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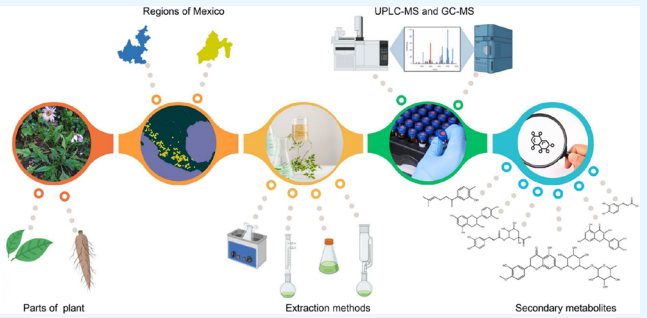
ABSTRACT: *Iostephane heterophylla* is a traditional Mexican medicinal plant and is an important source of secondary metabolites with antimicrobial and cytotoxic activity. The aim of this work was to conduct a comparative analysis of secondary metabolites of different roots and leaf extracts of *I. heterophylla* from two zones in Mexico using ultraperformance liquid chromatography (UPLC) and gas chromatography (GC) coupled with mass spectrometry (MS). Twelve secondary metabolites from roots were identified in the leaves. Five new molecular weight secondary metabolites not previously reported were found. Six bioactive metabolites were quantified (quercetin ≤0.151 mg/mL in root and ≤0.041 mg/mL in leaf; hesperidin ≤0.66 mg/mL in root and ≤0.173 mg/mL in leaf; epicatechin ≤0. 163 mg/mL in root and ≤0.664 mg/mL in leaf; caffeic acid ≤0.372 mg/mL in root and ≤0.393 mg/mL in leaf; chlorogenic acid ≤0.234 mg/mL in root and ≤0.328 mg/mL in leaf; and xanthorrhizol ≤0.667 mg/mL in root), and a selective extraction method was established: quercetin in root and leaf by reflux; hesperidin in leaf by Soxhlet and in leaf by reflux; chlorogenic acid in root by Soxhlet and in leaf by reflux; chlorogenic acid ≤0.234 mg/mL in root and ≤0.328 mg/mL in leaf by ultrasound-assisted extraction; epicatechin in root by ultrasound-assisted extraction; caffeic acid in root by reflux and in leaf by Soxhlet. The most efficient solvent was methanol. This study provides a new secondary metabolite profile found in the leaves of *I. heterophylla*, highlighting it is an essential source of three bioactive compounds: epicatechin, hesperidin, and quercetin.



1. INTRODUCTION

Natural products have contributed to the discovery and development of new drugs.[1] At the dawn of the 21st century, 11% of the 252 drugs considered basic and essential by the World Health Organization (WHO) were exclusively of plant origin.[2] Additionally, herbal medicines or plant extracts treatments are used by approximately 80% of the world population.[3]

The search for new therapeutic leads from natural resources has been going on for centuries and has led to several important discoveries. One of these discoveries was in 1803 with the isolation of alkaloids, such as morphine from *Papaver somniferum* L. (opium poppy) which is used as an analgesic.[4] Another example is acetylsalicylic acid, an anti-inflammatory agent known as aspirin, which is a derivative of the natural product salicin isolated from the bark of the willow tree *Salix alba* L. Other examples are pilocarpine [found in *Pilocarpus jaborandi* (*Rutaceae*)], an alkaloid derived from L-histidine, which has been used to treat chronic open-angle glaucoma and acute angle-closure glaucoma for over 100 years,[5] vincristine from the Madagascar periwinkle (*Catharanthus roseus*) with anticancer activity and artemisinin, a sesquiterpene from the Chinese herb *Artemisia annua* with antimalarial activity.[6]

Despite the availability of drugs from natural products, the use of medicinal plants (mainly extracts) continues to increase due to their therapeutic effects. *Psidium guajava*, *Azidarachta indica* (neem), *Ocimum sanctum*, *Momordica charantia*, and *Carica papaya* extracts are used for the treatment of gastrointestinal diseases, and as an antiparasitic, antifertility, anticancer, antidiabetic, antifungal, and antimicrobial.

In most cases, the chemical composition of the extracts is unknown; therefore, it is necessary to identify the secondary metabolites responsible for their therapeutic effect. In this sense, different qualitative and quantitative studies of the secondary metabolites of medicinal plant extracts search for an appropriate extraction method.[7] Different extraction methods were used to obtain secondary metabolites. These methods are classified as conventional (maceration, Soxhlet, and hydrodistillation) or nonconventional (ultrasound-assisted extrac-

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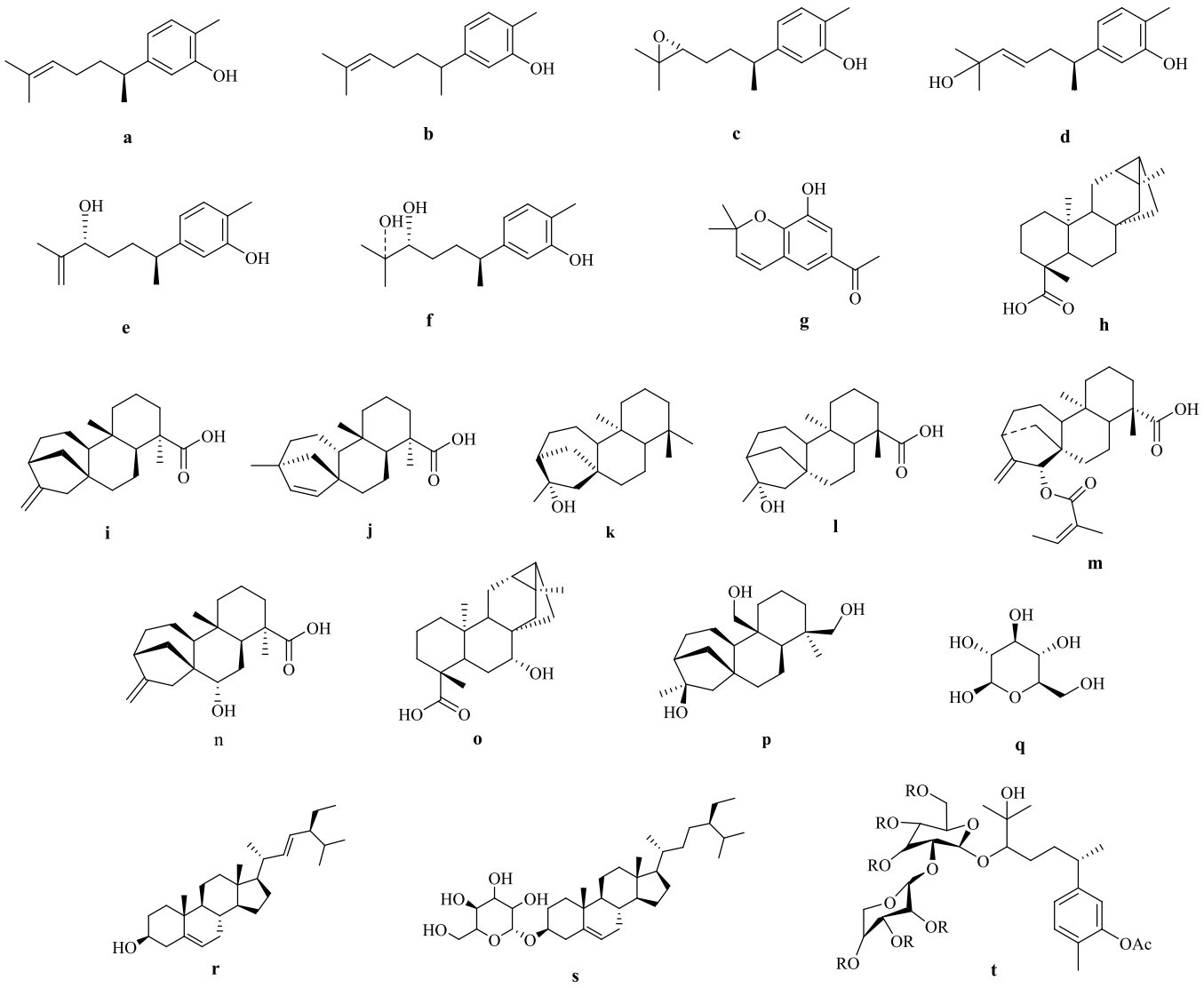

