Development of a DNA-based methodology for the identification of *Pfaffia glomerata* in herbal products

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ABSTRACT

Pfaffia glomerata (Brazilian ginseng) is a medicinal plant widely recognized for its adaptogenic, anti-inflammatory, and immunomodulatory properties. Due to its growing commercial value, as well as to its common name that includes the word "ginseng", and the morphological similarity to other ginsengs, it is increasingly prone to adulteration. This study aimed to develop and validate a species-specific real-time PCR method for the identification and quantification of *P. glomerata* in commercial herbal products. Primers targeting the ITS1 region were designed and evaluated both *in silico* and experimentally. Specificity was assessed against 51 medicinal plant samples, including other species known for their adaptogenic properties. Sensitivity assays confirmed an absolute detection limit of 0.001 ng of *P. glomerata* DNA. Quantitative analysis using binary mixtures with *Withania somnifera* and the ΔCq normalization method reliably detected *P. glomerata* at levels as low as 0.1% (w/w) and allowed the accurate estimate of the percentage of *P. glomerata* material in the range of 50% to 0.1% (m/m). The method was applied to 25 commercial products labeled as *P. glomerata, Pfaffia paniculata,* Pfaffia, Brazilian ginseng, or other possibly adulterant species of Brazilian ginseng, seven samples showed inconsistencies with the label and were considered as adulterated. *P. glomerata* DNA was also found to be present in nine other samples, but only at trace amounts (<LOQ of 0.1%), suggesting a cross-contamination rather than adulteration. These findings highlight the high sensitivity of the developed method but also evidence the importance of using the ΔCq normalization method to estimate the amounts of *P. glomerata* in mixtures. The results also showed that the molecular strategy developed was effective and sensitive for the authentication of *P. glomerata* and confirmed the method's capacity to authenticate *P. glomerata* and distinguish it from other adulterant species. The results reveal a high rate of adulteration (3 out of 6) among products marketed under the name *P. glomerata* or "Brazilian ginseng," with recurrent substitution of *P. glomerata* by *P. paniculata*. In addition, two samples labeled as *P. paniculata* were found to be *P. glomerata,* indicating a high level of mislabeling among the two Pfaffia species. These findings highlight weaknesses in labeling practices and support the need for molecular tools to ensure botanical authenticity, for a reliable traceability within the adaptogenic plant market and *Pfaffia* genus in particular.

Key words: *Pfaffia glomerata*; medicinal plants; authenticity; real-time PCR

RESUMO

Pfaffia glomerata (ginseng brasileiro) é uma planta medicinal amplamente reconhecida por suas propriedades adaptogénicas, anti-inflamatórias e imunomoduladoras. Devido ao seu crescente valor comercial, bem como ao facto de seu nome comum incluir a palavra "ginseng" e à sua semelhança morfológica com outros ginsengs, está cada vez mais sujeita a adulteração. Este estudo teve como objetivo desenvolver e validar um método de PCR em tempo real específico para a identificação e quantificação de *P. glomerata* em produtos fitoterápicos comerciais. Foram desenhados primers direcionados para a região ITS1, avaliados *in silico* e experimentalmente. A especificidade foi testada contra 51 amostras de plantas medicinais, incluindo outras conhecidas por suas propriedades adaptogénicas. Ensaios de sensibilidade confirmaram um limite absoluto de deteção de 0,001 ng de DNA de *P. glomerata*. A análise quantitativa, utilizando misturas binárias com *Withania somnifera* e o método de normalização ΔCq, detetou de forma confiável *P. glomerata* em níveis tão baixos quanto 0,1% (p/p) e permitiu estimar com precisão a percentagem de material de *P. glomerata* na gama de 50% a 0,1% (m/m). O método foi aplicado a 25 produtos comerciais rotulados como *P. glomerata*, *Pfaffia paniculata*, *Pfaffia*, ginseng brasileiro ou outras espécies possivelmente adulterantes de ginseng brasileiro. Sete amostras apresentaram inconsistências com o rótulo e foram consideradas adulteradas. DNA de *P. glomerata* também foi encontrado em outras nove amostras, mas apenas em quantidades residuais (<LOQ de 0,1%), sugerindo contaminação cruzada e não adulteração. Estes resultados destacam a alta sensibilidade do método desenvolvido, mas também evidenciam a importância da utilização do método de normalização ΔCq para estimar as quantidades de *P. glomerata* em misturas. Os resultados também mostraram que a estratégia molecular desenvolvida foi eficaz e sensível para a autenticação de *P. glomerata*, confirmando a capacidade do método para distinguir a espécie de possíveis adulterantes. Foi revelada uma taxa elevada de adulteração (3 em cada 6) entre os produtos comercializados sob o nome *P. glomerata* ou "ginseng brasileiro", com substituição frequente de *P. glomerata* por *P. paniculata*. Além disso, duas amostras rotuladas como *P. paniculata* foram identificadas como *P. glomerata*, indicando um elevado nível de erros de rotulagem entre as duas espécies de *Pfaffia*. Esses resultados evidenciam falhas nas práticas de rotulagem e reforçam a necessidade de ferramentas moleculares para garantir a autenticidade botânica, possibilitando uma rastreabilidade fiável no mercado de plantas adaptogénicas e, em particular, do género *Pfaffia*.

Palavras chave: *Pfaffia glomerata*; plantas medicinais; autenticidade; PCR em tempo real.

INDEX

ABSTRACT ..... **IV**

RESUMO ..... **V**

INDEX OF TABLES ..... **VIII**

INDEX OF FIGURES ..... **IX**

1 INTRODUCTION **1**

1.1 Medicinal Plants **1**

1.2 Plant Authenticity **3**

1.3 *Pfaffia glomerata* **5**

1.4 Approaches for Botanical Authentication **7**

1.4.1 Chemical Techniques - Chromatographic Processes 8

1.4.2 DNA-based Techniques for Plant Authentication 10

1.5 Objectives **17**

2 MATERIAL AND METHODS **18**

2.1 Plant Species, Commercial Samples and Sample Preparation **18**

2.2 DNA Extraction and Quality Assessment **20**

2.3 Primers Design for *Pfaffia glomerata* Identification **21**

2.4 Conventional PCR Assay Optimization **22**

2.4.1 Assessment of Primers Specificity 23

2.4.2 Electrophoresis of Qualitative PCR Products 24

2.5 Real-Time PCR Assay Optimization **25**

2.5.1 Assessment of Primers Sensitivity ..... **Error! Bookmark not defined.**

2.6 Development of a qPCR method for *Pfaffia glomerata* Detection in Herbal Products **25**

2.6.1 Application and validation of the qPCR method against commercial products 26

3 RESULTS AND DISCUSSION **28**

3.1 Evaluation of DNA extracts **28**

3.2 Conventional PCR Assay Optimization **30**

3.2.1 Primers Specificity 32

3.3 Real-Time PCR Assay Optimization 32

3.3.1 Primers Sensitivity 32

3.4 Development of a qPCR model for *Pfaffia glomerata* Detection in Herbal Products 36

3.4.1 Application of the qPCR model against commercial products 38

4 CONCLUSIONS 44

5 REFERENCES 46

APPENDIX I – LIST OF COMMERCIAL SAMPLES WITH IMAGES 63

INDEX OF TABLES

Table 1 - List of the 25 commercial plant samples used in this study. 18
Table 1 (continued) 19
Table 2 - Description of the primers selected in this study, targeting polymorphic sites in the ITS1 region of *P. glomerata*. 22
Table 3 - List of the 51 medicinal plants samples (previously authenticated through ITS2, matK and/or rbcL sequencing) used in the cross-reactivity tests..... 23
Table 3 (continued) 24
Table 4 – DNA concentration and purity ratios of commercial samples extracted by using the Nucleospin Plant II kit. 28
Table 4 (continued) 29
Table 5 - Results of the validation assays using the normalized quantitative PCR system applied to blind mixtures of *P. glomerata* in *W. somnifera*. 37
Table 6 - Results of the application by qPCR coupled with melting temperature analysis of the DNA extracts from commercial samples. ("+": amplification; "-": no amplification)..... 39
Table 6 (continued) 40
Table 7 - Normalized qPCR method for quantification of *P. glomerata* in commercial samples…… 41
Table 7 (continued)…… 42

INDEX OF FIGURES

Figure 1 - Agarose gel electrophoresis of PCR products using primers EG-F and EG-R, C40: *P. glomerata* (positive control); C23-C39: genomic DNA extracted from commercial samples; MM: 100 bp DNA ladder marker; CN: negative control. 30
Figures 2 – Results of PCR products at different annealing temperatures: **A:** 58 ºC, **B:** 60 ºC, **C:** 62 ºC and **D:** 65 ºC using serial dilutions of *P. glomerata* DNA (10 ng to 0.001 ng); MM: 50 bp DNA ladder marker (Bioron GmbH); CN: negative control. 31
Figure 3 - Agarose gel electrophoresis of PCR products using primers PFA3-F and PFA-R; C40: *P. glomerata*; P1 -P10: DNA mixtures; MM: 100 bp DNA ladder marker; CN: negative control. 32
Figure 4 – Results of the real-time PCR assay using the designed primers (PFA3-F and PFA3-R) targeting *P. glomerata*. The amplified extracts correspond to 10-fold serially diluted *P. glomerata* DNA ranging from 10 ng to 10 pg (n = 3 replicates). 33
Figure 5 – Melting curves obtained from the real-time PCR assay using the designed primers (PFA3-F and PFA3-R) targeting *P. glomerata*. The amplified extracts correspond to 10-fold serially diluted *P. glomerata* DNA ranging from 10 ng to 10 pg (n = 3 replicates). 34
Figure 6 – Standard curve obtained from the real-time PCR assay using the designed primers (PFA3-F and PFA3-R) targeting *P. glomerata*. The amplified extracts correspond to 10-fold serially diluted *P. glomerata* DNA ranging from 10 ng to 10 pg (n = 3 replicates). 35
Figure 7 – Normalized calibration curves obtained by real-time PCR, targeting the ITS1 region of *P. glomerata*, using the binary mixtures of *P. glomerata* in *W. somnifera* (50%, 10%, 5%, 1%, 0.5% and 0.1%, (w/w)). The normalized ΔCq method was performed by the parallel amplification of a eukaryotic sequence (18S rRNA) as reference (mean values of five independent assays with n = 3 replicates). 36
Figure 8 - Agarose gel electrophoresis of PCR products using PFA3-F and PFA3-R primers; C40: P. glomerata; **A:** C15-C31: commercial samples; **B:** C32-C39 commercial samples; MM: 100 bp DNA ladder marker; CN: negative control. 38

1 INTRODUCTION

1.1 Medicinal Plants

Over the centuries, humanity has turned to plants both to meet their dietary needs and to treat various illnesses. Natural products play a fundamental role in the development of modern medicine and continue to be essential elements in the search for new medicines. Many important discoveries, such as antibiotics, anticancer agents, anti-inflammatory compounds, and analgesics, originate from natural resources (Li *et al.*, 2022).

In their study, Almeida (2011) point out that medicinal plants are those that act in the fight against diseases. The author comment that they are capable of purifying the organism, helping the metabolism to function properly - stimulating and normalizing the activity of the organs, relieving local symptoms, increasing resistance and promoting the body's natural defenses, in addition to other benefits, which, in the long term, help in the recovery and maintenance of good health.

In this context, medicinal plants represent a unique and renewable source for the discovery of therapeutically active biomolecules, given their vast structural and biological diversity. Furthermore, the rich chemical diversity present in medicinal plants offers a range of possibilities for innovative research in the search for new treatments (Atanasov *et al*., 2015). The search for new chemical entities from nature remains a crucial source of structural diversity, while genomic research and biotechnology advance the identification of molecular targets for specific screening assays and assure the correct identification of botanical material (Kakkar *et al*., 2023).

"Ginseng" is historically used for its multitherapeutic effects, including its adaptogenic abilities (Potenza *et al*., 2023). The terminology "ginseng" refers to species of the genus Panax, such as *Panax ginseng* C.A. Meyer, known as Korean ginseng, or *P. quinquefolium* known as American ginseng, among others (Helms, 2004). However, species from other genera are also commonly designated as ginsengs, including *Eleutherococcus senticosus* Maxim, named as Siberian ginseng, and species of the genus Pfaffia such as *Pfaffia glomerata* (Spreng) Pedersen and *P. paniculata* (Mart.) Kuntze (*Hebanthe eriantha*) popularly sold as Brazilian ginseng (Shiobara *et al*., 1993). Among these plants, Brazilian ginseng (*P. glomerata*) emerges as a promising medicinal plant

due to its anti-inflammatory, anti-obesity, antidiabetic, central nervous system (CNS) depressant, leishmanicidal, and prebiotic actions (Cotrim Ribeiro *et al.*, 2024).

The integrity of plants like Brazilian ginseng is a crucial concern as their popularity increases. Throughout the distribution chain, from harvesting to the formulation of final products, medicinal plants are susceptible to adulteration and contamination that can compromise their effectiveness and, in some cases, pose health risks (Ichim & de Boer, 2021).

In this context, the lack of effective regulation, pressure for mass production, and the search for profits often lead to the decrease of the quality of medicinal plants. Raising awareness of the risks associated with adulteration and implementing traceability practices along the supply chain are crucial steps to ensuring that medicinal plants maintain their integrity and provide the sought-after therapeutic benefits (Wang *et al.*, 2023).

The role of biotechnology in enhancing the medicinal value of plants cannot be overlooked. Techniques such as genetic engineering, tissue culture, and metabolic engineering are being employed to increase the yield of active compounds and to produce plant-derived pharmaceuticals in a more sustainable and controlled manner (Ramawat *et al.*, 2009). In the case of Brazilian ginseng, these approaches can help ensure the consistent and high-quality production of its bioactive compounds, such as steroids, nortriterpenoids and triterpenoid saponins, which are responsible for many of its therapeutic effects (Zhenguang *et al.*, 2024). Furthermore, chemical and DNA analysis techniques, such as thin layer chromatography, high-performance liquid chromatography, real-time PCR, DNA barcoding, Barcode DNA High-Resolution Melting (BarHRM), molecular markers and microsatellites, have been used to differentiate morphologically similar species, either based on the identication of bioactive compounds in different parts of the plant or its genetic characteristics (Almeida *et al.*, 2023; Flores *et al.*, 2009; Figueira *et al.*, 2011).

