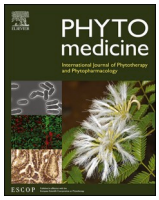
Phytomedicine 128 (2024) 155322

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed



Original Article

Development of a loop-mediated isothermal amplification assay for the rapid detection of *Styphnolobium japonicum* (L.) Schott as an adulterant of *Ginkgo biloba* (L.)

Vânia Rodrigues [a,b], Mónica Honrado [a,b,c], Joana Santos [a,b], M. Alice Pinto [a,b], Joana S. Amaral [a,b,*]

[a] Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[b] Laboratório Associado para a Sustentabilidade e Tecnologia em Regiões de Montanha (SusTEC), Instituto Politécnico de Bragança, Bragança, Portugal
[c] LAQV-REQUIMTE & Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, Aveiro, 3810-193, Portugal

ARTICLE INFO

Keywords:
LAMP
Colorimetric detection
Sophora japonica
Ginkgo biloba
Authenticity
Adulteration

ABSTRACT

Background: Species adulteration is a concern in herbal products, especially when plant substitutes of lower economic value replace valuable botanicals. *Styphnolobium japonicum* is well known as a potential adulterant of *Ginkgo biloba*, which is one of the most demanded medicinal plants due to its wide use in pharmaceuticals, food supplements, and traditional medicine. Despite bearing some resemblance to ginkgo's flavonol composition, *S. japonicum* lacks many of *G. biloba's* desired therapeutic properties. To prevent adulteration practices, it is crucial to implement rigorous quality control measures, including fast and simple diagnostic tools that can be used on-field.

Purpose: This study aims to develop for the first time a species-specific loop-mediated isothermal amplification (LAMP) method for the fast identification of *S. japonicum* in ginkgo-containing products.

Methods: A set of four specific primers (SjF3, SjB3, SjFIP, and SjBIP) and loop primers (SjLF and SjLB) were designed for a LAMP based assay using the 5.8S partial sequence and the internal transcribed spacer 2 of nuclear ribosomal DNA of *S. japonicum*.

Results: The successful amplification of the LAMP assay was inspected through visual detection, with the highest intensity recorded at the optimal conditions set at 68 °C for 40 min. The primers showed high specificity and were able to accurately discriminate *S. japonicum* from *G. biloba* and 49 other species of medicinal plants. Furthermore, the proposed LAMP assay proved to be fast, selective, and highly sensitive, as demonstrated by the absolute and relative limits of detection, which were reached at 0.5 pg for *S. japonicum* DNA and 0.01 % *S. japonicum* in *G. biloba*, respectively.

Conclusions: This novel approach allows easy identification and discrimination of *S. japonicum* as a potential adulterant of *G. biloba*, thus being a useful tool for quality control. Compared to chromatographic or PCR-based methods, the assay proved to be fast, sensitive and did not require expensive equipment, thus offering the possibly usage in field analysis.

Introduction

Ginkgo biloba (L.) is one of the most popular and scientifically scrutinized plant species used in herbal medicinal products and plant food supplements. Its leaves contain various bioactive compounds, including terpene lactones (ginkgolides and bilobalide) and flavonoids, with several beneficial effects such as antioxidant, inhibition of platelet aggregation, anti-inflammatory, and neuroprotective properties, among

Abbreviations: COI, Cytochrome c oxidase I gene; HDA, Helicase-dependent amplification; ITS, Internal transcribed spacer; LFS, Lateral flow strip assay; LOD, Limit of detection; LOQ, Limit of quantification; LAMP, Loop-mediated isothermal amplification; PCR, Polymerase chain reaction; RPA, Recombinase polymerase amplification; SAMRS, Self-avoiding molecular recognition systems; Tm, Temperature of melting.

* Corresponding author at: Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal

E-mail address: jamaral@ipb.pt (J.S. Amaral).

https://doi.org/10.1016/j.phymed.2023.155322
Received 30 September 2023; Received in revised form 20 December 2023; Accepted 25 December 2023
Available online 30 December 2023

others. *G. biloba* is often employed as an herbal medicine or food supplement to support cognitive function, enhance blood circulation, and alleviate symptoms associated with conditions such as dementia, cognitive decline, and peripheral vascular diseases (Diamond and Bailey, 2013). The increased value of Ginkgo products and the high demand for this plant in the market make it a potential target for adulteration. Since commercial leaf extracts are typically standardized to contain approximately 24 % flavonol glycosides (mainly derivatives of quercetin, kaempferol, and isorhamnetin), fraud can occur through the addition/partial replacement with other plant species containing high amounts of these compounds. This is the case of *Styphnolobium japonicum* (L.) Schott (syn: *Sophora japonica* L.), commonly known as the Chinese scholar tree or Japanese pagoda tree. Owing to the lower economic costs of *S. japonicum*, plant material of this species may be deliberately added as a *G. biloba* substitute to reduce manufacturing expenses and meet market demands (Bampali et al., 2021; Govindaraghavan, 2018). This practice has raised concerns regarding the quality and efficacy of ginkgo products, which has led to the implementation of several works mostly based on chromatography (Avula et al., 2015; Bampali et al., 2021; Budeč, 2019; Govindaraghavan, 2018; Ma et al., 2016).

In the last decades, DNA-centered techniques have been increasingly used to identify species in herbal products (Grazina et al., 2021, 2020; Liu et al., 2018). In this context, a recent study proposed the use of real-time polymerase chain reaction (PCR) to detect and quantify potential adulterations of *G. biloba* products with *S. japonicum* (Grazina et al., 2021). This species-specific real-time PCR method was proven accurate and cost-effective for authenticating ginkgo-containing herbal products, achieving a sensitivity down to 0.02 pg of *S. japonicum* DNA. However, the application of real-time PCR is not compatible with rapid on-site authentication as it is a time-consuming method, in addition to requiring expensive equipment with stringent temperature control, and professional expertise (Yan et al., 2014). In contrast, techniques based on DNA's isothermal amplification allow overcoming these PCR-associated drawbacks and, therefore, have been increasingly proposed as suitable tools for point-of-care or point-of-need applications (Oliveira et al., 2021). Among such techniques, loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), and helicase-dependent amplification (HDA) are promising for in-field applications and are becoming increasingly popular (Botella, 2022; Glökler et al., 2021; Moehling et al., 2021). While these approaches have been extensively exploited for clinical diagnosis of human pathogens (Oliveira et al., 2021) and are rapidly spanning to other fields, such as the detection of plant pathogens (Botella, 2022) and foodborne bacteria and fungi (Moon et al., 2022), their application to the identification of medicinal plants is still scarcely developed and largely unexplored (Grazina et al., 2020; Li et al., 2016).

Among the few published works, Liu et al. (2018) proposed the use of recombinase polymerase amplification combined with a lateral flow strip assay (RPA-LFS) as a rapid on-site method for the identification of *G. biloba* and detection of its adulterant *S. japonicum* in ginkgo herbal products. In this work, the Nfo kit (TwistAmp) developed for endpoint strip-based detection was used with an Nfo probe. The authors determined that approximately 1 ng of purified DNA was required for the RPA-LFS assay and reported that the entire detection process after DNA extraction was completed in 30 min. Despite being fast and sensitive, this method requires opening the tubes with the RPA product to load them on the strips, which increases the risk of contamination/carryover (Munawar, 2022). Moreover, the probe must be carefully designed and follow several specificities, namely, its 5′ end must be labeled with the FAM dye (antigenic label) and the 3′ end added with a polymerase blocker, while the primer opposing the probe must be labeled with biotin to allow for the anchorage on the strip (Munawar, 2022). While these primer and probe modifications negatively impact the global cost of the analysis, the main concern reported for RPA regards its possible nonspecific amplification and background noise due to its high mismatch tolerance (Botella, 2022; Munawar, 2022; Zhao et al., 2018). Different approaches have been proposed to circumvent this issue, such as a careful design to include possible mismatches at the 3′ end, where they are less tolerated, or using self-avoiding molecular recognition systems (SAMRS) primers (Munawar, 2022). Still, limited research has focused on RPA non-specificity.