Figure 1. Structure of 20 secondary metabolites reported from the root of *I. heterophylla*.

tion, enzyme-assisted extraction, microwave-assisted extraction, pulsed electric field-assisted extraction, supercritical fluid extraction, and pressurized liquid extraction). Although some methods are very efficient, they are expensive, so the simplest and most economical methods that are effective for extracting metabolites of interest are usually used.

Identifying secondary metabolites in plant extracts in the study of traditional medicine is important and crucial. Different factors affect the extraction processes such as the properties of the plant parts, matrix, solvent, temperature, pressure, and time.[8,9] These conditions must be optimized to obtain the best secondary metabolites yield. In relation to the above, several studies have focused on the selectivity of the extraction method and the use of different solvents for extracting specific compounds.

Secondary metabolites in the extracts are structurally elucidated after a postextraction investigation, using chromatographic techniques such as high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and other spectroscopic techniques.[10] The main advantage of MS is its high sensitivity, which allows the detection of low molecular weight compounds at concentrations below the nanogram per milliliter range if optimal MS conditions are provided.[11] This technique allowed different metabolomic analyses of plant extracts in *Cinnamomum* species (*C. verum*, *C. cassia*, *C. iners*, and *C. tamala*), which by UPLC-MS led to the annotation of 74 secondary metabolites and by GC-MS to 54 sequential metabolites belonging to different classes, including phenolic acids, tannins, flavonoids, and lignans.[12]

Mexico is the second country in the world with the greatest ancestral tradition and richness in the use of medicinal plants. There are records of 1032 medicinal plants, with 164 families registered with *Asteraceae*, *Fabaceae*, and *Rubiaceae* being the most used.[13] An example of the Asteraceae family is *Iostephane heterophylla* (*I. heterophylla*), a plant in central and northern Mexico[14,17] commonly known as "liga", "escorcionera", "raíz del manso", or "hierba del oso".[14−16] The roots of this plant, well-known in traditional Mexican medicine, are used to treat rheumatism, arthritis, diabetes, and gastrointestinal disorders.[18,19]

Previous studies have isolated and identified the secondary metabolites xanthorrhizol (a), 2-methyl-4-[(1*R*)-1,5-dimethyl-4-hexenyl]phenol (b), (12*R*/12*S*)-12,13-epoxy-xanthorrhizol (c), 12,13-dihydro-13-hydroxy-11-en-xanthorrhizol (d), 12,13-

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Table 1. Primary Yields (%) of the Extracts Obtained from Roots and Leaves of *I. heterophylla* from Two Regions of Mexico[a]

	root				leaves			
method/solvent	H (%)	D (%)	M (%)	W (%)	H (%)	D (%)	M (%)	W (%)
maceration	21.03**^{P}	17.00**^{P}	41.46**^{P}	54.96**^{P}	1.09*^{P}	2.89*^{P}	12.41*^{M}	21.50**^{M}
reflux	37.51**^{P}	23.37**^{P}	47.75*^{M}	47.34**^{M}	2.91**^{M}	2.17**^{P}	16.99*^{M}	30.42**^{P}
Soxhlet	27.46*^{P}	17.53**^{M}	43.91**^{M}	56.99**^{M}	7.76*^{P}	3.74*^{M}	29.90*^{M}	30.24 *^{P}
ultrasound	17.72**^{P}	10.76**^{P}	40.02**^{P}	57.72*^{P}	1.39**^{P}	2.58**^{M}	33.57**^{M}	31.35*^{M}

[a]Hexane: H, dichloromethane: D, methanol: M, water: W. *2019, **2020. M: Mexico, P: Puebla.

Table 2. Secondary Metabolites Identified from the Root of *I. heterophylla* by UPLC-MS and GC-MS from Two Regions of Mexico at Two Collection Times[a]

	method/solvent															
	maceration				reflux				Soxhlet				ultrasound			
SM	H	D	M	W	H	D	M	W	H	D	M	W	H	D	M	W
1	■/●	■/●	■	nd	●	●	■/●	●	●	●	■	●	●	nd	●	nd
2	nd	nd	nd	■	■	nd	nd	nd	nd	nd	nd	■	■	nd	■	nd
3	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
4	nd	nd	■	■	■	nd	■	■	nd	nd	■	■	nd	nd	■	■
5	●	●	■/●	nd	●	■/●	●	nd	●	●	■/●	■/●	■/●	●	●	●
*6	■/●	■/●	■/●	nd	■/●	■/●	■/●	■	■/●	■/●	■/●	nd	■/●	■/●	■/●	nd
7	■/●	■/●	■/●	■	■/●	■/	■/●	■	■/●	■/●	■/●	■	■/●	■/●	■/●	■
8	■/●	■/●	■/●	nd	■/●	■/●	■/●	nd	■/●	■/●	■	nd	■/●	■/●	■/●	nd
9	■/●	■/●	nd	nd	■/●	■/●	■	■	■/●	■/●	nd	nd	■/●	■/●	■	■
10	●	●	■/●	nd	●	●	■/●	nd	■/●	■/●	■/●	nd	■/●	■/●	■	nd
11	■/●	■/●	■/●	nd	■/●	■/●	■/●	nd	■/●	■	■	nd	■/●	■/●	■	■
12	■/●	■/●	■/●	nd	■/●	■/●	■/●	nd	■	■/●	■/●	nd	■/●	■/●	■/●	nd
13	■/●	■/●	■/●	■	■/●	■/●	■/●	■	■/●	■/●	■/●	■	■/●	■	■	■
14	■/●	■/●	■	nd	■/●	■/●	■	nd	■/●	■/●	■/●	nd	■/●	■/●	■/●	nd
15	nd	nd	■	nd	■	■	■	■	■	■	■	nd	■	■	■	nd
16	■/●	■/●	■/●	■	■/●	■/●	■/●	■	■/●	■/●	■/●	■	■/●	■/●	■/●	■

