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Qualitative and quantitative analysis of polyphenolic compounds in *Ilex* Sp.

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Abstract: Natural compounds are an important source of desired biological activity which help to improve nutritional status, enhance productivity and bring many health benefits. The leaves of the *Ilex paraguariensis* (Aquifoliaceae) are used for preparing a beverage known as yerba mate and represent a proven source of natural polyphenols which are known to foster biological activity with the emphasis on antioxidant properties. In present work we focused on the polyphenolic content of air-dried leaves of *Ilex aquifolium* L., *Ilex aquifolium 'Argentea Mariginata'*, *Ilex meserveae* '*Blue Angel*', and a commercially available mate as the reference product. Liquid chromatography combined with mass spectrometry (HPLC and LC-MS) and thin layer chromatography (TLC), were used to establish polyphenolic substances content in aqueous methanolic extracts obtained from the biological matter. Up to 20 polyphenolic compounds were identified in the extracts, including rutin, quinic acid and its caffeoyl esters, i.e. chlorogenic acid and its isomers as well as dicaffeoyl derivatives. We took chlorogenic acid and rutin as reference compounds to quantify their levels in the extracts. It was determined that in all tested plants, high levels of these antioxidants were present. This led us to the conclusion that their leaves might serve as valuable food additives.

Keywords: Ilex sp., extraction, polyphenols, HPLC MS

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1 Introduction

Yerba mate, an infusion prepared from leaves of *Ilex paraguariensis* A. St. Hilaire is widely consumed around the world. Traditionally it was used by the South American natives before the European colonization. In countries where it is produced (e.g. Argentina, Brazil),mate is not only an important branch of agriculture, but an important part of the economy as well. [1]. This species is exported worldwide, including Europe, the United States, and Japan, where it is marketed as a milled plant or extracts used in herbal formulations and functional food products. According to the Argentine Food Code, yerba mate is defined as the product constituted exclusively by the dried, slightly roasted, and milled leaves of *I. paraguariensis* which can contain fragments of young branches, pedicles, and floral peduncles [1,2].

The mate raw material presented as leaves and green stalks, is processed as a dye herb for the classical "Chimarrão", "Mate" or "Tereré," as a fine or soluble powder for beverage preparation, or as a base for different industrial purposes [3]. Basically, the mate beverages are prepared using either hot or cold water. There are also studies on the production of *I. paraguariensis* extracts using essential carbon dioxide [2]. However, regardless of the method, the resulting extracts are characterized by a distinct biological activity.

In many countries *I. paraguariensis* is used in folk medicine to treat different medical conditions. Literature studies indicate that the therapeutic efficacy focuses on diseases such as arthritis, inflammatory diseases, hemorrhoids, headache, hepatic disorders, and obesity [1,4,5]. A recent study conducted on rats indicates that *I. paraguariensis* plays an important role in the management of obesity by acting on the inflammatory profile [6]. Hydroethanolic mate extract reduced serum triglycerides in rats consuming a high fat diet, cholesterol, and also decreased the atherogenic index in treated animals [7]. These results support a potential therapeutic effect of the plant in cardiovascular disease.

It was also shown that the *I. paraguariensis* acts as an anti-inflammatory agent [2]. *In vivo* and *in vitro* research

demonstrated that the commercial extract of yerba mate has an antioxidant effect, due to the presence of caffeine and polyphenolic compounds like rutin and chlorogenic acid [8]. The plant also contains saponins which prevent a form of cancer [9]. There is a great probability that saponins may be contained in other *Ilex* species as well which gives way to further study.

Herb supplements may also be beneficial in animal production, such as in livestock nutrition, serving as an alternative to other chemical additives. Yerba mate ingredients undergo several physiological processes [10] and as such could be recommended as a natural and novel feed supplement with a potential for improving feed intake and in treating diseases. The yerba mate supplement given to dairy calves had significant effects on their metabolic and oxidative status, which resulted in lower liveweight [11].

Bioactive compounds present in *I. paraguariensis* undergo modification according to extractive methods, genetic and environmental variability, and harvest time [12,13]. The extracts contain mainly polyphenols like chlorogenic acid, purine alkaloids (methylxanthines) such as caffeine and theobromine, flavonoids, a combination of vitamins, tannins and numerous triterpenic saponins derived from ursolic acid [5,7,14]. The polyphenolic compounds of major importance in mate refer to caffeoyl derivatives, mainly monocaffeoyl quinic isomers and dicaffeoyl quinic isomers [7], and their level exceeds even that found in green tea, a typical 'antioxidant' product present on the market. Among *Ilex* species in terms of phytochemical research *I. paraguariensis* has been the subject of most intensive investigations [5].

