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Molecular approach for identification and validation of *Eurycoma longifolia*, *Labisia pumila*, and *Orthosiphon stamineus* in herbal products

Siti Nor Amira Mohd Azli[1], Adibah Abu Bakar[1], Salwa Shahimi[2], Bryan Raveen Nelson[3], Azi Azeyanty Jamaludin[1,4*] and Jameel R. Al-Obaidi[1*]

Abstract

Background *Eurycoma longifolia* (*E. longifolia*), *Labisia pumila* (*L. pumila*), and *Orthosiphon stamineus* (*O. stamineus*) are popular species known for their therapeutic properties. An increase in local demand for herbal products makes them susceptible to adulteration, which poses a risk to their safety and efficacy. Current identification methods, such as organoleptic, microscopic, and macroscopic analysis, need to be revised to identify plant species in highly processed herbal products due to their limited ability to detect morphological features and provide comprehensive plant taxonomy information.

Methods This research objective was to develop a simple, reliable, and accurate DNA molecular identification method based on polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) for *E. longifolia, L. pumila*, and *O. stamineus*, used to validate the species identification for herbal products. PCR–RFLP was developed for rapid identification using restriction enzymes *TaqI, BamH I, HinfI, EcoRI, EcoRV, MboI*, and *MspI*.

Results The nuclear DNA internal transcribed spacer 2 (ITS2) sequences were identified and compared between plant specimens of *E. longifolia, L. pumila*, and *O. stamineus* and 101 samples of commercial herbal products. Plant specimens of *E. longifolia, L. pumila,* and *O. stamineus* were successfully identified with high similarity of 100%, 100%, and 99.33%, respectively, based on National Center for Biotechnology Information (NCBI) GenBank. The recovery of DNA sequences from the herbal products was 60.4%, of which 81.97% were identified, and 18.03% showed no sequence through Basic Local Alignment Search Tool (BLAST) identification.

Conclusion A reliable approach for identifying and validating plant species in herbal products has been created using restriction enzymes. This simple and accurate PCR–RFLP approach efficiently identifies *E. longifolia*, *L. pumila*, and *O. stamineus* by analysing ITS2 sequences, assuring consumer health and safety.

Keywords DNA marker, Internal transcribed spacer 2 (ITS2), Molecular identification, PCR–RFLP, Plant-based products

*Correspondence:
Azi Azeyanty Jamaludin
azi_azeyanty@fsmt.upsi.edu.my
Jameel R. Al-Obaidi
jr_alobaidi@yahoo.com
Full list of author information is available at the end of the article

1 Background

Known plant extracts that potentially reduce the risk of illnesses have been added to foods and supplements to promote vitality aside from intentions to increase product marketability [1]. Herbs, classified as "beneficial" plant extracts could enhance the use of foods, cosmetics, drinks, or personal care products [2, 3]. Herbs from the tropical region such as *Eurycoma longifolia* (*E. longifolia*) known as Tongkat ali, *Labisia pumila* (*L. pumila*) known as Kacip fatimah, and *Ortosiphon stamineus* (*O. stamineus*) known as Misai kucing are considered botanical labels for good health [4]. However, the diverse availability of products and price range raises questions about the authenticity of the ingredients (especially plant components or extracts) in supply chains [5].

In the age of the fourth industrial revolution, necessities fuelled the generation, innovation, and research on dietary supplements [6]. Swift solutions through adulteration are promising to overcome resource shortages aside from the incremental revision in supply pricing [7]. Therefore, irresponsibility (label switching) could begin with the plant or extract itself either from the cultists, suppliers, or during production [6]. Herbal ingredients circulate as powders, dried materials, pills, capsules, and tea bags after being cleaned, dried, or minced and in this form, the source plant could not be directly identified [8, 9]. The use of traditional methods such as microscopy, spectrometry, and thin-layer chromatography has been introduced to identify the source plant in a crude [10]. However after modern processing techniques that alter the chemistry and biochemical profiles for long-term storage, the traditional identification means by morphological and microscopic identification seems irrelevant [11]. Similarly, methods that compare biochemical profiles with chemical standards are challenged by noise due to cultivation methods [7, 12].

Admixture can occur through several deliberate and accidental practices, such as blatant and obvious adulteration, typically done for benefit due to a lack of adequate quality control steps [13]. These practices are common in plant species that are in low supply but have great demand [14, 15]. If left uncontrolled, adulteration can severely impact the reputation of the place of origin and the export trade of medicinal plants in question [14]. Despite this, accidental contamination of herbal products can happen due to misidentification, confusion, or the use of vernacular names, underscoring the need for more care to assure purity and authenticity [16]. Misidentification of plant species is fairly common as macroscopic in the form of morphological identification of plant species is confusing and requires the expertise of a qualified specialist [9, 17]. Furthermore, it may be challenging to identify extracted natural product materials to the species level using morphology as the product is in processed form [18, 19].

Several genetic methods have been explored to enhance species recognition and move beyond the bounds of morphological identifications. With the help of molecular methods like the polymerase chain reaction (PCR), it is now possible to distinguish between different species based on their genetic information [20]. polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) is a method for generating polymorphic pieces that can be used as identifiers for identifying species by relying on the digestion of PCR amplicons with the appropriate restriction enzymes [21]. Following this, restriction enzymes are used to cleave the amplified DNA fragments at precise locations [22]. The generated fragments are size-separated by gel electrophoresis, and the pattern of the created fragments is compared with reference patterns to either identify the species or discover variations within the species [22]. PCR—RFLP has been utilised in various investigations to identify and authenticate herbal products. PCR–RFLP was used in various investigations to identify the species of *Ginkgo biloba*, *Panax ginseng*, and *Echinacea purpurea* in herbal products [23, 24].