[a](1) Quercetin; (2) hesperidin; (3) epicatechin; (4) caffeic acid; (5) chlorogenic acid; (*6) xanthorrhizol and derivatives [12,13-dihidro-12-hidroxi-13-en-xantorrizol, 12,13-dihidro-12.13-epoxi-xantorrizol, 12,13-dihidro-13-hidroxi-11-en-xantorrizol, 12,13-dihidro-12,13-dihidroxi-xantorrizol and 4-(1′,5′-dimethylhex-4′-enyl)-2-methylphenol]; (7) scopoletin; (8) 8-hydroxy-6-acetyl-2,2-dimethyl-chromene; (9) traquiloban-19-oic acid; (10) ent-15α-(3-methoxy-3-methyl-butanoyl)-<Kaur-16-en-19-oic acid; (11) ent-beyer-15-en 19-oic acid; (12) ent-kaur-16-en 19-oic acid; (13) 15α-tigloyloxy-ent-kaur-16-en-19-oic acid; (14) 16α hydroxy-ent-kaurane; (15) 16α-hydroxy-ent-kaur-11-en-19-oic acid; (16) 16α-hydroxy-ent-kaur-11-en-19-oic acid methyl ester. UPLC-MS: ■; GC-MS: ●; nd: not detected, SM: secondary metabolite, H: hexane, D: dichloromethane, M: methanol, W: water.

dihydro-12-hydroxy-13-en-xanthorrhizol (e), 12−13-dihydro-12−13-dihydroxy-xanthorrhizol (f), 1-(8-hydroxy-2,2-dimethyl-2*H*-chromen-6-yl)ethan-1-one (g), *ent*-trachyloban-19-oic acid (h), *ent*-kaur-16-en-19-oic acid (i), *ent*-beyer-15-en-19-oic acid (j), 16α-hydroxy-*ent*-kaurene (k), 16α-hydroxy-*ent*-kaur-11-en-19-oic acid (l), 15α-angeloyloxy-*ent*-kaur-16-en-19-oic acid (m), 15α-hydroxy-*ent*-kaur-16-en-19-oic acid (n), 15α-hydroxy-*ent*-trachyloban-19-oic acid (o), and *ent*-kauran-16β-19diol (p) in a chloroform extract obtained by root maceration of *I. heterophylla*.[16,17,19,20] Another study of an ethanolic extract obtained by root maceration identified metabolites such as β-D-glucopyranose (q), stigmasterol (r), sitosteryl β-D-glucopyranoside (s), and 1-*O*-[12-*O*-(12*S*)-dihydro-12,13 dihydroxyxanthorrhizol]-L-arabinopyranosyl-(1 → 2)-β-D-glucopyranoside (t) (Figure 1).[21,22] These studies suggest that different secondary metabolites of therapeutic importance (or yield improvement) may be obtained if other extraction methods and solvents are used. In this sense, as there is no record of the secondary metabolite profile of other parts of the plant, we did a comparative analysis of secondary metabolites of the root and leaves of *I. heterophylla* from two regions in Mexico at different collection times, evaluating the efficiency of the extraction method and solvent by ultraperformance liquid chromatography (UPLC) and gas chromatography (GC) coupled to MS.

2. RESULTS AND DISCUSSION

2.1. Molecular Identification of *I. heterophylla*.

Taxonomic plant identification is the most common method; however, it is expensive and requires a long time and ethnobotanical experts. The current molecular identification of plants offers advantages. It is fast, low cost, and uses trace fragments of samples such as pollen, leaves, or roots.[23,24] In this study, plant samples were collected from two regions of central Mexico and identified by homology analysis of the internal transcribed spacer (ITS) region. Plants from the city of Timilpan (M1) in the state of Mexico, and the city of Chignahuapan (P1) in the state of Puebla, were identified as *I. heterophylla* with 99.52% (Access number: HQ688821.1) and 99.32% (Access number: HQ688821.1) accuracy. Additionally, taxonomic identification by macroscopic observation and simple stereoscopic microscopy of diagnostic characteristics of the genus and species using a dichotomous key was done to conserve a representative plant sample (Supporting Information, Figure S1).

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Table 3. Secondary Metabolites Identified from Leaves of *I. heterophylla* by UPLC-MS and GC-MS from Two Regions of Mexico at Two Collection Times[a]

	method/solvent															
	maceration				reflux				Soxhlet				ultrasound			
SM	H	D	M	W	H	D	M	W	H	D	M	W	H	D	M	W
1	■	nd	●	●	nd	■	■	●	■/●	nd	■/●	●	■/●	●	●	nd
2	nd	nd	nd	nd	■	■	nd	nd	nd	nd	■	nd	nd	nd	nd	■
3	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
4	nd	nd	■	■	nd	nd	■	■	nd	nd	■	■	nd	nd	■	■
5	■/●	●	■/●	nd	●	■/●	■/●	■	●	■	■	■/●	■/●	■/●	■/●	nd
7	■/●	■/●	■/●	■	■/●	■/●	■/●	■	■/●	■/●	■/●	■	■/●	■/●	■/●	■
9	■/●	■/●	■	nd	■/●	■/●	■	nd	■	■	■	nd	■/●	■/●	■	■
10	nd	nd	nd	nd	nd	nd	■/●	nd	nd	nd	■	nd	nd	nd	■/●	nd
11	■	■	nd	nd	■	■	nd	nd	■	■	■	nd	■	■/●	■	nd
12	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	■/●	nd	●	nd
13	nd	■/●	nd	nd	nd	■/●	nd	nd	●	●	■/●	nd	■/●	■/●	■/●	■/●
16	nd	nd	■	nd	nd	■/●	nd	nd	nd	nd	■	■	nd	nd	■	■

[a](1) Quercetin; (2) hesperidin; (3) epicatechin; (4) caffeic acid; (5) chlorogenic acid; (7) scopoletin; (9) traquiloban-19-oic acid; (10) ent-15α-(3-methoxy-3-methyl-butanoyl)-<Kaur-16-en-19-oic acid; (11) ent-beyer-15-en 19-oic acid; (12) ent-kaur-16-en 19-oic acid; (13) 15α-tigloyloxy-ent-kaur-16-en-19-oic acid; (14) 16α hydroxy-ent-kaurane; (15) 16α-hydroxy-ent-kaur-11-en-19-oic acid; (16) 16α-hydroxy-ent-kaur-11-en-19-oic acid methyl ester. UPLC-MS: ■; GC-MS: ●; nd: not detected. SM: secondary metabolite, H: hexane, D: dichloromethane, M: methanol, W: water.

2.2. Extraction Efficiency. It is important to know the correct extraction method and the appropriate solvent to obtain each secondary metabolite from plants. Only maceration method with two solvents (chloroform and methanol) has been used in previous studies of *I. heterophylla* root; however, maceration has certain limitations, such as a low extraction yield, low efficiency, and the use of large quantities of solvents. Occasionally, the concentration of the extracted compounds decreases with prolonged maceration, possibly due to the precipitation or degradation of the secondary metabolites.[25] Therefore, in this study, we used three conventional extraction methods (maceration, reflux, and Soxhlet) and one nonconventional method (ultrasound-assisted extraction) to identify the appropriate method according to the secondary metabolites under study.[26] A solvent system of increasing polarity (hexane, dichloromethane, methanol, and water) was also used to determine the kind of secondary metabolites extracted according to their polarity. Root and leaf extracts of *I. heterophylla* were obtained from two regions of Mexico considering two collection years (2019 and 2020). Extraction yields ranged from 0.12 to 57.72% (Supporting Information, Table S1). Table 1 shows the primary yields of root and leaf extraction. Reflux extraction obtained the best yields in root using hexane, dichloromethane, and methanol. Considering the solvents used, the highest yield was in the following order: water > methanol > dichloromethane = hexane. Soxhlet and ultrasound (methanol and water) extraction were the most efficient in leaves. In the same way, as in the root, the methanol and water solvents recovered the highest leaf yield with the four methods. In summary, the root extracts had higher yields than leaf extracts.

