Qualitative and quantitative analysis of polyphenolic compounds in Ilex Sp.

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 “Tererê,” 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 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\textsubscript{18} column \( (100 \times 2.1 \text{ mm id, } 1.7 \mu m) \) 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\textsuperscript{TM} 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.

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

**Table 1:** Gradient program used in HPLC separations.

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

**Table 2:** Gradient program used in LC MS separations.

![Figure 1: The mean share of phenolic compounds in *Ilex* Sp.](image-url)
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 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                 |

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                    |
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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

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**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 meserveae '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  | 14.69          | 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 3: HPLC DAD chromatograms of Ilex sp. signal at 320 nm.
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<sub>t</sub> | 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)*       |
|                           | 17  | 8.53         | 515.1| 4.5-Dicaffeoylquinic acid       |
|                           | 18  | 8.84         | 515.1| Caffeoylferuloylquinic acid     |
|                           | 19  | 9.51         | 529.1| Caffeoylferuloylquinic acid     |
|                           | 20  | 9.82         | 515.1| 3.4-Dicaffeoylquinic acid       |
|                           | 21  | 9.82         | 515.1| *(Dicaffeoylquinic acid)*       |
| *Ilex aquifolium ’Argentea Mariginata’* | 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          |
|                           | 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         | 515.1| *(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)
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 antioxidant to 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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