Despite the promising potential of medicinal plants, several challenges need to be addressed to fully harness their benefits, including the implementation of practices that allow detecting botanical adulterations or accidental plant swaps. Additionally, standardization of herbal medicines is a significant issue, as the concentration of active compounds can vary widely depending on factors such as plant species, geographical location, and harvesting methods (Jordan, Cunningham & Marles, 2010). Ensuring

consistent quality and efficacy requires rigorous quality control measures and adherence to Good Agricultural and Collection Practices (GACP).

In summary, medicinal plants continue to be a vital resource for modern medicine, offering a rich source of bioactive compounds with diverse therapeutic effects. The integration of traditional knowledge with scientific research, coupled with advancements in biotechnology, holds great promise for the discovery of new treatments. However, addressing challenges related to standardization and quality control is crucial to fully realize the potential of medicinal plants in healthcare. As the demand for natural and sustainable therapies grows, the exploration and responsible use of medicinal plants will remain a key area of focus for researchers and healthcare providers.

1.2 Plant Authenticity

Plant authenticity is crucial to ensure that a plant is genuine and corresponds to the declared species or variety (Silva & Rabelo, 2010). In areas such as agriculture, botany, food industry and the pharmaceutical industry, accurate identification of plants is essential to guarantee productivity, quality and effectiveness of treatments and products (Secretaria de Infraestrutura e Meio Ambiente do Estado de São Paulo, 2015).

In the agricultural and horticultural context, ensuring the authenticity of plants can directly affect crop productivity and quality. Specific varieties may be resistant to pests, diseases or adverse weather conditions, making verification very important to maximize agricultural efficiency (Wadood *et al*., 2020).

Correct species identification is also essential for conservation and ecological restoration strategies, helping to preserve threatened ecosystems and species. DNA-based methods, such as DNA barcoding, have proven effective in accurately identifying plant species, helping to conserve and monitor biodiversity (Nazar *et al*., 2025).

In the context of phytotherapy and the pharmaceutical industry, the authenticity of medicinal plants is essential to guarantee the safety and effectiveness of treatments since adulterated or incorrectly identified products can lead to adverse health consequences. Ichim (2019) conducted a literature review that analyzed data reporting medicinal plants' authenticity established by DNA-based methods. Of the 5957 commercial products analyzed, sold in 37 countries, 27% of these products were considered adulterated since the labeled ingredients were not according to the identified plant species.

In the case of “ginsengs”, several varieties are sold at different stages of processing, whether as raw material or highly processed products that have already lost their morphological characteristics, which makes authentication difficult (Yang *et al*., 2017). However, even for fresh products, the ginseng cultivar, location, growth conditions, and age of the plant directly affect the active ingredients and healing effects (Kim *et al*., 2012). These factors directly influence the sales price of plants (Kim *et al*., 2011), and the price difference is the primary factor for intentional adulteration (Choi *et al*., 2007). Adulterations using lower-cost species discredit the entire supply chain and ginseng-based products (Zhao *et al*., 2020).

In the European Union (EU), food supplements and traditional herbal medicinal products (THMPs) should follow different legislation, despite both being structured to ensure the safety of these products. In the EU, food supplements are legally considered as foods under Directive 2002/46 /EC of the European Parliament and of the Council, dated June 10, 2002, which defines food supplements as “… foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills (…) and other similar forms designed to be taken in measured small quantities”. This directive sets specific requirements for these products, which should follow the regulations applied to foods. Additionally, despite not being mandatory, Good Manufacturing Practices (GMP) are considered essential to ensure the purity and authenticity of food supplements and herbal infusions, preventing contamination and adulteration (European Parliament and Council, 2002).

For traditional herbal medicinal products (THMPs), the relevant legislation is Directive 2004/24/EC, which amends Directive 2001/83/EC on the Community code relating to medicinal products for human use. This legislation introduced a simplified registration procedure for THMPs, allowing these products to be evaluated based on their traditional and historical use. Directive 2004/24/EC stipulates that THMPs must have a safe history of use for at least 30 years, with 15 years in the European Union. Monographs from the European Medicines Agency (EMA) are used for standardization and evaluation, and adherence to GMP is crucial to prevent contamination and ensure product purity. Additionally, rigorous quality control tests should be implemented, including the verification of the authenticity of the ingredients (European Parliament and Council, 2004).

However, some products can raise doubts regarding their classification, being considered borderline products (Infarmed, 2025). For example, some medicinal plants can be used either in products classified as food supplements or THMP. In both cases, issues related to the adulteration of plant species represent an important safety problem for the market of supplements and herbal medicines.

Adulteration occurs when products are mislabeled or added with inferior or different species than those stated, leading to significant public health risks and economic consequences (Booker, Johnston & Heinrich, 2012). This malpractice can undermine consumer trust and compromise the therapeutic efficacy of these products. Despite the stringent regulations, economic motivations often drive some companies to create false or adulterated products, undermining the integrity of the market (Ichim, 2019).

Therefore, rigorous analytical methods and quality control procedures to verify the authenticity of the products are required.

1.3 *Pfaffia glomerata*

Pfaffia glomerata (Spreng.) Pedersen, commonly known as "Brazilian ginseng," is a perennial plant from the Amaranthaceae family, widely distributed in Brazil's *Cerrado* and *Pantanal* biomes. Traditionally used in folk medicine, it is valued for its adaptogenic, tonic, aphrodisiac, anti-inflammatory, and antioxidant properties (Rates & Gosmann, 2002). Given its established medicinal use, the plant has attracted growing interest from the pharmaceutical and nutraceutical industries.

Taxonomically, *P. glomerata* belongs to the kingdom Plantae, order Caryophyllales, and genus *Pfaffia*. Morphologically, *P. glomerata* is an erect or subscandent shrub that can reach up to two meters in height. It has rounded stems, opposite leaves with short petioles and oval or elongated blades, and globular inflorescences that range in color from whitish to yellowish. The roots are thick, tuberous, and light brown, and they are the primary part used in traditional medicine and commercial applications (Corrêa Jr, 2003).

The roots contain a wide range of bioactive compounds, including ecdysteroids such as 20-hydroxyecdysone, triterpenoids such as pfaffic, glomeric, and oleanolic acids, flavonoids, tannins, inulin, and fructooligosaccharides. These secondary metabolites are associated with various pharmacological activities, including adaptogenic, analgesic,

anti-inflammatory, hepatoprotective, and immunomodulatory effects (Cotrim Ribeiro *et al*., 2024; Neto *et al*., 2005).

Due to its phytochemical profile, *P. glomerata* is widely used as a functional ingredient in dietary supplements and herbal medicines. However, rising commercial demand, combined with morphological similarities to other species in the genus and with other genera such as *Panax*, raises concerns about the authenticity of raw materials used. Cases of adulteration and substitution have been documented, which can compromise the efficacy and safety of final products (Almeida *et al*., 2023). This difficulty in visual differentiation is accentuated when the plant is marketed in the form of powder, extract or capsules, making morphological recognition of the plant raw material very difficult or even unfeasible (Vigo *et al*., 2004).

The species is often confused with others from the same genus, such as *P. paniculata* (synonym, *Hebanthe eriantha*), due to strong morphological similarities (Almeida *et al*., 2023). *P. glomerata* and *P. paniculata* roots have a very similar morphologyand both species are popularly known as "Brazilian ginseng" (Almeida *et al.*, 2023). Despite sharing some bioactive constituents such as saponins and phytoecdysteroids, the two species have distinct chemical profiles: *P. glomerata* contains higher levels of β-ecdysterone (20-hydroxyecdysone), a compound associated with adaptogenic, anabolic and antioxidant activities (Terhaag *et al*., 2025) while *H. eriantha* presents lower concentrations of this substance, being characterized mainly by the presence of pfaffic acid and pfaffosides as chemical markers (Vigo *et al*., 2004).

Also, the genera *Eleutherococcus*, *Panax* and *Withania* stand out as particularly relevant in the study of possible adulteration of *P. glomerata*, not only due to the overlapping therapeutic uses, but also due to the similarity of bioactive compounds with adaptogenic action (Yuk *et al*., 2016; Wang *et al*., 2023; Petrovska, 2012). While *P. glomerata* has saponins and phytoecdysteroids as its main bioactive metabolites (Vigo *et al.*, 2004), the genus *Panax* is known for the presence of ginsenosides, responsible for neuroprotective and immunomodulatory effects (Yuk *et al*., 2023). *Eleutherococcus*, such as *E. senticosus* often called "Siberian ginseng", contains eleutherosides, also associated with tonic and anti-stress action (Petrovska, 2012), while *Withania*, such as *W. somnifera* (ashwagandha), presents withanolides, steroidal lactones with anxiolytic and immunomodulatory effects (Singh *et al*., 2011).

Several studies have already documented substitutions and frauds involving these genera, especially in products marketed as "ginseng", where the term can refer indistinctly

to species of *Panax*, *Pfaffia*, *Eleutherococcus*, contributing to taxonomic confusion and adulterations in international trade (Wang *et al*., 2023; Kim *et al*., 2025). In a survey based on genetic analysis by DNA Bar-HRM (High Resolution Melting), Almeida *et al*. (2023) observed that only 28.6% of commercial samples labeled as *P. glomerata* were correct. The majority of samples, approximately 71.4%, presented adulteration or substitution by *P. paniculata*, which highlights the frequency of this practice.

From an economic standpoint, *P. glomerata* represents a high-value plant in the medicinal and functional plant market. It is estimated that around 300 tons of roots were traded between 2019 and 2020, with a significant share exported to Asia and Europe (FAEP/SENAR-PR, 2021). Its market value stems from its application as a substitute for Asian ginseng and the growing demand for natural adaptogenic ingredients (De Paris *et al*., 2000). Economic feasibility studies suggest that producing concentrated extracts using intensified methods, such as supercritical fluid extraction, yields positive returns, particularly when combined with product standardization and quality control (Leal *et al*., 2010; Carulo, 2012). Therefore, strengthening the *P. glomerata* supply chain depends on adopting technologies that ensure the plant's authenticity and the quality of its commercial derivatives in both domestic and international markets.

The integrated application of morphological, chemical, and molecular techniques constitutes a robust strategy to ensure the authenticity of *P. glomerata*, promoting consumer safety and the ethical and effective use of botanical resources in medicine and nutrition.

1.4 Approaches for Botanical Authentication

Authentication of botanical materials is a critical aspect to ensure the quality, safety, and efficacy of herbal products. Given the diversity of plant species and the complexity of plant matrices, multiple complementary techniques have been developed. These include morphological and microscopic identification, chemical profiling, and molecular genetic methods, each with specific advantages and limitations (Jiang *et al*., 2010).

Traditional methods of botanical authentication, rooted in pharmacognosy, rely on macroscopic and microscopic examination, as well as chemical assays, to identify plant materials. These approaches are foundational and still employed in many pharmacopeias; however, they have significant limitations, particularly when dealing

with processed or powdered samples in which key morphological features may no longer be visible (Wallace *et al.*, 2012). Morphological analysis involves the macroscopic examination of plant organs, including their leaf shape, venation, flower structure, and seed morphology. The procedure includes collecting plant specimens, cleaning and drying them, and analyzing key diagnostic features under standardized lighting conditions using botanical keys and atlases (Salmeri, 2019). However, this method is constrained by phenotypic plasticity, developmental stage, and the fact that processed materials (e.g., fragmented plants or powders) often lack distinguishable characteristics (Wallace *et al*., 2012). Microscopic analysis further aids in identifying cellular structures, such as trichomes, stomata, calcium oxalate crystals, and fiber types, by preparing slides stained with reagents (e.g., chloral hydrate or phloroglucinol-HCl) and analyzing them under a light microscope (Jiang *et al.*, 2010).

To overcome these challenges, more advanced chemical techniques such as chromatographic fingerprinting have been developed. HPLC coupled to different detection methods, for instance, enables the detection of complex chemical profiles that can serve as signatures for specific plant species. These chemical fingerprints improve reliability compared to visual methods but can still be influenced by factors such as plant age, part used, harvesting time, and processing conditions, which may alter the chemical composition (Alaerts *et al*., 2007).

In recent years, DNA-based techniques have emerged as highly precise tools for botanical authentication. Methods like PCR (polymerase chain reaction) and DNA sequencing allow for the identification of species-specific genetic markers that remain stable regardless of processing. These molecular tools provide a reliable solution for verifying plant identity, especially in complex formulations or processed herbal products where traditional and chemical methods fall short (Sucher & Carles, 2008).

1.4.1 Chemical Techniques - Chromatographic Processes

High-Performance Liquid Chromatography (HPLC), Gas Chromatography (GC), and Capillary Electrophoresis (CE) coupled to different detectors are commonly employed to separate, identify, and quantify phytochemicals in botanical samples. The general procedure for HPLC includes sample extraction using solvents such as methanol or ethanol, followed by filtration and injection into an HPLC system. The system includes a pump, injector, chromatographic column (typically C18 reverse phase), and detectors

such as Diode-Array Detector (DAD) or Mass Spectrometer (MS). Mobile phases are generally composed of water and acetonitrile or methanol with gradient elution. Chromatograms are analyzed for retention time and peak area to determine the presence and concentration of compounds (Zhang *et al.*, 2012; Zhang *et al*., 2018).

For GC, which is especially suited for volatile compounds, the procedure includes derivatization of non-volatile metabolites, sample injection into a heated inlet, and separation through a capillary column using inert carrier gases (e.g., helium). Detection is typically achieved with Flame Ionization Detector (FID) or MS (Dettmer-Wilde & Engewald, 2014). CE separates molecules based on their charge and size using narrow capillaries under an electric field and is useful for polar and charged secondary metabolites (Masár *et al*., 2020).