On the contrary, LAMP offers an interesting molecular alternative for amplifying DNA under isothermal conditions. This technique relies on the use of four to six specifically designed primers to hybridize six or eight different parts of the target DNA sequence, thus conferring a high level of specificity (Li et al., 2016). LAMP is well-established and widely applied due to its simplicity, ability to generate prompt results, ease of use, and straightforward visual interpretation employing turbidity, fluorescent dyes, or colorimetric methods (Soroka et al., 2021; Park, 2022). For these reasons, LAMP has been successfully used in diverse applications, such as to diagnose viral diseases, to detect fungi and bacteria (Moehling et al., 2021; Oliveira et al., 2021), and, to a lesser degree, to detect herbal medicine adulterants (Chaudhary et al., 2012; Lai et al., 2015; Li et al., 2013; Wu et al., 2016). However, to our knowledge, there is still no report on the use of LAMP to rapidly identify potential adulterant species of *G. biloba*. Therefore, the present work aims to fill this gap by developing a colorimetric LAMP assay for *S. japonicum* detection that is sensitive and specific and outperforms the existing PCR-based methods in its speed, ease of use, and potential on-site application.

Materials and methods

Plant samples, model mixtures, and DNA extraction

Voucher leaf samples of *S. japonicum* and *G. biloba* were kindly provided by the following botanical gardens: Botanische Gärten der Universität Bonn, Germany (samples of *S. japonicum* were collected from the specimen Ipen-Nr.: XX-0-BONN-12,020, while those of *G. biloba* were collected from the specimen Ipen-Nr.: XX-0-BONN-1894), from the Museo Botanico della Università degli Studi di Siena, Italy (samples of *S. japonicum* were collected from the specimen located in aiuola del Parco XIX 8, while those of *G. biloba* were collected from the specimen located in aiuola del Parco I Bis 1). Samples of the single specimen of *S. japonicum* were also obtained from Jardi Botanic de Sóller, Spain (samples collected from ES-0-SOLLE-10,112) and from the Jardim Botânico da Universidade do Porto, Portugal. These samples were deposited in the herbarium of the Polytechnic Institute of Bragança under the references BRESA 6723 to BRESA 6728. To further confirm their authenticity, DNA was extracted from these voucher samples using the NucleoSpin® Plant II kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions, PCR amplified for the matK and rbcL barcode regions, and then sent to STABVIDA Inc. (Portugal) for Sanger sequencing. PCR amplification was carried out with the primers matK-KIM3F: 5′- CGTACAGTACTTTTGTGTTTACGAG-3′ and matK-KIM1R: 5′- ACCCAGTCCATCTGGAAATCTTGGTTC-3′ for matK, and rbcLa-F: 5′-ATGTCACCACAAACAGAGACTAAAGC-3′ and rbcLa-R: 5′-GTAAAATCAAGTCCACCRCG-3′ for rbcL, and respective conditions proposed by the Consortium for the Barcode of Life (CBOL Plant Working Group et al., 2009). PCR products were sequenced in both directions after purification to remove possible interferents. The consensus sequence was built from the sequencing results of both strands (Rausch and Fritz, 2020), and then submitted to the NCBI Basic Local Alignment Search Tool (BLAST, https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The results obtained corresponded to 100 % identity for matK and varied from 99.31 % to 100 % identity for rbcL, therefore confirming the species identity of the 5 voucher samples sequenced.

Binary mixtures containing known amounts of powdered *S. japonicum* leaves (25 %, 10 %, 5 %, 1.5 %, 0.15 %, 0.05 %, 0.01 %) in *G. biloba* leaves were prepared to establish the relative limit of detection of the LAMP assay. Besides *G. biloba*, 49 other different species of

Table 1
Description of the primers (set 2) selected in this study for detecting *S. japonicum* using the colorimetric LAMP assay.

Primer name	Sequence (5′- 3′)	Tm (°C)	Primer Length (nt)	Amplicon Size (bp)
SjF3	CGAAGCCATTAGGCCGAG	59.38	18	235
SjB3	TCCGTGGGTCACATAGTCC	60.17	19	
SjFIP(F1c+F2)	CAACATTCGCCCCGCTCAGG-GCCTGGGTGTCACACATC	–	38	
SjBIP(B1c+B2)	TGTCTGTGGTGGAGAGCACCA-AAGAGACACACGCGATTGG	–	40	
SjLF	GCACTGGCATTGGGGCAAC	64.24	19	
SjLB	TGGTGGCTGAGTAAAATCTCGA	61.23	22	

medicinal plants previously identified through DNA barcoding (Supplementary material, Table S1) were used for cross-reactivity assessment. Cross-reactivity was also assessed against another species from the same genus of the potential adulterant, namely *Styphnolobium davidii*, which was provided by the Botanical Garden of the Université Louis Pasteur (Strasbourg, France).

Total genomic DNA was extracted from 50 to 100 mg of powdered plant material using the NucleoSpin® Plant II kit following the manufacturer's instructions. Yield and purity of the DNA extracts were evaluated by UV spectrophotometry on a SPECTROstar® Nano microplate reader (BMG Labtech, Offenburg, Germany) with an LVis plate accessory. The 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 evaluation, the samples were stored at −20 °C until required.

LAMP primers design

The LAMP primers were designed using the PrimerExplorer V5 software (http://primerexplorer.jp/lampv5e/), based on the sequence of the internal transcribed spacer 2 (ITS2) of the nuclear ribosomal DNA of *S. japonicum* identified by the GenBank accession: HQ229005.1, which also covers the partial sequence of 5.8S (155 bp) and 28S (31 bp) regions. A set of six primers were selected, including two outer primers (forward SjF3 and backward SjB3), two inner primers (forward SjFIP and backward SjBIP), and two loop primers (forward SjLF and backward SjLB). The specificity of the selected primer set was tested *in silico* for possible cross-reactions with other species using the NCBI Primer-BLAST online tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). The properties of the primers were checked for primer dimers and hairpin structures using the OligoAnalyser software (https://www.idtdna.com/calc/analyzer). The selected primers (Table 1) were synthesized and purified by HPLC by STAB VIDA Inc., Portugal.

LAMP reaction

A series of LAMP reactions were performed on a T100 thermocycler (Bio-Rad Laboratories, USA) considering different reaction temperatures and times to obtain optimal conditions and ensure the best amplification. After optimization, all LAMP reactions were carried out in a 10 µl reaction mixture containing 0.1 µM of each SjB3 and SjF3, 0.8 µM of each SjFIP and SjBIP, and 0.2 µM of each SjLF and SjLB primers, 5 µl of WarmStart® Colorimetric LAMP 2x Master Mix (DNA&RNA) (New England Biolabs, MA, USA), 1 µl of genomic DNA (5 ng/µl), and 3 µl nuclease-free water. In each assay, a positive (1 µl of *S. japonicum* DNA extract template) and a negative control (1 µl of molecular biology grade water template) were included. As the mix contains phenol red as a pH indicator, the color will change from pink (negative) to yellow, if positive amplification is achieved.

Specificity of the LAMP assay

To determine the specificity of the LAMP assay, the designed outer primers (SjF3 and SjB3) were first tested *in silico* and then by PCR amplification against a total of 50 medicinal plants typically used to improve cognition or other cerebral functions, such as mood or sleep (Supplementary material, Table S1). The conventional PCR was conducted using only the LAMP outer primers SjF3 and SjB3, amplifying a 235 bp target fragment within the selected *S. japonicum* sequence (GenBank accession: HQ229005.1). PCR was carried out using 1 µl (10 ng) of DNA extract, 5 µl of Q5® High-Fidelity 2X Master Mix (New England Biolabs Ltd.), 0.5 µM of each primer SjF3/SjB3, and the remaining of nuclease-free water to perform a total reaction volume of 10 µl. The reactions were performed on a T100 thermocycler (Bio-Rad Laboratories, USA) with the following temperature program: initial denaturation at 98 °C for 3 min; 34 cycles of amplification at 98 °C for 10 s, 67 °C for 30 s, and 72 °C for 30 s; final extension at 72 °C for 2 min. In addition, to corroborate that the proposed LAMP assay correctly amplified the selected target, the LAMP products were digested with MseI restriction enzyme. The LAMP amplification products contain the MseI restriction endonuclease site (243rd base of the HQ229005.1 sequence) and can be digested into 126 bp and 109 bp fragments. The experiment was carried out using 4 µl of the LAMP assay products, 2 µl of MseI restriction enzyme (New England Biolabs Ltd.), 2 µl of MseI reaction buffer, and the remaining of nuclease-free water for a total reaction volume of 20 µl. The digestion reaction was performed on a T100 thermocycler (Bio-Rad Laboratories, USA) using the following temperature program: activation at 37 °C for 2 h and denaturation at 65 °C for 20 min.

