

Phytochemical, physicochemical, microbiological study and anticholinesterase activity of *Ginkgo biloba L.* and *Bacopa monnieri L.* used in phytotherapy

Estudo fitoquímico, físico-químico, microbiológico e atividade anticolinesterásica de *Ginkgo biloba L.* e *Bacopa monnieri L.* usados em fitoterapia

Actividad fitoquímica, físico-química, microbiológica y anticolinesterasa de *Ginkgo biloba L.* y *Bacopa monnieri L.* utilizados en fitoterapia

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Abstract

Herbal medicines based on *Ginkgo biloba* L. and *Bacopa monnieri* L. are used to improve memory and cognitive function. The quality control and anticholinesterase activity of the herbal medicines prepared from *G. biloba* and *B. monnieri* were evaluated which are commercialized in handling pharmacies. Samples of herbal medicines based on *G. biloba* and *B. monnieri* were obtained from handling pharmacies in Teresina-PI, submitted to labeling analysis and anticholinesterase activity. The phytochemical study was performed by preliminary prospecting, TLC and HPLC. The tests physicochemical and microbiological analyses were made according to the Pharmacopoeia 2010. It was observed that the labeling, foreign material, disintegration, and microbiological parameters were in accordance with ANVISA standards. The colorimetric tests were uniform in the samples of *B. monnieri* and variable of *G. biloba*. The average weight evaluation shows that the G1 and B3 samples have capsular content above that described in the labeling and presented pH values different. Among the thermogravimetric profiles, the mass losses of samples G1 and B3 showed values with high ash content, suggesting adulterations. Phytochemical prospecting showed flavonoids as common secondary metabolites in both species, corroborating with TLC and HPLC analysis, which identified the compounds chlorogenic acid, rutin, myricetin and quercetin. The samples G4, B2 and B5 present metabolites capable of inhibiting the enzyme acetylcholinesterase with IC₅₀ of 0.8540 mg/mL, 0.9650 mg/mL and 1.8221×10⁻⁵ mg/mL, respectively. The samples G1 and B3 of *G. biloba* and *B. monnieri*, did not obey some parameters of quality control for herbal medicines according to the criteria of the Brazilian Pharmacopoeia.

Keywords: Quality control; Phytochemical analysis; Anticholinesterase activity; *Ginkgo biloba* L.; *Bacopa monnieri* L.

Resumo

Os medicamentos fitoterápicos à base de *Ginkgo biloba* L. e *Bacopa monnieri* L. são usados para melhorar a memória e a função cognitiva. Foram avaliados o controle de qualidade e a atividade anticolinesterásica dos fitoterápicos preparados a partir de *G. biloba* e *B. monnieri* comercializados em farmácias de manipulação. Amostras de medicamentos fitoterápicos à base de *G. biloba* e *B. monnieri* foram obtidas nas farmácias distribuidoras de Teresina-PI, submetidas à análise de rotulagem e atividade anticolinesterásica. O estudo fitoquímico foi realizado por prospecção preliminar, CCD e CLAE. Os testes físico-químicos e análises microbiológicas foram realizados de acordo com a Farmacopéia 2012. Observou-se que a rotulagem, material estranho, desintegração e parâmetros microbiológicos estavam de acordo com as normas da ANVISA. Os testes colorimétricos foram uniformes nas amostras de *B. monnieri* e variáveis de *G. biloba*. A avaliação do peso médio mostrou que as amostras G1 e B3 possuem conteúdo capsular acima do descrito na rotulagem e apresentaram valores de pH diferentes. Dentre os perfis termogravimétricos, as perdas de massa das amostras G1 e B3 apresentaram valores com alto teor de cinzas, sugerindo adulterações. A prospecção fitoquímica apresentou flavonóides como metabólitos secundários comuns em ambas as espécies, corroborando com análises de CCD e CLAE, que identificaram os compostos ácido clorogênico, rutina, miricetina e quercetina. As amostras G4, B2 e B5 apresentam metabólitos capazes de inibir a enzima acetilcolinesterase com IC₅₀ de 0,8540 mg / mL, 0,9650 mg / mL e 1,8221 × 10⁻⁵ mg / mL, respectivamente. As amostras G1 e B3 de *G. biloba* e *B. monnieri*, não obedeceram a alguns parâmetros de controle de qualidade para medicamentos fitoterápicos segundo os critérios da Farmacopéia Brasileira.

Palavras-chave: Controle de qualidade; Análise fitoquímica; Atividade anticolinesterásica; *Ginkgo biloba* L.; *Bacopa monnieri* L.

Resumen

Los medicamentos a base de hierbas a base de *Ginkgo biloba* L. y *Bacopa monnieri* L. se utilizan para mejorar la memoria y la función cognitiva. Se evaluó el control de calidad y la actividad anticolinesterasa de los medicamentos herbarios preparados a partir de *G. biloba* y *B. monnieri* vendidos en farmacias de manipulación. Se obtuvieron muestras de medicamentos herbales a base de *G. biloba* y *B. monnieri* de las farmacias distribuidoras de Teresina-PI,

sometidas a análisis de etiquetado y actividad anticolinesterasa. El estudio fitoquímico se realizó mediante prospección preliminar, CCD y CLAE. Se realizaron pruebas físico-químicas y análisis microbiológicos de acuerdo con Farmacopeía 2012. Se observó que el etiquetado, material extraño, desintegración y parámetros microbiológicos estaban de acuerdo con las normas ANVISA. Las pruebas colorimétricas fueron uniformes en las muestras de *B. monnieri* y variables de *G. biloba*. La evaluación del peso medio mostró que las muestras G1 y B3 tienen un contenido capsular superior al descrito en la etiqueta y presentan diferentes valores de pH. Entre los perfiles termogravimétricos, las pérdidas de masa de las muestras G1 y B3 presentaron valores con alto contenido en cenizas, sugiriendo adulteraciones. La prospección fitoquímica mostró flavonoides como metabolitos secundarios comunes en ambas especies, corroborando con análisis de CCD y HPLC, que identificaron los compuestos ácido clorogénico, rutina, miricetina y quercetina. Las muestras G4, B2 y B5 presentan metabolitos capaces de inhibir la enzima acetilcolinesterasa con IC50 de 0.8540 mg / mL, 0.9650 mg / mL y 1.8221×10^{-5} mg / mL, respectivamente. Las muestras G1 y B3 de *G. biloba* y *B. monnieri*, no obedecieron algunos parámetros de control de calidad para medicamentos herbarios según los criterios de la Farmacopea Brasileña.

Palabras clave: Control de calidad; Análisis fitoquímico; Actividad anticolinesterasa; *Ginkgo biloba* L.; *Bacopa monnieri* L.

1. Introduction

Phytotherapy is a powerful natural resource for the prevention and treatment of diseases through medicinal plants, used as complementary therapy (Chakraborty, 2018). The Agência Nacional de Vigilância Sanitária (ANVISA), defines herbal medicine as the medicine produced exclusively with derivatives of plant drugs, without the addition of isolated substances in its composition (Brasil, 2010).