2.3. Qualitative Identification of Secondary Metabolites in Roots by UPLC-MS and GC-MS. Initially, according to the m/z reported in previous studies,[16,17,19−21] 16 secondary metabolites were qualitatively identified by UPLC-MS and GC-MS in root extracts obtained from *I. heterophylla* using different extraction methods and solvents (Supporting Information, Table S2). Five secondary metabolites not reported in the literature were also detected (Supporting Information, Table S3). A summary of this identification is shown in Table 2. The metabolites epicatechin, scopoletin, 15α-tigloyloxy-ent-kaur-16-en-19-oic acid, and 16α-hydroxy-ent-kaur-11-en-19-oic acid methyl ester were identified with all extraction methods and solvents used for UPLC-MS or GC-MS and in some instances with both techniques. The least detected compound was hesperidin.

Regarding the type of solvent used, most of the metabolites were identified in the hexanoic extract using the four extraction methods. In contrast, the least number of secondary metabolites was identified in the aqueous extract. Considering the analytical technique, liquid chromatography was sensitive only to the secondary metabolite's hesperidin, epicatechin, caffeic acid, and 16-α-hydroxy-ent-kaur-11-en-19-oic acid. In general, it is necessary to have a medium or high polarity solvent to extract phenolic compounds; therefore, the extraction yield is higher with polar solvents such as ethanol, methanol, and mixtures with water (v/v).[27−29]

2.4. Qualitative Identification of Secondary Metabolites in Leaves by UPLC-MS and GC-MS. Secondary metabolites were screened in *I. heterophylla* leaf extracts (Supporting Information, Table S2). A summary of their identification is shown in Table 3. In this case, 12 secondary metabolites were detected. Xanthorrhizol, 8-hydroxy-6-acetyl-2, 2-dimethyl-chromium, 16α-hydroxy-ent-kauran, and 16α-hydroxy-ent-kaur-11-acid were not detected in leaves. Six secondary metabolites not reported in the literature were detected (Supporting Information, Table S3). Epicatechin and scopoletin were identified with all solvents and methods, and ent-kaur-16-en-19-oic acid was the least detected metabolite. The metabolites hesperidin, epicatechin, and caffeic acid were detected by only UPLC-MS with equal identification in the root.

2.5. Quantification of Secondary Metabolites by UPLC-MS. In accordance with previous results and considering their pharmacological importance, six of the secondary metabolites (quercetin, hesperidin, epicatechin, caffeic acid, chlorogenic acid, and xanthorrhizol) were quantified. The analytical standards were analyzed by UPLC-MS to determine

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Table 4. Quantification of Six Secondary Metabolites of *I. heterophylla* Root by UPLC-MS[a]

	quercetin		hesperidin		epicatechin		caffeic acid		chlorogenic acid		xanthorrhizol	
	Puebla	Mexico	Puebla	Mexico	Puebla	Mexico	Puebla	Mexico	Puebla	Mexico	Puebla	Mexico
solvent	2019/2020		2019/2020		2019/2020		2019/2020		2019/2020		2019/2020	
maceration												
H	0.079^{b}	0.044^{b}	nd	nd	0.081^{b}	nd	nd	nd	nd	nd	0.225^{b}	0.296^{b}
	0.064^{b}	nd	nd	nd	nd	nd	nd	nd	nd	nd	0.184^{b}	0.303^{a}
D	nd	nd	nd	nd	0.074^{b}	nd	nd	nd	nd	nd	0.118^{b}	0.485^{a}
	nd	0.028^{c}	nd	nd	nd	0.024^{c}	nd	nd	nd	nd	0.055^{c}	0.194^{b}
M	0.013^{c}	nd	nd	nd	nd	nd	0.202^{a}	0.372^{a}	0.075^{b}	nd	0.513^{a}	0.417^{a}
	nd	nd	nd	nd	0.019^{c}	0.163^{a}	0.124^{a}	0.195^{a}	nd	0.008^{c}	0.653^{a}	0.461^{a}
W	nd	nd	nd	0.054^{b}	0.069^{b}	0.040^{b}	0.102^{a}	0.358^{a}	nd	nd	nd	nd
	nd	nd	nd	nd	0.020^{c}	0.026^{c}	0.256^{a}	0.377^{a}	nd	nd	nd	nd
reflux												
H	nd	nd	0.066^{b}	nd	0.024^{c}	0.046^{b}	nd	0.067^{b}	nd	nd	nd	0.179^{b}
	nd	nd	nd	nd	nd	0.058^{b}	0.183^{a}	nd	nd	nd	0.229^{b}	0.158^{b}
D	nd	nd	nd	nd	0.013^{c}	0.030^{c}	nd	nd	nd	nd	0.090^{b}	0.384^{a}
	nd	nd	nd	nd	0.086^{b}	0.030^{c}	nd	nd	0.081^{b}	nd	0.402^{a}	0.092^{b}
M	nd	nd	nd	nd	nd	0.127^{a}	0.051^{b}	0.115^{a}	nd	nd	0.483^{a}	0.582^{a}
	nd	nd	nd	nd	0.079^{b}	0.151^{a}	0.144^{a}	0.260^{a}	nd	nd	0.535^{a}	0.401^{a}
W	nd	nd	nd	nd	0.107^{b}	0.043^{b}	0.210^{a}	0.248^{a}	nd	nd	nd	nd
	nd	nd	nd	nd	0.055^{b}	0.053^{b}	0.314a	0.307^{a}	nd	nd	0.024^{c}	nd
Soxhlet												
H	nd	nd	nd	nd	0.066^{b}	nd	nd	nd	nd	nd	0.198^{b}	0.398^{a}
	nd	nd	nd	nd	0.067^{b}	nd	nd	nd	nd	nd	0.241^{b}	0.245^{b}
D	nd	nd	nd	nd	0.081^{b}	0.046^{b}	nd	nd	nd	nd	0.206^{b}	0.564^{a}
	nd	nd	nd	nd	0.051^{b}	nd	nd	nd	nd	nd	0.179^{b}	0.233^{b}
M	0.151^{a}	nd	nd	nd	0.092^{b}	nd	0.302^{a}	0.111^{a}	0.234^{a}	nd	0.026^{c}	0.647^{a}
	0.064^{b}	0.017^{c}	nd	nd	0.097^{b}	0.094^{b}	0.248^{a}	0.199^{a}	nd	nd	0.667^{a}	0.595^{a}
W	nd	nd	0.010^{c}	nd	0.078^{b}	0.075^{b}	0.287^{a}	0.061^{b}	0.015^{c}	nd	nd	nd
	nd	nd	nd	nd	0.032^{c}	0.040^{b}	0.129^{a}	0.110^{a}	0.009^{c}	0.011^{c}	nd	nd
ultrasound												
H	nd	nd	nd	nd	0.087^{b}	0.020^{c}	nd	nd	nd	nd	0.165^{b}	0.362^{a}
	nd	nd	nd	0.011^{c}	0.078^{b}	0.071^{b}	nd	nd	0.014^{c}	nd	0.207^{b}	0.172^{b}
D	nd	nd	nd	nd	0.055^{b}	0.036^{c}	nd	nd	nd	nd	0.412^{a}	0.303^{a}
	nd	nd	nd	nd	0.036^{c}	0.079^{b}	nd	nd	nd	nd	0.086^{c}	0.148^{b}
M	nd	nd	nd	0.023^{b}	0.058^{b}	0.090^{b}	nd	0.115^{a}	nd	nd	0.485^{a}	0.620^{a}
	nd	nd	nd	nd	0.033^{c}	0.062^{b}	0.234^{a}	0.290^{a}	nd	nd	0.593^{a}	0.206^{b}
W	nd	nd	nd	nd	0.089^{b}	0.038^{c}	0.050^{b}	0.099^{b}	nd	nd	nd	nd
	nd	nd	nd	nd	0.058^{b}	0.054^{b}	0.202^{a}	0.381^{a}	nd	nd	nd	nd