Sensitivity of the LAMP assay

To assess the sensitivity of the LAMP assay, the limit of detection (LOD) was measured using serial 10-fold dilutions of 5 ng/µl of *S. japonicum* DNA, ranging from 5 ng to 0.005 pg per reaction. In addition, real-time PCR was conducted with the LAMP outer primers SjF3 and SjB3 for comparison purposes. The reactions were performed on a fluorometric thermal cycler CFX96 Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) using a total volume of 10 µl per reaction containing 1 µl of DNA extract at 10 ng/µl, 1× of SsoFast™ Evagreen® Supermix (Bio-Rad Laboratories, USA) and 500nM of each outer primer SjB3/SjF3. The following conditions were used: 98 °C for 5 min, 40 cycles at 98 °C for 5 s, 67 °C for 5 s, with the collection of fluorescence signal at the end of each cycle. For melting curve analysis, the PCR products were denaturated at 98 °C for 1 min and then annealed at 67 °C for 5 min. After, the temperature was increased from 67 °C to 95 °C with increments of 0.2 °C every 5 s, with fluorescence data acquired at the end of each melting temperature. Data were processed using the software Bio-Rad CFX Maestro 1.1 (Bio-Rad Laboratories, Hercules, CA, USA). Each sample was analyzed in triplicate. For the construction of the calibration curve and subsequent determination of the absolute limits of detection (LOD) and quantification (LOQ), a 10-fold serially diluted *S. japonicum* DNA extract (10 ng - 0.01 pg) was amplified by real-time PCR. The relative limit of detection of the LAMP reaction was also determined by using binary mixtures containing known amounts of powdered *S. japonicum* (25 %, 10 %, 5 %, 1.5 %, 0.15 %, 0.05 %, 0.01 %) in *G. biloba*.

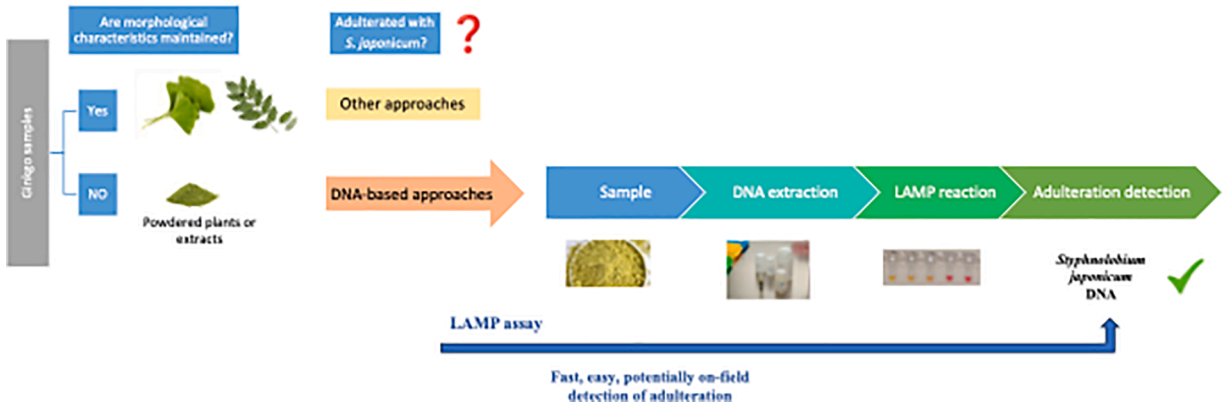


Fig. 1. Summary of the principle and approach based on LAMP reaction for detecting *S. japonicum* as an adulterant of *G. biloba*.

Electrophoresis of LAMP, PCR, and restriction enzyme products

The LAMP reaction products were analyzed via electrophoresis on a 2 % agarose gel prepared with 1x TAE and stained with GelRed® (Biotium, Fremont, CA, USA) at 85 V for 1 h. For conventional PCR products, 1 % agarose gel containing 1x Gel Red was used, and electrophoresis was carried out in 1x TAE buffer running 20 min at 90 V. In both cases, the 100 bp DNA Ladder ready-to-use (Bioron, Frankfurt, Germany) was used as a molecular standard for comparing molecular size. The products of the MseI restriction enzyme digestion were analyzed with the 50 bp DNA Ladder ready-to-use (Bioron, Frankfurt, Germany) via electrophoresis on a 2.5 % agarose gel made with 1× TBE buffer (Tris/Borate/EDTA: 89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) stained with GelRed®, and run at 90 V for 1h30m. 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).

Results and discussion

Until now, different techniques have been proposed for the authentication of medicinal plant species. Those can vary from low-cost morphological identification by macroscopic and/or microscopic analysis when samples maintain their botanical features, such as in bulk or fragmented plant leaves, to more advanced techniques such as DNA analysis when those characteristics are lost, as happens in plant powders and extracts (Fig. 1) (Smillie and Khan, 2010; Grazina et al., 2020). Among DNA-based approaches, LAMP has been proposed as a simple and fast tool with high potential to be used in-field applications since it enables fast, specific, and robust DNA amplification under isothermal conditions, without requiring sophisticated equipment (Li et al., 2016). Therefore, in this study, specific LAMP primers were designed, and the reaction was optimized towards developing a simple methodology for the specific identification of *S. japonicum* DNA.

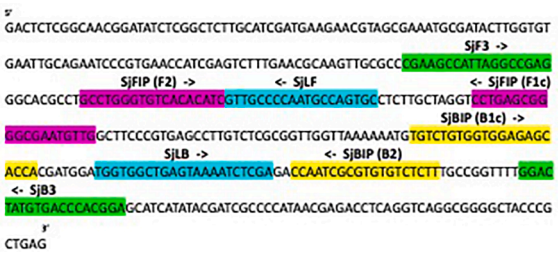


Fig. 2. Location of LAMP primers designed on the HQ229005.1 NCBI accession number. SjF3 and SjB3 are shown in green, SjFIP (F1c+F2) in pink, SjBIP (B1c+B2) in yellow, and the two loop primers (SjLF and SjLB) in blue. The arrow symbols indicate the direction of DNA polymerization from the LAMP primers.

LAMP primers design

While for animals, the mitochondrial cytochrome c oxidase I gene (COI) has been established and well accepted as the universal DNA barcoding marker (Hebert et al., 2003), no single barcode has demonstrated similar effectiveness for plants. Thus, various chloroplastidial and nuclear regions, single or combined, have been evaluated and proposed for plants. Among those, the non-coding internal transcribed spacer (ITS) of nuclear ribosomal DNA has been suggested as a core barcode due to its better species discriminating power over other regions (China Plant BOL Group et al., 2011). For this reason, ITS, particularly ITS2, has been frequently selected as a region for developing PCR species-specific primers aiming for medicinal plant identification (Doganay-Knapp et al., 2018; Grazina et al., 2020). Herein, ITS2 was selected for designing the LAMP primers, aiming for high specificity. For that purpose, the GenBank accession HQ229005.1, corresponding to the partial sequence of the 5.8S ribosomal RNA gene, the complete sequence of ITS2, and partial 26S ribosomal RNA gene, was used, as previously described by Grazina et al. (2021) when developing a species-specific real-time PCR approach. In addition, key parameters were considered when designing the LAMP primers, including melting temperatures (Tm), stability at the end of each primer, GC content, and the formation of secondary structures (Parida et al., 2008; Soroka et al., 2021). Taking these parameters into account, the best primer sets proposed by the PrimerExplorer V5 software were analyzed in detail, and the best two options were selected: one targeting a sequence comprising only the ITS2 (primer set 1) and the other targeting a sequence expanding to the 5.8S rRNA region (primer set 2), where the outer forward primer was located. The two selected primer sets exhibited similarities for most parameters, except in forming secondary structures, including primer-dimers and hairpin loops. Hairpin loops are formed due to intramolecular interactions, which can impact the amplification step and produce nonspecific amplicons or even no amplicon yield. Primer-dimers are formed due to complementary sequences within a primer (self-dimer), or complementary sequences shared by two primers (hetero-dimer), thereby inhibiting the primers from effectively annealing to the target sequence. Although there is limited information regarding the impact of these structures on the LAMP reaction (Meagher et al., 2018), secondary structures are generally recognized features to be avoided when designing nucleic acid amplification techniques.