Ginkgo biloba L. (Ginkgoaceae), is among the most consumed medicinal plants in the world, has neuroprotective, vascular and cardiological applications, acting against depression, Alzheimer's disease (AD) and ischemic stroke (Yang et al., 2016; Tian et al., 2017; Dai et al., 2018). It has been used as a herbal product in the treatment of patients with AD, as it is considered a potent inhibitor of acetylcholinesterase (AChE) (Kim et al., 2016). Some studies indicate that secondary metabolites isolated from the leaves of this plant, present several biological activities (Singh et al., 2008). In the group of flavonoids stand out, canferol, quercetin, isorhamnetin, myricetin, 3'-methyl-myricetin, catechins and proanthocyanidins (Ding et al., 2008). It also contains terpenic lactones, particularly ginkgolides A, B, C, J and M and bilobalide (Ding et al., 2008; Song et al., 2010). The standardized extract of *G. biloba* L. EGb761, according to RDC 89/2004 (Brasil, 2004), contains at least 24.0% flavonoids and 6% terpenoids.

Bacopa monnieri L. (Scrophulariaceae), also known as Brahmi, is a medicinal plant, with branched leaves and purple flowers, known as "memory booster" (Russo & Borrelli, 2005; Rao et al., 2012) This plant has activity against Parkinson's disease, Alzheimer's, epilepsy, behavioral deficit, stress and has an antioxidant effect (Mathew et al., 2010; Jadiya et al., 2011; Saini et al., 2012). The compounds responsible for pharmacological effects include alkaloids, flavonoids, saponins and sterols (Azad et al., 2012; Le et al., 2013). Bacoid saponins A and B are the components responsible for *B. monnieri* ability to increase the transmission of nerve impulses. It is also responsible for the competence of neuronal repair, increased kinase activity, neurogenesis and restoration of synaptic activity (Azad et al., 2012; Dutta & Chakraborty, 2020). The use of herbal medicines in the treatment of specific cognitive problems search for the identification of AChE enzyme inhibitors.

In this study, the phytochemical, physical-chemical, microbiological profile and anticholinesterase activity of herbal medicines based on *G. biloba* and *B. monnieri* commercialized in handling pharmacies in Brazil in the city of Teresina - PI were evaluated.

2. Methodology

2.1 Sample Selection

Samples of the herbal medicines *G. biloba* (120 mg) and *B. monnieri* (225 mg) were purchased from four master pharmacies located in the city of Teresina-PI. The manipulation pharmacies chosen were those that supplied the two herbal

medicines. Each sample obtained contained 60 capsules, totaling four samples of *G. biloba* (G1, G2, G3, G4) and four samples of *B. monnieri* (B1, B2, B3, B4). The G5 and B5 samples were dry leaves and aerial parts of the respective medicinal plants and purchased at a natural products store in the city of Teresina - PI (Brazil), which served as a standard for comparing results with herbal medicines, as shown in Table 1.

Table 1: Samples codes, dosage form and plants parts and shapes, respectively.

Code	Pharmaceutical form	Plants parts and shapes
G1	Capsule	Dry extract
G2	Capsule	Dry extract
G3	Capsule	Dry extract
G4	Capsule	Dry extract
G5	Vegetable parts	Leaves
B1	Capsule	Dry extract
B2	Capsule	Dry extract
B3	Capsule	Dry extract
B4	Capsule	Dry extract
B5	Vegetable parts	Aerial parts

Source: Authors.

2.2 Labeling Analysis

The analysis of the sample labels was performed by comparing the information contained in the sample labels with those recommended by RDC No. 67/2007 (Brasil, 2007).

2.3 Physicochemical Analysis

All samples were subjected to the analysis of foreign material, determination of pH, disintegration time, and average weight, according to the general methods of the Brazilian Pharmacopoeia (2012).

2.4 Thermal Analysis by Thermogravimetry

The dynamic thermogravimetric (TG) curves of *G. biloba* and *B. monnieri* samples were obtained in a thermogravimetric apparatus TGA-50 model from Shimadzu, with a heating rate of 10 °C/min, in the range temperature of 25 °C to 700 °C. The analyzes were carried out under a air atmosphere, with a flow of 50 mL.min⁻¹. 5 mg samples (± 0.05) were placed in a platinum crucible. The TG curves were analyzed using Shimadzu's TA 50 software, to characterize the stages of mass loss. The moisture content was identified from the first stage of mass loss between 25 and 200 °C. The ash contents were obtained directly from the percentage of the thermal decomposition product at 700 °C.

2.5 Colorimetric Analysis

The colorimetric analysis was made with a portable digital colorimeter with an 8 mm caliber (model FRU®, WR-10QC) in the CIE 10° standard observer, luminance measurement range (L) from 0 to 100 and using the difference formula of color: $\Delta E a^* b^*$ in the CIELAB color space defined by the International Lighting Commission (ILC) in 1976 (McLaren, 2008).

2.6 Phytochemical Prospecting

For the phytochemical characterization of the samples, classical tests were carried out to identify the main groups of active ingredients (Matos, 2009): flavonoids; saponins; tannins; steroids/ triterpenoids and alkaloids. For the analysis by thin layer chromatography (TLC), the ethanolic extracts were obtained using 500 mg of the samples with 10 mL of ethanol and applied on silica gel chromatographic plates. Together with the standards of epicatechin, rutin, quercetin, lupeol and sitosterol, the samples was eluted in the following solvent systems: hexane/ethyl acetate (80:20), chloroform/methanol (90:10) and chloroform/methanol/water (65:30:5). The spots were visualized by spraying with ceric sulfate and placed on a hot plate (Chaves, 1997).

2.7 Phytochemical Profile by HPLC Analysis

The phytochemical profile of *G. biloba* and *B. monnieri* samples were investigated by High-Performance Liquid Chromatography (HPLC). These HPLC analyses were performed in an analytical chromatograph Shimadzu® model LC-20AT, CBM-20A controller, SPD-20 with an Ultraviolet-Visible (UV-Vis) detector, CTO-20A oven, SIL-20A HT auto-injector, DGU-20A 5R degasser, reverse phase column (C18 Shim-pack VP-ODS, 4.6mm × 150 mm, 4.6 µm).

Initially, ethanolic extracts were prepared from the samples of *G. biloba* and *B. monnieri* using 500 mg of the samples diluted in 10 mL of ethanol. Ethanol extracts obtained from herbal medicines and standard solutions of gallic acid, catechol, catechin, chlorogenic acid, caffeic acid, (-) epicatechin, syringaldehyde, cumaric acid, coumarin, rutin, myricetin, and quercetin were filtered on a Macherey-Nagel chromabond® C18-ec solid-phase extraction cartridge and 0.45 µm filter disc. The samples were filtered on 25 mm Chromafil® Xtra PTFE-20/25 filter membrane with 0.20 µm pore. Elution process was performed with a 0.1% acetic acid solution in water (Solvent A) and methanol (Solvent B).