Chromatographic fingerprinting allows for the creation of unique chemical profiles that reflect the presence and concentration of multiple constituents. These fingerprints serve as reference standards for quality control. LC-MS can utilize both targeted and untargeted approaches. Targeted analysis focuses on known marker compounds, while untargeted approaches aim to capture the overall chemical complexity of a sample. In targeted analysis, known marker compounds specific to a medicinal plant are detected with high sensitivity and selectivity. However, the limited number of unique chemical markers for individual species often necessitates the use of multiple markers to create a characteristic profile. Untargeted analysis aims to detect as many unspecified compounds as possible, generating a comprehensive fingerprint of the sample. High-resolution MS, which provides accurate mass measurements, facilitates the identification and structural elucidation of unknown compounds. Advanced statistical tools, such as hierarchical cluster analysis and principal component analysis, are employed to process the large datasets generated by untargeted approaches, enabling sample classification based on chemical fingerprints without the need for complete identification of all components (Blaženović *et al*., 2018; Hostettmann, Wolfender, & Rodriguez, 1997).

Studies such as Chen *et al*. (2022) used UPLC fingerprinting combined with chemometrics to authenticate *P. notoginseng*, demonstrating high reproducibility and species specificity. Felipe *et al*. (2014) conducted a phytochemical investigation of *P. glomerata* inflorescences using LC-ESI-MS/MS. The study successfully identified key bioactive compounds such as β-ecdysone, flavonoid glycosides (e.g., quercetin-3-O-glucoside), and oleanane-type saponins, establishing a robust chemical basis for the authentication of this species.

1.4.2 DNA-based Techniques for Plant Authentication

1.4.2.1 DNA Barcodes as Molecular Markers

In living beings, some regions offer a combination of DNA conservation at higher taxonomic levels (such as family and genus) and enough variability among species to allow for precise distinction between them (Hollingsworth, 2011). These regions are frequently used for species identification based on a short, standardized sequence, usually referred to as a DNA barcode. A DNA barcode must meet specific criteria: (i) be easily amplified using universal primers, (ii) contain both conserved and variable regions, (iii) have a sequence length compatible with sequencing platforms (less than 1000 base pairs (bp)), and (iv) be associated with a reliable reference database for comparison, such as BOLD (Barcode of Life Data Systems) or GenBank (Ratnasingham & Hebert, 2007).

The DNA barcode concept was proposed by Hebert *et al*. (2005), initially for animals, using the COI (cytochrome c oxidase I) gene. In plants, identifying an optimal universal marker has been revealed to be complex due to the relatively slow evolutionary changes in plant genomes compared to those of animals (Hollingsworth *et al*., 2009). Numerous genetic markers have been assessed individually and in combinations for their effectiveness in plant species barcoding. Commonly evaluated markers include matK, rbcL, ITS1 and ITS2, trnH-psbA, atpF-atpH, ycf5, trnL-F, rpoB, and rpoC1. However, the search for a region equivalent to COI in animals revealed that no single plastid region possessed both the required substitution rate and the necessary technical robustness.

The Consortium for the Barcode of Life (CBOL) examined 907 samples from 550 plant species and found that a combination of the chloroplast genes matK and rbcL is particularly effective for plant species identification. The rbcL (ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit) region is a coding gene found in the chloroplast genome. It is a highly conserved sequence, which allows for efficient amplification using universal primers and makes it suitable for phylogenetic studies at higher taxonomic levels, such as orders and families (Chase *et al*., 1993; Kress & Erickson, 2007). The matK (maturase K) gene, also located in the plastid genome, evolves more rapidly than rbcL. This higher substitution rate provides better resolution at lower taxonomic levels, such as between closely related species or within genera (Hilu *et al*., 2003).

In addition to chloroplast markers, nuclear regions are also used, particularly the Internal Transcribed Spacer (ITS), which is part of the nuclear ribosomal DNA (nrDNA),

located between the 18S, 5.8S, and 26S genes. The ITS region includes ITS1, the 5.8S gene, and ITS2. Due to its rapid evolutionary rate and high degree of variability, ITS is widely used to distinguish very closely related species (Baldwin *et al*., 1995; Álvarez & Wendel, 2003). Different studies suggest that the ITS2 region serves as a universal barcode for plants, demonstrating the ability to identify related species across different families and genera (Yao *et al*., 2010).

1.4.2.2 Molecular Techniques

Different molecular techniques have been used in several studies aiming for plant authentication. However, before DNA amplification, different steps must be performed, with extraction being a crucial one. The first step is to extract the genetic material from the samples. DNA/RNA extraction typically involves three main stages: cell envelope disruption, deproteinization, and DNA recovery (Nascimento *et al*., 2022). When aiming to extract RNA, it is crucial to take several precautions to ensure its integrity and quality. One of the main challenges in this process is the presence of RNases, which are highly stable and active enzymes capable of rapidly degrading RNA. To prevent this degradation, it is essential to add an extraction buffer at the very beginning of the procedure, as it aids in RNA precipitation and helps preserve it throughout the subsequent steps (Nascimento *et al*., 2022).

The choice of the most suitable extraction method depends on the sample type, the resources available in the laboratory, and the required level of purity. In many cases, to simplify handling reagents and solutions, commercial kits are used. These kits generally work with filter columns that retain the genetic material, allowing impurities to be eliminated at each step by switching microtubes between stages (Nascimento *et al*., 2022).

In this context, it is very important to evaluate the type of sample to be analyzed. For example, molecular analysis of food supplements in the form of capsules and tablets is considered complex due to the presence of pharmaceutical excipients, such as talc, silica, and metal oxides, which hinder DNA recovery through adsorption (Costa *et al*., 2015). Moreover, these products can also contain amplification inhibitors like magnesium stearate, carbonates, and/or synthetic dyes (Coghlan *et al*., 2012; Raclariu *et al*., 2017). Therefore, kits or protocols with greater capacity to remove these excipients and recover DNA should be used.

After extraction, it is essential to quantify the genetic material to assess its quality, purity and determine the appropriate concentration for amplification. This step can be performed in three ways: by agarose gel electrophoresis, which uses molecular weight to separate the genetic material; by fluorometry, where a fluorescent dye binds to nucleic acids; and by spectrophotometry, which is based on the absorption of light by nucleic acids at wavelengths of 260 and 280 nanometers (nm). In spectrophotometry, the A260/A280 ratio is an indication of purity: values between 1.75–1.80 for DNA and close to 2.0 for RNA are considered ideal (Ma *et al*., 1996). Values below or above indicate the presence of proteins or organic solvents. The A260/A230 ratio is used as an additional indicator of purity. This metric allows identifying the presence of contaminants that absorb around 230 nm, such as phenol, guanidine, carbohydrates, polyphenols, and saline residues (Matlock, 2015; Koetsier & Cantor, 2019). Under ideal conditions, A260/A230 ratio values in the range of 2.0–2.2 for DNA/RNA samples are considered indicative of adequate purity (Matlock, 2015; Lucena-Aguilar *et al*., 2016).

After these steps, the amplification of the extracted DNA by polymerase chain reaction (PCR) is generally carried out for most of the techniques.

1.4.2.3 Polymerase Chain Reaction (PCR) and Real-Time PCR

PCR is a central technique in molecular biology, allowing for the targeted amplification of specific DNA regions from even trace amounts of genetic material. Since its development in the 1980s, PCR has been instrumental in advancing areas such as medical diagnostics, forensic analysis, biotechnology, and the authentication of biological ingredients. Within the context of verifying plant species in dietary supplements or traditional herbal medicinal products, both being products often subjected to high levels of processing, PCR provides a highly sensitive method for identifying botanical DNA, even when morphological features are no longer present (Mullis & Faloona, 1987; Saiki *et al*., 1988).

The reaction is based on thermal cycling that separates the DNA strands (denaturation), allows primers to bind to complementary regions (annealing), and enables enzymatic synthesis of new strands (extension). For the reaction to proceed effectively, a carefully formulated mixture is used, containing ultrapure water, reaction buffer, magnesium chloride ($MgCl_2$), deoxyribonucleotide triphosphates (dNTPs), synthetic

oligonucleotide primers, and a thermostable DNA polymerase such as Taq polymerase. Details on the PCR components are described as follows:

Reaction buffer – The buffer maintains the optimal pH and provides suitable conditions for enzymatic activity. Its composition may vary but typically includes salts that stabilize the enzyme and promote efficient primer binding (Green & Sambrook, 2012).

Magnesium chloride ($MgCl_2$) – Magnesium ions (Mg^{2+}) are essential cofactors for DNA polymerase activity and also to form soluble complexes with dNTPs, facilitating their incorporation into the growing DNA strand. Magnesium concentration is critical: low levels reduce enzyme activity, while excessive concentration may reduce specificity and promote nonspecific amplification (Lorenz, 2012).

Primers (oligonucleotide initiators) – Primers are the most influential elements in determining the specificity of the PCR. These are short, single-stranded DNA sequences – typically between 18 and 25 nucleotides – designed to bind flanking regions of the target sequence. Designing primers for plant authentication involves identifying species-specific genetic markers. Commonly targeted DNA regions include chloroplast genes such as *rbcL* and *matK*, as well as nuclear ribosomal spacers like ITS1 and ITS2. These sequences are widely accepted in the DNA barcoding community due to their variability among species and conservation within species (Hollingsworth *et al*., 2009). Reference sequences are usually retrieved from curated databases such as GenBank or the Barcode of Life Data System (BOLD).

Once a target region is selected, bioinformatics tools like Primer3 and BLAST are employed to generate candidate primers. These are evaluated based on length, GC content, melting temperature (Tm), and the absence of secondary structures such as hairpins and primer-dimers (Untergasser *et al*., 2012). Importantly, specificity is verified *in silico* through alignment searches (e.g., using BLAST) to ensure the primers do not hybridize with non-target taxa. Additionally, for degraded DNA, which is common in highly processed supplements, primers are optimized to amplify short fragments between 100 and 300 base pairs, increasing the likelihood of successful amplification (Chen *et al*., 2022).

After computational validation, primers undergo preliminary testing in the laboratory to ensure the absence of cross-reactivity against other non-target species.

dNTPs (deoxyribonucleotide triphosphates) – These are the building blocks used by DNA polymerase to synthesize the new DNA strand. An equimolar balance of dATP, dTTP, dCTP, and dGTP is essential to ensure replication fidelity and avoid base misincorporation (Innis *et al*., 1990).

Thermostable DNA polymerase – The most commonly used enzyme is Taq polymerase, derived from the thermophilic bacterium *Thermus aquaticus*, due to its ability to withstand high temperatures. It extends the primers in the 5′→3′ direction, with an optimal working temperature of approximately 72–75 °C (Terpe, 2013). Although Taq is widely used, it has a relatively high error rate. For high-fidelity applications, alternative enzymes that possess proofreading (3′→5′ exonuclease) activity are preferred (Chien, Edgar & Trela 1976; Saiki *et al*., 1988).

PCR typically involves 30 to 40 thermal cycles composed of three main steps. In the denaturation step, the temperature is raised to approximately 95 °C to break hydrogen bonds between DNA strands, exposing the template for primer binding (Brown, 2020). During annealing, the temperature is reduced to 50–65 °C to allow primers to hybridize with their complementary sequences. The exact annealing temperature depends on the primers' Tm and must be optimized to promote primer-template binding over re-annealing of the template strands. In the extension step, the reaction is held at 72 °C, the optimal temperature for Taq polymerase activity, to synthesize the new DNA strand by incorporating dNTPs (Terpe, 2013). Within a few hours, this exponential amplification process can produce millions of copies of the target DNA sequence. The speed, sensitivity, and specificity of PCR make it an indispensable tool in molecular biology, particularly in applications requiring accurate detection of plant ingredients in processed products.

The amplified PCR products are commonly analyzed using agarose gel electrophoresis, which allows for the visualization and size confirmation of the DNA fragments. This method represents one of the most straightforward approaches for detecting the presence of a specific plant species in a sample. Alternatively, more refined techniques such as DNA sequencing may be employed to verify the identity of the amplicon, providing higher resolution and reliability when confirming species-level identification (Mafra, Ferreira, & Oliveira, 2008).

Real-time PCR, also known as quantitative PCR (qPCR), represents an evolution of conventional PCR as it allows for the monitoring of DNA amplification in real time with high sensitivity and precision. This technique combines the basic principles of PCR with continuous detection of the fluorescent signal generated during DNA synthesis, eliminating the need for post-PCR analyses such as gel electrophoresis (Kubista *et al*., 2006).

Fluorescence detection can be achieved through specific fluorophore-labeled probes, such as TaqMan probes, or through non-specific intercalating dyes like SYBR Green I or Evagreen, which bind to double-stranded DNA. The increase in fluorescent signal is proportional to the amount of DNA amplified, enabling both detection and quantification of the genetic material present in the sample (Heid *et al*., 1996; Arya *et al*., 2005). Each amplification cycle is accompanied by fluorescence measurement, which allows for the construction of an amplification curve. The point at which fluorescence crosses the detection threshold, known as the cycle threshold (Ct) value, is inversely proportional to the initial quantity of target DNA in the reaction. Thus, a lower Ct value indicates a higher initial DNA concentration (Bustin *et al*., 2009).

In qualitative analysis, qPCR is widely used to confirm the presence or absence of a target species in a sample, which is particularly useful for authenticating botanical ingredients in processed products. Additionally, its high specificity can be enhanced by using primers and probes designed for conserved and diagnostic regions of a specific plant species, thereby reducing false positives or non-specific amplification (Newmaster *et al*., 2019). Furthermore, its sensitivity allows the detection of trace amounts of genetic material, which is essential for identifying species substitutions or cross-contamination (Wu & Shaw, 2022).

Another relevant advantage of real-time PCR is the possibility of multiplex analysis, that is, the simultaneous detection of multiple genetic targets in a single reaction. This approach is especially efficient in the analysis of multicomponent supplements where the presence of several plant species must be verified (Bustin & Nolan, 2004).

Finally, qPCR can also be combined with High Resolution Melting (HRM), a post-PCR technique that analyzes the melting profile of the amplified DNA. This strategy enables the detection of small nucleotide variations, which can be useful for distinguishing closely related species or identifying genetic variants (Reed *et al*., 2007).

1.4.2.4 DNA Sequencing and Next-Generation Sequencing (NGS)

The process that determines the sequence of nucleotides in a DNA fragment is termed DNA sequencing (Watson *et al*., 2013). The first technique developed for this purpose is known as the Sanger method. This methodology uses modified nucleotides, called dideoxynucleotides (ddNTPs), to deliberately interrupt DNA synthesis. ddNTPs are labeled with radiation or fluorescence and lack the 3'-hydroxyl (3'-OH) group, which is the necessary site for the next nucleotide to attach to the strand. Thus, when ddNTPs are incorporated into the chain, elongation is interrupted, resulting in a defined DNA fragment (Sanger, Niclen & Cousons, 1977).

