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Analytical methods of phytochemicals from the *Cuphea* genus - A review

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Cuphea genus (Lythraceae) comprises about 260 species. The dispersion of the genus occurs in two mains geographic centers: North and South America, with Brazil being the most *Cuphea* species-rich country, with approximately 104 identified species. Still poorly studied, the number of papers about genus has been growing considerably. However, a review of its analytical methods has not been previously performed. Therefore, this review aims to provide studies about different chromatographic methods used for the separation, elucidation, and identification of metabolites present in species of the *Cuphea* genus. Research in scientific databases like Scopus, PubMed, and Science Direct were managed, and all references were analyzed. This review covers the relevant literature until May 2021, totalizing 22 studies described on 12 species of *Cuphea*. Most methods were employed for chemical analysis, and just one of them was validated for quantification purposes. Thus, this review provides a brief overview of the different chromatographic methods used in the separation, elucidation, and identification of compounds on different species of the *Cuphea* genus.

Keywords: *Cuphea*; Lythraceae; analytical methods, phytochemicals; phenolic compounds

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Introduction

Cuphea is the largest genus belonging to the Lythraceae family, covering around 260 species of perennial herbaceous plants and small shrubs. A large number of species of this genus are often found on degraded land [1, 2]. In Brazil, popularly known as "*sete-sangrias*", *Cuphea* genus is used in folk medicine as diuretic, antipyretic, anti-inflammatory, laxative, and anti-hypertensive [3, 4]. Brazil is the richest country in *Cuphea* species, contemplating approximately 104 identified species [5]. The Brazilian species inhabit all the biomes, with an affinity for humid environments, found in all types of vegetation, however are richer in the *Cerrado* (similar to savannah biome) [6].

Chromatography is an important technique that enables the separation, identification, and purification of the components of a mixture for qualitative and quantitative analyses. This technique consists of two phases, a stationary phase (composed of a solid phase or a layer of a liquid adsorbed on the surface solid support), and a mobile phase (composed of liquid or gaseous components). Moreover, the purpose of chromatography is to achieve a satisfactory separation within a suitable time interval [7].

Polyphenols, a group of small organic molecules that protect plants. Several polyphenols have been reported in higher plants and this structural diversity contributes to the complexity of their analyses [8,9]. Therefore, the need for sensitive and accurate methods for the analysis of polyphenols is essential. Thus, classical techniques such as high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC), gas chromatography (GC), and capillary electrophoresis (CE), have been used in polyphenol profiles analysis [9].

A large number of phytochemical studies performed with *Cuphea* genus report several polyphenolic compounds [10], and a wide diversity of flavonoid structures [15-18]. Moreover, has been identified the presence of tannins [15-18], medium-chain fatty acids [19], steroids [20-22], and triterpenes [12,13,20,21]. Therefore, the purpose of this review is to provide an update of studies about different chromatographic methods used for the separation, elucidation, isolation, and identification of metabolites present in species of the *Cuphea*.

Methodology

A comprehensive literature search was performed utilizing scientific databases including Scopus, PubMed, and Science Direct were employed using selected keywords, until May 2021. All references were transferred to Mendeley, a reference manager software, and were checked. The inclusion criteria were: (a) *Cuphea* genus, (b) chromatography methods, (c) analytical methods, and (d) isolation studies. The excluded criteria were: (a) pharmacological properties, (b) traditional uses, (c) toxicological, (d) botanical studies, (e) synthesis, and (f) biotechnology. On the basis of these criteria, 22 references were selected. Figure 1 shows an organizational chart with the number of articles initially found, and the included and excluded articles in relation to the used criteria.