The extract and standards were eluted according to solvent gradient B: 0 to 5 min (7-11%); 5 to 10 min (11-16%); 10 to 15 min (16-25%); 15 to 30 min (25%); 30 to 34 min (25-38%); 34 to 38 min (38-50%); 42 to 46 min (60-65%); 46 to 50 min (65-70%); 54 to 58 min (75-85%); 58 to 62 min (85-25%); 62 to 63 min (25-7%); 63 to 80 min (7%), 0.6 mL/min flow, 20 µL injection volume for standards and extract. The oven temperature was set at 35 °C. The chromatographic measurements were monitored at $\lambda \sim 290$ nm.

The compounds present in the ethanolic extracts were identified by comparing retention time (R_i) with those obtained by injecting standards prepared under the same conditions. For quantification, an analytical curve (10 points) was constructed from a solution containing a mixture of all standards (Mix). For this, solutions with concentrations ranging from 1 to 10 ppm in water: ethanol solution (70:30 v/v) were prepared. The quantification of compounds was executed by correlating the signal area and added standards volume, being the concentration expressed in g.Kg⁻¹.

2.8 Microbiological Analysis

Previously, Sabouraud Agar (fungi), Xylose Lysine Deoxycholate Agar (*Salmonella*), MacConkey Agar (*Escherichia coli*), and Salt Mannitol Agar (*Staphylococcus aureus*), the buffer solution and the dilution of the samples (1:10) were prepared. This was followed by the inoculation on the surface of the media in plates and their incubation for 24 hours at 35 °C (bacteria) and 7 days (fungi). After the indicated period, macroscopic observation and colony counting was performed according to the Brazilian Pharmacopeia (2012).

2.9 Enzymatic Assay of Acetylcholinesterase

The enzymatic assays for AChE inhibition were carried out according to the methodology proposed by Ellman et al. (1961), modified by Rhee et al. (2001). The procedures were performed in triplicate. The percentage of inhibition, a

concentration that inhibits 50% of the enzyme (IC_{50}), was calculated using the equation: % inhibition = $100 - (A_{\text{sample}}/A_{\text{white}}) \times 100$; Where, A is the change in absorbance at the beginning and end of the readings. Data were calculated using the GraphPad Prism Data Editor for Windows, version 6.0 (GraphPad Software Inc., San Diego, CA).

3. Results and Discussion

3.1 Labeling Analysis

The RDC nº 67/2007 (ANVISA), establishes several operational procedures for the labeling of developed products, such as every preparation must contain reliable and correct information, that would be provided to the patient (Silva and Silva, 2014). The results for the labeling investigation of *G. biloba* and *B. monnieri* phytotherapeutics from the referred study showed that all samples are in compliance with the current legislation, showing no inconsistencies with the information presented and required.

3.2 Physico-chemical analysis

The purity and quality of the samples of *G. biloba* and *B. monnieri* showed that the characteristics of the samples of *G. biloba* did not show similarities with the vegetal sample G5, whereas the samples of *B. monnieri* were more homogeneous in relation to the plant sample B5. The analysis of the foreign material revealed that only the G3 sample had 0.03% impurities, corresponding to structures similar to white crystals, as shown in Table 2.











However, this sample passed the quality test, since the material index strange present did not exceed that allowed by the Brazilian Pharmacopeia (2012), which is a maximum of 2% for plant medicines. By the values of the phytotherapeutic contents, of *G. biloba* and *B. monnieri* samples, described in Table 3, it is observed that the G2 sample had an average phytotherapeutic content close to 120 mg, while G1 presents twice the concentration expressed on the label.

Through values of herbal contents, the average weight, relative standard deviation, and limit of variance were calculated for *G. biloba* and *B. monnieri* samples. Table 3 shows that the G2 sample had an average phytotherapeutic content close to 120 mg, while G1 shows twice the amount of the concentration expressed on the label. Regarding Relative Standard Deviation (RSD), G2 and G3 samples have values above 4%, not meeting ANVISA RDC 67/2007 (Figure 1a). The results of *B. monnieri* samples have an average weight different from the concentration value (225 mg) indicated on the label, as will be shown later in Table 3, whereas the values of RSD were less than 4% (Figure 1b), according to the RDC 67/2007 of ANVISA. The acceptable limits for weight variation are established in the Brazilian Pharmacopeia, (2012), which may be 10% if the average weight is less than or equal to 300 mg and 7.5% if the average weight is greater than 300 mg. All results of the unit weights of *G. biloba* and *B. monnieri* samples are in accordance with current legislation.

The pH values of the samples of *G. biloba* and *B. monnieri* ranged from 5.07 to 12.85. The G1 sample showed a basic characteristic with a pH value of 9.08, suggesting adulteration of the sample different from the other samples of *G. biloba*, which showed an acidic behavior. Regarding the samples of *B. monnieri*, sample B3 had an extremely basic character with a pH value of 12.85, differing from the other samples that had a pH around 7.0, as shown in Table 3, indicating that this sample is possibly adulterated.

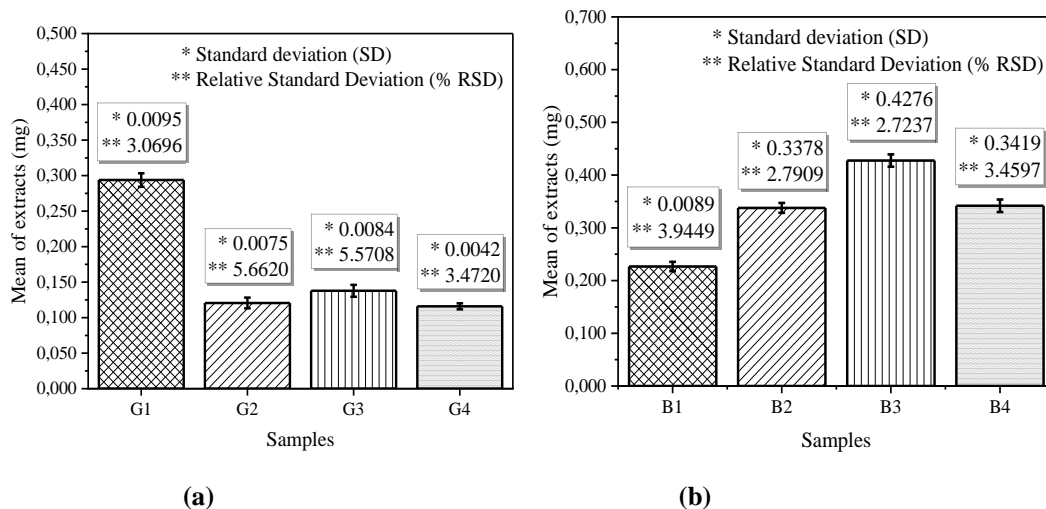
The disintegration time of the *G. biloba* and *B. monnieri* samples showed the maximum disintegration time of the *G. biloba* capsules of 8 min and 5 s, while the *B. monnieri* capsules, the B4 sample showed the longest disintegration time. (12 min and 2 s). Therefore, the samples are in accordance with current legislation (RDC 67/2007), as shown in Table 3.