The OligoAnalyzer tool was used to analyze the two selected primer sets, particularly the ΔG values, which consider the longest stretch of complementary bases, of any secondary structure. When comparing the LAMP outer primers of both selected sets, although primer SjF3 of set 2 exhibited a higher potential for self-dimer formation (ΔG of −9.28 kcal/

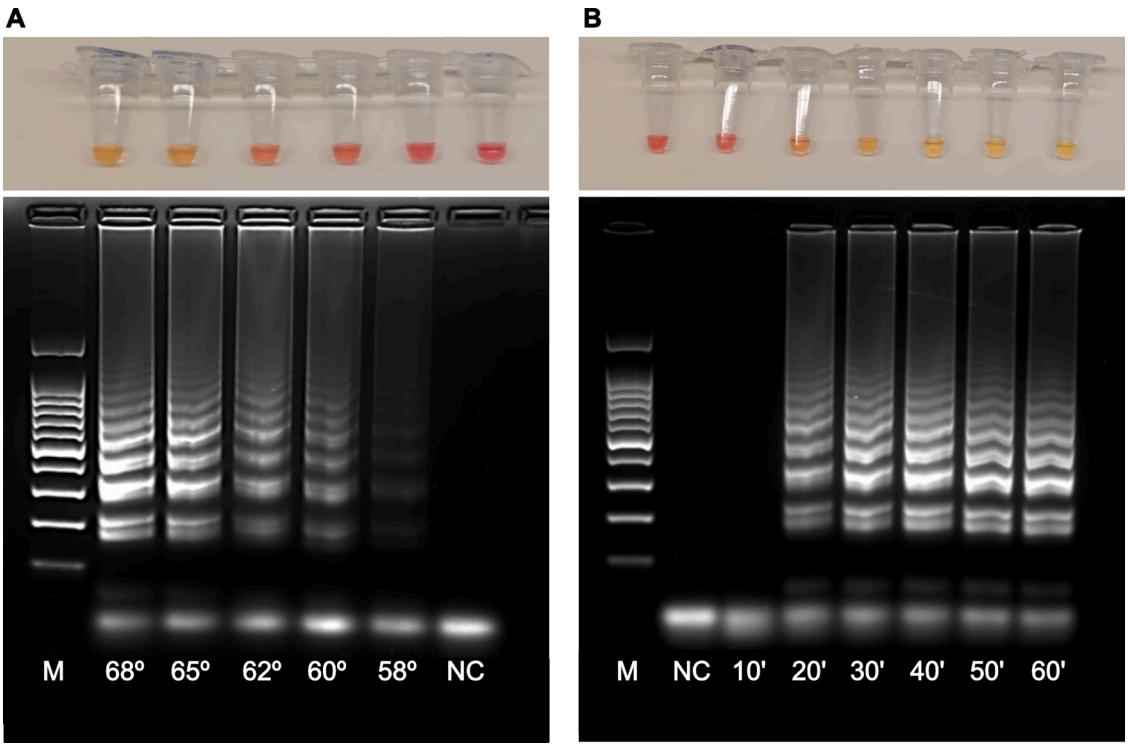


Fig. 3. Results of the LAMP reactions when testing the amplification temperature and reaction time. A – Color changes and electrophoresis results of the products amplified at 68 ºC, 65 ºC, 62 ºC, 60 ºC, and 58 ºC for 30 min. B – Color changes and electrophoresis results of the products amplified at 68 ºC for 10, 20, 30, 40, 50, and 60 min. M: 100 bp DNA ladder marker; NC: negative control.

mol, slightly below the maximum recommended value of −9 kcal/mol), primer set 1 showed the potential formation of five hairpins and a likelihood of primer dimer formation. In addition, the LAMP inner primers (FIP and BIP) of the primer set 1 showed the potential formation of seven and five hairpins, respectively, and both exhibited a greater likelihood of primer dimer formation in both self-dimers and hetero-dimers secondary structures (ΔGs: −10.36 kcal/mol). In contrast, the inner primers of set 2 (SjFIP and SjBIP) showed a lower potential formation of hairpins and a reduced likelihood of hetero-dimers formation (ΔGs: −8.33 kcal/mol), despite SjFIP showing some potential formation of self-dimers (ΔG: −9.28 kcal/mol). Based on these results and to minimize the potential formation of secondary structures, primer set 2 was chosen for detecting *S. japonicum* using the colorimetric LAMP assay. The sequences and binding sites of this primer set are provided in Table 1 and Fig. 2, respectively.

LAMP reaction and assay optimization

The LAMP method, originally proposed by Notomi (2000), can amplify sequences of nucleic acids based on DNA strand displacement of a DNA polymerase, generally *Bst* polymerase. The reaction requires at least four different primers, which recognize six independent regions of the target sequence, and may be improved by the use of two additional loop primers (Notomi, 2000; Moehling et al., 2021; Soroka et al., 2021). Herein, the addition of the loop primers showed to be crucial for the efficiency of the reaction as the four primers alone (SjF3, SjB3, SjFIP and SjBIP) did not lead to amplification after 1-hour reaction at 65 ºC, requiring a 2 h 30 min reaction to achieve a positive result (data not shown). However, when the six primers were used (Table 1) the reaction proceeded faster, generating the expected stem-loop DNA structures, as evidenced by the characteristic ladder pattern in the agarose gel electrophoresis (Fig. 3). Moreover, because loop primers allow to further increase the specificity of the LAMP assay, this option was selected and further optimized. In addition, considering that the formation of primer-dimers in LAMP assays may randomly lead to false-positive results, precautionary steps were considered to mitigate nonspecific amplification and minimize the occurrence of undesired interactions.

The first trials for assessing the performance of the designed primers (with and without the addition of the loop primers) were carried out using the concentrations recommended by the manufacturer of the colorimetric mix (www.neb.com/en/protocols/2016/08/15/warmstart-colorimetric-lamp-2x-master-mix-typical-lamp-protocol-m1800), namely: 1.6 μM for BIP and FIP, 0.2 μM for F3 and B3 and 0.4 μM for loop primers. However, given the high cost of the HPLC-purified primers that are required for the LAMP assay, two different concentrations maintaining the primers' ratios were assessed under the same amplification temperature and reaction time: 1.2 μM for BIP and FIP, 0.15 μM for F3 and B3 and 0.3 μM for loop primers (75 % reduction) and 0.8 μM for BIP and FIP, 0.1 μM for F3 and B3 and 0.2 μM for loop primers (50 % reduction). Moreover, it is known that higher concentrations of primers in LAMP assays increase the formation of primer-dimmers or other mismatched structures, which in turn increases the chances for false positive results (Kim et al., 2023; Ku et al., 2022). The outputs showed that similar performances were still achieved when using half of the concentration for all primers (data not shown), representing a considerable reduction in the global cost of the LAMP assay and minimizing the chances for nonspecific amplification arising from primer-dimers. Therefore, all subsequent trials were performed using 50 % of the primers' initial concentration.

The subsequent trials for optimizing the colorimetric LAMP assay concerned the amplification temperature and reaction time (Fig. 3). To examine the influence of the temperature on the LAMP success, the reaction time was set at 30 min while the temperature ranged from 58 ºC to 68 ºC (Fig. 2A). In addition to the gel electrophoresis analysis, the outcomes of the LAMP reaction were visually inspected for color change, as allowed by the presence of phenol red within the reaction mixture. Positive DNA amplification was indicated by a color change from pink (no amplification) to yellow (amplification). As shown in Fig. 3A, the