In *in vitro* replication, DNA fragments of varying lengths ending in specific bases are generated. These fragments can be separated by high-resolution gel electrophoresis due to their differences in length and terminal bases. The sequence is then read either through autoradiography (when ddNTPs are radioactively labeled) or through an electropherogram in the case of fluorescent ddNTPs (Alberts *et al*., 2017).

However, this technique is limited in throughput and scalability, as it sequences only one molecule at a time (Mardis, 2008). To address the market need for a technique capable of large-scale sequencing, the method known as Next-Generation Sequencing (NGS) was developed.

NGS is based on the addition of adapters that allow attachment to the sequencing surface, clonal amplification, and subsequent sequencing. This enables massive and parallel sequencing of genetic material, with greater speed, scalability, and cost reduction (Voelkerding, Dames & Durtschi, 2010; Linnarsson, 2010). The classical NGS workflow consists of: (I) extraction and fragmentation of the genetic material; (II) library preparation with adapter insertion; (III) clonal amplification of the fragments; (IV) sequencing; and (V) bioinformatic analysis, which includes sequence assembly, alignment, variant detection, and functional analysis (Goodwin, McPherson & McCombie, 2016). Each bioinformatics platform differs in read length, throughput, and error profile; therefore, the choice of technology depends on the specific goals of each study (Goodwin, McPherson & McCombie, 2016).

NGS has wide applicability and, in plant authentication, it is used to identify species through DNA barcodes sequencing. The use of metabarcoding, which combines barcoding and large-scale sequencing, allows the analysis of complex mixtures such as herbal products, detecting adulterants or contaminants (Raclariu *et al*., 2017).

1.5 Objectives

Given the relevance of *P. glomerata* as a medicinal plant that presents problems of adulteration with contaminating species, and considering the potential of DNA-based techniques to perform plant authentication - the objectives of the work were:

- Develop specific primers for *P. glomerata,* through analysis of available genomic sequences and *in silico* assessment of specificity;
- Validate the primers's specificity by analyzing the optimal amplification conditions, and by performing PCR cross-reactivity tests with other medicinal plants and known historical adulterants of *P. glomerata*;
- Develop a real-time PCR technique and confirm the primers's analytical sensitivity;
- Establish a quantitative detection limit for *P. glomerata* in mixtures with potential adulterants using real-time PCR and the ΔCq method (difference between the quantification cycles of the species under study with an endogenous reference control);
- Apply the validated primers and methods to test the authenticity of commercial products labeled as *P. glomerata* or in other similar species. Compare the molecular results with the information declared on the packaging to identiy discrepancies and potential adulteration.

2 MATERIAL AND METHODS

2.1 Plant Species, Commercial Samples and Sample Preparation

A total of 26 samples were analyzed in this study, consisting of 25 commercial samples and one sample supplied by the Universidade Tecnológica Federal do Paraná (UTFPR), Brasil, which was authenticated by ITS2 metagenomics and used as a positive control (Table 1: C40). The sample set included the target species of *Pfaffia glomerata* and the adulterant species, namely *Pfaffia paniculata*, *Eleutherococcus senticosus* (Siberian ginseng), *Panax ginseng* (Asian ginseng) and *Withania somnifera* (Indian ginseng). The plant samples were purchased from herbal stores, e-commerce platforms, and natural product shops in the form of raw material, (fragmented roots/straws, root powder, leaves, barks) and processed products (herbal infusions, powders, and gelatin capsule supplements). Visual documentation of the original packaging, including product labels and sample characteristics, is provided in Appendix I.

Table 1 - List of the 25 commercial plant samples used in this study.

Internal ID	Label information	Origin	Sample Form
C15	*Panax ginseng*	Commercial sample - Bulgaria - Healthy life	Root powder
C16	*Pfaffia paniculata*	Commercial sample - Bulgaria - Healthy life	Root/Straw
C17	*Eleutherococcus senticosus*	Commercial sample - Bulgaria - Healthy life	Root/Straw
C18	*Withania somnifera*	Commercial Sample - Jordan	Root/Leaves/Seeds
C19	*Withania somnifera*	Commercial sample - Bulgaria - Healthy life	Root powder
C20	*Withania somnifera*	Commercial sample - Portugal - Iswari	Root powder
C21	*Eleutherococcus senticosus*	Commercial sample - Portugal - Iswari	Root powder
C22	*Panax ginseng*	Commercial sample - Brazil - local health food store	Powder
C23	*Pfaffia paniculata*	Commercial sample - Brazil - BioVittare	Powder capsule
C24	*Panax ginseng*	Commercial sample - Brazil - Gerovital	Gelatin capsule
C25	*Panax ginseng C. A. Mey*	Commercial sample - Brazil - Gerilon	Gelatin capsule
C26	*Panax ginseng C. A. Mey*	Commercial sample - Brazil - NatusGerin	Gelatin capsule

Table 1 (continued)

Internal ID	Label information	Origin	Sample Form
C27	*Pfaffia glomerata*	Commercial sample - Brazil - Chamel	Root/Straw
C28	*Pfaffia paniculata*	Commercial sample - Brazil - Mercadão Natural	Straw
C29	*Pfaffia paniculata*	Commercial sample - Brazil - Mercadão Natural	Powder
C30	*Pfaffia glomerata*	Commercial sample - Brazil - Chamel	Root powder
C31	*Pfaffia glomerata*	Commercial sample - Brazil - Chamel	Root/Straw
C32	*Pfaffia glomerata*	Commercial sample - Brazil - Fitoessência	Straw
C33	*Pfaffia iresinoides*	Commercial sample - Brazil - Flora Saúde Comércio de Produtos Naturais	Straw/infusion
C34	*Pfaffia paniculata*	Commercial sample - Brazil - Empório Natural Mix	Powder
C35	*Brazilian ginseng*	Commercial sample - Brazil - Malagueta Produtos Naturais	Powder
C36	*Pfaffia infusion*	Commercial sample - Brazil - Malagueta Produtos Naturais	Bark
C37	*Panax ginseng*	Commercial sample - Brazil - Malagueta Natural Products	Bark
C38	*Pfaffia paniculata*	Commercial sample - Bulgaria - Healthy life	Straw
C39	*Eleutherococcus senticosus*	Commercial sample - Bulgaria - Healthy life	Dry root/Straw
C40	*Pfaffia glomerata*	UTFPR authenticated sample	Leaves

Plant materials, including roots, leaves, barks, straws, and herbal infusions, were ground using a Moulinex grinder. To prevent cross-contamination between samples, the equipment was thoroughly washed with water and soap, followed by a five-minute soak in sodium hypochlorite (NaOCl) solution, rinsing with distilled water, and oven-drying. For the capsule samples, the powder capsule (C23) was opened using previously sterilized tools, and its content was collected. For the gelatin capsules (C24, C25 and C26), 1 mL of each sample was extracted and mixed with 1 mL of ethanol. The mixture was then centrifuged at 11000 x g for 20 minutes to achieve phase separation, after which the supernatant was discarded to isolate the solid phase.

To confirm the authenticity of the *P. glomerata* sample supplied as authentic (C40), DNA was extracted as described in 2.2, diluted to 10 ng/μL and submitted to PCR reaction for amplification of ITS2 region using the primers ITS-S2F (Chen *et al.*, 2010) and ITS-S4R (White, 1990) modified to contain the overhang adapters (in bold) proposed

by Illumina (Illumina, 2025), namely ITS-S2F-adp1 5'-**TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG**ATGCGATACTTGGTGTGAAT-3′ and ITS-S4R-adp1 5′-**GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG**TCCTCCGCTTATTGATATGC-3′. The PCR was carried out as described by Quaresma *et al*. (2021). The obtained amplicons were sent to the Centre for Molecular Analyses of the Research Centre in Biodiversity and Genetic Resources (CIBIO, Vairão, Portugal), where the second-stage PCR was performed to incorporate the custom-made unique indexes developed by CIBIO. The obtained indexed products were purified, quantified, pooled and submitted to next generation sequencing on the Illumina MiSeq using the 2×250 cycles v2 nano chemistry, according to the manufacturer's instructions.

For method validation purposes, binary mixtures were prepared using plant material of sample C40 authenticated as *P. glomerata* by ITS2 metabarcoding and *W. somnifera*. Sample C20, used as *W.* somnifera was also submitted to ITS2 metabarcoding, confirming its authenticity.

2.2 DNA Extraction and Quality Assessment

Total genomic DNA was extracted from 100 mg of plant materials, including roots, leaves, seeds, barks, straws, and herbal infusions dried or in powder, using the NucleoSpin Plant II kit (Macherey-Nagel, Düren, Germany), following the manufacturer's instructions with some changes as previously described by Costa *et al* (2016).

For the capsule samples (Table 1), DNA was extracted using the NucleoSpin Soil kit (Macherey-Nagel, Düren, Germany), also according to the supplier's protocol. This kit was chosen for the capsule samples instead of the NucleoSpin Plant II because it contains a specific inhibitor removal system based on specialized columns and includes the Enhancer SX additive, which aids in DNA purification. Moreover, it combines mechanical and chemical lysis making it more suitable for compacted and highly processed matrices.

The yield and purity of DNA extracts were assessed by UV spectrophotometry on a SPECTROstar® Nano microplate reader (BMG Labtech, Offenburg, Germany) with an LVis plate accessory. Absorbance was measured at 260, 280, and 230 nm to estimate DNA content and purity using the Multi-user Reader Control and MARS Data Analysis

Software (LVis) (BMG Labtech, Offenburg, Germany). After quality assessment, all extracts were stored at -20 °C until further analysis.

Following DNA extraction and normalization, PCR assays were performed to verify DNA integrity and suitability for downstream analysis. Thus, to ensure the presence of amplifiable DNA, DNA extracts were submitted to PCR targeting a conserved 18S rRNA nuclear region using a universal eukaryotic primer pair EG-F and EG-R, as previously described by Villa *et al* (2017). PCR assays were carried out in a total reaction volume of 10 µL, containing 1 µL (10 ng) of DNA extract, 5 µL of OneTaq 2X Master Mix with Standard Buffer (New Englan Biolabs Ltd.), 0.2 micromolar µM of each primer EG-F/EG-R, and 3.6 µL of nuclease-free water to complete the total volume. The reactions were performed on a T100 thermocycler (Bio-Rad Laboratories, USA) with the following program: initial denaturation at 95 °C for 5 minutes (min); 37 cycles of amplification at 95 °C for 30 seconds (s), 55 °C for 30 s, and 72 °C for 30 s; final extension at 72 °C for 5 minutes.

PCR products were verified with the 100 bp DNA Ladder ready to use (Bioron, Frankfurt, Germany) by electrophoresis in a 1% agarose gel made with 1× TAE buffer stained with GelRed® (Biotium, Fremont, CA, USA) and run at 90 volts (V) for 1 h. After electrophoresis, all the gels were visualized under a UV light tray Gel Doc™ EZ System (Bio-Rad Laboratories, Hercules, CA, USA) and a digital image was recorded using the Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.3 Primers Design for *Pfaffia glomerata* Identification

The species-specific primers used for *P. glomerata* identification were designed based on sequences from the internal transcribed spacer 1 (ITS1) region. ITS1 sequences of *P. glomerata* available in the NCBI GenBank database, were aligned and analyzed identified using BioEdit v7. 2.5 (Ibis Biosciensces, Carlsbad, CA, USA) to identify conserved yet polymorphic regions.

The specificity of the selected primer pair was evaluated *in silico* using the NCBI Primer-BLAST online tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) to detect potential cross-reactivity with other plant species. Additionally, the primers were checked for secondary structures, such as, primer dimers and hairpins, using OligoAnalyser software (https://www.idtdna.com/calc/analyzer).

The selected primer pair PFA3-F (5'-GGAGGTGTTGGCTCGTTCAG-3') and PFA3-R (5'-CAGGCACGAATGGGCATACT-3') amplifies a 132 bp fragment (Table 2) ideal for real-time PCR of possibly degraded samples due to its short length, which enhances amplification from processed or degraded DNA samples while preserving discriminatory power among related species. The GC content of the forward and reverse primers was 60% and 55%, respectively, with melting temperatures (Tm) of 60.95 °C and 60.46 °C, indicating thermodynamic compatibility and ensuring efficient and specific annealing.

These parameters align with established primer design principles, which recommend a GC content between 40 % - 60 % and a Tm between 50 - 65 °C, a maximum Tm difference of 5 °C to optimize specificity and minimize secondary structure formation (Dieffenbach, Lowe, & Dveksler, 1993; Feeney, Murphy, & Lopilato, 2014; Bio-Rad Laboratories, 2025). The primers were synthesized and purified by HPLC by STAB VIDA Inc. (Portugal).

Table 2 - Description of the primers selected in this study, targeting polymorphic sites in the ITS1 region of *P. glomerata.*

PFA3	Sequence (5'->3')	Primer Length (nt)	Tm (ºC)	GC%	Amplicon Size (bp)
Forward primer	GGAGGTGTTGGCTCGTTCAG	20	60.95	60	132
Reverse primer	CAGGCACGAATGGGCATACT	20	60.46	55	

2.4 Conventional PCR Assay Optimization

A series of qualitative PCR was performed on a T100 thermocycler (Bio-Rad Laboratories, USA), using serial dilutions of of *P. glomerata* DNA template and different annealing temperatures (58ºC, 60ºC, 62ºC and 65ºC) to determine the optimal conditions for effective amplification. After optimization, all PCR reactions were performed using 1 µL (10 ng) of DNA extract, 5 µL of OneTaq 2X MasterMix with Standard Buffer (New England Biolabs Ltd.), 0.2 µM of each primer PFA3-F and PFA3-R, and the remaining of nuclease-free water to perform a total reaction volume of 10 µL. The reactions were performed with the following program: initial denaturation at 94 °C for 3 min; 35 cycles of amplification at 94 °C for 30 s, annealing for 30 s at 58ºC, 60ºC, 62ºC or 65ºC, and 68 °C for 30 s; final extension at 68 °C for 5 min.