[a]nd: not detected. H: hexane, D: dichloromethane, M: methanol, W: water. Values with different letters (a, b, and c) in superscript denote significant differences with LSD test ($p \leq 0.05$).

their retention time, experimental mass, and fragmentation pattern (Supporting Information, Table S4 and Figures S2–S13). After the parameters of the six analytical standards were determined, the secondary metabolites were quantified by UPLC-MS in the extracts obtained from the roots of the two regions of Mexico in different years (Supporting Information, Table S5). A summary is shown in Table 4.

Quercetin in *I. heterophylla* root was quantified only with the maceration and Soxhlet methods, obtaining the highest concentration (0.151 mg/mL) in the sample from Puebla with Soxhlet in methanol. This quantity was significantly different from all of the quantified amounts. Quercetin was not quantified in any aqueous extract, and in the case of Soxhlet, it was quantified only with methanol.

Hesperidin in the root was quantified with all of the extraction methods. The highest concentration was obtained in hexanoic extract with the reflux method. Although this concentration was not significantly different from those obtained in water and methanol by maceration and ultrasound, respectively. Hesperidin was not quantified in the dichloromethane, hexane, and methanol extracts using Soxhlet and maceration methods.

Epicatechin was the only metabolite quantified in root extracts using all extraction methods with the four solvents. The highest concentration (0.163 mg/mL) was obtained by maceration with methanol, although this amount was not significantly different from the concentrations obtained by refluxing with methanol.

Caffeic acid was quantified in the extracts obtained by the four extraction methods but only with methanol and water, except with hexane with the reflux method. The highest concentration in the root was 0.381 mg/mL determined by ultrasound-assisted extraction with water. This concentration was not significantly different from those obtained in maceration, reflux, and Soxhlet with methanol and water. This concentration was higher than that detected in shoots and seeds of *Helianthus annuus* L. (0.110 g/100 g of fresh material).[30]

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Table 5. Quantification of Secondary Metabolites of *I. heterophylla* Leaves by UPLC-MS[a]

	quercetin		hesperidin		epicatechin		caffeic acid		chlorogenic acid		xanthorrhizol	
	Puebla	Mexico	Puebla	Mexico	Puebla	Mexico	Puebla	Mexico	Puebla	Mexico	Puebla	Mexico
Solvent	i2019/2020		2019/2020		2019/2020		2019/2020		2019/2020		2019/2020	
maceration												
H	nd	nd	nd	nd	0.101^{a}	0.157^{a}	nd	nd	nd	nd	nd	nd
	nd	0.022^{c}	nd	nd	0.030^{c}	0.086^{b}	nd	nd	nd	0.011^{c}	nd	nd
D	nd	nd	nd	nd	nd	0.077^{b}	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	0.161^{a}	0.055^{b}	nd	nd	nd	nd	nd	nd
M	nd	nd	nd	nd	0.616^{a}	0.312^{a}	0.071^{b}	0.102^{a}	0.085^{b}	0.091^{b}	nd	nd
	nd	nd	nd	nd	0.292^{a}	0.070^{b}	0.147^{a}	0.135^{a}	0.043^{b}	0.016^{c}	nd	nd
W	nd	nd	nd	nd	0.029^{c}	nd	0.076^{b}	0.067^{b}	nd	nd	nd	nd
	nd	nd	nd	nd	0.015^{c}	0.048^{b}	nd	0.040^{b}	nd	nd	nd	nd
reflux												
H	nd	nd	nd	nd	0.764^{a}	0.061^{b}	nd	nd	nd	nd	nd	nd
	nd	nd	0.051^{b}	nd	0.110^{a}	0.109^{a}	nd	nd	nd	nd	nd	nd
D	nd	nd	0.028^{b}	0.040^{c}	0.292^{a}	0.181^{a}	nd	nd	nd	0.015^{c}	nd	nd
	0.017^{c}	nd	nd	nd	0.136^{a}	0.061^{b}	nd	nd	nd	nd	nd	nd
M	nd	nd	nd	nd	0.569^{a}	0.098^{b}	0.092^{a}	0.055^{b}	0.047^{b}	0.084^{b}	nd	nd
	nd	0.041^{b}	nd	nd	0.266^{a}	0.294^{a}	0.382^{a}	0.139^{a}	0.026^{c}	0.063^{b}	nd	nd
W	nd	nd	nd	nd	0.057^{b}	0.086^{b}	0.151^{a}	0.094^{b}	nd	0.009^{c}	nd	nd
	nd	nd	nd	nd	0.106^{a}	0.073^{b}	0.145^{a}	0.112^{a}	0.011^{c}	0.026^{c}	nd	nd
Soxhlet												
H	nd	0.021^{c}	nd	nd	0.195^{a}	0.244^{a}	nd	nd	nd	nd	nd	nd
	nd	nd	nd	nd	0.070^{b}	0.155^{a}	nd	nd	nd	nd	nd	nd
D	nd	nd	nd	nd	0.253^{a}	0.071^{b}	nd	nd	0.071^{b}	nd	nd	nd
	nd	nd	nd	nd	0.084^{b}	0.104^{a}	nd	nd	nd	nd	nd	nd
M	0.010^{c}	nd	0.173^{a}	nd	0.500^{a}	0.171^{a}	nd	0.204^{a}	0.052^{b}	0.095^{b}	nd	nd
	nd	nd	nd	nd	0.348^{a}	0.289^{a}	0.440^{a}	0.388^{a}	0.050^{b}	0.081^{b}	nd	nd
W	nd	nd	nd	nd	0.064^{b}	nd	0.260^{a}	0.051^{b}	0.006^{c}	0.012^{c}	nd	nd
	nd	nd	nd	nd	0.070^{b}	nd	0.099^{b}	0.152^{a}	nd	nd	nd	nd
ultrasound												
H	nd	nd	nd	nd	0.333^{a}	0.278^{a}	nd	nd	0.016^{c}	nd	nd	nd
	nd	0.009^{c}	nd	nd	0.061^{b}	0.047^{b}	nd	nd	nd	nd	nd	nd
D	nd	nd	nd	nd	0.172^{a}	0.281^{a}	nd	nd	0.024^{b}	nd	nd	nd
	nd	nd	nd	nd	0.074^{b}	0.074^{b}	nd	nd	nd	nd	nd	nd
M	nd	nd	nd	nd	0.090^{b}	0.106^{a}	0.294^{a}	0.152^{a}	0.328^{a}	0.119^{a}	nd	nd
	nd	nd	nd	nd	0.240^{a}	0.248^{a}	0.393^{a}	0.348^{a}	0.041^{b}	0.051^{b}	nd	nd
W	nd	nd	nd	nd	0.068^{b}	nd	0.115^{a}	0.023^{b}	nd	nd	nd	nd
	nd	nd	0.003^{c}	0.024^{b}	0.086^{b}	0.075^{b}	0.110^{a}	0.080^{b}	nd	nd	nd	nd

[a]Pue: Puebla, Mex: Mexico, nd: not detected. H: hexane, D: dichloromethane, M: methanol, W: water. Values with different letters (a, b, and c) in superscript denote significant differences with LSD test ($p \leq 0.05$).