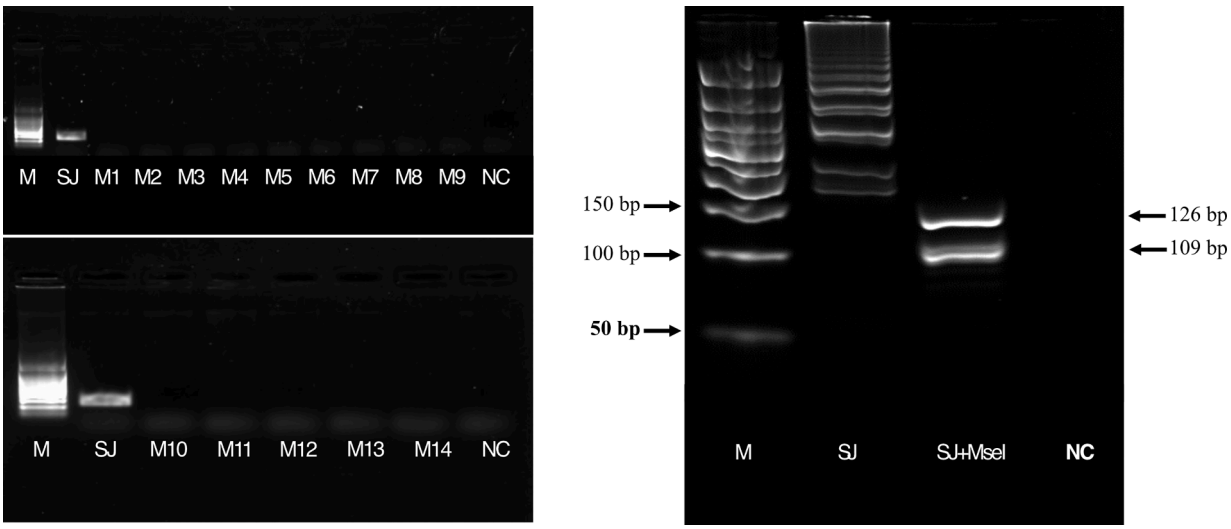


Fig. 4. Specificity of the LAMP reaction for the authentication of S. japonicum. A – Conventional PCR using SjF3 and SjB3 primers and the DNA extracts of 51 medicinal plants (SJ: *S. japonicum*, M1–14: mixtures of 50 medicinal plants including *G. biloba*, as described in Supplementary Table S1); M: 100 bp DNA ladder marker. B – LAMP amplification product and corresponding MseI digestion product; M: 50 bp DNA ladder marker; SJ: positive control (*S. japonicum*). SJ+MseI: MseI digestion product; NC: negative control.

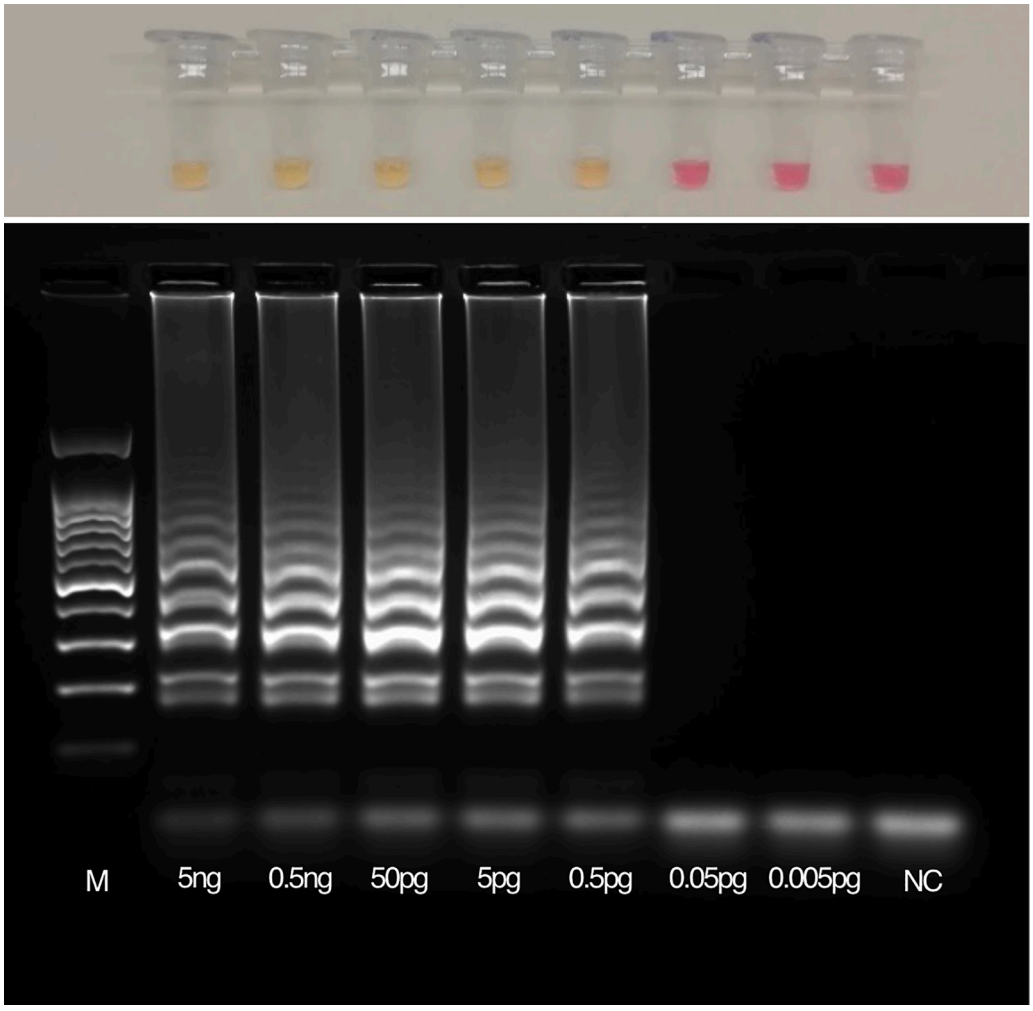


Fig. 5. Limit of detection (LOD) of the LAMP reaction for the authentication of *S. japonicum* using a 10-fold serial dilution of *S. japonicum* DNA varying from 5 ng to 0.005 pg. M: 100 bp DNA ladder marker; NC: negative control.