To the best of our knowledge, amongst the *Ilex* genera, only *I. paraguariensis* was the subject of intensive phytochemical studies. Therefore, we concentrated on other available *Ilex* varieties: *I. aquifolium* L., *Ilex aquifolium* '*Argentea Mariginata*', and *Ilex meserveae*. This study focuses on the polyphenolic content of these shrubs.

2 Materials and methods

2.1 Plant materials and reagents

Commercial Argentinian mate packed in ready-to-use bags containing three-gram portions of finely ground material, were purchased from a local shop. Relevant specimens of selected plants were deposited at the Wrocław University of Environmental and Life Sciences herbarium. Seedlings of *I. aquifolium* L., *I. aquifolium* '*Argentea Mariginata*' and *I. meserveae* '*Blue Angel*' were purchased from a local supplier. All seedlings were planted in the ground, the leaves were hand-picked, and air-dried. Solvents of suitable grades used in this experiment were bought from Archem (Poland). Merck TLC silica gel 60 F_{254} aluminium plates were used for thin layer chromatography (TLC).

2.2 Extraction of polyphenols

Extracts from commercial mate and the other *Ilex* sp. dried leaves were prepared using the method presented by Erol et al. [15], with slight modifications using 80% methanol: a ground 10 g sample was mixed with 200 mL of the solvent and extracted for 24 h using a rotatory shaker at 25°C in the dark. After extraction, the mixture was filtrated by using a vacuum filtration and then evaporated at a reduced pressure until it was dry. The dry extract was weighed to calculate the yield.

2.3 TLC

Crude extracts were initially analyzed on TLC plates using a quaternary solvent mixture (chloroform/acetone/acetic acid/water, 3:7:1:1) and cerium (IV) sulphate with phosphormolybdenic acid in 10% aqueous sulphuric acid as the staining solution.

2.4 HPLC analysis

HPLC analyses were performed by using the Dionex Ultimate 3000 chromatograph equipped with a PDA detector. Separation was achieved using the Cadenza column C18. Two eluents were used in gradient mode: 4.5% formic acid (A) and 100% acetonitrile (B). The detailed gradient program is listed in Table 1. The flow rate was 1 mL min^{-1}, and the injection volume was 20 µL. Flavonols were monitored at a wavelength of 360 nm, and phenolic acids at 320 nm.

2.5 LC MS analysis

Samples were dissolved in HPLC grade methanol at a concentration of 5 mg mL^{-1}. Phenolic constituents were identified with the aid of analytical standards using UPLC chromatograph coupled with a mass spectrometer Q-TOF-MS (XEVO-G2QTOF, Waters). Separation was achieved

injecting 5 µL of samples using AcquityTM BEH C_{18} column (100 × 2.1 mm id, 1.7 µm) thermostated at 30°C in gradient elution with 0.5% formic acid in water (solvent A) and 100% acetonitrile (solvent B) and constant flow rate of 0.45 mL min^{-1}.The detailed gradient program is listed in Table 2.

MS parameters were as follows: capillary voltage – 2.0 kV; a sampling cone voltage – 45 V; the gas flow on the cone – 11 L h^{-1}, the collision energy of 50 eV. The camera was set to positive and negative ion scanning modes, *m/z* 100 to 2500. The system worked with software MassLynx™ V 4.1.

3 Results

3.1 Extraction yield of *Ilex* species

The amount of dry matter extracted with the use of aqueous methanol constituted more than 20% of the air-dried plant material with the exception of *I. meserveae*, for which we obtained only about half of the values recorded for the other three plants. The results are summarized in Table 3.

Table 1: Gradient program used in HPLC separations.

Time [min]	Eluent A	Eluent B
0	95%	5%
1	75%	25%
20	0%	100%
27	95%	5%

Table 2: Gradient program used in LC MS separations.