Chlorogenic acid was not quantified in the root extract obtained with hexane and dichloromethane in any extraction method, except the reflux method with dichloromethane and ultrasound with hexane. The highest concentration of chlorogenic acid was 0.234 mg/mL when applying Soxhlet extraction with methanol. This finding represents a significant difference concerning the extract concentrations obtained by maceration, reflux, and ultrasound-assisted extraction.

Xanthorrhizol is the major compound in the extracts of the root samples. The highest concentration (0.667 mg/mL) was obtained by the Soxhlet method with methanol. However, this was not significantly different from the concentrations obtained with dichloromethane and hexane by maceration, reflux, and Soxhlet. Xanthorrhizol was not quantified in any aqueous extract except with the Soxhlet method. The concentration of xanthorrhizol in the root of *I. heterophylla* reported by Aguilar et al.[21] and Bernal[31] represents 11.54% of the content of compounds in the root, a similar value obtained in the root from Mexico and Puebla (Table 4).

According to the results in Table S4, the quantification of quercetin, hesperidin, epicatechin, caffeic acid, chlorogenic acid, and xanthorrhizol from *I. heterophylla* leaves by UPLC-MS was also considered (Table 5).

Quercetin in the sample from Mexico presented the highest concentration (0.041 mg/mL) using the reflux method with methanol. Hesperidin was not determined by the maceration method. In the sample from Puebla, hesperidin has the highest concentration (0.173 mg/mL) using the Soxhlet method with methanol, with a significant difference concerning the concentrations obtained in the extracts using the reflux and ultrasound methods.

Epicatechin was quantified at a high concentration (0.616 mg/mL) by maceration with methanol. This amount did not present significant differences concerning the reflux method with hexane and dichloromethane and the Soxhlet method with methanol.

Caffeic acid was obtained at a higher concentration (0.440 mg/mL) using a Soxhlet with methanol. This concentration

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Table 6. Method, Solvent, Region, and Sample Type with the Highest Concentrations of Six Secondary Metabolites from *I. heterophylla*

metabolite	method	solvent	region	sample
quercetin	reflux	methanol	Puebla	root
hesperidin	Soxhlet	methanol	Puebla	leaf
epicatechin	maceration, reflux, Soxhlet, ultrasound	hexane, dichloromethane, methanol	Puebla, Mexico	root, leaf
caffeic acid	maceration, reflux, Soxhlet, ultrasound	methanol, water	Puebla, Mexico	root, leaf
chlorogenic acid	Soxhlet, ultrasound	methanol	Puebla, Mexico	root, leaf
xanthorrizol	maceration, reflux, Soxhlet, ultrasound	hexane, dichloromethane, methanol	Puebla, Mexico	root

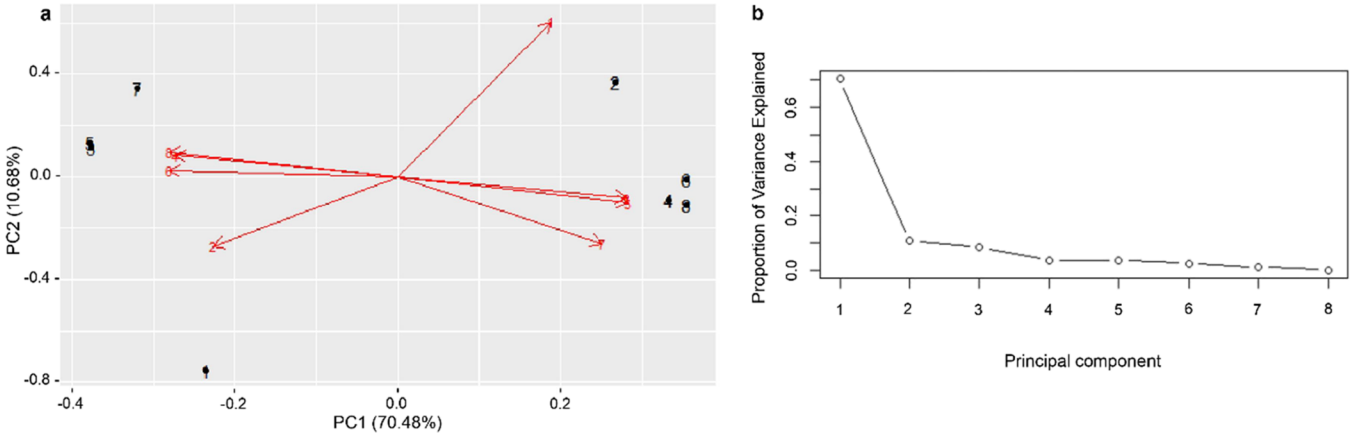

Figure 2. (a) PCA (PC1 vs PC2). (b) Proportion of explained variance of six secondary metabolites from *I. heterophylla*.

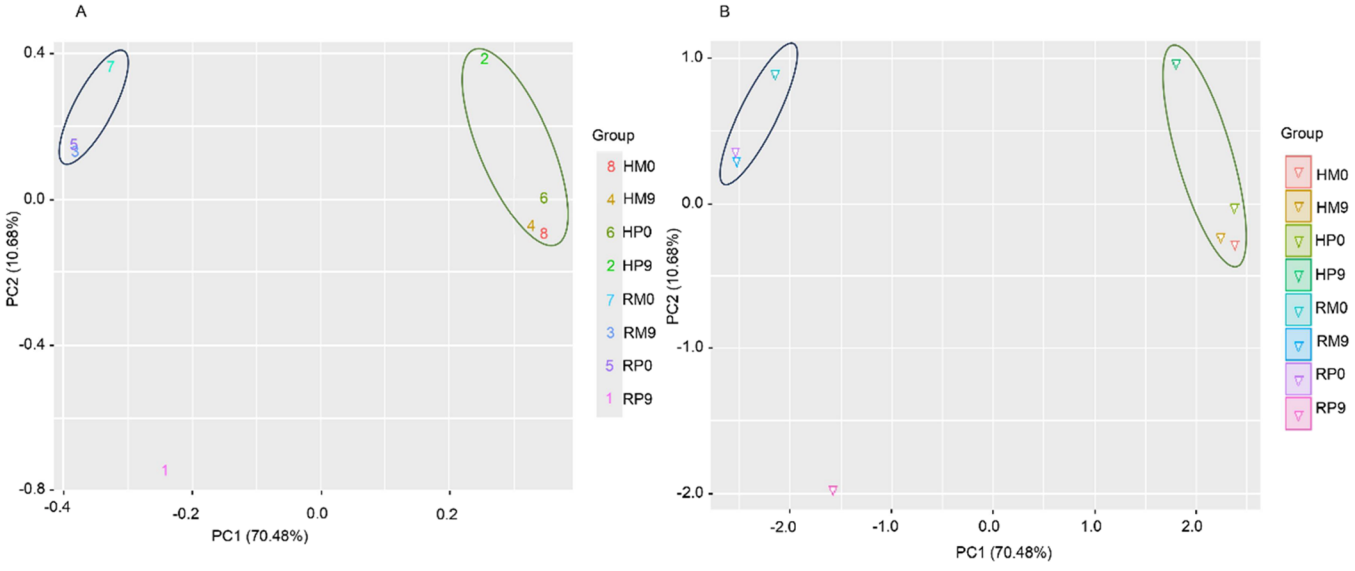

Figure 3. (A) Graph of punctuation of PC1 and PC2 with respect to PC3, PC4, PC5, and PC6. (B) Scoring graph of PC1 and PC2 with respect to PC7 and PC8.