Table 2: Foreign material identification, sample codes, and colorimetric coordinates values (L*, a*, and b*) for *G. biloba* and *B. monnieri* solid samples.

Codes	Color digital photo	L*	a*	b*
G1		50.33	2.09	18.52
G2		39.89	7.95	18.43
G3		42.43	9.23	22.03
G4		49.78	6.09	17.94
G5		32.58	5.50	18.89
B1		50.82	5.89	13.84
B2		56.06	5.14	16.16
B3		57.72	4.02	13.06
B4		57.06	4.93	16.21
B5		44.39	5.42	18.73

L = Luminosity, a* (+a* indicates the red color and -a* indicates the green color); b* (+b* indicates the yellow color and -b* indicates the blue color). Source: Authors.

Figure 1: Relative standard deviation of the mean weight of *G. biloba* (a) and *B. monnieri* (b) samples.



Source: Authors.

Table 3: Samples codes, average weight, pH values and disintegration time for commercial capsules of *G. biloba* and *B. monnieri* capsules.

Codes	Average weight (mg)	pH* and SD	Capsules disintegration time (s*)
G1	0.2938±0.085 ^a	9.08± 1.642 ^a	316
G2	0.1206±0.085 ^a	5.67± 1.642 ^a	245
G3	0.1378±0.085 ^a	5.62± 1.642 ^a	485
G4	0.1159±0.085 ^a	5.07± 1.642 ^a	321
G5	-	5.42± 1.642 ^a	-
B1	0.2267±0.008 ^b	7.40± 2.447 ^b	650
B2	0.3378±0.008 ^b	7.17± 2.447 ^b	535
B3	0.4275±0.008 ^b	12.85± 2.447 ^b	510
B4	0.3419±0.008 ^b	7.08± 2.447 ^b	722
B5	-	8.25± 2.447 ^b	-

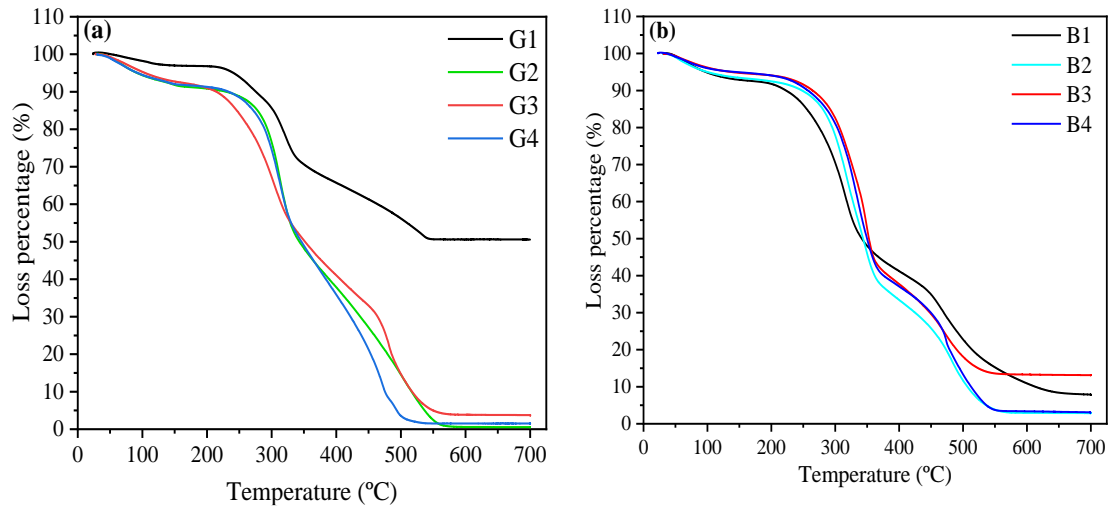
* Average of three repetitions ± standard deviation (SD) and s* = unit of time in seconds. ^{a,b}. Source: Authors.

3.3 Thermal analysis by thermogravimetry (TG) curves

The thermogravimetric characteristics of samples G2, G3 and G4 showed similar profiles, which suggests that the samples have the same composition (Figure 2a). However, the G1 sample differs from the others, suggesting the presence of other constituents in addition to the encapsulated composition. This result corroborates the discrepant data of mean weight and pH (Table 3), presented by the G1 sample. The analyzes of samples B1, B2 and B4 suggest uniformity between the profiles, evidenced by the decomposition steps, indicating classes of similar organic compounds for the samples of *B. monnieri* (Figure 2b). Only sample B3 had different capsular content and pH than other samples of *B. monnieri* (Table 3).

The study by Macêdo (2020), states that thermal analysis is an important tool used in the quality control of herbal products, allowing the simultaneous verification of moisture content, ash and access to the entire thermoanalytical profile of the samples. According to the values established by the Brazilian Pharmacopeia (2012), it was observed that the moisture content, obtained by thermogravimetry, for all samples of *G. biloba* and *B. monnieri* were below the maximum limit of 14.0%. On the other hand, regarding the ash contents of the samples of *G. biloba* and *B. monnieri*, only samples G2 and G4 were approved, with values below the maximum limit of 2.0%. Samples G1 and B3 had the highest ash content of 50.59% and 13.15%, respectively, suggesting adulteration or production error (Table 4).

Figure 2. TG curve for (a) *G. biloba* and (b) *B. monnieri* solid samples, respectively.



Source: Authors.

Table 4: Results of the thermogravimetric curve of *G. biloba* and *B. monnieri* samples at a heating rate of 10 °C/min under air atmosphere.

Codes	Mass decomposition steps (%)				Residual mass (%)
	1 st	2 nd	3 rd	4 th	
G1	3.36	25.04	21.01	-	50.59
G2	8.60	35.18	56.22	-	0
G3	8.07	60.13	28.02	-	3.78
G4	8.34	37.37	52.76	-	1.53
B1	8.03	38.43	18.61	27.08	7.85
B2	8.30	53.75	14.28	20.89	2.78
B3	6.43	50.28	30.14	-	13.15
B4	6.41	52.21	19.19	19.03	3.16

Source: Authors.