Time [min]	Eluent A	Eluent B
0	99%	1%
12	75%	25%
12.5	0%	100%
13.5	99%	1%

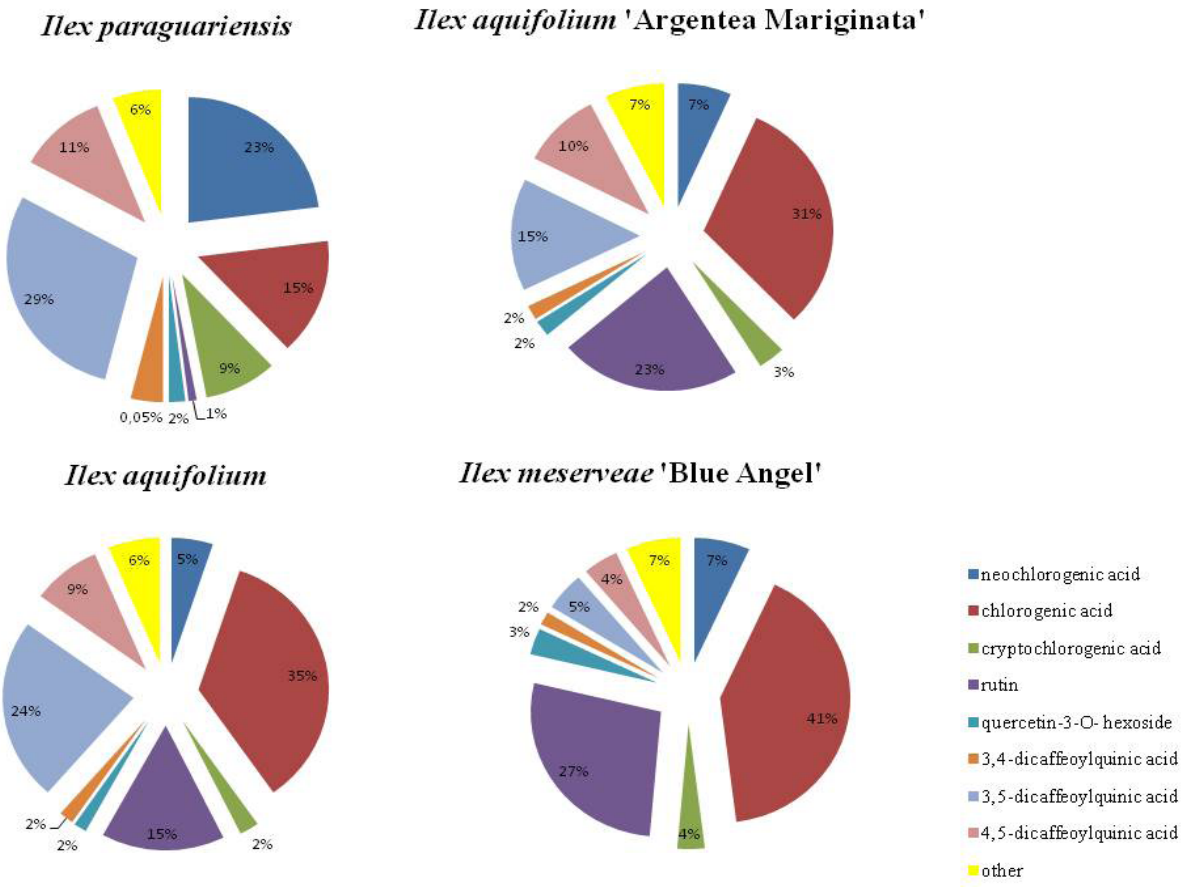


Figure 1: The mean share of phenolic compounds in *Ilex* Sp.

Table 3: The per cent extraction yield of *Ilex* species.

Ilex sp.	Extraction yield [%]
Ilex paraguariensis	24.3
Ilex aquifolium 'Argentea Mariginata'	25.3
Ilex aquifolium L.	21.8
Ilex meserveae	12.8

3.2 TLC

TLC of resulting extracts redissolved in methanol indicated the presence of a complex mixture of compounds with a broad spectrum of polarity. The TLC plate is shown on Fig.1

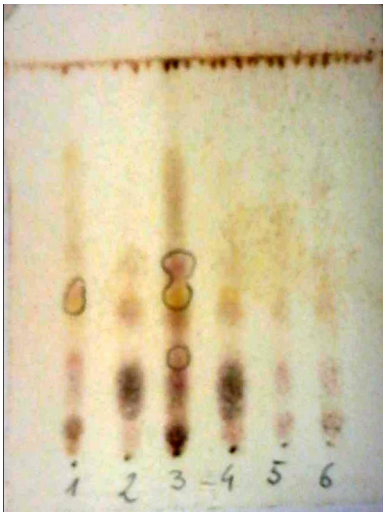
Table 4: HPLC DAD characteristics of phenolic compounds in *Ilex sp.*