2.4.1 Assessment of Primers Specificity

Following the optimization of conventional PCR, the designed primers for *Pfaffia glomerata* (PFA3-F and PFA3-R) were evaluated for specificity, as predicted by *in silico* analysis. The primers were tested for cross-reactivity against 51 medicinal plant samples. These included known common adulterants of *P. glomerata*, such as *Withania somnifera*, *Eleutherococcus senticosus*, *Panax ginseng*, as well as 48 additional medicinal plant samples traditionally used for brain activity (cognition, mood, anxiety, etc).

For this purpose, DNA mixtures were prepared by combining the DNA extracts from each species to a final concentration of 5 ng/µL per extract. The list of the 51 medicinal plants samples and the corresponding mixtures is presented in Table 3.

Table 3 - List of the 51 medicinal plants samples (previously authenticated through ITS2, matK and/or rbcL sequencing) used in the cross-reactivity tests.

Mixtures	Internal ID	Commom name	Species
P1	P01	St. John's Wort (Gêres)	*Hypericum androsaemum*
	P02	Condurango vine	*Marsdenia condurango*
	P03	Passiflora	*Passiflora incarnata*
	P04	Lemon balm	*Melissa officinalis*
	P05	Mugwort	*Artemisia vulgaris*
P2	P06	Jasmine	*Jasminum grandiflorum*
	P07	Chamomile	*Matricaria chamomilla*
	P08	Cipress	*Cupressus sempervirens*
	P09	Virginia snakeroot	*Aristolochia esperanzae*
	P10	Brazilian pepper tree	*Pistacia Lentiscus*
P3	P11	Aristolochia (Dutchman's pipe)	*Aristolochia esperanzae*
	P12	Ginseng siberiano	*Eleutherococcus senticosus*
	P13	Motherwort	*Leonurus cardiaca*
	P14	Periwinkle	*Vinca minor*
	P15	Calamint	*Calamintha nepeta*
P4	P16	Rosemary	*Rosmarinus officinalis*
	P17	Erva Moura	*Solanum nigrum*
	P18	Cidreira	*Aloysia citrodora*
	P19	Fennel	*Foeninculum vulgare*
	P20	Espinheiro Alvar	*Crataegus monogyna*
P5	P21	Gerivão / Verbena	*Verbena officinalis*
	P22	Ginkgo Biloba	*Ginkgo biloba*
	P23	Field horsetail	*Equisetum arvense*
	P24	Hipericão Kneip	*Hypericum androsaemum*

Table 3 (continued)

Mixtures	Internal ID	Commom name	Species
P5	P25	Bistorta	*Persicaria bistorta*
	P26	Valeriana	*Valeriana officinalis*
P6	P27	Angelica	*Angelica archangelica*
	P28	White ginseng	*Panax ginseng*
	P29	Guaraná	*Paullinia cupana*
	P30	Noz de Kola	*Cola nitida*
	P31	Siberian ginseng	*Ginkgo Biloba*
P7	P32	Yerba mate	*Eleutherococcus senticosus*
	P33	Rooibos Relax Anti-Stress	*Ilex paraguariensis*
	P34	Tisana Ginseng e Vitalidade	*Aspalathus linearis*
	P35	Hipericão Premium	*Panax ginseng/Eleutherococcus senticosus*
	P36	Passiflora Premium	*Hypericum perforatum*
	P37	Moringa Oleifera	*Passiflora incarnata*
P8	P38	Marapuama	*Moringa oleifera*
	P39	Tisana Bom Sono	*Ptychopetalum olacoides*
	P40	Mulungu	*Melissa officinalis/Valeriana officinalis/Passiflora incarnata*
	P41	Insulina Vegetal	*Erythrina mulungu*
	P42	Bitter orange	*Myrcia citrifolia*
P9	P43	Linden	*Citrus aurantium*
	P44	Centella Asiatica Gotu Kola	*Tilia cordata*
	P45	Lemongrass	*Centella asiatica*
	P46	Tisana Calmante	*Cymbopogon citratus*
	P47	Lemon tree	*Melissa officinalis/Valeriana officinalis/Passiflora incarnata*
P10	P48	Nervcalmflora	*Citrus limon*
	P49	Bom Sono	*Melissa officinalis/Valeriana officinalis/Passiflora incarnata*
	P50	Calamint	*Melissa officinalis/Valeriana officinalis/Passiflora incarnata*
	P51	Japanese pagoda tree	*Calamintha nepeta*

2.4.2 Electrophoresis of Qualitative PCR Products

PCR products from the optimization reactions were analyzed by electrophoresis on a 1% agarose gel prepared with 1X TAE stained with GelRed (Biotium, Fremont, CA, USA) and run at 90 V for 1 h. Subsequent PCR assays were analyzed using 2% agarose gels prepared under the same conditions. In both cases, a 50 bp DNA Ladder ready-to use (Bioron, Frankfurt, Germany) was used as a molecular standard for comparing molecular size. Following electrophoresis, all gels were visualized using a UV light tray Gel Doc™

EZ System (Bio-Rad Laboratories, Hercules, CA, USA), with digital images captured using Image Lab software version 5.2.1 (Bio-Rad Laboratories, Hercules, CA, USA).

2.5 Real-Time PCR Assay Optimization for Assessment of Primers Sensitivity

After demonstrating the suitability of the designed primers (PFA3-F and PFA3-R) for the specific identification of *P. glomerata* DNA using conventional PCR, a real-time PCR assay was developed using the EvaGreen® dye. This assay aimed to determine the analytical sensitivity, defined as the lowest concentration of pure *P. glomerata* genomic DNA that could be reliably detected and amplified by the primer pair.

To establish the limit of detection (LOD), a calibration curve was constructed, preparing a 10-fold serial dilution series of *P. glomerata* DNA, ranging from 10 ng to 0.01 picogram (pg), across seven concentration levels: 10 ng; 1 ng; 100 pg; 10 pg; 1 pg; 0.1 pg; 0.01 pg.

The real-time PCR reactions were carried out in 10 µL of total reaction volume, containing 1 µL of DNA (20 ng), 1x of SsoFast® Evagreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 200 nmol/L of each primer (PFA3-F/PFA3-R), using four replicates. The reactions were performed on fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the following conditions: 98 °C for 5 min; 40 cycles at 98 °C for 5 s and at 62 °C for 5 s, with collection of fluorescence signal at the end of each cycle. For melting curve analysis, the PCR products were denaturated at 98 ºC for 1 min and then annealed at 62 ºC for 5 min. After, the temperature was increased from 62 ºC to 95 ºC with increments of 0.2 ºC every 5 s, with fluorescence data acquired at the end of each melting temperature. Data acquisition was performed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). Each sample was analyzed in triplicate.

2.6 Development of a qPCR method for *Pfaffia glomerata* Detection in Herbal Products

To establish a quantitative model for detecting *P. glomerata* in plant-based herbal products, a normalized real-time PCR assay was developed using the ΔCq method. This approach accounts for variations in DNA recovery and quality across different extracts, which are common in processed botanical matrices (Villa *et al*., 2018; López-Andreo *et*

al., 2005; Soares *et al*., 2014; Costa *et al*., 2017). The method involved constructing a normalized calibration curve by comparing quantification cycle (Cq) values of the target region (IT2) with those of a nuclear endogenous reference gene (18S rRNA), using the formula ΔCq = Cq (*Pfaffia glomerata*) − Cq (reference gene).

The calibration curve was generated by plotting ΔCq values against the logarithm of the *P. glomerata* concentration in binary mixtures with *W. somnifera*, prepared at known proportions. The choice of *W. somnifera* as the background plant matrix was based on its frequent inclusion in herbal formulations and supplements, making it a relevant and realistic model for assessing assay performance.

For method development, model mixtures using known amounts of *P. glomerata* (C40) in *W. somnifera* (C20), with concentrations of 50%, 10%, 5%, 1%, 0.5%, and 0.1% (w/w). Firstly, a reference mixture with 50% of *P. glomerata* was prepared by adding 300 mg of *P. glomerata* to 300 mg of *W. somnifera*. All the subsequent mixtures were prepared by sequential additions of *W. somnifera* plant material up to the level of 0.1% (w/w). For method validation, blind mixtures were independently prepared as described for the reference mixtures, with the proportion of 20%, 8% and 2% (w/w) of *P. glomerata in W. somnifera* plant material and were further analyzed as unknown samples.

The real-time PCR assays were carried out in 10 µL of total reaction volume, containing 1 µL of DNA of the known amounts, 1x of SsoFast® Evagreen® Supermix (Bio-Rad Laboratories, Hercules, CA, USA) and 200 nmol/L of each primer (PFA3-F/PFA3-R), using four replicates. The reactions were performed on fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) using the following conditions: 98 °C for 5 min; 40 cycles at 98 °C for 5 s and at 65 °C for 5 s, with collection of fluorescence signal at the end of each cycle. Data were processed using the software Bio-Rad CFX Manager 3.1 (Bio-Rad Laboratories, Hercules, CA, USA). The normalized curve, mean ΔCq, absolute errors, and estimated percentage of *P. glomerata* were calculated using Microsoft Excel (Microsoft Corporation, Version 2013).

2.6.1 Application and validation of the qPCR method against commercial products

After the development and validation of the primers, as well as the establishment of a quantitative model for *P. glomerata*, conventional PCR and qPCR were performed

on the commercial samples (each analyzed in triplicate) to evaluate the applicability of the methods. All assay parameters followed those previously described during method validation, with the exception of the annealing temperature, where 65°C was chosen - both in conventional PCR and in qPCR as it was found that this temperature amplifies with greater clarity and specificity. Calculations of mean Cq values, ΔCq, $\log_{10}(\%)$, and the estimated percentage of *P. glomerata* DNA were performed using Microsoft Excel (Microsoft Corporation, Version 2013).

3 RESULTS AND DISCUSSION

3.1 Evaluation of DNA extracts

Following the DNA extraction procedures described in Section 2.2, the resulting extracts were evaluated for concentration, purity, and quality. Table 4 presents average DNA concentrations for each extract and respective purity value ratios.

Table 4 – DNA concentration and purity ratios of commercial samples extracted by using the Nucleospin Plant II kit.

Internal ID	Labelled information	Sample Form	Concentration (ng/ µL)	Ratio (260/280)	Ratio (260/230)
C15	*Panax ginseng*	Root powder	16.42	1.6	0.7
C16	*Pfaffia paniculata*	Straw	24.54	1.8	1.8
C17	*Eleutherococcus senticosus*	Root/Straw	20.07	1.6	0.9
C18	*Withania somnifera*	Root/Leaves/Seeds	146.18	1.8	2.3
C19	*Withania somnifera*	Root powder	10.23	2.1	1.2
C20	*Withania somnifera*	Root powder	92.73	1.8	2.0
C21	*Eleutherococcus senticosus*	Root powder	7.17	1.3	0.6
C22	*Panax ginseng*	Powder	16.83	1.9	1.5
C23	*Pfaffia paniculata*	Powder capsule	3.56	1.9	0.5
C24	*Panax ginseng*	Gelatin capsule	Undet.	-	-
C25	*Panax ginseng C. A. Mey*	Gelatin capsule	Undet.	-	-
C26	*Panax ginseng C. A. Mey*	Gelatin capsule	Undet.	-	-
C27	*Pfaffia glomerata*	Root/Straw	34.36	1.8	1.8
C28	*Pfaffia paniculata*	Straw	10.62	1.9	1.2
C29	*Pfaffia paniculata*	Powder	25.28	1.7	2.0
C30	*Pfaffia glomerata*	Root powder	10.80	1.9	1.6
C31	*Pfaffia glomerata*	Root/Straw	7.38	2.1	1.0
C32	*Pfaffia glomerata*	Straw	6.63	2.2	1.3

Table 4 (continued)

Internal ID	Labelled information	Sample Form	Concentration (ng/ µL)	Ratio (260/280)	Ratio (260/230)
C33	*Pfaffia iresinoides*	Straw/Infusion	4.16	3.2	1.7
C34	*Pfaffia paniculata*	Powder	20.16	1.9	2.0
C35	*Pfaffia paniculata*	Powder	44.58	1.7	2.0
C36	*Pfaffia paniculata*	Bark	5.05	1.7	0.6
C37	*Panax ginseng*	Bark	4.33	2.5	0.6
C38	*Pfaffia paniculata*	Straw	9.16	1.9	1.7
C39	*Eleutherococcus senticosus*	Dry root/Straw	1.82	3.3	0.7
C40	*Pfaffia glomerata*	DNA extract (positive control)	51.35	1.8	1.8

Undet = undetermined

Table 4 shows that DNA concentration of the extracts ranged from 1.82 to 146.2 ng/µL and for most extracts the purity ranged from 1.6 to 2.0. The DNA concentration of samples C24, C25 and C26, all being gelatin capsules, was undetermined as the spectrophotometric readings for these samples were inconsistent. Although DNA was detectable in some replicates, the values varied and failed to to be reproducible across measurements.

Several readings were performed on these samples to correct possible experimental errors, but the lack of reproducibility persisted, which suggests only a trace presence of DNA. The challenge in quantifying these samples persisted even for the extracts obtained by using the commercial kit NucleoSpin Soil, which is specifically designed for complex samples. The pharmaceutical formulation of these samples appears to be the primary limiting factor, as supported by previous studies demonstrating that herbal capsule excipients may significantly impair DNA recovery efficiency (Costa *et al.*, 2015; Abdel-Latif & Osman, 2017).

Quality assessment revealed that samples C21, C33, C37 and C39 exhibited purity values ratios (260/280 and 260/230) outside the optimal range, suggesting the potential presence of proteins or polysaccharides. The DNA extracts from the remaining samples yielded sufficient concentration and qualityfor subsequent analysis.

To validate the DNA extraction efficiency, PCR amplification using universal eukaryotic primers (EG-F and EG-R) was performed. This assay confirmed the presence of amplifiable DNA in most samples, including those with inconsistent or unexpected spectrophotometric readings, except for samples C25 and C26, which showed no amplification. The bands observed in the electrophoresis (Figure 1) indicate successful amplification of intact DNA, with no evidence of carryover.