3.4 Colorimetric Coordinates Analysis

In the colorimetric data presented in this work, it was indicated that the variation of shades of the solid samples of *G. biloba* and *B. monnieri* are confirmed by the gradual reduction in the L* coordinate, presenting phytotherapics with a darker or more intense color (Tosun et al., 2008) It was observed that the L* values were not discrepant in the sampling evaluation of herbal medicines based on *B. monnieri*, showing uniform color and that the samples have the same formulation pattern, as shown in Table 2. The values obtained for the chromatic coordinate or blue-yellow matrix (b*) indicate that the samples of *G. biloba* and *B. monnieri* show uniformity and tendency to develop yellow color (Esteves et al., 2008; Pincelli et al., 2012).

3.5 Phytochemical study

In the phytochemical analysis of ethanolic extracts, the samples of *G. biloba* revealed the presence of flavonoids and the samples of *B. monnieri* showed positive results for flavonoids and triterpenes, as is evidenced by Figures 3 (c) and 4 (c,d). Ibrahim and Nuhu (2016), showed that the main classes of bioactive metabolites identified in *G. biloba* extracts were the flavonoids and terpenes that are responsible for the antioxidant activity of this plant. The secondary metabolites that confer the pharmacological activities of *B. monnieri* are saponins, flavonoids, phenols, alkaloids, terpenoids, tannins and steroids (Azad et al., 2012). The chromatographic profile allows, in many cases, confirmation of the identity of herbal medicine and even the detection of forgeries (Sherma & Rabel, 2020).

In Figure 3 (a,b), the TLC analyzes of *G. biloba* samples showed that the main constituents identified were the flavonoids. Samples G1, G2, G3 and G4 demonstrated yellow spots with Rf close to the standard quercetin flavonoid (Figure 3a). However, in Figure 3b, it can be observed that samples G2, G3 and G4 displayed yellow spots with Rf close to the standard rutin flavonoid. These results corroborate the findings of the preliminary phytochemical analysis (Figure 3c). Banov et al., (2006) and Guo et al., (2020), reported the identification of flavonoids in the dry extract of *G. biloba*, confirming the presence of the quercetin and rutin.

The B1, B2, B3 and B4 samples of *B. monnieri* revealed similar chromatographic characteristics, showing that they have the same chemical composition. In addition to having Rf close to the epicatechin flavanol, characterized by reddish-orange color, makes it possible to identify the existence of this chemical constituent (Figure 4b). In Figure 4a, samples B1, B2, B3 and B4 showed Rf close to the steroid sitosterol, showing the results of the preliminary phytochemical analysis of the samples of *B. monnieri* (Figure 4c, d). Flavonoids are considered pharmacological markers of the species *G. biloba*, the standardized extract GbE 761 must contain at least 24.0% ginkgo flavonoides (Singh et al., 2008, Silva, 2017).

The comparison of chromatographic profiles determined by HPLC of herbal medicines (G1, G2, G3 and G4) and the vegetable sample (G5) of *G. biloba*, demonstrated the difference in the phytochemical composition of these samples. The chromatograms of samples G1, G2, G3 and G4 of *G. biloba* present chromatographic profiles of phytopharmaceuticals, with retention time for the flavonoids rutin and quercetin. The compound rutin (**1**) was found in greater concentration in samples G2 (544.30 g/kg), G3 (1023.14 g/kg) and G4 (1003.72 g/kg) and the quercetin (**2**) compound in sample G1 (608, 00 g/kg) (Figure 5), corroborating the TLC results of this study (Figure 3a, b).

The comparison of the chromatographic profiles determined by HPLC of the herbal medicines (B1, B2, B3 and B4) and the plant sample (B5) of *B. monnieri* showed similar phytochemical composition, which is evidenced by the presence of several peaks with times of distinct retentions. In the samples of *B. monnieri*, traces of phenolic compounds were identified and quantified, such as chlorogenic acid, rutin, myricetin and quercetin (Figure 6). Sample B1 stood out with the presence of the compounds rutin (**1**) (3.71 g/kg), quercetin (**2**) (3.50 g/kg), chlorogenic acid (**3**) (2.36 g/kg) and myricetin (**4**) (4.57 g/kg). The remaining samples had only two or one of these compounds.

The flavonoids rutin and quercetin, identified in the samples of *G. biloba* and *B. monnieri*, as well as steroidal saponins may also be the secondary metabolites responsible for the efficacy of these herbal medicines in the treatment of memory loss. Rutin has pharmacological and therapeutic importance because it inhibits the process of free radical formation, in addition to preventing damage to the nervous system by modulating the activity of enzymes such as the enzyme acetylcholinesterase (Youdim et al., 2003; Soobrattee et al., 2005; Park, 2010). Quercetin has broad therapeutic benefits that involve its antioxidant and anti-inflammatory effects (Oliveira & Ropke, 2016) and its neuroprotective activity (Lu et al., 2006). Quercetin is capable of preventing memory and learning loss and reversing cognitive deficits induced by AD (Wang et al., 2018; Gayoso et al., 2017).