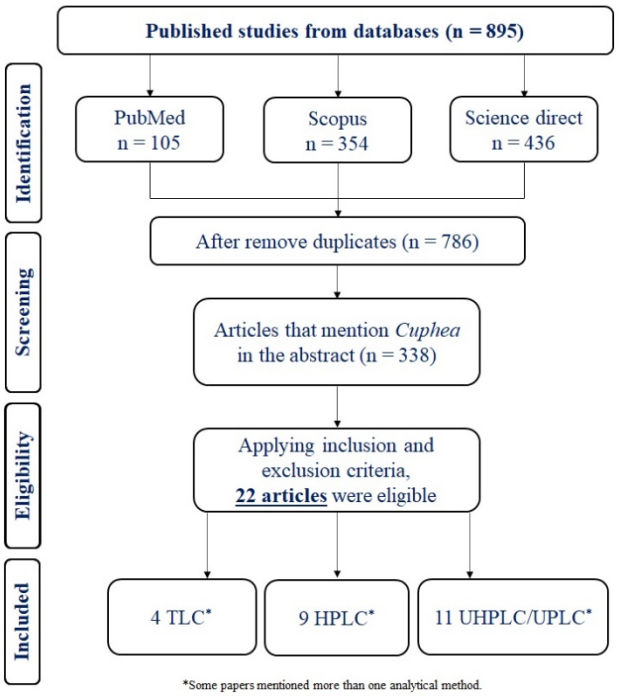

Figure 1. Organizational chart of included and excluded articles in this review

Results and Discussion

Analytical methods

Twenty-two studies were found about the analyses and development of analytical methods for compounds from the *Cuphea* genus. Among these four papers about TLC, nine studies using HPLC, four methods with ultra-high-performance liquid chromatography (UHPLC), and seven methods with ultraperformance liquid chromatography (UPLC). The HPLC, UHPLC, and UPLC techniques were coupled to different detectors such as PDA and MS. In addition, most methods were employed for chemical analyses, and just one of them was validated for quantification purposes. A summary of the methods, species, extraction, and compounds can be seen in Table 1.

TLC

In this review, four studies on TLC methods were found in three species of the genus *Cuphea*. Barboza et al. [23] developed a TLC method in 7 cm long silica-gel plates (Merck), where the standards and sample were applied on the plate and eluted with ethyl acetate, n-propanol, acetic acid, and water (4:2:2:1, v/v) and, after of dried and stained by orcinol-H_2SO_4 reagent, *C. carthagenensis* extracts were analyzed. Furthermore, through the TLC analysis was possible to confirm the presence of glucuronic acid, identifying the quercetin-glucuronide the compound major on the *C. carthagenensis* sample of this study.

The compounds of *C. hyssopifolia* were separated and purified by column chromatography and TLC. TLC was used to purify the cuphiin D1 compound, using Kieselgel PF254 (Merck) plates, mobile phase n-hexane-chloroform-acetone (5:4:1), and visualized under UV irradiation [15]. In another study, also with *C. hyssopifolia*, the fraction IV (eluted with 40% Methanol, 0.75 g) was fractioned and purified on preparative TLC Cellulose PEI-F Sheets 200 x 200 mm using n-BuOH:AcOH:H_2O, 4:1:5, v/v to afford hydrolyzable tannin valoneic acid dilactone[24].

Nevertheless, Mallco et al. [25] used TLC out on a preparative scale about a chromatoplate of silica gel 60 G of 15x20 with a chloroform-methanol solvent system (3.5:1) for the elucidation of six flavones and one flavonol in *C. ciliate* extracts following: 7-hydroxy flavone, 7-hydroxy-4'-5-dimethoxy flavone, 4',5,7-trimethoxy flavone, 4',8-dihydroxy flavone, 4',5-dihydroxy-6,7,8-trimethoxy flavone, 5,7-dihydroxy-4'-methoxy flavonol, 5,6,7-trihydroxy flavone.

HPLC

Nine HPLC methods were found in this revision, for characterization, purification, isolation, identification, and elucidation of different samples. Krepsky et al. [17] developed an HPLC method to analyze a precipitate from aerial parts of *C. carthagenensis* on Shimadzu system composed of UV–Vis detector model SPD-6AV, on an ODS column (250 × 9.4 mm i.d., 5 µm, Agilent Zorbax SB), using MeOH and H_2O 7:3 as eluent, at a flow rate of 3.0 mL/min. HPLC was used for purification and later as one of the identification techniques of quercetin-3-sulfate.

Posteriorly, Krepsky et al. [13] proposed an HPLC method to analyze extracts and fractions of *C. carthagenensis*, on a Waters alliance 2695 HPLC system composed of a PDA detector, and an ODS column (125 × 4.0 mm i.d., 5 µm; Merck), the mobile phase consisted of water (A) and methanol (B) at a flow rate of 1.0 mL/min, using a gradient as follows: 0 min 95% A, 5% B; 2 min 88% A, 12% B; 18 min 80% A, 20% B; 20 min 72% A, 28% B. According to HPLC profiles observed the presence of quercetin-5-O-β-glucopyranoside, quercetin-3-O-α-arabinofuranoside, and quercetin-3-sulfate.