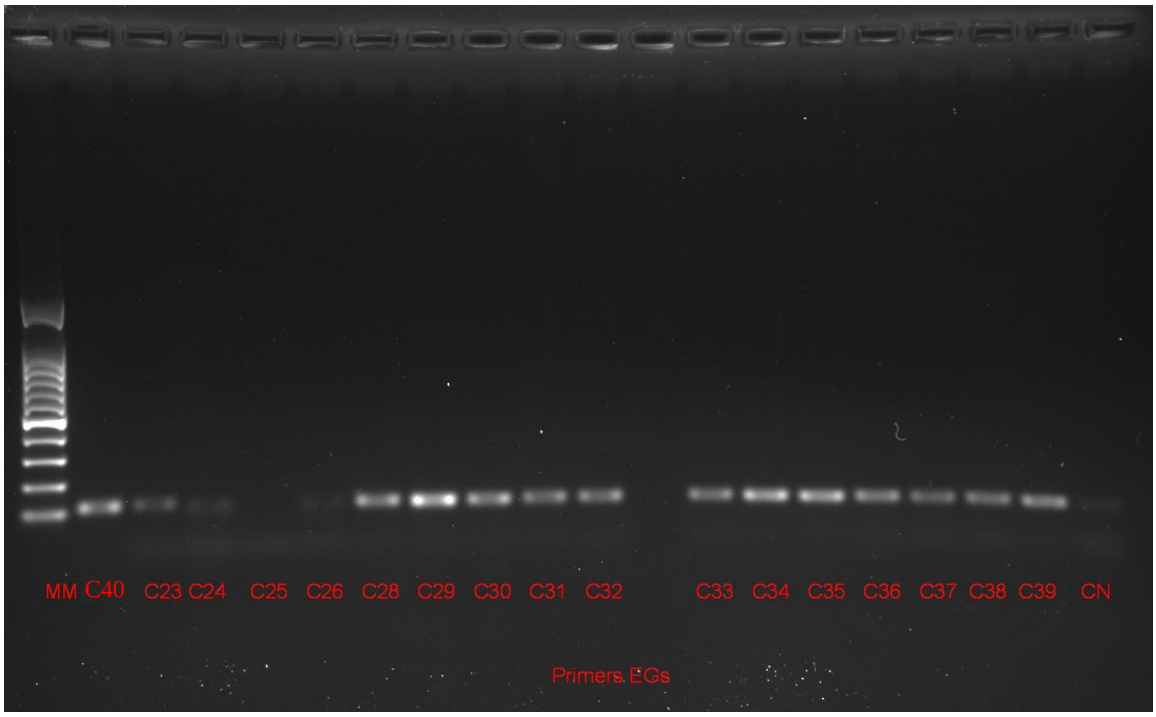
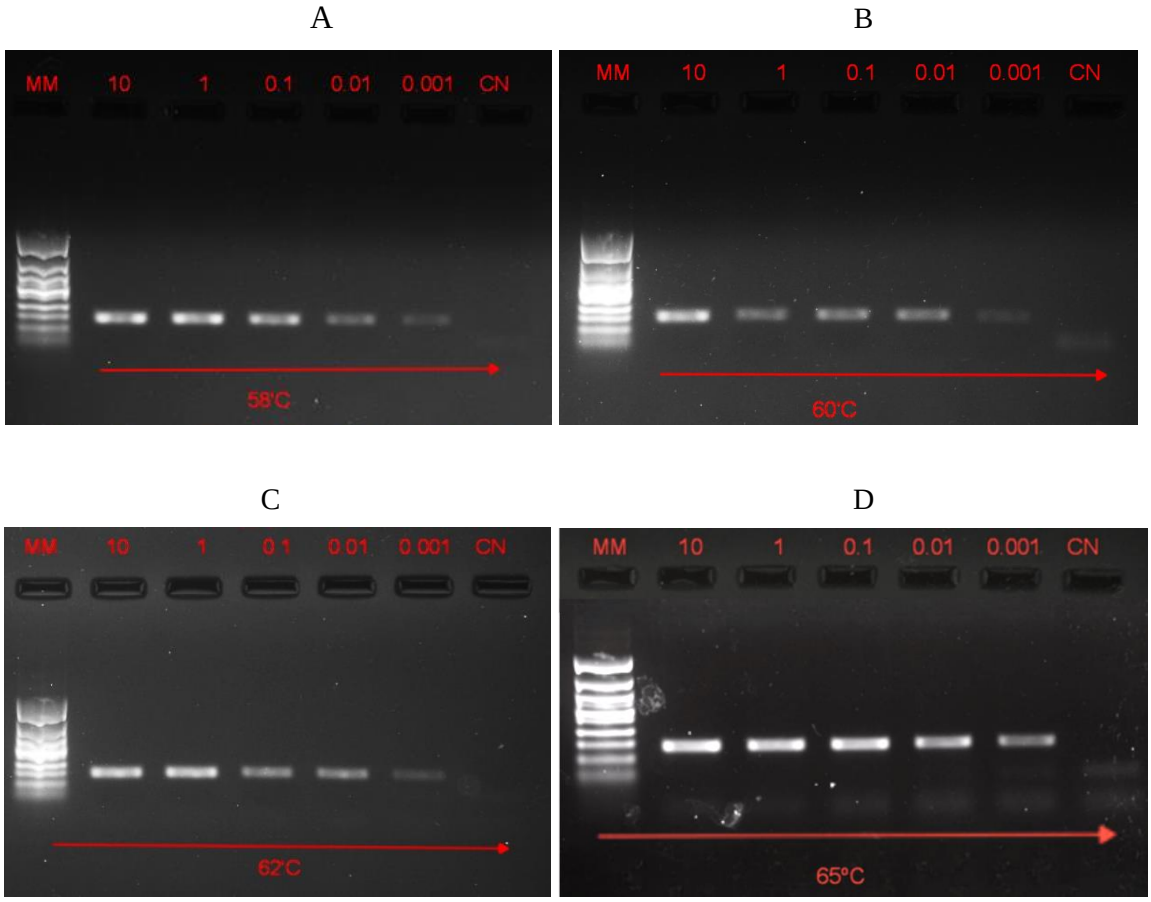


Figure 1 - Agarose gel electrophoresis of PCR products using primers EG-F and EG-R, C40: *P. glomerata* (positive control); C23-C39: genomic DNA extracted from commercial samples; MM: 100 bp DNA ladder marker; CN: negative control.

3.2 Conventional PCR Assay Optimization

The trials to assess the performance of the designed primers (PFA3-F and PFA3-R) were conducted by testing different annealing temperatures, namely 58ºC, 60ºC, 62ºC and 65ºC, and evaluating their robustness using serial dilutions of *P. glomerata* DNA to determine the optimal temperature that provided the lowest limit of detection (Figures 2).



Figures 2 – Results of PCR products at different annealing temperatures: **A:** 58 ºC, **B:** 60 ºC, **C:** 62 ºC and **D:** 65 ºC using serial dilutions of *P. glomerata* DNA (10 ng to 0.001 ng); MM: 50 bp DNA ladder marker (Bioron GmbH); CN: negative control.

As shown in Figure 2, the electrophoresis of the PCR products amplified using the designed primers exhibited clear and specific bands with the expected size (123 bp) across all tested annealing temperatures, confirming their effectiveness in amplifying *P. glomerata* DNA down to a concentration of 0.001 ng. Among the tested conditions, amplification at 65 °C produced the most intense bands on the agarose gel (Figure 2D), indicating a higher quantity of amplified DNA. Since all temperatures allowed DNA detection down to 0.001 ng, and considering that higher annealing temperatures help minimize primer-dimer formation and enhance specificity of primer-template binding (Wang *et al.*, 2015), thereby reducing the risk of nonspecific amplification, and the use of higher temperatures decrease the chances of non-specific amplifications, 65 °C was selected as the optimal annealing temperature for subsequent qPCR assays.

3.2.1 Primers Specificity

The specificity of the PFA3-F/PFA3-R primer pair was evaluated by testing the possible cross-reactivity against DNA extracted from 51 medicinal plantsamples, particularly focusing on medicinal plants used for similar purposes as the Brazilian ginseng. To perform this test, mixtures containg DNA from different plant species (5 ng/µl) were prepared by admixing the DNA extracts obtained from those species and subjected to conventional PCR. The results, visualized by agarose gel electrophoresis (Figure 3), showed amplification only for *P. glomerata*. No bands were observed in any of the non-target mixtures, confirming the specificity of the PFA3-F and PFA3-R primers to *P. glomerata* DNA.

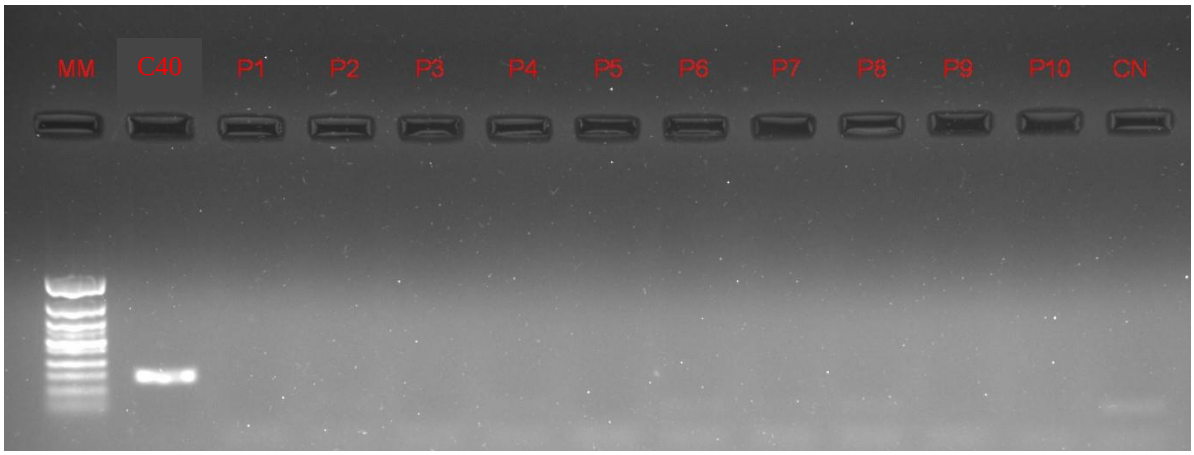


Figure 3 - Agarose gel electrophoresis of PCR products using primers PFA3-F and PFA3-R; C40: *P. glomerata*; P1 -P10: DNA mixtures; MM: 50 bp DNA ladder marker; CN: negative control.

This absence of amplification across diverse species supports the primer's suitability for reliable identification of *P. glomerata* DNA in herbal products.

3.3 Real-Time PCR Assay Optimization

3.3.1 Primers Sensitivity

Following the successful amplification of *P. glomerata* DNA, the sensitivity of the primers was evaluated using real-time PCR. A 10-fold serial dilution of *P. glomerata* DNA extract was tested, generating well-defined amplification curves with progressively increasing values of cycle quantification (Cq) corresponding to decreasing DNA

concentrations (Figure 4). These results showed the assay's high sensitivity and reproducibility, reliably detecting even low concentrations of target (0.01 pg).

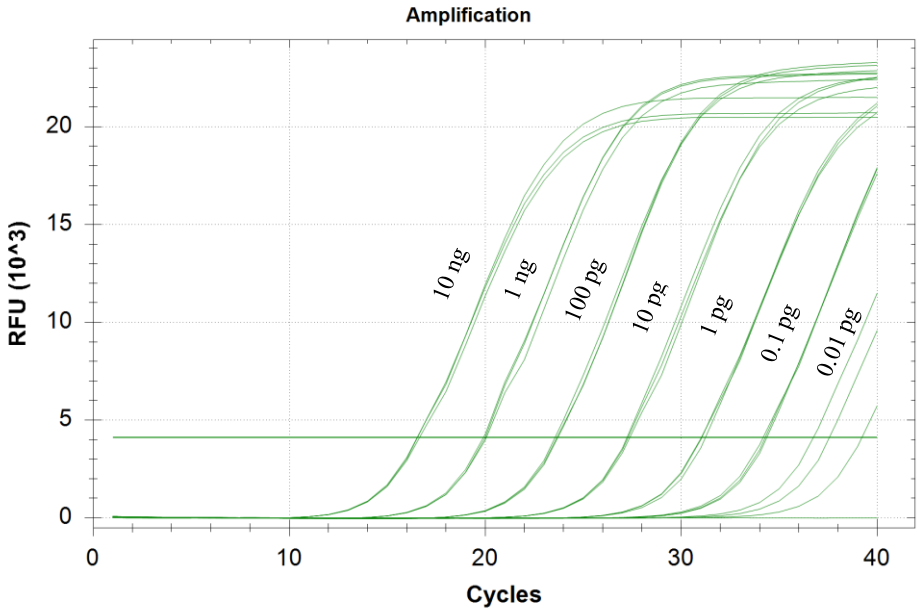


Figure 4 – Results of the real-time PCR assay using the designed primers (PFA3-F and PFA3-R) targeting *P. glomerata*. The amplified extracts correspond to 10-fold serially diluted *P. glomerata* DNA ranging from 10 ng to 0.01 pg (n = 3 replicates).

Melting curve analysis indicated highly consistent melting peaks at 84.40 ± 0.20 °C across all tested concentrations, with no evidence of nonspecific products or primer-dimer formation (Figure 5). These results further confirm the high specificity of the primers and demonstrate the robustness and reliability of the developed real-time PCR.