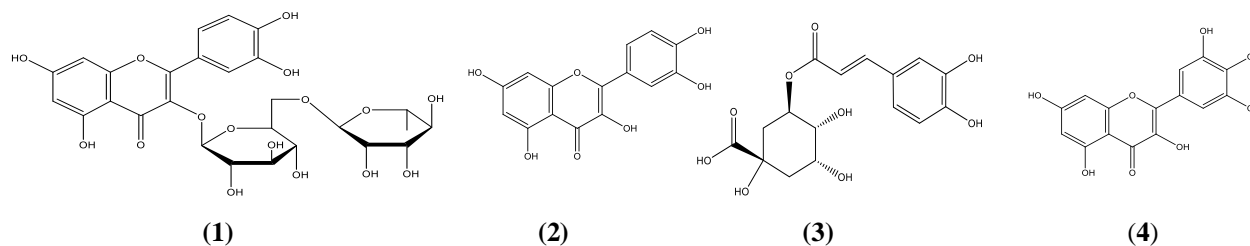
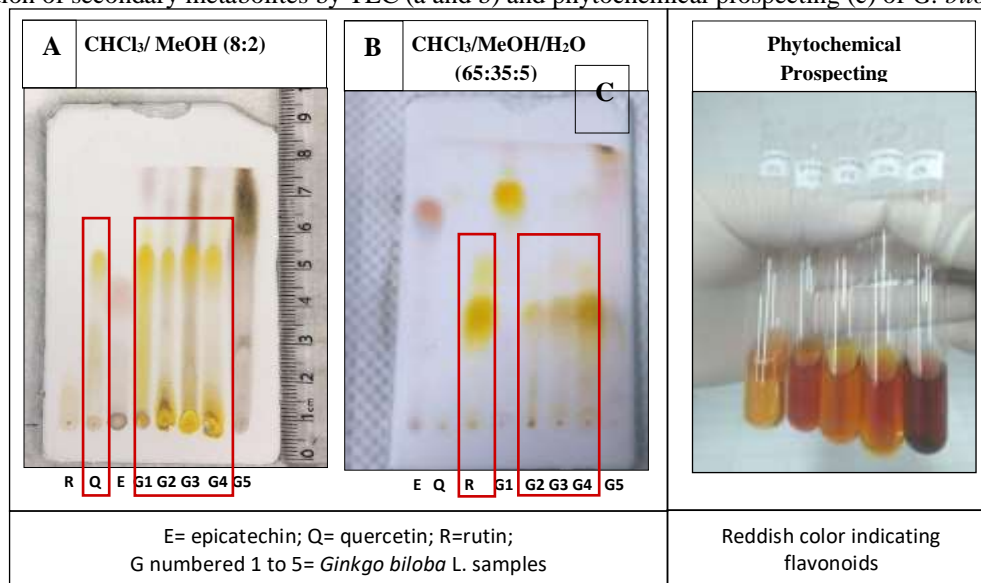
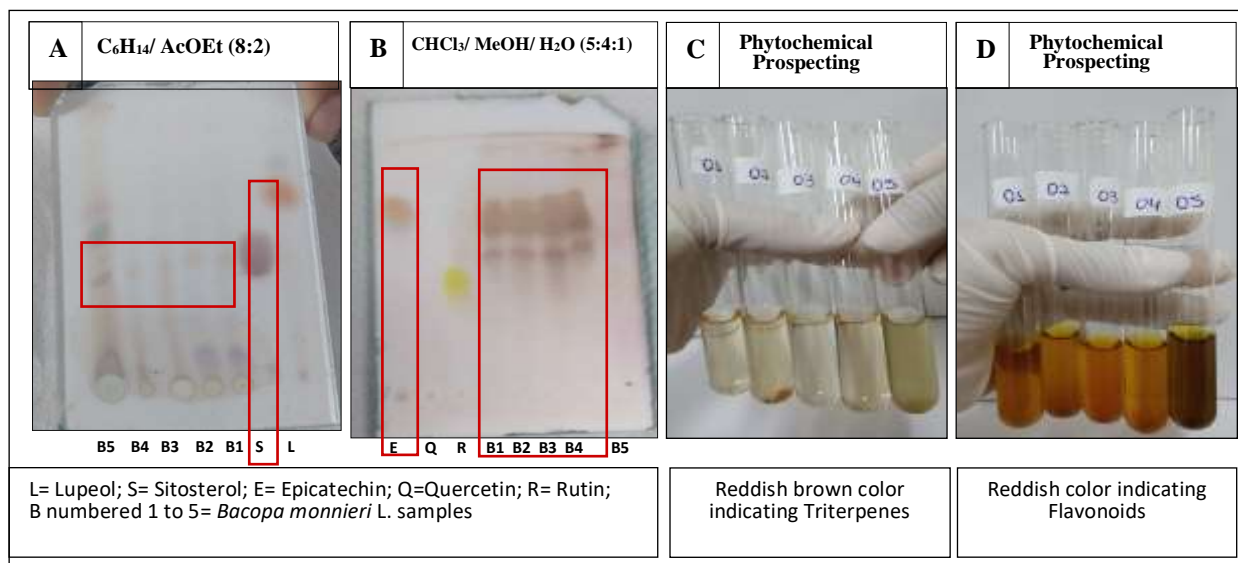


Figure 3. Identification of secondary metabolites by TLC (a and b) and phytochemical prospecting (c) of *G. biloba* samples.



Source: Authors.

Figure 4. Identification of secondary metabolites by TLC (a and b) and phytochemical prospecting (c and d) of *B. monnieri* samples.



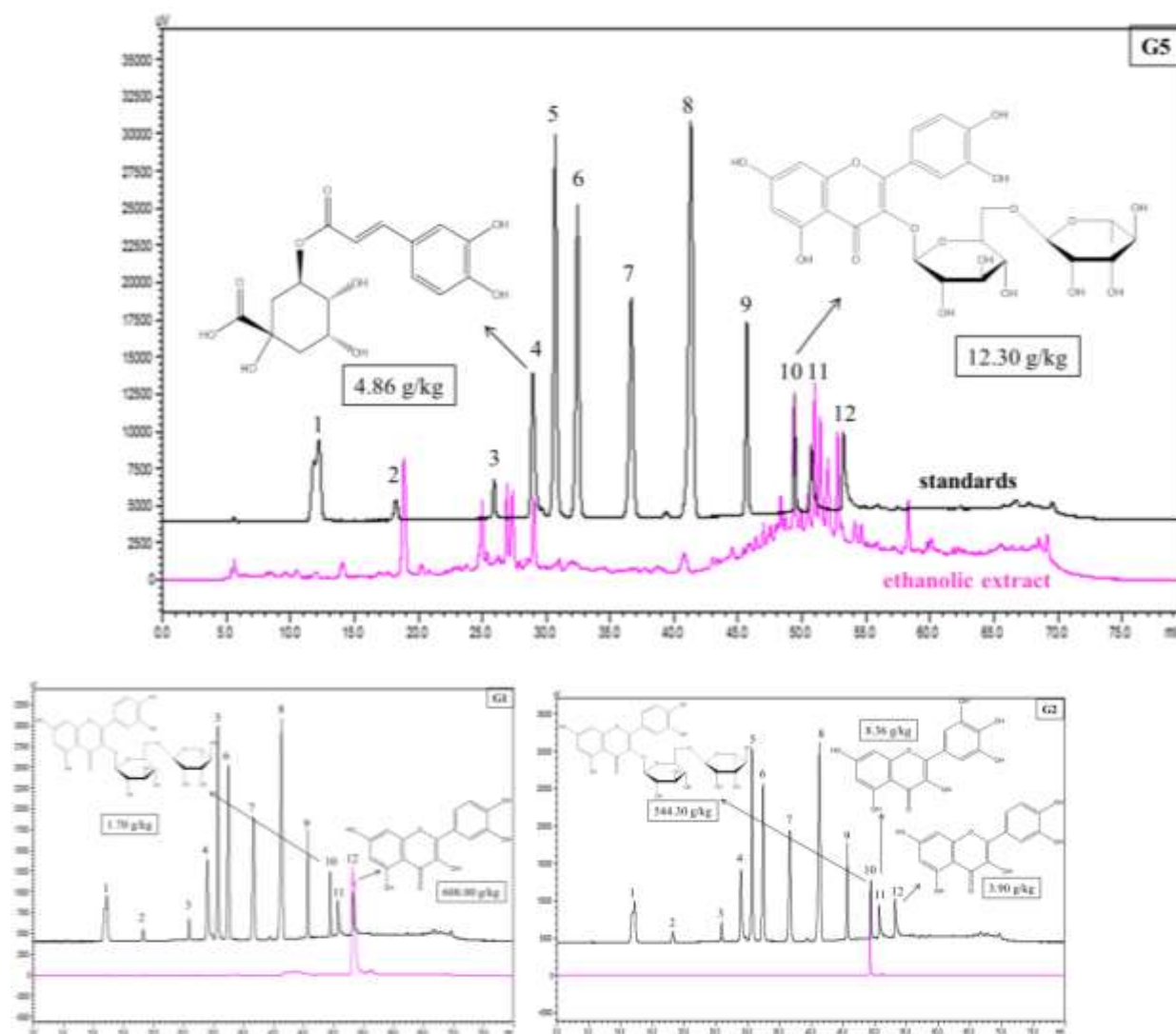
Source: Authors.