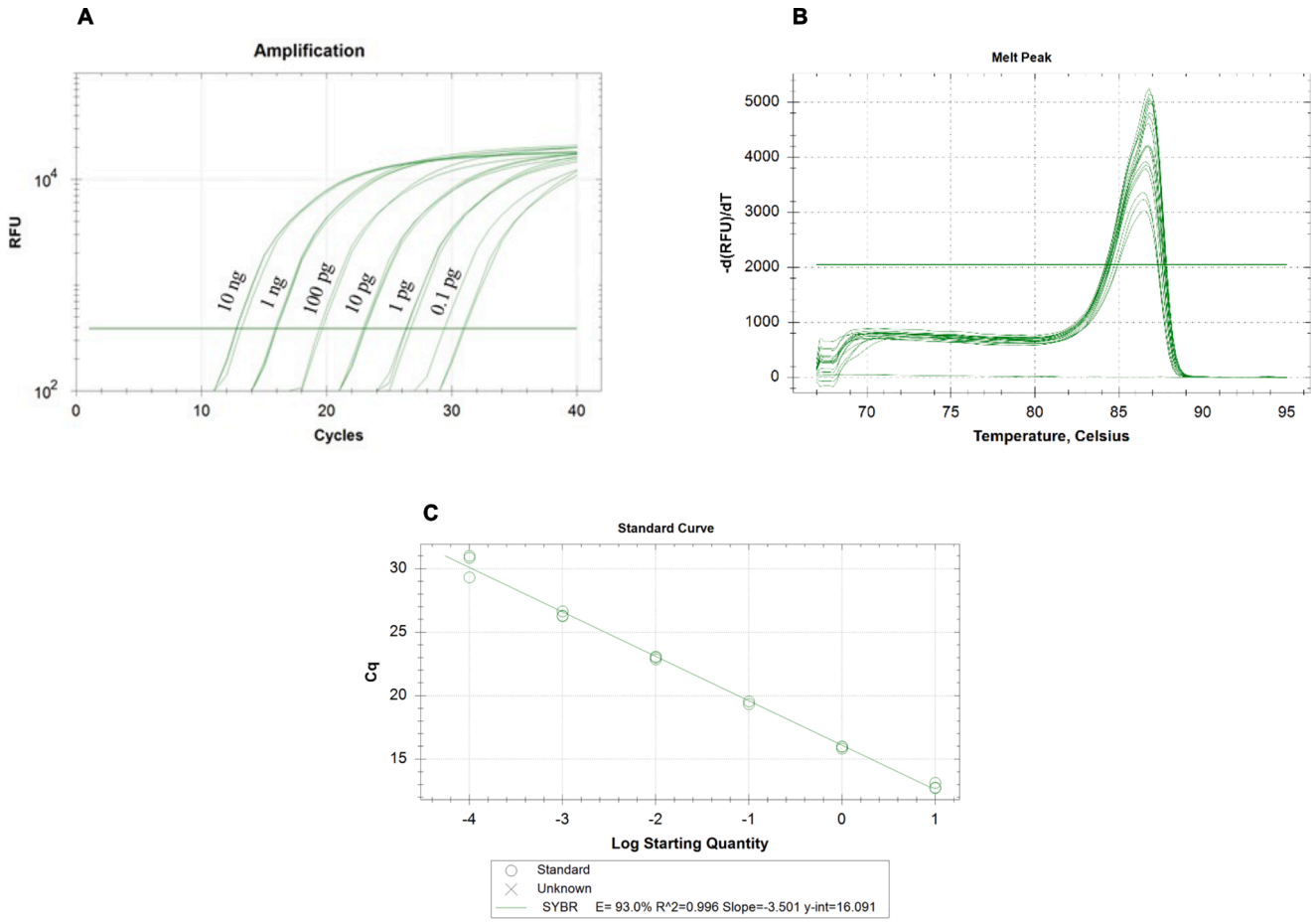


Fig. 6. Results of the real-time PCR assay using the LAMP outer primers (SjF3 and SjB3) targeting *S. japonicum*. A – Amplification curves, B – melting curves, C – calibration curve. The amplified extracts correspond to 10-fold serially diluted *S. japonicum* DNA ranging from 10 ng to 0.01 pg (n = 3 replicates).

electrophoresis of the LAMP-amplified products exhibited a characteristic ladder pattern for most of the tested temperatures, demonstrating the effectiveness of the designed primer set. Nevertheless, a color change to yellow was only clearly observed for the reactions carried out at 68 ºC and 65 ºC, while at 62 ºC an orange color was obtained. Because the electrophoresis was brightest at 68 ºC, evidencing a higher content of amplified products, the optimal amplification temperature for the LAMP assay was set at 68 ºC. Moreover, the higher temperature contributes to inhibiting the random formation of primer-dimers and promoting the correct combination of primers and template (Wang et al., 2015), therefore decreasing the possibility of nonspecific amplification. In this regard, also to minimize possible nonspecific amplifications, all reactions were performed in a pre-heated thermocycler using the Bst 2.0 WarmStart® DNA Polymerase. Despite this modified version of the original Bst enzyme exhibits low levels of activity at low temperatures, according to Ye et al. (2018) annealing and extension of eventual primer-dimers can still occur while the heating block is reaching the target temperature, thus promoting nonspecific amplification and the production of false positives. Subsequently, the influence of the reaction time was evaluated by carrying out the reaction at this temperature for reaction times varying between 10 and 60 min with increments of 10 min (Fig. 3B). The shortest reaction time required for developing the yellow-colored solution was 20 min, although from 30 min onward the solution exhibited a progressively enhanced yellow luminosity. Electrophoresis revealed the characteristic ladder pattern for all tested reaction times, except for the shortest 10 min reaction (Fig. 3B). After comparing the amplified products at the various reaction times, the most favorable outcomes in terms of color visual inspection were achieved when the reaction was conducted at 68 °C for 40 min. Therefore, this parameterization was used in all subsequent analyses.

Specificity of the LAMP assay

To further verify the specificity of the LAMP primers for *S. japonicum* as first determined by *in silico* analysis, cross-reactivity tests were performed by conventional PCR with the designed outer primers SjF3 and SjB3 against a total of 51 different species comprising the three samples of *G. biloba* obtained from the botanical gardens, *S. davidii* and 50 samples of medicinal plants previously identified through ITS2, matK and/or rbcL sequencing (supplementary table S1). To that purpose, mixtures containing DNA from different plant species (5 ng/µl) were prepared by admixing the DNA extracts obtained from those species. As shown in Fig. 4A, none of the mixtures comprising a total of 50 medicinal plants (represented by Ml from 1 to 14) exhibited amplification, confirming the specificity of the LAMP outer primers.

The specificity of the LAMP assay can be further corroborated by using restriction enzymes that cleave the amplicons at a specific location (DI Felice et al., 2019). Based on the *in silico* analyses, *S. japonicum* amplicons are 235 bp long and include the restriction site for the MseI enzyme. Fig. 4B shows the gel electrophoresis of the LAMP products (line SJ) and their digestion using the MseI enzyme (line SJ+MseI). Two bands of approximately 109 and 126 bp can be observed in the gels, indicating the successful amplification of the target region containing the MseI restriction site and, therefore, confirming the high specificity achieved by the primer set designed herein for detecting *S. japonicum* DNA.

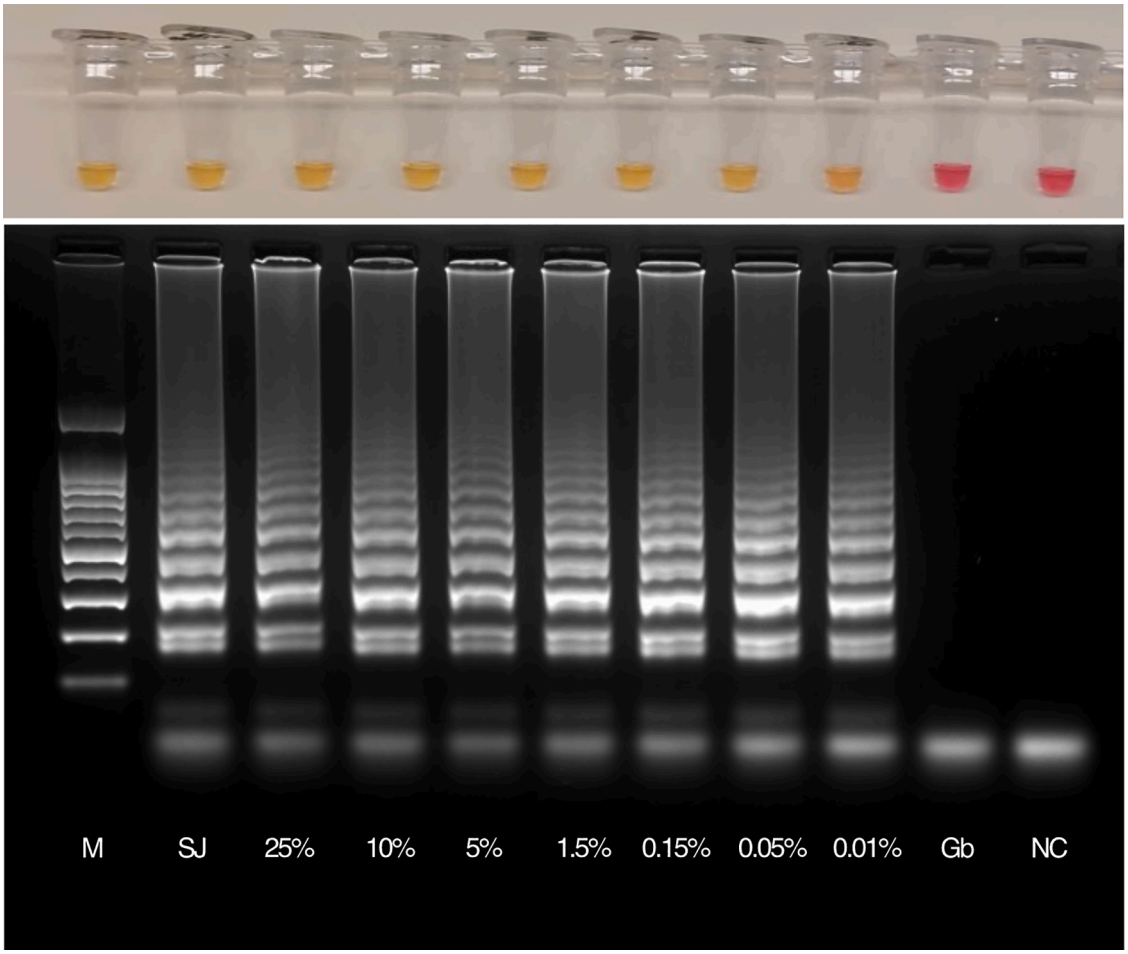


Fig. 7. Binary mixtures containing known amounts of powdered *S. japonicum* (25 %, 10 %, 5 %, 1.5 %, 0.15 %, 0.05 %, 0.01 %) in *G. biloba*. M: 100 bp DNA ladder marker; SJ: *S. japonicum*; Gb: *G. biloba*; NC: negative control.