was not significantly different from the concentrations obtained by the reflux and ultrasound methods. This concentration is higher than that obtained in shoots and seeds of *Helianthus annuus* L. (0.093/100 g of fresh material).[30]

Chlorogenic acid was detected with all four extraction methods, using methanol. In other, solvents were detected at a lower concentration. The highest concentration (0.328 mg/mL) was obtained by using the methanol ultrasound method. Finally, xanthorrhizole was not detected. This secondary metabolite has only been identified in rhizomes of *I. heterophylla* and *Curcuma xanthorrhiza*.[32]

In summary, Table 6 shows the method, solvent, region, and part of the plant with the highest concentrations of each secondary metabolite from *I. heterophylla*. The highest concentrations were quantified in methanolic root extracts from the Puebla region. The preceding agrees with that described for phenols, flavonoids, and anthocyanins since most of them are water-soluble; thus, polar and moderately polar solvents, such as water, ethanol, methanol, and propanol, are widely used for their extraction[33−36] and even combinations have been used in previous studies for extracting phenolics from plant materials, often with different proportions of water, to establish their extractive efficiency.[27,29,37−40] The least

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efficient method for the quantification of the six metabolites was maceration.

Finally, a principal component analysis (PCA) was applied to determine the significant difference in the concentration of secondary metabolites between the leaf and root of *I. heterophylla*, including as variables region, year, extraction method, and solvent (Figure 2a). The PCA was performed with eight components (PC1−8), PC1 represents the six quantified metabolites; PC2 the four extraction methods and solvents used; PC3 and PC4 the collection region (Puebla and Mexico, respectively); PC5 and PC6 the collection years (2019 and 2020); and PC7 and PC8 the leaves and root, respectively (Figure 2b). The analysis was based on the first two principal components, PC1 and PC2, since they explain most of the observed variance, 70.48 and 10.68%, which together with the other components, provide us with an approximate explained variance of more than 95% (Figure 2b).

The difference in the content of the six quantified metabolites according to the type of sample and region is shown in Figure 3A. The metabolites obtained from the root using the different extraction methods and solvents from Puebla 2020 (5:RP0) and Mexico 2019 (3:RM9) and 2020 (7:RM0) had not a significantly different. Only, samples from Puebla 2019 (1:RP9) show a significantly different with the other root samples. In this sense, the leaf samples from Puebla 2020 (6:HP0) and Mexico 2019 (4:HM9) and 2020 (8:HM0) had not significantly different, except samples from Puebla 2019 (2:HP9). Additionally, the secondary metabolite profile of *I. heterophylla* indicates a significant difference between the compounds contained in the leaves (HM0, HM9, HP9, and HP0) concerning those of the root (RM0, RM9, RP0, and RP9) (Figure 3B).

Finally, an analysis was performed of the heat map of the secondary metabolite profile of *I. heterophylla* (Figure 4). It was found that the different metabolites contained in the leaf and root of *I. heterophylla* are extracted in higher concentration using the Soxhlet and ultrasound-assisted extraction methods with methanol, and it is worth noting that the use of solvents such as dichloromethane and water favor the extraction of metabolites but in lower concentration compared to methanol.

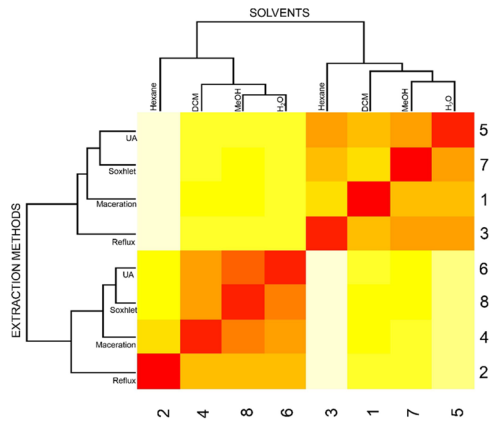

Figure 4. Heat map of the secondary metabolite profile of *I. heterophylla* by UPLC-MS. The row shows the extraction method, and the column represents the solvent used. The lowest concentration of metabolites (quercetin, hesperidin, epicatechin, caffeic acid, chlorogenic acid, and xanthorrhizol) is shown in beige, while those with significantly higher concentrations are shown in red. The brightness of each color corresponds to the magnitude of the difference compared with the mean value. DCM: dichloromethane, USA: ultrasound-assisted extraction, H_2O: water, and MeOH: methanol.

3. MATERIALS AND METHODS

3.1. Plant Materials. *I. heterophylla* was collected from two regions of Mexico: Chignahuapan, Puebla (19°49′57.3″N 97°59′31.8′′ W) and Timilpan, State of Mexico (19°50′14.7′′N 99°44′56.1′′ W) in December 2019 and August 2020. The root and leaves were disinfected with a commercial detergent solution (1% w/v), cut, and dried at 58 °C for 3 days; both materials were pulverized and stored in the dark at room temperature until use.

3.2. Molecular Identification. The leaf tissue was frozen with liquid nitrogen and macerated to carry out the gDNA extraction later using the QUIAGEN extraction protocol from the DNeasy Plant commercial kit. The DNAg obtained was visualized in an electrophoresis chamber in a 1% agarose gel with 0.5× TBE buffer (0.45 M Tris-base, 0.45 M boric acid, and 0.5 M EDTA) as running buffer. Five microliters of DNAg with 3 μL of SYBR Green and lambda phage were used as molecular mass markers. The products were analyzed using the UV light photo documenter (Logic I) with the Kodak Molecular Imaging program, and the sample was quantified in a Nanodrop Thermo Scientific 2000. Subsequently, the amplification of the ITS was performed by PCR end point. To amplify the ITS, the primers ITS1 (5′-TCCGTAGGT-GAACCTGCGG-3′) and ITS4 (5′TCCTCCGCTTATTGA-TATGC-3′) were used.[41] PCR conditions were standardized by adjusting the reagent concentrations; the following reaction was prepared: 5 μL of 5× Taq DNA polymerase buffer, 1.5 μL of 25 mM MgCl2, 1 μL of 5 μM ITS1 primer, 1 μL of ITS4 primer, 5 μM, 0.5 μL dNTP's 10 mM, 0.25 μL 5 U GoTaq polymerase, ≤10 ng DNA, adjust final volume to 25 μL with sterile MiliQ water. Subsequently, the reaction was run in the thermal cycler (Applied Biosystems SimpliAmp by Life Technologies) according to the following amplification conditions: it started with a temperature of 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min and the final step of 72 °C for 5 min. The visualization of the DNA fragments obtained was performed by 2% agarose gel electrophoresis. Five microliters of the PCR product was loaded on the agarose gel with 3 μL of SYBR Green 10× and 3 μL of the molecular mass marker (Ladder 100 bp) with 3 μL of SYBR Green 10×. The agarose gel was visualized in a photo documenter with UV light using the Kodak Molecular Imaging program.