Plant material	No.	Peak name	Ret.time [min]	Content [μg g^{-1} d.m.]
Ilex paraguariensis	2	Neochlorogenic acid	3.12	11810
	3	*(Dicaffeic acid)*	4.18	193
	4	*(Dicaffeic acid)*	4.59	116
	5	*(Dicaffeic acid)*	4.95	122
	6	Chlorogenic acid	5.35	7755
	7	Cryptochlorogenic acid	5.65	4460
	8	*(Dicaffeic acid)*	6.00	131
	9	Feruloylquinic acid isomer	9.14	134
	10	Feruloylquinic acid isomer	9.35	162
	12	Rutin	12.5	648
	13	Quercetin-3-O-hexoside	12.93	1063
	14	*(Dicaffeoylquinic acid)*	13.27	28
	15	3,4-Dicaffeoylquinic acid	13.78	1956
	16	3,5-Dicaffeoylquinic acid	14.28	14858
	17	Kaempferol-3-O-rhamnoglucoside	14.69	220
	18	*(Quercetin)*	15.33	174
	19	4,5-Dicaffeoylquinic acid	16.08	5533
	20	Feruloylquinic acid	17.87	263
	21	Tricaffeoylquinic acid	22.05	132
Ilex aquifolium 'Argentea Mariginata'	2	Neochlorogenic acid	3.08	2601
	4	*(Dicaffeic acid)*	4.55	211
	5	*(Dicaffeic acid)*	4.95	4
	6	Chlorogenic acid	5.31	12041
	7	Cryptochlorogenic acid	5.61	1318
	8	*(Dicaffeic acid)*	6.18	208
	11	Feruloylquinic acid	10.26	54
	12	Rutin	12.48	8803
	13	Quercetin-3-O-hexoside	12.92	814
	14	*(Dicaffeoylquinic acid)*	13.29	24
	15	3,4-Dicaffeoylquinic acid	13.77	726
	16	3,5-Dicaffeoylquinic acid	14.28	5697
	17	Kaempferol-3-O-rhamnoglucoside	14.66	247
	19	4,5-Dicaffeoylquinic acid	16.07	3896
	20	Feruloylquinic acid	17.85	70

Continued **Table 4:** HPLC DAD characteristics of phenolic compounds in *Ilex sp.*

Plant material	No.	Peak name	Ret.time [min]	Content [μg g^{-1} d.m.]
Ilex aquifolium	2	Neochlorogenic acid	3.07	1381
	4	*(Dicaffeic acid)*	4.52	238
	6	Chlorogenic acid	5.28	9577
	7	Cryptochlorogenic acid	5.58	644
	12	Rutin	12.43	4159
	13	Quercetin-3-O-hexoside	12.86	446
	15	3,4-Dicaffeoylquinic acid	13.72	490
	16	3,5-Dicaffeoylquinic acid	14.23	6414
	17	Kaempferol-3-O-rhamnoglucoside	14.59	205
	19	4,5-Dicaffeoylquinic acid	16.03	2331
Ilex meservea 'Blue Angel'	1	Quinic acid	2.64	31
	2	Neochlorogenic acid	3.06	619
	4	*(Dicaffeic acid)*	4.52	51
	6	Chlorogenic acid	5.28	3693
	7	Cryptochlorogenic acid	5.58	306
	12	Rutin	12.39	2449
	13	Quercetin-3-O-hexoside	12.83	307
	15	3,4-Dicaffeoylquinic acid	13.69	152
	16	3,5-Dicaffeoylquinic acid	14.2	445
	17	Kaempferol-3-O-rhamnoglucoside	14.58	65
	18	*(Quercetin)*	15.26	62
	19	4,5-Dicaffeoylquinic acid	16.01	397

(Preliminary identification indicates the presence of isomers of these compounds)



1 – Ilex paraguariensis
3 – Ilex aquifolium 'Argentea Mariginata'
5 – Ilex aquifolium L.
6 – Ilex meserveae 'Blue Angel'

Figure 2: TLC of *Ilex* sp.

3.3 HPLC and MS

The phenolic compounds present in the four samples of *Ilex* sp. were analyzed in LC-MS using a soft ionization technique which provided M-1 ions for the oxygenated molecules. To distinguish the phenolic acids from flavonols and to receive quantitative results, chromatograms were recorded using DAD at 320 nm and 360 nm, respectively. LC patterns of these extracts are shown in Fig. 3. The extracts of the four *Ilex* varieties showed similar qualitative phenolic compounds profile.