Santos et al. [14] studied from leaves and roots of *C. glutinosa* by a Prominence-i® LC-2030C integrated Liquid Chromatograph system Shimadzu, with C18 column XTerra® (150 x 4.6 mm, 5.0 µm); gradient elution by (A) water 0.08% trifluoroacetic acid and (B) acetonitrile 0.08% trifluoroacetic acid, and flow rate of 1.0 mL/min. Additionally, this system also was used to verify the chemical profiles of different species of *Cuphea* in the current study by Santos et al. [10, 26]. The species *C. calophylla*, *C. carthagenensis*, *C. glutinosa*, *C. lindmaniana*, *C. racemosa*, and *C. urbaniana* showed some similarities, as compounds detected between 12 min and 22 min in the HPLC chromatograms.

Chromatographic fingerprints from *C. calophylla* extracts, studied by Atehortúa et al. [27] which developed an HPLC method coupled to a photodiode-array detector

Agilent series 1200, with reverse phase column Agilent Zorbax SB RRHT C18 (50 mm × 4.6 mm, 1.8 µm), and a flow rate of 1.0 mL/min. The mobile phase consisted of water with 0.1% acetic acid (solvent A) and acetonitrile (solvent B), following solvent gradient: 0–5 min, 5% (B), 5–35 min 5–25% (B), 35–55min, 25–55% (B). The injection volume was 5 μL.

Table 1. Chemical analysis of *Cuphea* species using technique different.

Technique	Species	Part	Extraction	Analytes	Reference
TLC	*C. carthagenensis*	Leaves	Infusion	quercetin-glucuronide	[23]
	C. hyssopifolia	Aerial	Maceration	cuphiin D1	[15]
	C. hyssopifolia	Whole pant	Decoction	valoneic acid dilactone	[24]
	C. ciliate	Leaves and stems	Maceration	six flavones and one flavonol	[25]
HPLC	*C. carthagenensis*	Aerial	Decoction	quercetin-3-sulfate	[17]
	C. carthagenensis	Aerial	Percolation	quercetin derivatives	[13]
	C. glutinosa	Leaves	Maceration and Infusion	quercetin derivatives	[14]
	C. calophylla, *C. carthagenensis*, *C. glutinosa*, *C. lindmaniana*, *C. racemosa*, and *C. urbaniana*	Leaves	Ultrasound-assisted	phenolic compounds	[10,26]
	C. calophylla	Whole pant	Sonication bath	Unidentified[1]	[27]
	C. hyssopifolia	Aerial	Maceration	ellagitannins	[15]
	C. aequipetala	Whole pant	Maceration	flavonols	[28]
	C. calophylla	Aerial	Infusion	gallic acid derivatives, ellagitannins, and flavonoids	[29]
UHPLC/ UPLC	*C. ingrata*	Leaves	Infusion	phenolic acids	[30,31]
	C. carthagenensis	Leaves	Maceration	ellagic, ascorbic and hippuric acids	[32]
	C. glutinosa	Leaves	Ultrasound-assisted	miquelianin	[33]
	C. carthagenensis	Leaves	Infusion	flavonol glycosides	[23,34]
	C. carthagenensis	Aerial	Infusion	flavonol glycosides	[35]
	C. glutinosa	Leaves	Maceration and Infusion	quercetin derivatives	[14]
	C. calophylla, *C. carthagenensis*, *C. glutinosa*, *C. lindmaniana*, *C. racemosa*, and *C. urbaniana*	Leaves	Ultrasound-assisted	phenolic compounds	[10,26]
	C. ingrata	Aerial	Maceration	phenolic constituents	[36]

[1]Method used only for separation.

Together with other techniques, the HPLC method was used in the characterization and elucidation of compounds of *C. hyssopifolia* on normal-phase conducted on YMC-pack SIL-A003 column (4.6 mm 250 mm) using the solvent systems: n-hexane:methanol:tetrahydrofuran:formic acid (60:45:15:1) contained oxalic acid (at a concentration of 500 mg/1.2 L) and another system containing, n-hexane:ethyl acetate (2:1), with a flow rate of 1.5 mL/min. The analysis in reversed-phase was performed on a YMC-pack J'sphere ODS H-80 (4.6 mm 250 mm) column, using the following solvent systems: 0.1 M phosphoric acid:0.1 M monopotassium phosphate:ethanol:ethyl acetate (42.5:42.5:10:5), and another system 0.1 M phosphoric acid:0.1 M monopotassium phosphate:ethanol: ethyl acetate (44:44:7:5) with the flow rate of 1.0 mL/min [15].

HPLC-MS

C. aequipetala methanolic extracts were analyzed and flavonoids were separated in the HPLC method, coupled to a mass spectrometric, on a Shimadzu LC-MS system consisting of a CBM-20A system controller and an SPD-M20A detector PDA. The samples were analyzed on an Alltima HP C18 HL column (7 mm x 53 mm, 3 µm), and a flow rate of 1 mL/min. Mass spectrometric analyses were in LCMS-2020 mass spectrometer interfaced with an electrospray ionization source in negative ion mode [28].