Sensitivity of the LAMP assay

To confirm the sensitivity of the LAMP primers, the absolute limit of detection (LOD) was determined using a 10-fold dilution, with DNA templates ranging from 5 ng to 0.005 pg per reaction. The colorimetric LAMP assay was detectable up to 0.5 pg of *S. japonicum* DNA template, in which the positive reactions turned yellow whereas the negative control samples remained pink (Fig. 5). All reaction products were subsequently analyzed on gel electrophoresis to check for the typical ladder pattern of LAMP. Based on the results, the genomic DNA required to detect *S. japonicum* reliably was determined to be 0.5 pg per reaction.

In addition, for comparison purposes, a real-time PCR assay using the EvaGreen fluorescent dye was performed using the outer primers SjF3 and SjB3. After establishing the optimal conditions regarding the annealing temperature and time of the extension step, real-time PCR was applied to amplify an *S. japonicum* DNA extract 10-fold serially diluted from 10 ng to 0.01 pg for method evaluation and absolute LOD determination (Fig. 6A). The acceptance criteria previously established for real-time PCR assays, namely the slope between −3.6 and 3.1, PCR efficiency between 90 and 110 %, and correlation coefficient (R^2)>0.98, were considered (Bustin et al., 2009). Fig. 5C shows the obtained real-time PCR calibration curve, presenting a slope of −3.501, PCR efficiency of 93 %, and a R^2 of 0.996, demonstrating its good performance. The corresponding melting curves showed close profiles and melt peaks, which is characteristic of the formation of one single type of fragment and indicates the absence of unspecific amplicons (Fig. 6B). Based on the obtained results, the absolute LOD was determined to be 0.1 pg of *S. japonicum* DNA, which was lower than the one obtained by the proposed LAMP assay (0.5 pg).

Furthermore, the relative LOD for the colorimetric LAMP assay was assessed through the amplification of the DNA extracted from the laboratory-prepared binary mixtures encompassing predetermined quantities of powdered *S. japonicum* (25 %, 10 %, 5 %, 1.5 %, 0.15 %, 0.05 %, 0.01 %) added to powdered *G. biloba*. The relative LOD achieved 0.01 % of *S. japonicum* plant material (Fig. 7), demonstrating the high sensitivity of the LAMP assay. Overall, considering that adulteration by the substitution of plant material generally is done in higher amounts to be profitable, the sensitivity of the LAMP assay developed here was demonstrated to be adequate for detecting this type of fraud. Nevertheless, for control purposes, it should be taken into account that the developed assay is based on a colorimetric assay only, without the use of any additional specific probe (e.g. molecular beacon). The option for colorimetric instead of fluorescence determination was selected to offer a fast, sensitive, and low-cost approach with the possibility of being used as a screening tool for in-field analysis. Therefore, it is advisable that after screening commercial samples using the herein-proposed approach, positive results are further confirmed at the laboratory by using the conventional real-time PCR approach.

Conclusions

The proposed colorimetric LAMP assay for the identification of *S. japonicum* showcased its rapidity, sensitivity, and specificity. This approach allows for visual inspection of results based on the color change from pink to yellow and eliminates the need for a PCR thermocycler and gel electrophoresis of the amplified product, which makes it

suitable for on-site detection of *S. japonicum*. Compared to other methods previously proposed to detect the fraudulent addition of *S. japonicum* in *G. biloba*-based products, including chromatographic and molecular ones, the LAMP assay developed here was shown to be faster and/or simpler.

Therefore, this species-specific LAMP assay can be a suitable tool for manufacturers (e.g., to be used on-field for quality control screening of raw materials such as bulk or powdered plants) and regulators to authenticate and ascertain the quality of ginkgo-based products that potentially incorporate *S. japonicum* as an adulterant. This work significantly contributes to upholding the safety and efficacy of medicinal plant products and plant food supplements.

Authors contribution

All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Funding

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support through the project "POIROT: novel methods and approaches for detecting the illegal addition of Pharmaceutical drugs and bOtanIcal adulteRatiOn in planT food supplements" (PTDC/SAU-PUB/3803/2021) and through national funds FCT/MCTES (PIDDAC) to CIMO (UIDB/00690/2020 and UIDP/00690/2020) and SusTEC (LA/P/0007/2020). M. Honrado is grateful to FCT for PhD grant 2021.08119.BD financed by POPH-QREN (subsidised by FSE and MCTES).

CRediT authorship contribution statement

Vânia Rodrigues: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Mónica Honrado:** Formal analysis, Investigation, Methodology. **Joana Santos:** Investigation, Methodology. **M. Alice Pinto:** Supervision, Writing – review & editing. **Joana S. Amaral:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are grateful to the Botanical Garden of the University of Porto (Portugal), Botanical Garden of the University of Bonn (Germany), and Botanical Museum of the Department of Life Sciences, University of Siena (Italy), and Botanical Garden of Sóller (Spain) for supplying leaves of authentic G. biloba and/or S. japonicum, and to the Botanical Garden of the Université Louis Pasteur (Strasbourg, France) for supplying leaves of authentic S. davidii.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2023.155322.

References

Avula, B., Sagi, S., Gafner, S., Upton, R., Wang, Y.H., Wang, M., Khan, I.A., 2015. Identification of *Ginkgo biloba* supplements adulteration using high performance thin layer chromatography and ultra high performance liquid chromatography-diode array detector-quadrupole time of flight-mass spectrometry. Anal. Bioanal. Chem. 407, 7733–7746. https://doi.org/10.1007/s00216-015-8938-1.

Bampali, E., Germer, S., Bauer, R., Kulić, Ž., 2021. HPLC-UV/HRMS methods for the unambiguous detection of adulterations of *Ginkgo biloba* leaves with *Sophora japonica* fruits on an extract level. Pharm. Biol. 59, 436–441. https://doi.org/10.1080/13880209.2021.1910717.

Botella, J.R., 2022. Point-of-care DNA amplification for disease diagnosis and management. Annu. Rev. Phytopathol. 60, 1–20. https://doi.org/10.1146/annurev-phyto-021621-115027.

Budeč, M., 2019. Verification of authenticity of *Ginkgo biloba* L. Leaf extract and its products present on the croatian market by analysis of quantity and ratio of ginkgo flavone glycosides (Quercetin, Kaempferol and Isorhamnetin) to terpene trilactones to the effect of unmasking counterfeit drugs endangering patient health. Acta Clin. Croat. 58 https://doi.org/10.20471/acc.2019.58.04.15.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., Vandesompele, J., Wittwer, C.T., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clin. Chem. 55, 611–622. https://doi.org/10.1373/clinchem.2008.112797.

Chaudhary, A.A., Hemant, Mohsin, M., Ahmad, A., 2012. Application of loop-mediated isothermal amplification (LAMP)-based technology for authentication of *Catharanthus roseus* (L.) G. Don. Protoplasma 249, 417–422. https://doi.org/10.1007/s00709-011-0293-2.

China Plant BOL Group, Li, D.Z., Gao, L.M., Li, H.T., Wang, H., Ge, X.J., Liu, J.Q., Chen, Z.D., Zhou, S.L., Chen, S.L., Yang, J.B., Fu, C.X., Zeng, C.X., Yan, H.F., Zhu, Y. J., Sun, Y.S., Chen, S.Y., Zhao, L., Wang, K., Yang, T., Duan, G.W., 2011. Comparative analysis of a large dataset indicates that Internal Transcribed Spacer (ITS) should be incorporated into the core barcode for seed plants. Proc. Natl. Acad. Sci. 108, 19641–19646. https://doi.org/10.1073/pnas.1104551108.

DI Felice, F., Micheli, G., Camilloni, G., 2019. Restriction enzymes and their use in molecular biology: an overview. J. Biosci. 44, 38.

Diamond, B.J., Bailey, M.R., 2013. Ginkgo biloba: indications, mechanisms, and safety. Psychiatr. Clin. N. Am. 36, 73–83. https://doi.org/10.1016/j.psc.2012.12.006.

Doganay-Knapp, K., Orland, A., König, G.M., Knöss, W., 2018. The potential of three different PCR-related approaches for the authentication of mixtures of herbal substances and finished herbal medicinal products. Phytomedicine 43, 60–67. https://doi.org/10.1016/j.phymed.2018.03.062.