Table 4 gives the MS characteristics of eluted compounds, along with their proposed structure, comparing M-1 values (in negative MS mode) with recorded retention times for the standard samples. Up to 20 different phenolic compounds were identified in the extracts. Chlorogenic acid was the main component in all four plant varieties of, for which other characteristic *m/z* values (i.e. 191, 179 and 707) were also observed. The LC retention times, high UV absorption at 320 nm, and MS spectra of

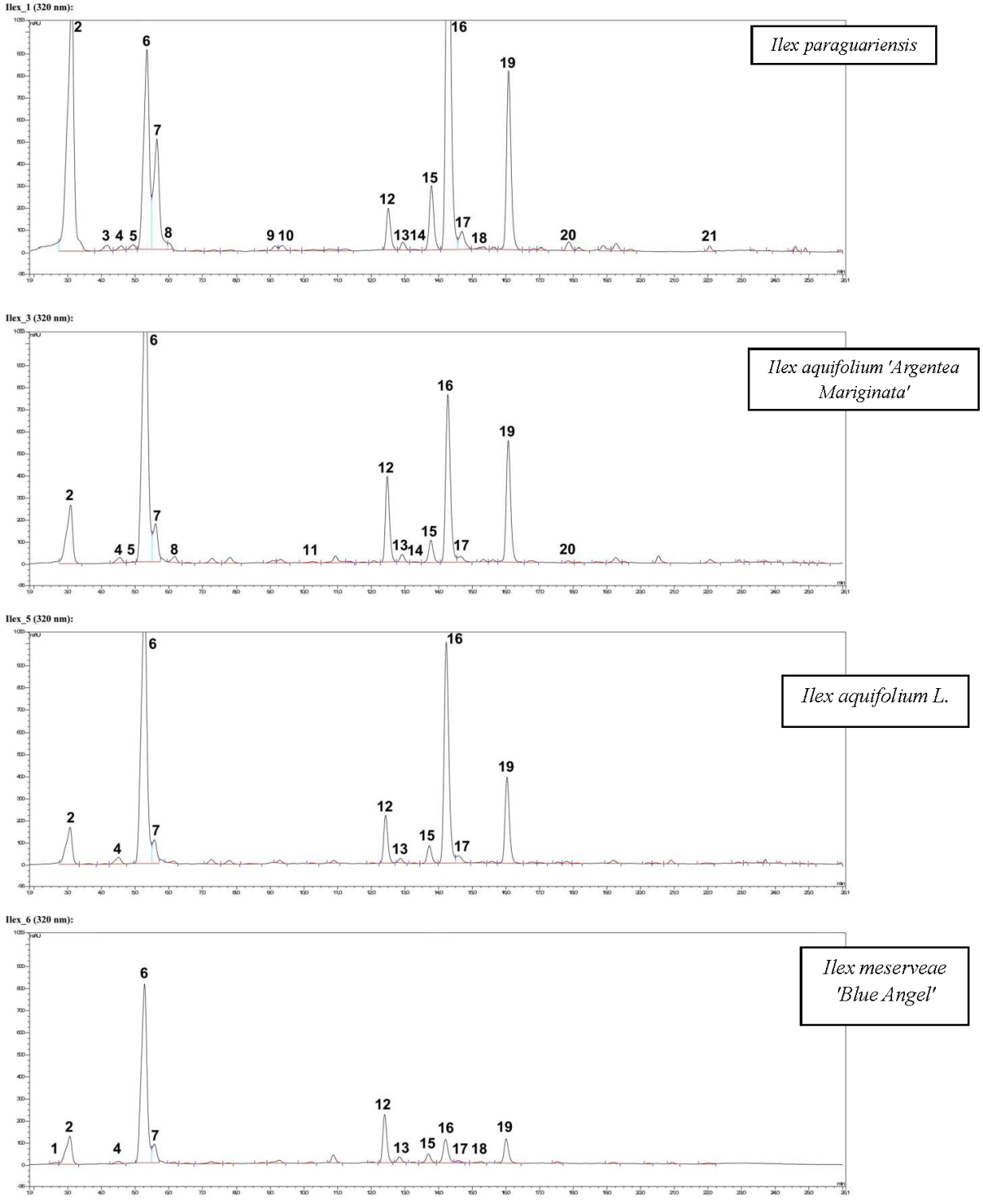


Figure 3: HPLC DAD chromatograms of *Ilex* sp. signal at 320 nm.