Moreover, an ethanol-soluble fraction from *C. calophylla* extract, for identification of the chemical composition by LC-DAD-MS, was injected into the equipment Prominence Shimadzu LC-20AD HPLC chromatography, which was coupled to a DAD and a mass spectrometer (MicrOTOF-Q III; Bruker Daltonics, Billerica, MA,

USA). The analyses were monitored between 240 and 800 nm and MS analyses were acquired in negative and positive ion mode. The stationary phase was a Kinetex C18 chromatography column (2.6 µm, 150 x 2.1 mm; Phenomenex). The flow rate was 0.3 mL/min. Of the 41 compounds, the main identified were gallic acid derivatives, ellagitannins, and flavonoids [29].

UHPLC/UPLC

In this review, were found 11 UHPLC/UPLC methods most frequently coupled to MS detectors. A UPLC method (Acquity Waters®) was used to detect bioactive compounds from *C. ingrata* (infusion leaves) with a diode-array detector, reversed-phase C18 column (100 mm x 2.1 mm, 1.7 µm, Phenomenex). The mobile phase was composed of water acidified with formic acid 0.1% (A) and acetonitrile (B), with a flow rate of 0.3 mL/min [30, 31].

Methanol extract of *C. carthagenensis* was analyzed by UHPLC (Thermo Scientific) with HYPERSIL GOLD C18 (5 µm 250×4.6 mm) analytical column, a quaternary pump (Thermo Scientific LPG-3400SDN UltiMate, 3000), and a UV–vis detector (Thermo Scientific, DAD-3000), using gradient elution method, to identify the constituents of extract. The flow rate of 0.8 mL/min, column temperature of 28°C, and wavelength of 280 nm for UV–vis detector. The UHPLC analysis demonstrated the presence of ellagic acid, ascorbic acid, and hippuric acid [32]

Recently, Santos et al. [33] validated the method for miquelianin quantification in *C. glutinosa* leaves, with an Acquity® UPLC system (Waters Co., MA, USA) equipped with a detector PDA. The analysis conditions were: fast C18 analytical column BEH (2.1 x 50 mm, 1.7 µm) a mobile phase 0.08% TFA (A) and acetonitrile (B), a flow rate of 0.11 mL/min, the injection volume was 2 µL, and the analysis was at 356 nm.

UHPLC-MS/UPLC-MS

C. carthagenensis samples also were investigated for mass spectrometry, using UPLC - Acquity™ Waters, PDA detector, coupled to high-resolution mass spectrometry (HR-MS). On C18 column HSS T3 (100 x 2.1 mm, 1.7 mm, Waters) using ultrapure water (A) and acetonitrile (B), both containing 0.1% formic acid, with a gradient increasing solvent B: 0–30% in 7 min, 30–80% in 12 min, returning to initial condition in 15 min [23, 34], and a flow rate of 400 µL/min [34].

Equally, Prando et al. [35] explored *C. carthagenensis* samples by UPLC - Acquity™ Waters, modifying the mobile phase (0.1% aqueous formic acid (A) and methanol (B)) and the gradient at a flow rate of 0.4 mL/min. Phytochemical investigation for *C. carthagenensis* leaves was performed mass spectrometry in LTQ-Orbitrap XL (Thermo Scientific), with electrospray ionization in the negative and positive modes [23, 34, 35]. Similarly, in the three studies, the samples from *C. carthagenensis* showed the same compounds, mainly flavonol glycosides [23, 34, 35].

Quercetin derivatives from *C. glutinosa* were identified by an Acquity® UPLC system (Waters) with two detectors: DAD-UV and Q-TOF Micro-Micromass (Waters Co., MA, USA). In the conditions as: fast C18 analytical column Shim-pack XR-ODS column (50 x 2 mm, 2.1 µm); mobile phase acetonitrile:methanol (4:1) (A) and 0.1 % formic acid (B). The mass spectrometry analyses were in positive-ion mode [14].

In other recent studies of Santos et al. [10, 26] investigated the chemical composition of *C. calophylla*, *C. carthagenensis*, *C. glutinosa*, *C. lindmaniana*, *C. racemosa*, and *C. urbaniana* via Ultra-High-Performance Liquid Chromatograph Nexera X2 (UHPLC-Shimadzu, Japan). Chromatographic analyses following conditions: BEH C-18 (1.7 µm x 2.1 mm x 50 mm), mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile (B) at a flow rate of 0.20 mL/min, using a gradient system. The mass spectrometry (MS) ESI-MS/MS analyses were performed on a micrOTOF-Q III (Bruker Daltonics, Germany) equipped with an ESI interface operating in positive ion mode. In these studies, phenolic compounds, mainly flavonols, predominated [10, 26].