The PCR products obtained from *I. heterophylla* (700−800 bp) were purified using ExoSAP-IT PCR Product Cleanup Reagent, where for every 10 μL of product, 4 μL of ExoSAP-IT reagent was added, and the reaction was carried out into 0.2 μL microtubes. The samples were placed in a thermal cycler (Applied Biosystems SimpliAmp by Life Technologies) under the following reaction conditions; a cycle at 37 °C for 15 min and at 80 °C for 15 min. The samples were quantified in the Thermo Scientific Nanodrop 2000/2000C Spectrophotometers equipment, and their concentration was adjusted between 60 ng μL^{-1}, and they were sequenced in the company Eurofins GTM Operon LLC (Louisville KY, USA). Finally, the sequence alignment was carried out where from the sense and antisense nucleotide sequences obtained, a consensus sequence was built using the SeqMan-Pro program. The consensus sequences in the Basic Local Alignment Search Tool (BLAST) database of the National Center for Biotechnology

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Information (NCBI) and through its local alignment, the genus, and species of the sequenced plant were determined.

3.3. Extraction Procedure. The extraction of secondary metabolites was done using four methods and solvents by triplicate. Maceration: 2 g of leaf or root were added in 50 mL of solvent and stored for 8 days at controlled room temperature in the dark; Soxhlet: 2 g of leaf or root on filter paper were used to be placed in the Soxhlet apparatus using 100 mL of solvent for 6 h; Reflux: 2 g of leaf or root were added in 50 mL of solvent and refluxed for 4 h; and Ultrasound: 2 g of leaf or root were added in 50 mL of solvent and subjected to a Brasonic ultrasound bath for 40 min. In all procedures, at the end of the time, a filtration process was carried out, and the liquid extract was collected. Four solvents with different polarities were used: hexane, dichloromethane, methanol, and water. The solvent (hexane, dichloromethane, and methanol) was removed under reduced pressure at a temperature not higher than 50 °C with the help of a Rotavapor R-100. The water was removed over 24−48 h in a LabconcoTM 77540-00 freeze-dryer. The extracts were stored in the dark at 4 °C until use. This procedure was performed in triplicate with each extraction method and solvent used.

3.4. Analysis by Ultraperformance Liquid Chromatography (UPLC-MS). Standards from Sigma-Aldrich (chlorogenic acid, caffeic acid, hesperidin, quercetin, epicatechin) and Cayman Chemical (xanthorrhizol) at concentrations of 0.125, 0.25, 0.5, 0.75, and 1 mg/mL were vortexed for 1 min, then filtered with a 0.22-μm membrane, and transferred to standard vials for analysis. Instrumentation and methods: UPLC-MS analysis was performed using liquid chromatography equipment with an ACQUITY UPLC system coupled to a QDA mass detector from Waters (Milford, MA, USA). ACQUITY UPLC CORTECS C18 1.6 μm, 3.0 mm × 100 mm column. Column and autosampler temperatures of 40 and 15 °C were used, respectively. Elution was achieved with 0.1% formic acid in water (Phase A), acetonitrile (Phase B), and 5 mM ammonium acetate (Phase C). The composition of the solvents over time was as follows: initial A, 5%; B, 85%; C, 10%, at 3.0 min increase A: 15%; B: 75%; C: 10%, changing at 10.0 min A: 5%; B: 85%; C: 10% with a flow rate of 0.3 mL/min. Sample preparation: 5 μL of extracts (1 mg/mL) were injected. Results: the phenolic compounds and xanthorrhizol were identified by comparing the mass spectra and retention times with the corresponding standards (Figures S2−S7) and quantifying the compounds according to the results of the concentrations analyzed according to the standards.

3.5. Analysis by GC-MS. Standards from Sigma-Aldrich (chlorogenic acid, caffeic acid, hesperidin, quercetin, epicatechin) and Cayman Chemical (xanthorrhizol) at concentrations of 0.25, 0.5, and 1 mg/mL were vortexed for 1 min, then filtered with a 0.22 μm membrane, and transferred to standard vials for analysis. Instrumentation and methods: the GC-MS analysis was performed according to the methodology described by Trivedi et al.[42] with slight modifications. It was carried out on an Agilent Technologies model 7890B Network Series GC System (Palo Alto, CA, USA) equipped with a triple-axis mass selective detector model 5975C (Agilent Technologies, Palo Alto, CA, USA). With HP-5 ms 19091S-433 capillary column (30 m × 0.25 mm, 0.25 μm, J & W Scientific, Folsom, CA, USA) in brief, inlet and detector temperatures were 250 and 300 °C, respectively. The oven temperature program started at 70 °C for 2 min and increased to 250 °C. The rate of increase was 5 °C/min and was maintained for 15 min; 2 μL of samples and standards (1 mg/mL) were injected using the mode. Helium was used as carrier gas (0.65 mL/min). The mass selective detector (MSD) (EI mode) was operated at 70 eV, the ion source temperature was 250 °C, and the m/z detection ranged from 50 to 650 amu. The identification of the metabolites was carried out by the retention time and by the comparison of the mass spectra of the standards (Figures S8−S13) and together with the database NIST (a similarity index >90%) 11 MS Database and Ms Search Program v2.0

3.6. Statistical Analysis. PCA was used to define both similarities and differences between the compound identified by UPLC, collection site, sample type (leaf and root), extraction method, and solvent type. The resulting data set was exported to R-studio software to perform the PCA, complementary to this an ANOVA and LSD test ($P < 0.05$) were performed using Statgraphics centurion XV version 15.2.06.

4. CONCLUSIONS

In this study, a comparative analysis of the secondary metabolite profile of *I. heterophylla* showed a difference in their content according to the part of the plant analyzed (root and leaves); however, considering the regions (Mexico and Puebla) and the collection year, no significant differences were found. Highlighting six metabolites in high amounts: quercetin ≤0.151 mg/mL in root and ≤0.041 mg/mL in leaf; hesperidin ≤0.66 mg/mL in root and ≤0.173 mg/mL in leaf; epicatechin ≤0. 163 mg/mL in root and ≤0.664 mg/mL in leaf; caffeic acid ≤0.372 mg/mL in root and ≤0.393 mg/mL in leaf; chlorogenic acid ≤0.234 mg/mL in root and ≤0.328 mg/mL in leaf; and xanthorrhizol ≤0.667 mg/mL in root. Therefore, these results show that the leaves are an important source of hesperidin, quercetin, and epicatechin, suggesting a potential use for this underutilized plant part. Additionally, the obtention of secondary metabolites using different methods and solvents allows selective extraction of each metabolite: quercetin in root and leaf by reflux; hesperidin, in leaf by Soxhlet and in root by reflux; chlorogenic acid, in root by Soxhlet and in leaf by ultrasound-assisted extraction; and epicatechin, caffeic acid and xanthorhizol, in leaf and root by reflux, maceration, Soxhlet, and ultrasound-assisted extraction. The most effective solvent is methanol. Finally, the secondary metabolite profile of the leaves suggests their potential use as a source of bioactive compounds such as hesperidin, quercetin, and especially epicatechin.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06800.

Extraction yields of each sample; qualitative identification of secondary metabolites by UPLC-MS and GC-MS; experimental data obtained by UPLC-MS; taxonomic information; spectral information on the standards by UPLC-MS and GC-MS; and calibration curves of the analytic standards (PDF)

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Notes

The authors declare no competing financial interest.

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