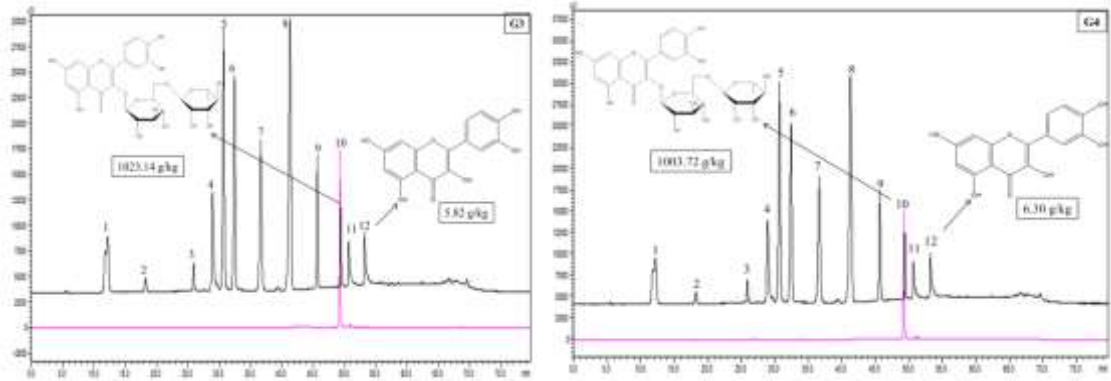
3.6 Microbiological Analysis

Microbiological tests assess the quality of the finished product in order to rule out possible contamination by bacteria and fungi, which are extremely pathogenic, and may irreversibly worsen the clinical condition of the use of herbal medicine (Andrade et al., 2013; Bonfilio et al., 2013). *G. biloba* and *B. monnieri* samples did not show growth of bacteria such as *Escherichia coli*, *Staphylococcus aureus* and *Salmonella* spp., therefore they are in accordance with the parameters described in the Brazilian Pharmacopoeia, which recommends a value of up to 10^4 CFU.mL⁻¹ for approval. In relation to fungal growth, all samples analyzed were within the required quality standards (up to 10^2 CFU/mL).

Therefore, both samples analyzed are in accordance with RDC 67/2007- ANVISA. The acceptable values of microorganisms in the analyzed samples may be associated with the antimicrobial activity described for the vegetable species *G. biloba* and *B. monnieri*. According to Ibrahim and Nuhu (2016), the GbE 761 extract has a considerable inhibitory activity against pathogenic bacteria and fungi, which may be due to the presence of varieties of active compounds in the extract such as flavonoids and tannins. The study by Fazlul et al. (2019) revealed that extracts of *B. monnieri* have demonstrated broad-spectrum antimicrobial activity, with flavonoids, tannins and phenolic compounds responsible for this inhibitory action.

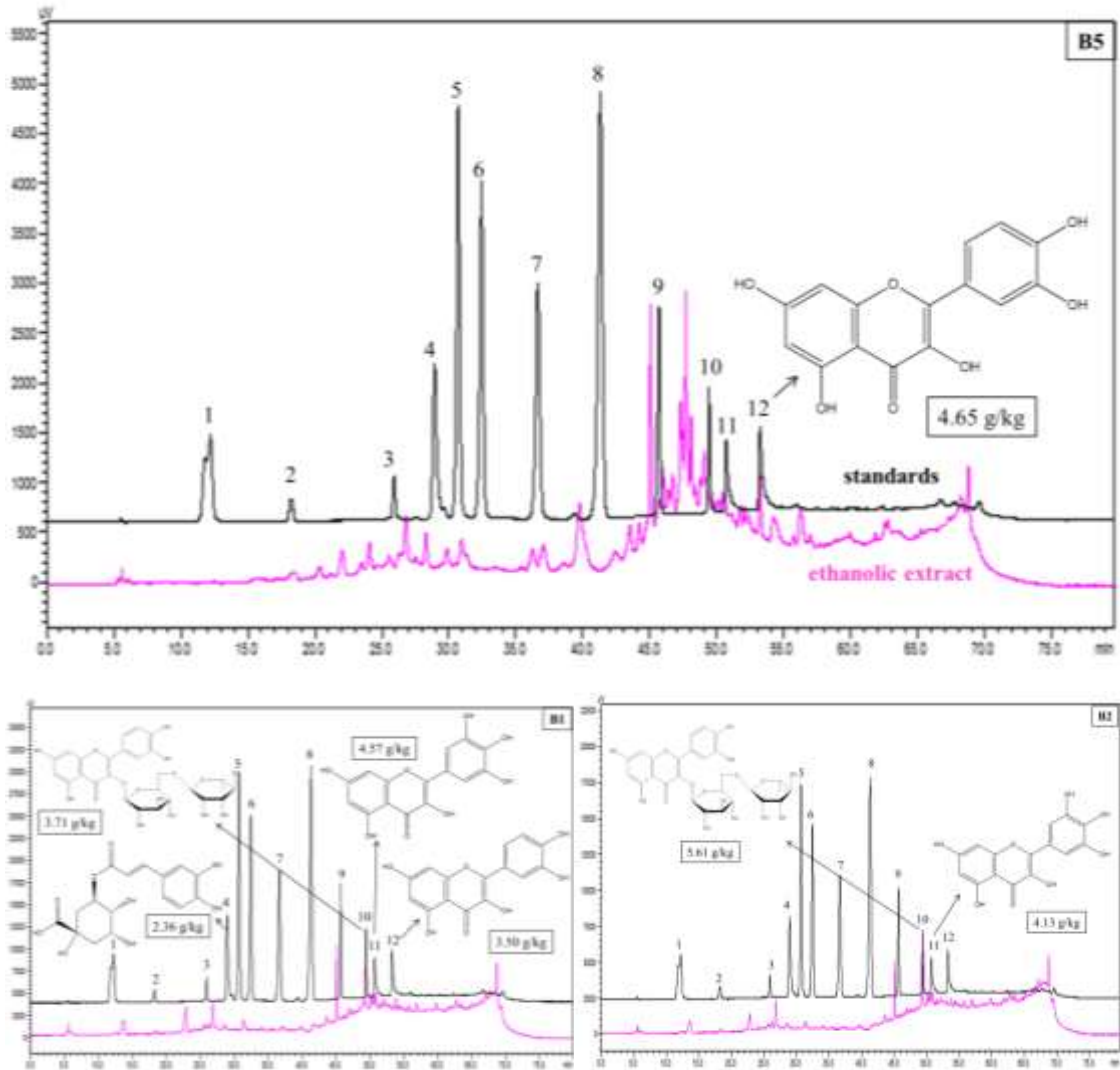
Figure 5. Mix chromatogram of 1-Gallic acid, 2- Catechol, 3- Catechin, 4- Chlorogenic acid, 5- Caffeic acid, 6- (-) epicatechin, 7- Syringaldehyde, 8- Cummaric acid, 9- Coumarin, 10- Rutin, 11- Myricetin, 12- Quercetin standards and ethanolic extracts from *G. biloba* samples (G5; G1; G2; G3 and G4 at 290 nm).