The ethyl acetate and n-butanol fractions from *C. ingrata* methanolic extract were analyzed on a UHPLC-3000 RS Dionex system equipped with a dual low-pressure gradient pump, an autosampler, a diode array detector, and an AmaZon SL ion trap mass spectrometer with an ESI interface Bruker Daltonik. A Kinetex XB-C18 column (1.7 µm, i.d.; Phenomenex, Torrance, CA, USA) at 25°C was used, on mobile phase consisting of solvent A – water/formic acid (100:0.1, v/v), and solvent B – acetonitrile/formic acid (100:0.1, v/v), with a flow rate of 0.3 0.3 mL/min [36].

The ESI parameters follow: the nebulizer pressure was 40 psi; dry gas flow 9 L/min; dry temperature 300°C; and capillary voltage 4.5 kV, in a negative ion mode. The analyses led to the detection of over sixty phenolic constituents as phenolic acids and their derivatives, tannins, and flavonoids in fractions from *C. ingrata* methanolic extract [36].

Analytical methods applied to isolation of compounds

In a study with extracts and fractions of *C. carthagenensis*, a method was used to isolate compounds, on the HPLC Shimadzu system, an ODS column (250 × 21.1 mm i.d., 10 µm, Agilent Prep) eluted with methanol/water acidified with 1% HAc (3:7) at a flow rate of 8.0 mL/min. The isolation resulted in the compounds quercetin-5-O-β-glucopyranoside, and quercetin-3-O-α-arabinofuranoside from the n-BuOH and EtOAc fractions, respectively [13].

In a similar study, for the isolation of quercetin-3-sulfate from *C. carthagenensis*, the aqueous extract was sequentially partitioned with n-hexane, ethyl acetate, n-BuOH, and aqueous fractions. These fractions were subjected to preparative HPLC on an ODS column, using MeOH and H_2O 7:3 as eluent. Precipitation from the n-BuOH fraction, followed by RP-HPLC purification was

repeated several times. The solvent was removed under vacuum in a rotatory evaporator with careful control of temperature (<50 C), and the solid was identified as quercetin-3-sulfate according to UV, IR, 1H, and 13C NMR spectral data and by comparison with literature [17].

Flavonoids were isolated from *C. pinetorum* roots after the exhaustive extraction with methanol. The methanol was evaporated and a part was further fractionated over a cellulose column (C6H10O5 Avicel, Merck). The elution was performed by using 100% EtOAc (F1 and F2); 50:50 Me2CO/MeOH (F3); 100% MeOH (F4) and 75:25 MeOH/H_2O (F5 and F6). The EtOH fraction afforded a mixture of flavonoids, which was chromatographed on a Silica gel column (Silica gel, 30–70 mesh, Merck) with EtOAc, allowing the isolation of kaempferol and quercetin. The compounds were characterized by the reported 13C and 1H NMR data, and by direct comparison with authentic samples [37].

Isolation and identification of the phenolics from *C. ignea* aerial parts, were detected by HPLC/ESI-MS, whose dried aqueous EtOH extract was loaded onto a Sephadex LH-20 (900 g) column (120 × 7.5 cm). Elution was then started using H_2O, followed by isocratic elution with MeOH/H_2O mixture of decreasing polarities, and 10 fractions were obtained. The isolation, occurred by means of consecutive polyamide S6, MCI gel, and repeated Sephadex LH-20 column fractionation, and Prep. Finally, the isolation and purification of phenolics were monitored by two-dimensional paper chromatography. The ESI-MS, 1H, and 13C NMR spectra were then recorded, completely interpreted, and confirmed by HR-ESI-MS and 2D NMR spectroscopy [38].

Conclusions

In this review, 22 studies were described distributed in 12 species of the *Cuphea* genus. These species have complex chemistry, and they present some variations in their chemical markers, emphasizing the importance of quality control to distinguish the species. Studies with TLC, HPLC, UHPLC, and UPLC with variations in the system, and different detectors, demonstrated to assist in the separation, purification, identification, isolation, and elucidation of compounds from distinct species of the *Cuphea* genus. However, studies are still few analytical methods developed for *Cuphea* species, and until now, only one method has been validated for quantification purposes.

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Conflict of interest

The authors declare no conflicts of interest.

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