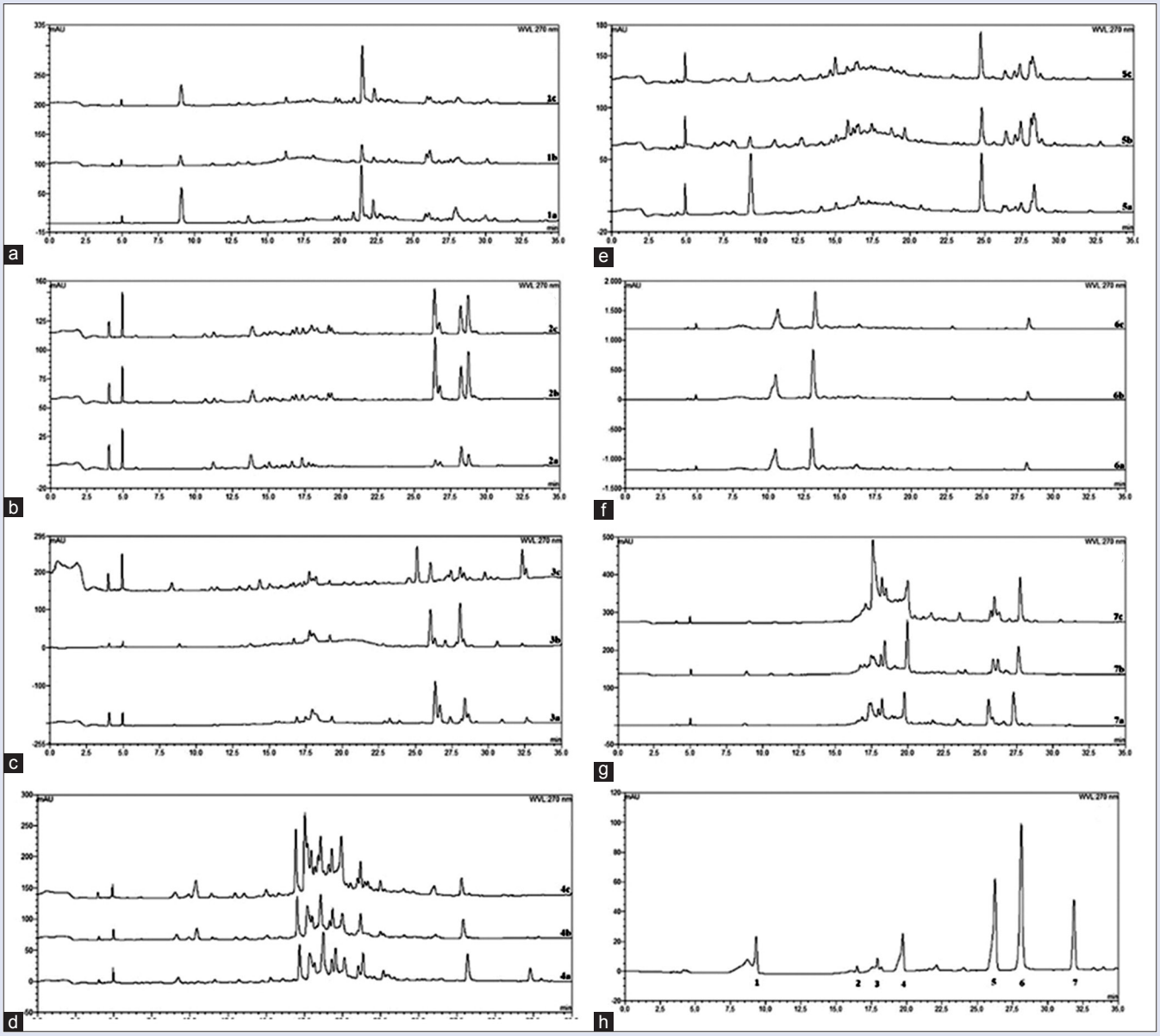

Figure 2: LC fingerprints at 270 nm for the herbal samples of *Anacardium occidentale* (a); *Annona muricata* (b); *Guazuma ulmifolia* (c); *Phyllanthus niruri* (d); *Psidium guajava* (e); *Punica granatum* (f) and *Spondias mombin* (g), and standards (h): gallic acid (1), catechin (2), chlorogenic acid (3), caffeic acid (4), rutin (5), ellagic acid (6), and quercetin (7)

and 197.2, 265.7, and 348.6 nm, respectively). Despite the absence of known markers, the fingerprints obtained for *A. muricata* presented a similarity and together with the TLC data, are important platforms for the evaluation of the chemical quality of the species.

Guazuma ulmifolia

In regard to the fingerprint of leaves from *G. ulmifolia*, the experimental conditions confirmed the presence of rutin as an important marker [Figure 2c]. In addition, two similarity zones, the first at 3–5 min and second at 26–29 min, were observed in all samples. The presence of rutin was also reported previously,[46] reinforcing its relevance as a chemical marker for leaves from *G. ulmifolia*.

Phyllanthus niruri

The presence of hydrolysable tannins was previously reported for aerial parts from *P. niruri*[47] and the samples analyzed in this study showed gallic and ellagic acids [Figure 2d]. The similarity zones among chromatograms occur at 3–5 min and again at 15–23 min. The presence of flavonoids could not be confirmed under the chromatographic conditions used in our study.

Psidium guajava

The chromatograms of leaves from *P. guajava* were rich in tannins and the peaks of gallic acid, ellagic acid, and catechin were identified in all samples [Figure 2e]. Notwithstanding the detection of at least two bands for flavonoid by TLC, only one important peak at 25 min could be satisfactorily separated and showed a representative spectrum for flavonoid (maximum at 204.4, 256.4, and 352.2 nm).

Punica granatum

The analysis of fruit peels from *P. granatum* by HPLC provided very similar chemical profiles for the samples, suggesting that the

fingerprint approach can be a robustness tool for the herbal material analysis [Figure 2f]. The fruit peels of *P. granatum* showed an important amount of ellagic acid. The presence of ellagic acid is closely related to the punicalagins and ellagitannins previously reported in pomegranates[48,49] and pomegranate Juice.[50]

Spondias mombin

In the literature, there are reports of the presence of ellagic acid in hydroethanolic[30] and methanolic[51] extracts from leaves of *S. mombin*, in agreement with the results of this study. Besides the presence of ellagic acid, it was possible to separate and identify by HPLC, the gallic acid, and rutin. The compounds were observed in all samples of leaves from *S. mombin*. Furthermore, the comparison among the chemical profiles showed a high degree of similarity of sample profiles.

CONCLUSION

The chemical qualitative analysis of several herbal materials from Brazilian medicinal species showed that standardized analytical conditions by either TLC or HPLC were able to separate relevant substances which can be used for the purpose of quality control. In addition, the chemical profiles obtained can provide reliable references for the evaluation of the complex composition of the matrices.

Since the herbal materials were botanically authenticated and identified, the fingerprints (TLC and/or HPLC) allowed for the detection of slight differences due to their intrinsic variability from biological matrices and/or conditions of growth. On the other hand, most of the fingerprints showed important similarities due to several common signs (bands/peaks), which can be used as references to standardize the herbal products.

Thus, this chromatographic study contributes to the development of additional quality parameters for the chemical standardization of such herbal materials, to ensure the reproducibility of activity during the biological assays.

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Conflicts of interest

There are no conflicts of interest.

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