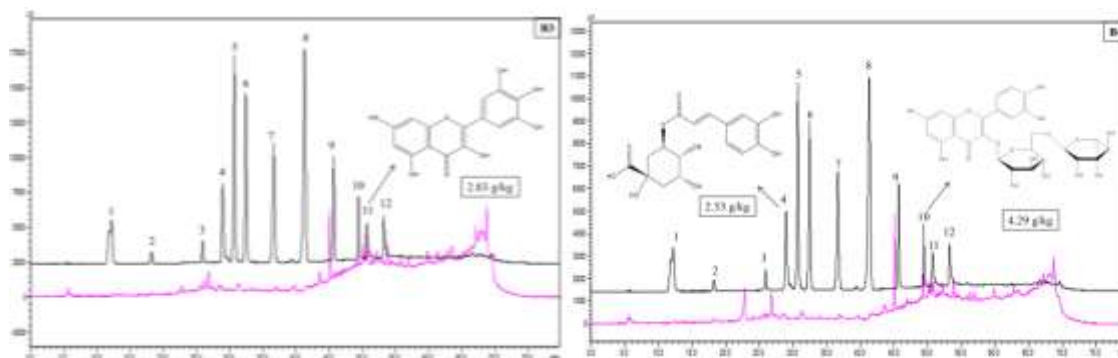




Source: Authors.

Figure 6. Mix chromatogram of 1-Gallic acid, 2- Catechol, 3- Catechin, 4- Chlorogenic acid, 5- Caffeic acid, 6- (-) epicatechin, 7- Syringaldehyde, 8- Cummaric acid, 9- Coumarin, 10- Rutin, 11- Myricetin, 12- Quercetin standards and ethanolic extracts from *B. monnieri* samples (B5; B1; B2; B3 and B4 at 290 nm).





Source: Authors.

3.7 Anticholinesterase activity

Several reversible AChE inhibitors are used in clinical trials as drugs to treat AD (Zhanga et al., 2018). Standardized extract of the leaves of *G. biloba*, labeled GbE 761, has been widely used since its introduction in the market to improve cognitive deficits in a wide range of conditions, from aging to dementia (Müller et al., 2019). Pre-clinical *in vitro*, *in vivo* and clinical studies, have confirmed the neuroprotective effects of GbE 761, being considered the most sold herb for various health disorders (Ibrahim & Nuhu, 2016).

Studies suggest that aerial parts of *B. monnieri* have the potential to improve cognitive function, possibly by reducing AChE activity, increasing antioxidant function, improving cerebral blood flow and neurotransmitter modulation (Kongkeaw et al., 2014). Vinutha et al. (2007), in their study of AChE inhibition with extracts of medicinal plants, proposed the classification of the inhibitory potential of the samples: strong inhibitors (> 50% inhibition), moderate inhibitors (30-50% inhibition) and weak inhibitors (<30% inhibition).

Thus, samples G4, B2 and B5 with percentage values above 50%, were classified as strong AChE inhibitors. In this research, the study of the anticholinesterase inhibitory activity showed that of the five ethanolic extracts of *G. biloba* tested, only G4 showed an inhibition above 50% ($59.92\% \pm 14.00$) with IC_{50} of 0.8540 mg/mL (Table 5). The promising values of quantitative AChE inhibition were observed in the ethanol extracts of *B. monnieri*, for samples B2 ($56.03\% \pm 5.96$) and B5 ($98.61\% \pm 0.46$), with an IC_{50} of 0.9650 mg/mL and 1.8221×10^{-5} mg/mL, respectively (Table 5).

These results are relevant, with the B5 sample being more effective when compared to rivastigmine $IC_{50} = 1.87 \times 10^{-3}$ mg/mL (Cavalcante et al., 2018), which is an AChE inhibitor drug widely used in the treatment of people with AD. AChE was more inhibited in the presence of *G. biloba* compared to *B. monnieri*, and none of the extracts of *B. monnieri* showed more than 50% inhibition, which differs from our results suggesting that the quantitative AChE inhibition may be an improvement in disturbed cholinergic function (Ramasamy et al., 2015). *B. monnieri* has been reported as a direct inhibitor of AChE activity tested by an *in vitro* enzymatic assay (Le et al., 2013). In the report by Yamchuen et al. (2017), *B. monnieri* extract and all tested compounds did not alter the basal cell activity of AChE.

Table 5. Inhibition and classification of inhibition intensity of *G. biloba* and *B. monnieri* samples at a concentration of 1mg/mL.

Samples	Percentage of inhibition (%)	Inhibition intensity	Inhibitory concentration (IC ₅₀) mg/mL
G1	ND	-	-
G2	31.99% ± 0,19	Moderate	-
G3	31.17% ± 13,99	Weak	-
G4	59.92% ± 2,45	Strong	0.8540
G5	47.92% ± 5,80	Moderate	-
B1	33.89% ± 1,39	Moderate	-
B2	56.03% ± 0,92	Strong	0.9650
B3	35.28% ± 0,00	Moderate	-
B4	29.78% ± 0,71	Weak	-
B5	98.61% ± 0,46	Strong	1.8221x10 ⁻⁵

ND – Not Detect. Source: Authors.

4. Conclusion

Samples of herbal medicines based on samples of *G. biloba* (G1) and *B. monnieri* (B3) revealed contradictory physical-chemical parameters with the current laws of ANVISA and Brazilian Pharmacopoeia, indicating the need for standardization in filling capsules, validating unit doses, adding inorganic compounds, errors in the storage and handling of these herbal medicines. The preliminary phytochemical analysis and the CCD of *G. biloba* and *B. monnieri*, indicated the presence of flavonoids. In the investigation and quantification of bioactive compounds by HPLC, the presence of phenolic compounds, chlorogenic acid, quercetin, myricetin and rutin, was demonstrated, with all samples of *G. biloba* showing high concentrations of quercetin or rutin. In *B. monnieri* samples, only sample B1 showed traces of the four phenolic compounds. The AChE inhibition test showed that the G3 sample is promising when compared to the G5 vegetable sample of *G. biloba*. In relation to the samples of *B. monnieri*, the vegetal sample B5 showed an inhibition potential of 98.61%, reinforcing the importance of this phytotherapy in the improvement of cognitive processes.

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