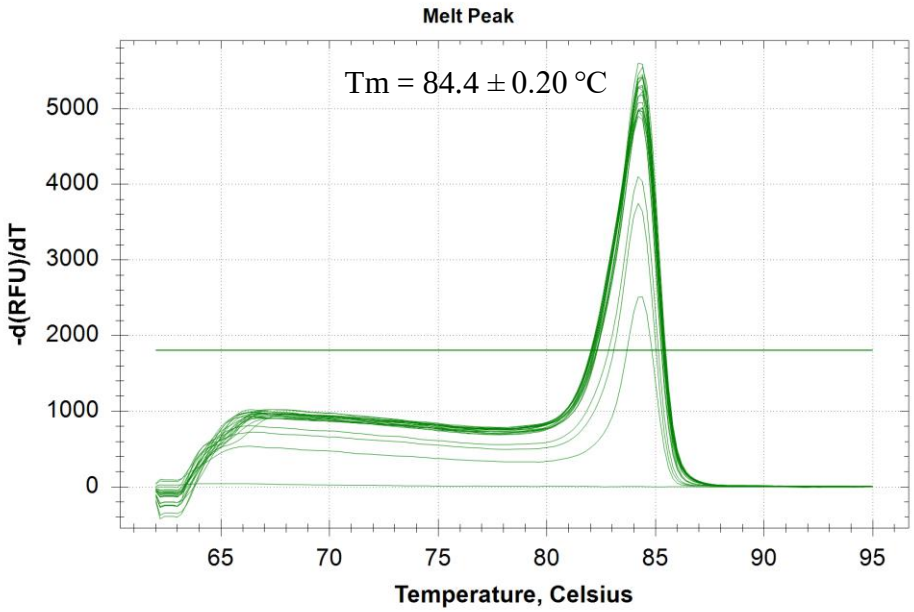


Figure 5 – Melting curves obtained from the real-time PCR assay using the designed primers (PFA3-F and PFA3-R) targeting *P. glomerata*. The amplified extracts correspond to 10-fold serially diluted *P. glomerata* DNA ranging from 10 ng to 0.01 pg (n = 3 replicates).

The calibration curve generated from the dilution series exhibited excellent linearity over six orders of magnitude (from 10 ng to 0.01 pg of *P. glomerata* DNA) (Figure 6).

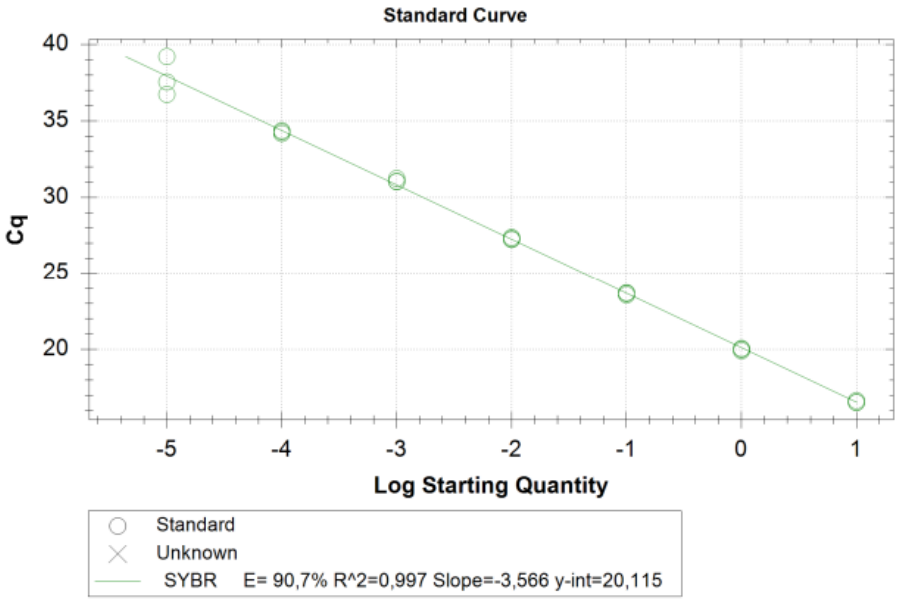


Figure 6 – Standard curve obtained from the real-time PCR assay using the designed primers (PFA3-F and PFA3-R) targeting *P. glomerata*. The amplified extracts correspond to 10-fold serially diluted *P. glomerata* DNA ranging from 10 ng to 0.01 pg (n = 3 replicates).

The performance of the assay was evaluated according to the guidelines established by Bustin *et al.* (2009) and ENGL (2015), which recommend a slope between −3.6 and −3.1, a PCR efficiency between 90% and 110%, and a correlation coefficient (R^2) of at least 0.98. These criteria ensure reliable efficiency and linearity of quantitative detection across a wide dynamic range. The developed assay demonstrated high performance, since the analytical parameters were within the acceptable criteria, namely the PCR efficiency of 90.7% (90-110%); a R^2 of 0.997 (≥98 %) and the slope of -3.566 (-3.6 to -3.1). The dynamic range covered six orders of magnitude of the target analyte from 10 ng to 0.01 pg of *P. glomerata* DNA.

The absolut limit of detection (LOD) was determined as the lowest DNA concentration consistently amplified in all technical replicates (n = 3), corresponding to 0.01 pg (0.00001 ng) of *P. glomerata* DNA, with corresponding Cq values below or around 37 cycles. This threshold aligns with Bustin *et al.* (2009) and ENGL (2015) guidelines, which recommend interpreting results above Cq 35 with caution, unless melting curve analysis confirms specificity. At 0.01 pg, all replicates were amplified (mean Cq = 37.84 ± 1.26), corresponding to a coefficient of variation (CV) of 3.3%, which is below the 25% threshold recommended by ENGL. Therefore, this concentration

was also accepted as the absolut limit of quantification (LOQ), as it fell within the linear range of the calibration curve.

3.4 Development of a qPCR model for *Pfaffia glomerata* Detection in Herbal Products

To support the development of a quantitative model for *P. glomerata* detection in herbal products using qPCR, a standardized calibration curve was generated. This strategy enabled the evaluation of the assay's sensitivity and linearity under conditions that mimic commercial products.

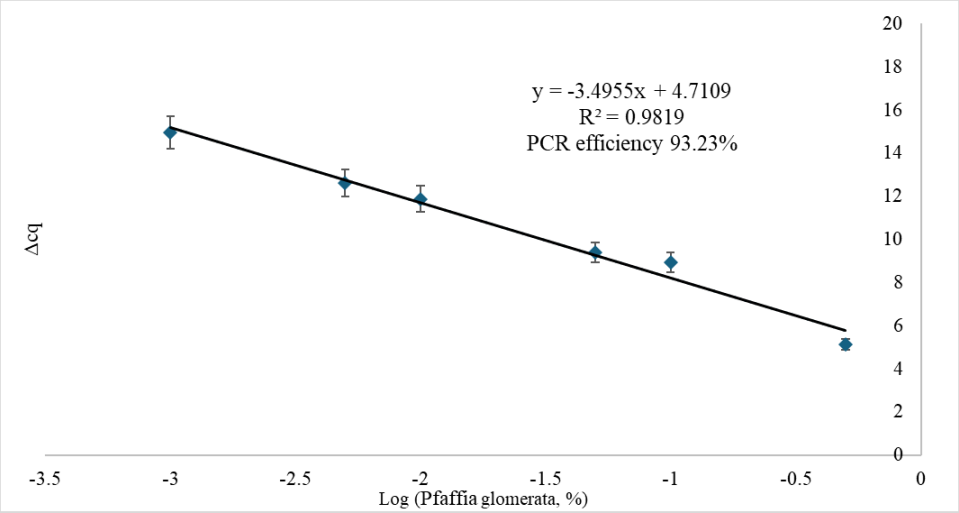


Figure 7 – Normalized calibration curves obtained by real-time PCR, targeting the ITS1 region of *P. glomerata*, using the binary mixtures of *P. glomerata* in *W. somnifera* (50%, 10%, 5%, 1%, 0.5% and 0.1%, (w/w)). The normalized ΔCq method was performed by the parallel amplification of a eukaryotic sequence (18S rRNA) as reference (mean values of five independent assays with n = 3 replicates).

The resulting curve showed a slope of −3.4955, a PCR efficiency of 93.23%, and a R^2 of 0.9819, (mean values from five independent assays), covering six orders of magnitude. All parameters met the performance criteria recommended by Bustin *et al.* (2009) and ENGL (2015). Therefore, 0.1% was established as the operational relative LOD and LOQ in this work.

The normalized real-time PCR method was further validated for precision and accuracy using blind mixtures. These samples contained *P. glomerata* mixed into *W. somnifera* at known proportions of 20.0%, 8.0%, and 2.0% (w/w), and were analyzed as unknowns to simulate real-world applications.

Quantification was performed using ΔCq method, and the estimated concentrations were compared with the real values (Table 5). The CV ranged from 2.91% to 16.54%, remaining well below the 25% threshold considered acceptable for qPCR assays. These results confirm the method's high repeatability and reliability, consistent with the requirements for quantitative analysis in botanical products (Bustin *et al*., 2009; Grazina *et al*., 2020).

Table 5 - Results of the validation assays using the normalized quantitative PCR system applied to blind mixtures of *P. glomerata* in *W. somnifera*.

Blind Sample	Real Value (%)	Estimated Value (%)	Error (%)	CV (%)
1	20.0	21.08 ± 0.022	−5.38	2.91
2	8.0	8.94 ± 0.005	−11.79	6.39
3	2.0	1.50 ± 0.0001	25.06	16.54

In terms of accuracy, the absolute deviation between estimated and true values ranged from −5.38% to 25.06%, which remains within the generally accepted ±25% threshold for qPCR-based quantification (Grazina *et al*., 2020; Talkington, 2013). These results support the method's reliability for determining the proportion of *P. glomerata* in herbal mixtures, particularly at moderate to low concentrations.

Generally, adulterations are associated with the admixture or substitution of a reasonable percentage of plant material to be economically advantageous, thus values of 0.1% are not expected to be found in food supplements or medicinal plants' fraud. However, the lowest value of the calibration curve (0.1%) was chosen because it may indicate potential unintentional contamination. Although values lower than the end of the calibration curve (for instance 0.01% w/w) were not assessed in blind validation, the LOD of the absolute calibration curve and the normalized calibration curve spanning from 50% to 0.1% (w/w) showed excellent linearity ($R^2 = 0.982$) and high PCR efficiency (~93%), suggest that the method is potentially capable of detecting trace levels with sufficient replicates and experiments.

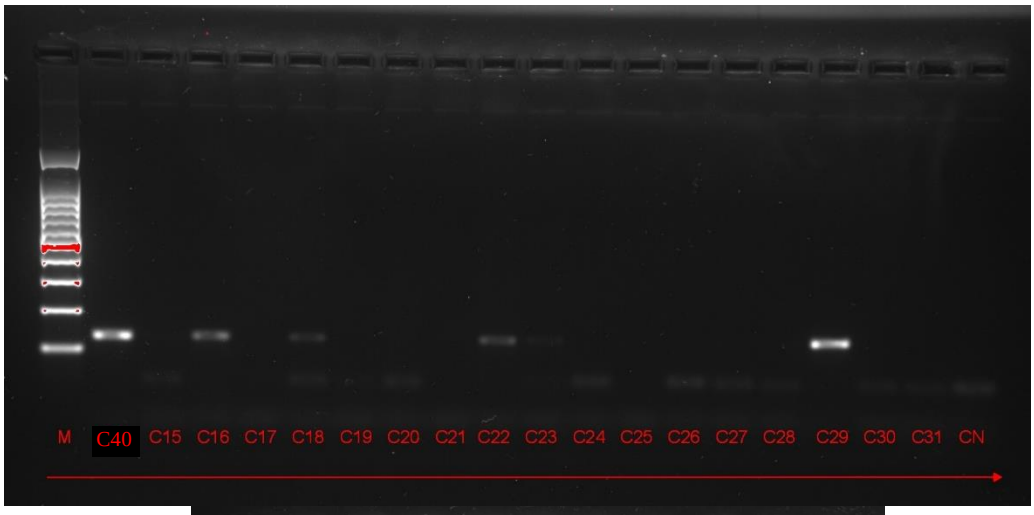
This finding is consistent with the results of Grazina *et al*. (2020), who also reported high accuracy and precision for the detection of *Ginkgo biloba* using a normalized quantification approach.

3.4.1 Application of the qPCR model against commercial products

Following the qPCR method development and validation, it was applied for differentiating *P. glomerata* from its main botanical contaminants, namely *P. paniculata* and *P. irisinoides*, *W. somnifera*, *E. senticosus* and *P. ginseng*, and to assess the authenticity of 25 commercial samples. The DNA extracted from commercial samples was first amplified by conventional PCR followed by qPCR assays using the PFA3-F and PFA3-R primers.

The results of the conventional PCR assays electrophoresis gels are presented in Figures 8A and 8B for samples C15 to C31, and for samples C32 to C39, respectively.

A



B

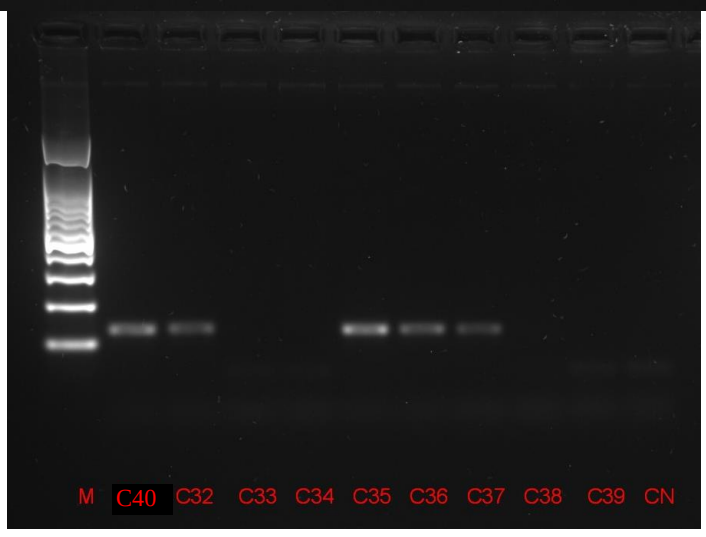


Figure 8 - Agarose gel electrophoresis of PCR products using PFA3-F and PFA3-R primers; C40: *P. glomerata*; **A:** C15-C31: commercial samples; **B:** C32-C39 commercial samples; MM: 100 bp DNA ladder marker; CN: negative control.

Amplification was observed in the target sample used as positive control (C40) as well as in commercial samples C16, C18, C22, C23, C29, C32, C35, C36 and C37. The remaining samples showed no amplification.

According to the information provided on the product labels, samples C27, C30, C31 and C32 were expected to amplify as they were labeled as being *P. glomerata*. Among these, only sample C32 showed the expected result. The amplification observed in the other samples, despite not being labeled as containing *P. glomerata*, may suggest possible contamination, adulteration or primer cross-reactivity with phylogenetically related species not included in the cross-reactivity assays.