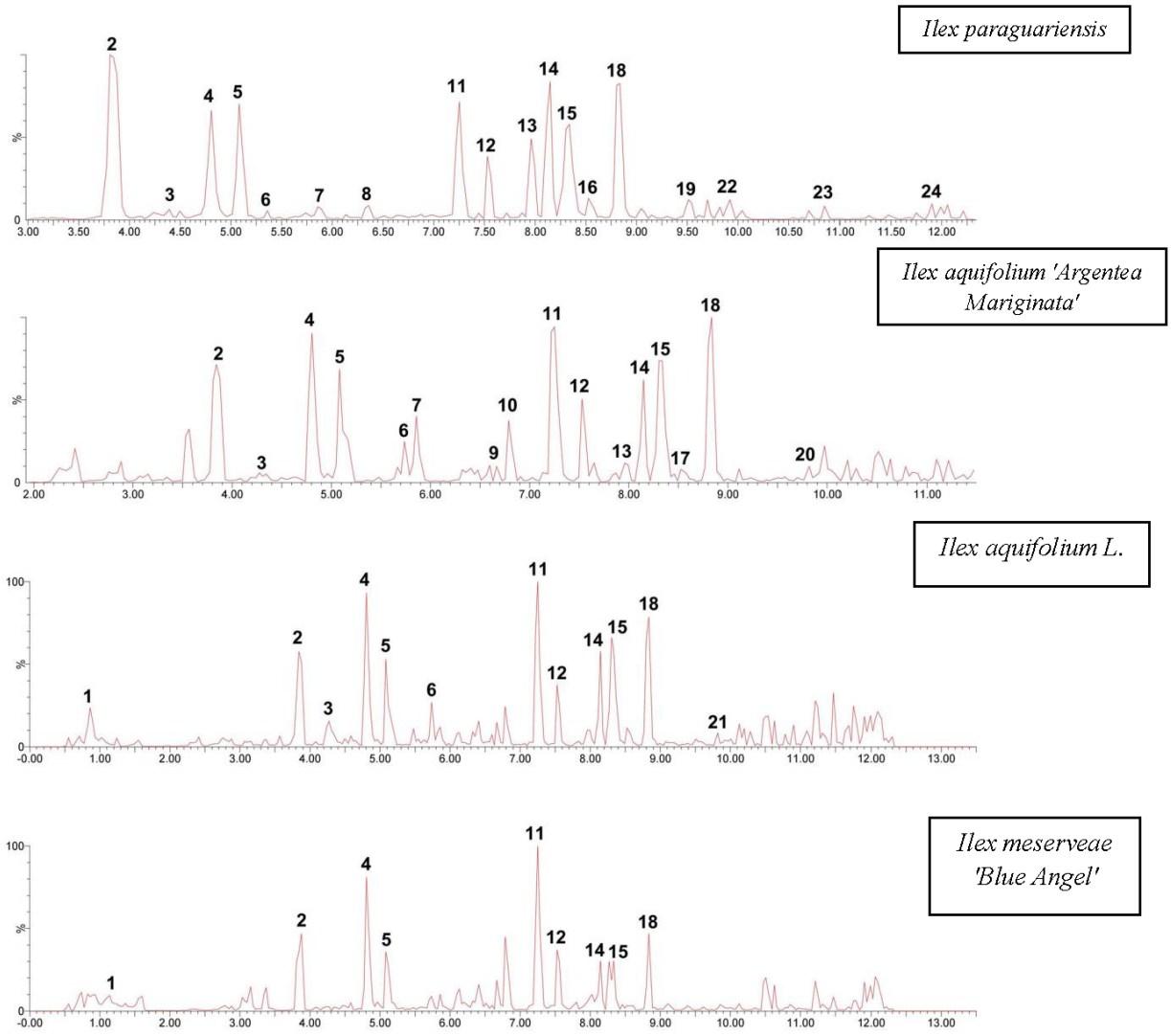


Figure 4: LC/MS chromatograms of *Ilex sp*. signal at 320nm.