Glökler, J., Lim, T.S., Ida, J., Frohme, M., 2021. Isothermal amplifications – a comprehensive review on current methods. Crit. Rev. Biochem. Mol. Biol. 56, 543–586. https://doi.org/10.1080/10409238.2021.1937927.

Govindaraghavan, S., 2018. Increasing sophistication in adulteration of commercial Ginkgo biloba leaf products: detection using existing methods of analysis of ginkgoflavone glycosides. Fitoterapia 131, 146–159. https://doi.org/10.1016/j.fitote.2018.10.024.

Grazina, L., Amaral, J.S., Costa, J., Mafra, I., 2021. Tracing *Styphnolobium japonicum* (syn: sophora japonica) as a potential adulterant of ginkgo-containing foods by real-time PCR. J. Food Compos. Anal. 100, 103891 https://doi.org/10.1016/j.jfca.2021.103891.

Grazina, L., Amaral, J.S., Mafra, I., 2020. Botanical origin authentication of dietary supplements by DNA-based approaches. Compr. Rev. Food Sci. Food Saf. 19, 1080–1109. https://doi.org/10.1111/1541-4337.12551.

Hebert, P.D.N., Cywinska, A., Ball, S.L., deWaard, J.R., 2003. Biological identifications through DNA barcodes. Proc. R. Soc. Lond. B Biol. Sci. 270, 313–321. https://doi.org/10.1098/rspb.2002.2218.

Kim, S.H., Lee, S.Y., Kim, U., Oh, S.W., 2023. Diverse methods of reducing and confirming false-positive results of loop-mediated isothermal amplification assays: A review. Anal. Chim. Acta 1280, 341693. https://doi.org/10.1016/j.aca.2023.341693.

Ku, J., Chauhan, K., Hwang, S.H., Jeong, Y.J., Kim, D.E., 2022. Enhanced Specificity in Loop-Mediated Isothermal Amplification with Poly(ethylene glycol)-Engrafted Graphene Oxide for Detection of Viral Genes. Biosensors 12 (8), 661. https://doi.org/10.3390/bios12080661.

Lai, G.H., Chao, J., Lin, M.K., Chang, W.T., Peng, W.H., Sun, F.C., Lee, M.S., Lee, M.S., 2015. Rapid and sensitive identification of the herbal tea ingredient taraxacum formosanum using loop-mediated isothermal amplification. Int. J. Mol. Sci. 16, 1562–1575. https://doi.org/10.3390/ijms16011562.

Li, J., Xiong, C., Liu, Y., Liang, J., Zhou, X., 2016. Loop-mediated isothermal amplification (LAMP): emergence as an alternative technology for herbal medicine identification. Front. Plant Sci. 7 https://doi.org/10.3389/fpls.2016.01956.

Li, M., Wong, Y.L., Jiang, L.L., Wong, K.L., Wong, Y.T., Lau, C.B.-S., Shaw, P.C., 2013. Application of novel loop-mediated isothermal amplification (LAMP) for rapid authentication of the herbal tea ingredient Hedyotis diffusa Willd. Food Chem. 141, 2522–2525. https://doi.org/10.1016/j.foodchem.2013.05.085.

Liu, Y., Wang, X., Wei, X., Gao, Z., Han, J., 2018. Rapid authentication of ginkgo biloba herbal products using the recombinase polymerase amplification assay. Sci. Rep. 8, 8002. https://doi.org/10.1038/s41598-018-26402-8.

Ma, Y.C., Mani, A., Cai, Y., Thomson, J., Ma, J., Peudru, F., Chen, S., Luo, M., Zhang, J., Chapman, R.G., Shi, Z.T., 2016. An effective identification and quantification method for Ginkgo biloba flavonol glycosides with targeted evaluation of adulterated products. Phytomedicine 23, 377–387. https://doi.org/10.1016/j.phymed.2016.02.003.

Meagher, R.J., Priye, A., Light, Y.K., Huang, C., Wang, E., 2018. Impact of primer dimers and self-amplifying hairpins on reverse transcription loop-mediated isothermal amplification detection of viral RNA. Analyst 143, 1924–1933. https://doi.org/10.1039/C7AN01897E.

Moehling, T.J., Choi, G., Dugan, L.C., Salit, M., Meagher, R.J., 2021. LAMP diagnostics at the point-of-care: emerging trends and perspectives for the developer community. Expert Rev. Mol. Diagn. 21, 43–61. https://doi.org/10.1080/14737159.2021.1873769.

Moon, Y.J., Lee, S.Y., Oh, S.W., 2022. A review of isothermal amplification methods and food-origin inhibitors against detecting food-borne pathogens. Foods 11, 322. https://doi.org/10.3390/foods11030322.

Munawar, M.A., 2022. Critical insight into recombinase polymerase amplification technology. Expert Rev. Mol. Diagn. 22, 725–737. https://doi.org/10.1080/14737159.2022.2109964.

Notomi, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28. https://doi.org/10.1093/nar/28.12.e63, 63e–663.

Oliveira, B.B., Veigas, B., Baptista, P.V., 2021. Isothermal amplification of nucleic acids: the race for the next "Gold standard. Front. Sens. 2, 752600 https://doi.org/10.3389/fsens.2021.752600.

Parida, M., Sannarangaiah, S., Dash, P.K., Rao, P.V.L., Morita, K., 2008. Loop mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Rev. Med. Virol. 18, 407–421. https://doi.org/10.1002/rmv.593.

Park, J.W., 2022. Principles and applications of loop-mediated isothermal amplification to point-of-care tests. Biosensors 12, 857. https://doi.org/10.3390/bios12100857.

Plant Working Group, CBOL, Hollingsworth, P.M., Forrest, L.L., Spouge, J.L., Hajibabaei, M., Ratnasingham, S., van der Bank, M., Chase, M.W., Cowan, R.S., Erickson, D.L., et al., 2009. A DNA barcode for land plants. PNAS 106 (31), 12794–12797. https://doi.org/10.1073/pnas.0905845106.

Rausch, T., Fritz, M.H.Y., Untergasser, A., Benes, V., 2020. Tracy: basecalling, alignment, assembly and deconvolution of sanger chromatogram trace files. BMC Genom. 21, 230. https://doi.org/10.1186/s12864-020-6635-8.

Smillie, T., Khan, I., 2010. A comprehensive approach to identifying and authenticating botanical products. Clin. Pharmacol. Ther. 87 (2), 175–186. https://doi.org/10.1038/clpt.2009.287.

Soroka, M., Wasowicz, B., Rymaszewska, A., 2021. Loop-Mediated Isothermal Amplification (LAMP): the better sibling of PCR? Cells 10, 1931. https://doi.org/10.3390/cells10081931.

Wang, D-G., Brewster, J.D., Paul, M., Tomasula, P.M., 2015. Two Methods for Increased Specificity and Sensitivity in Loop-Mediated Isothermal Amplification. Molecules 20 (4), 6048–6059. https://doi.org/10.3390/molecules20046048.

Wu, L., Wang, B., Zhao, M., Liu, W., Zhang, P., Shi, Y., Xiong, C., Wang, P., Sun, W., Chen, S., 2016. Rapid identification of officinal akebiae caulis and its toxic adulterant aristolochiae manshuriensis caulis (Aristolochia manshuriensis) by loop-mediated isothermal amplification. Front. Plant Sci. 7 https://doi.org/10.3389/fpls.2016.00887.

Yan, L., Zhou, J., Zheng, Y., Gamson, A.S., Roembke, B.T., Nakayama, S., Sintim, H.O., 2014. Isothermal amplified detection of DNA and RNA. Mol. Biosyst. 10, 970. https://doi.org/10.1039/c3mb70304e.

Ye, X., Fang, X., Li, X., Kong, J., 2018. Gold nanoparticle-mediated nucleic acid isothermal amplification with enhanced specificity. Anal. Chim. Acta. 1043, 150–157. https://doi.org/10.1016/j.aca.2018.09.016.

Zhao, Y., Chen, F., Qin, J., Wei, J., Wu, W., Zhao, Y., 2018. Engineered Janus probes modulate nucleic acid amplification to expand the dynamic range for direct detection of viral genomes in one microliter crude serum samples. Chem Sci 9 (2), 392–397. https://doi.org/10.1039/C7SC03994H.