Subsequently, qPCR assays coupled with melting temperature analysis were conducted. The results are shown in Table 6, which presents for each sample the amplification results of the reaction as well as the corresponding Cq values and melting temperature (Tm) obtained for the commercial extracts.

Table 6 - Results of the application by qPCR coupled with melting temperature analysis of the DNA extracts from commercial samples. ("+": amplification; "-": no amplification).

Internal ID	Labelled Species	Amplified to qPCR	Cq Value (Mean±SD)	Melt Temp (°C)
C15	*Panax ginseng*	+	37.64±1.94	84.4
C16	*Pfaffia paniculata*	+	16.42±0.05	84.6
C17	*Eleutherococcus senticosus*	+	38.12±0.90	84.4
C18	*Withania somnifera*	+	34.62±0.43	84.4
C19	*Withania somnifera*	+	37.45±0.00	84.2
C20	*Withania somnifera*	-	N/A	None
C21	*Eleutherococcus senticosus*	-	N/A	None
C22	*Panax ginseng*	+	23.5±0.41	84.4
C23	*Pfaffia paniculata*	+	33.69±0.28	84.2
C24	*Panax ginseng*	-	N/A	None
C25	*Panax ginseng*	-	N/A	None
C26	*Panax ginseng*	-	N/A	None
C27	*Pfaffia glomerata*	-	N/A	None
C28	*Pfaffia paniculata*	+	37.23±0.00	84.2
C29	*Pfaffia paniculata*	+	16.76±0.10	84.6
C30	*Pfaffia glomerata*	+	39.32±0.00	None
C31	*Pfaffia glomerata*	-	N/A	None
C32	*Pfaffia glomerata*	+	18.34±0.02	84.4
C33	*Pfaffia iresinoides*	-	N/A	None
C34	*Pfaffia paniculata*	-	N/A	None
C35	*Brazilian ginseng*	+	18.49±0.04	84.4
C36	*Pfaffia infusion*	+	23.74±0.05	84.4
C37	*Panax ginseng*	+	21.03±0.05	84.4

Table 6 (continued)

Internal ID	Labelled Species	Amplified to qPCR	Cq Value (Mean±SD)	Melt Temp (°C)
C38	*Pfaffia paniculata*	+	37.7±0.46	84.2
C39	*Eleutherococcus senticosus*	+	37.35±1.49	84.2
C40	*Pfaffia glomerata*	+	20.81±0.21	84.4

Overall, qPCR confirmed the results of all the samples that were positive on qualitative PCR. Furthermore, amplification was also obtained for samples C15, C17, C19, C28, C30, C31, C38 and C39. Of these, only C30 and C32 were labeled as *P. gomerata*, however, sample C30 showed a very high Cq value indicating only trace amounts of the species. The presence of *P. glomerata* was also confirmed in samples C35 and C36 labelled as "Brazilian ginseng" and "Pfaffia infusion", respectively.

Samples C16, C22, C29, C32, C35, C36, and C37 showed Cq values between 16.4 and 23.7 and Tm similar to that of the positive control (*P. glomerata*). Samples C16 and C29 labeled as being *P. paniculata* and C22 and C37 labeled as *Panax ginseng* revealed to be adulterated by the admixture or substitution with *P. glomerata*. This result is in line with Almeida *et al*. (2023), who reported a high level of substitution between *P. glomerata* and *P. paniculata* as well as with studies that reported a high risk of substitution, contamination or adulteration in the commercial market of herbal products (Newmaster *et al*., 2013; Ichim, 2019).

No specific amplification was observed in samples C20, C21, C24-C27, C31, C33, and C34, indicating the absence of *P. glomerata* DNA. This outcome was consistent with the labeling of most of these samples, which listed other species. However, the lack of amplification in sample C31 was unexpected, as its label indicated the presence of *P. glomerata*. Samples C15, C17-C19, C23, C28 and C30 showed a veryhigh Cq which was interpreted as indicative of possible contamination.

The normalized quantitative PCR method, using the previously developed standard curve, was applied to estimate the proportion of *P. glomerata* DNA in each commercial sample. Quantitative evaluation was based on the calibration model developed from binary mixtures of *P. glomerata* and *W. somnifera*. This model, which employed the ΔCq approach, enabled the estimation of the relative amount of *P. glomerata* DNA present in the tested products.

The ΔCq method involved calculating the difference between the Cq value of the target gene (amplified using the *P. glomerata* specific primers PFA3-F/PFA3-R) and the

Cq value of the endogenous reference gene (18S rRNA), obtained from the amplification of same extracts.

The resulting ΔCq values were then inserted into the standard curve equation previously validated in this study (Figure 7) and the the relative proportion of *P. glomerata* in each product was estimated by applying Equation 1.

Equation 1:

$$\log_{10}(Pfaffia) = \frac{(4.7109 - \Delta Cq)}{3.4955} \Rightarrow \%\, Pfaffia = 10^{\left[\frac{(4.7109 - \Delta Cq)}{3.4955}\right]} \times 100$$

Table 7 shows the detailed results including ΔCq values and estimated percentages of *P. glomerata* of the commercial samples.

Table 7 - Normalized qPCR method for quantification of *P. glomerata* in commercial samples.

Internal ID	**Labelled Species**	**ΔCq**	**% Pfaffia (estimated)**
C15	*Panax ginseng*	20.58	<LOQ
C16	*Pfaffia paniculata*	-0.64	>50
C17	*Eleutherococcus senticosus*	21.06	<LOQ
C18	*Withania somnifera*	17.56	<LOQ
C19	*Withania somnifera*	20.39	<LOQ
C20	*Withania somnifera*	-	-
C21	*Eleutherococcus senticosus*	-	-
C22	*Panax ginseng*	6.44	32.0
C23	*Pfaffia paniculata*	16.63	<LOQ
C24	*Panax ginseng*	-	-
C25	*Panax ginseng*	-	-
C26	*Panax ginseng*	-	-
C27	*Pfaffia glomerata*	-	-
C28	*Pfaffia paniculata*	20.17	<LOQ

Table 7 (continued)

Internal ID	Labelled Species	ΔCq	% Pfaffia (estimated)
C29	*Pfaffia paniculata*	-0.30	>50
C30	*Pfaffia glomerata*	22.26	<LOQ
C31	*Pfaffia glomerata*	-	-
C32	*Pfaffia glomerata*	1.28	>50
C33	*Pfaffia iresinoides*	-	-
C34	*Pfaffia paniculata*	-	-
C35	Brazilian ginseng	1.43	>50
C36	Pfaffia infusion	6.68	27.3
C37	*Panax ginseng*	3.97	16.3
C38	*Pfaffia paniculata*	20.64	<LOQ
C39	*Eleutherococcus senticosus*	20.29	<LOQ

Samples C16 and C29 exhibited high estimated concentrations of *P. glomerata* above 50% (the higher limit of the calibration curve), consistent with strong amplification (low Cq values) and both with melting temperatures of 84.6 °C. These samples, though labeled as *P. paniculata*, were confirmed to be *P. glomerata* or to contain a high content of this species, and were therefore classified as adulterated.

Samples C35 and C36 labeled as "Brazilian ginseng" and "Pfaffia infusion", respectively, both presented positive amplification results, being compatible with *P. glomerata*. However, sample C35 was estimated to contain >50% *P. glomerata,* and considering the low Cq value, it probably contains only this species. On the contrary, sample C36 was estimated to contain only 27% of *P. glomerata* and is possibly a mixture of *Pfaffia* species. Samples C27, C30 and C31, labeled as *P. glomerata* were also considered adulterated by the full substitution of this species.

In Brazil, *P. glomerata* and *P. paniculata* are popularly known as "Brazilian ginseng", but the morphology of the roots and the chemical similarity between them make their botanical identification difficult, which facilitates adulteration (Vigo *et al.*, 2004). Recently, Almeida *et al.* (2023) demonstrated, using the Bar-HRM technique targeting the matK gene, that only 56% of the samples sold as Brazilian ginseng actually contained

P. glomerata, while the others were predominantly *P. paniculata* - highlighting discrepancies in labeling and the need for reliable molecular methods for authentication. These studies support the conclusion that the presence of *P. glomerata* DNA in products labeled as containing "Brazilian ginseng" does not always correspond to the correct labeling, reinforcing the importance of methodologies such as qPCR-HRM and sequencing to ensure the true botanical identity of the material.

Samples C22 and C37, both labelled as *P. ginseng*, were estimated to contain 32% and 16% of *P. glomerata* material, respectively. Thus, these two samples were also considered adulterated. Sample C30, despite giving a positive amplification, revealed to contain only trace levels (below LOQ) of *P. glomerata*, and was also considered adulterated, as well as sample C31, which failed to amplify *P. glomerata* DNA.

Mislabeling in herbal products has been extensively reported. For example, Newmaster *et al.* (2013) reported that 59% of herbal supplements evaluated in the USA contained undeclared species, indicating substitution, contamination, or unlisted additives. This type of misidentification can compromise efficacy and pose significant health risks. This example reinforces both the prevalence of mislabeling and the effectiveness of molecular methods for authentication.

Finally, samples C15, 17, 18, 19, 23, 28, 38 and 39 gave values below 0.1%. Therefore, the positive amplification of these samples most probably corresponds to a cross-contamination rather than an adulteration. It should be mentioned that to account for possible non-intended contamination during production, different pharmacopoeial monographies of plant raw materials usually allow a content of up to 2% of foreign (Parveen *et al*., 2016).

From the evaluated samples, we can conclude that seven samples were found to be adulterated, either by the fraudulent addition of *P. glomerata* material (C16, C22, C29, C37) or by the substitution of this species by others (C27, C30, C31), thus highlighting the relevance of the normalized qPCR model for quantification of *P. glomerata* as a screening tool for verifying botanical authenticity in natural products.

4 CONCLUSIONS

This study aimed to develop and validate a DNA-based methodology using PCR and qPCR for the authentication of *P. glomerata*, a medicinal plant of pharmacological importance that is frequently adulterated with morphologically similar species.

Specific primers (PFA3-F/PFA3-R) were successfully developed, and their specificity was confirmed both through *in silico* analyses and experimental testing against a broad panel of plant species, most being used traditionally for their similar properties. The developed technique demonstrated high sensitivity and robustness: qPCR assays consistently detected pure *P. glomerata* DNA at concentrations as low as 10 picograms. This value was established as both the absolute limit of detection (LOD) and limit of quantification (LOQ), as it fell within the linear amplification range and yielded specific melting profiles.

Furthermore, the methodology proved effective in detecting *P. glomerata* in binary mixtures with possible adulterants, using a relative quantification strategy based on the ΔCq method normalized by a reference gene (18S rRNA). The calibration model showed excellent linearity and precision across a wide range, from 50% to 0.1% (w/w) of *P. glomerata* DNA in *W. somnifera*. Blind validation assays demonstrated accurate quantification down to 2% (w/w), with absolute errors and coefficients of variation within internationally accepted standards (<25%). At lower concentrations (0.2%), the method showed a higher variability, with CV% higher (27%) than the recommended threshold.

When applied to commercial herbal products, the method revealed several inconsistencies between the declared species on the product's label and actual genetic composition. Moreover, it successfully distinguished *P. glomerata* from species such as *W. somnifera*, *E. senticosus*, and *P. ginseng*, confirming its value as a traceability and quality control tool.

Overall, the results of this study highlight the value of DNA-based methods for the authentication of medicinal plant products and demonstrate the potential of qPCR as a practical tool for quality control laboratories, regulatory agencies, and the phytopharmaceutical industry. The methodology developed herein represents an important advance toward the authentication of *P. glomerata* and consumer protection, though improvements are still needed to enhance discrimination among closely related species.

Future work could include the NGS analysis of *P. glomerata* samples that yielded negative results, as well as the samples that showed evidence of fraudulent addition of this species, in order to clarify their true composition. In addition, extending the evaluation to a broader range of commercial products would provide a more comprehensive assessment of market authenticity.

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APPENDIX I – LIST OF COMMERCIAL SAMPLES WITH IMAGES

Appendix I - List of the 25 commercial samples under study, containing the information declared on the packaging, the physical characterization of these samples and images (continue).

Internal sample code	Labelled information	Sample characterization	Images
C15	*Panax ginseng*	Root powder	
C16	*Pfaffia paniculata*	Straw	
C17	*Eleutherococcus senticosus*	Root/Straw	
C18	*Withania somnifera*	Root/Leaves/Seeds	

C19	*Withania somnifera*	Root powder	

Appendix I - List of the 25 commercial samples under study, containing the information declared on the packaging, the physical characterization of these samples and images (continue).

Internal sample code	Labelled information	Sample characterization	Images
C20	*Withania somnifera*	Root powder	
C21	*Eleutherococcus senticosus*	Root powder	
C22	*Panax ginseng*	Powder	

C23	*Pfaffia paniculata*	Powder capsule	
C24	*Panax ginseng*	Gelatin capsule	

Appendix I - List of the 25 commercial samples under study, containing the information declared on the packaging, the physical characterization of these samples and images (continue).

Internal sample code	Labelled information	Sample characterization	Images
C25	*Panax ginseng C. A. Mey*	Gelatin capsule	
C26	*Panax ginseng C. A. Mey*	Gelatin capsule	
C27	*Pfaffia glomerata*	Root/Straw	

C28	*Pfaffia paniculata*	Straw	
C29	*Pfaffia paniculata*	Powder	
C30	*Pfaffia glomerata*	Root powder	

Appendix I - List of the 25 commercial samples under study, containing the information declared on the packaging, the physical characterization of these samples and images (continue).

Internal sample code	Labelled information	Sample characterization	Images
C31	*Pfaffia glomerata*	Root/Straw	
C32	*Pfaffia glomerata*	Straw	

C33	*Pfaffia iresinoides*	Straw	
C34	*Pfaffia paniculata*	Powder	
C35	*Brazilian Ginseng*	Powder	

Appendix I - List of the 25 commercial samples under study, containing the information declared on the packaging, the physical characterization of these samples and images (conclusion).

Internal sample code	Labelled information	Sample characterization	Images
C36	*Pfaffia infusion*	Bark	
C37	*Panax infusion*	Bark	

C38	*Pfaffia paniculata*	Straw	
C39	*Eleutherococcus senticosus*	Dry root/Straw	
C40	*Pfaffia glomerata*	Leaves