the compound matched the standard chlorogenic acid. Ions with *m/z* 191 and 179 corresponded to deprotonated quinic acid and caffeic acid fragments, respectively, while a highly intensive ion with *m/z* 707 could be ascribed to a dimeric adduct of the caffeoylquinic acid molecule. Three other compounds corresponded to isomers of chlorogenic acid. Considering the elution profile of chlorogenic acid isomers from plant foods reported in the literature on C18 HPLC columns [16], compounds at 4.8, 4.81, and 5.08 min were identified as 5-*O*-caffeoylquinic acid (neochlorogenic acid), 3-*O*-caffeoylquinic acid (chlorogenic acid), and 4-*O*-caffeoylquinic acid (cryptochlorogenic acid), respectively. Compounds at 8.15 min all shared the same high UV absorption at 320 nm characteristic as that of chlorogenic acid, although the MS spectra showed a $[M-H]^-$ ion at *m/z* 515 and a fragment with *m/z* 353. The molecular ion at *m/z* 515 is indicative of dicaffeoylquinic acid isomers, which has been reported as a major constituent of the phenolic fraction of mate [17]. In *I. aquifolium* a peak present at 9.82 min characterized with *m/z* 515 strongly suggests a tricaffeoylquinic acid isomer. The presence of tricaffeoylquinic acid derivatives were previously reported in the leaves of sweet potato *Ipomoea batatas* L. [18,19]. Compounds with an ion with *m/z* 367 and fragment ions with *m/z* 193 and 191 are a feruloylquinic acid isomers. Feruloylquinic acid isomers are also known as natural products and have been previously described as constituents of burr parsley *Caucalis platycarpos* L. [20]. The MS spectrum of the compound at 7.25 min showed the pseudo-molecular ion with *m/z* 609, suggesting the deprotonated molecular ion of rutin (quercetin-3-*O*-rutinoside). The structure of this

Table 5: LC/MS characteristics of phenolic compounds in *Ilex sp*.

Plant material	No.	R_t	M-1	Compound
Ilex paraguariensis	2	3.81	353.1	Neochlorogenic acid
	3	4.39	341.1	*(Dicaffeic acid)*
	4	4.81	353.1*	Chlorogenic acid
	5	5.08	353.1	Cryptochlorogenic acid
	6	5.36	353.1	*(Dicaffeic acid)*
	7	5.86	517.2	*(Dicaffeic acid)*
	8	6.36	367.1	Feruloylquinic acid
	11	7.25	609.1	Rutin
	12	7.525	463.1	Quercetin-3-*O*-hexoside
	13	7.96	593.2	Kaempferol-3-*O*-rhamnoglucoside
	14	8.15	515.1	3.4-Dicaffeoylquinic acid
	15	8.34	515.1	3.5-Dicaffeoylquinic acid
	16	8.53	353.1	*(Dicaffeoylquinic acid)*
	18	8.84	515.1	4.5-Dicaffeoylquinic acid
	19	9.51	529.1	Caffeoylferuloylquinic acid
	22	9.92	529.1	Caffeoylferuloylquinic acid
	23	10.85	677.2	Tricaffeoylquinic acid
	24	11.9	1119.6	*unknown*
Ilex aquifoliumn ‚Argentea	2	3.84	353.1	Neochlorogenic acid
Mariginata'	3	4.27	353.1*	*(Dicaffeic acid)*
10	4	4.81	353.1*	Chlorogenic acid
11	5	5.08	353.1	Cryptochlorogenic acid
12	6	5.74	353.1	*(Dicaffeic acid)*
13	7	5.86	517.2	*(Dicaffeic acid)*
14	9	6.6	367.1	Feruloylquinic acid
15	10	6.79	595.1	*unknown*
	11	7.25	609.1	Rutin
	12	7.53	463.1	Quercetin-3-*O*-hexoside
	13	7.96	593.2	Kaempferol-3-*O*-rhamnoglucoside
	14	8.15	515.1	3.4-Dicaffeoylquinic acid
	15	8.34	515.1	3.5-Dicaffeoylquinic acid
	17	8.53	515.1	*(Dicaffeoylquinic acid)*
	18	8.84	515.1	4.5-Dicaffeoylquinic acid
	20	9.82	353.1	*(Dicaffeoylquinic acid)*
Ilex aquifolium L.	1	0.86	191.1	Quinic acid
	2	3.84	353.1	Neochlorogenic acid
	3	4.27	353.1*	*(Dicaffeic acid)*
	4	4.81	353.1*	Chlorogenic acid
	5	5.08	353.1	Cryptochlorogenic acid
	6	5.74	353.1	*(Dicaffeic acid)*
	11	7.25	609.1	Rutin
	12	7.53	463.1	Quercetin-3-*O*- hexoside
	14	8.149	515.1	3.4-Dicaffeoylquinic acid
	15	8.3	515.1	3.5-Dicaffeoylquinic acid
	18	8.84	515.1	4.5-Dicaffeoylquinic acid
	21	9.82	515.1	*(Dicaffeoylquinic acid)*
Ilex meserveae ‚Blue Angel'	1	1.14	191	Quinic acid
	2	3.88	353.1	Neochlorogenic acid
	4	4.81	707.2	Chlorogenic acid
	5	5.08	353.1	Cryptochlorogenic acid
	11	7.25	609.1	Rutin
	12	7.53	463.1	Quercetin-3-*O*- hexoside
	14	8.15	515.1	3.4-Dicaffeoylquinic acid
	15	8.25	515.1	3.5-Dicaffeoylquinic acid
	18	8.84	515.1	4.5-Dicaffeoylquinic acid

*Dimeric adduct

(Preliminary identification indicates the presence of isomers of these compounds)

compound was confirmed by comparing it to the authentic standard, with an identical retention time and the MS and UV spectra under the same chromatographic conditions. Some authors have reported that flavonoids such as rutin and quercetin show antioxidant activity [21,22].

HPLC chromatograms of the polyphenolic compounds from *Ilex* leaves are shown in Fig. 3. Quantitative results are based on chromatographic runs in which standard samples containing 0.1 μg mL^{-1} of chlorogenic acid and 0.1 μg mL^{-1} rutin were recorded. The chromatographic HPLC DAD data used for quantification of the compounds are presented in Table 5.

Thus for the extracts, the following components were quantified: neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, rutin, quercetin-3-*O*-hexoside, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid. The mean share of polyphenolic compounds in *Ilex sp.* are shown in Fig. 2. According to the results obtained from chromatographic analyses, the total content of phenolic compounds was calculated which varied in the range from 51066 μg g^{-1} d.m. for *I. paraguariensis*, 38789 μg g^{-1} d.m. for *I. aquifolium* '*Argentea Mariginata*', 27169 μg g^{-1} d.m. for *I. aquifolium* L. to 8984 μg g^{-1} d.m. for *I. meserveae* '*Blue Angel*'. In general, it was observed that *I. paraguariensis* has more phenolic acids than other *Ilex* species used in the present study. Chlorogenic acid was the main component in all four plant varieties.. Our study showed that the amount of chlorogenic acid was the highest in the *I. aquifolium* L. extract with a value of 12041 μg g^{-1} d.m. However, the mean share of chlorogenic acid was highest in *I. meserveae* '*Blue Angel*' and found to be 46%. Rutin was the second most abundant phenolic compound in the extracts that were studied. The highest value was found in *I. aquifolium* '*Argentea Mariginata*' as 5593 μg g^{-1} d.m. However the mean share of rutin was highest in *I. meserveae* '*Blue Angel*' and found to be 46%.

4 Discussion

The leaves of *Ilex* sp. are a rich source of polyphenols possessing health-protective properties. Polyphenols through their antioxidant and free radical scavenger properties are becoming more and more popular among scientists, nutritionists and consumers. The extraction process used in this work made it possible to obtain extracts with a satisfactory content of phenolic compounds even though we did not use any extraneous antioxidantsto protect the samples from deterioration. Polyphenolic compounds found in mate differ significantly from green tea because mate leaves contain high concentrations of chlorogenic acid and no catechins [23]. Literature shows that *I. paraguariensis* is especially rich in chlorogenic acids [12,24], and in our work we proved that the selected plant belonging to the genus *Ilex*, possess a similar antioxidant pattern with high amounts of chlorogenic acid as well as its isomers and similar in chemical character diesters. It is worth noting that similar results were obtained by other researchers who studied yerba mate [17].

Another abundant phenolic compound found in extracts was rutin which is a glycosilated quercetin. Its presence in the selected plants is valuable because of its biological activity as it antagonizes the increase of capillary fragility associated with hemorrhagic disease, reduces high blood pressure [25], decreases the permeability of the vessels, has an antiedema effect, and reduces the risk of arteriosclerosis and shows antioxidant activity [26].

5 Conclusion

Polyphenols through their antioxidant and free radical scavenger properties are becoming more and more popular among scientists, nutritionists and consumers. Our research shows that leaves of *Ilex* sp. are a rich source of polyphenols.

It has been shown that among the tested cultivars of *Ilex* sp. the high content of chlorogenic acid was characterized by a variety of *Ilex meserveae 'Blue Angel' Ilex aquifolium* L., and *Ilex aquifolium 'Argentea Mariginata'*. The content was higher than in *Ilex paraguariensis*. In addition, these species were found to contain very high concentrations of rutin.

While there is still a need for more research on the isolation and identification of bioactive compounds, evidence seems to support the *Ilex* species as a plant with a variety of compounds that can be used in human health and animal production.

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