In light of the absence of standardised quality assessment techniques and the fierce competition within the herbal product market, there is an increasing drive to employ undisclosed fillers and substitutes [16]. To address this issue, DNA molecular diagnostics has emerged as a powerful tool for ongoing market analysis [25]. Thus, this study aimed to develop a straightforward, dependable, and precise PCR–RFLP DNA molecular method to supervise *E. longifolia*, *L. pumila*, and *O. stamineus* herbal products, guaranteeing consumer health and efficacy without resorting to time-consuming and expensive DNA sequencing. Ultimately, the goal of this PCR–RFLP authentication method is to verify the presence of *E. longifolia*, *L. pumila*, and *O. stamineus* in herbal products, ensuring their integrity.

2 Methods

2.1 Plant samples and herbal products samples collection

The fresh leaves of three plant species, *E. longifolia*, *L. pumila*, and *O. stamineus* were obtained from a local nursery in Shah Alam and the botanical gardens in Seri Kembangan, Selangor, Malaysia. Morphologica confirmation and species labelling were conducted with the help of local expert taxonomists. The voucher specimen from each collections were deposited in the herbarium of the Universiti Pendidikan Sultan Idris (UPSI), Perak Malaysia. To prevent DNA degradation, a twig of leaves was placed in a zip lock bag, transported back to the laboratory, and kept in the −20°C freezer for further analysis.

In the present study, 106 herbal products were successfully acquired from different retail stores, pharmaceutical companies, and e-commerce in Malaysia. Product samples were collected according to accessibility, ensuring the most comprehensive range possible covering different parts of Malaysia. The product was in the form that includes (62 powders, four pastes, 16 liquids, four pills, nine capsules, and 11 teabags) (Table 1) representing three plant species: *E. longifolia, L. pumila* and *O. stamineus*. An overview of the products was listed according to the front of their pack name, the name presented in the list of ingredients containing the target species, *E. longifolia, L. pumila*, and *O. stamineus*, and their product form. To retain sample identification and prevent sample mixing between the time of collection and DNA sequencing, each sample was assigned a coded number.

2.2 DNA extraction

Approximately ~ 300 mg of each plant specimen was subjected to total genomic DNA (tgDNA) extraction using the Nucleospin Plant II Kit (Macherey–Nagel™, Düren, Germany), following minor modifications to the manufacturer's instructions. Likewise, approximately ~ 200 mg of each herbal product underwent extraction using the Nucleospin® Food kit (Macherey–Nagel), with slight adjustments to the manufacturer's guidelines. The quality of the extracted gDNA from both the plant specimens and herbal products was assessed using 1% agarose gel electrophoresis, incorporating GelRed as the gel stain. Subsequently, the obtained genomic data were utilised for PCR amplification.

2.3 ITS2 Barcode amplification

Internal transcribed spacer 2 (ITS2) non-coding region DNA was amplified with 10 µM primer ITS2_F: GGG GCGGATATTGGCCTCCCCTTGC and primer ITS2_R: GACGCTTCTCCAGACTACAAT [26]. PCR reactions were carried out with an Applied Biosystems Thermal Cycler, in a total volume of 50 µl reaction mixtures containing 25 µl of Green Taq Mix, 0.5 µl of 10 µM bovine serum albumin (BSA), and 2.5 µl of DNA template. Thermal cycling was performed under the following conditions: initial denaturation at 95 °C for 2 min, then 30 cycles of denaturation at 95 °C for 1 min, annealing at 56 °C for 15 s, extension at 72 °C for 1 min, and finally elongation at 72 °C for 5 min. The PCR products were visualised on 2% agarose gel electrophoresis. Fragment sizes were estimated using a 50 bp and 100 bp [27] size ladder (GeneDirex, Inc, Taiwan). The amplicons that were successfully obtained were forwarded to Apical Scientific Sdn Bhd in Malaysia for sequencing.

2.4 Product identification and analysis

The acquired sequences were manually modified in MEGA 11 and used as a query sequence in NCBI to identify the closest match with a minimum cut-off of 97% to the GenBank nucleotide database. For identifying each DNA sequence obtained from this investigation, the NCBI Basic Local Alignment Search Tool (BLAST) search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used.

2.5 Restriction analysis of the PCR products

Seven restriction enzymes (*TaqI*, *BamHI*, *HinfI*, *EcoRI*, *EcoRV*, *Mbol*, and *Mspl*) were used to analyse the PCR-amplified ITS2 region products. NEBcutter V2.0 web server [28] was used to predict potential restriction sites by performing restriction mapping on the ITS2 sequences. The 20 l reaction volume used for the digesting procedure contained 15 l of ddH2O, 2 l of PCR products, 1 l of restriction enzymes, and 2 l of enzyme buffers. The digestions were conducted at 37 °C for an hour, as instructed by the manufacturer. Using a 50 bp DNA ladder from GeneDirex, Inc., Taiwan, and 1 × Tris–Borate-EDTA buffer (TBE buffer), the resultant DNA fragments were separated by electrophoresis on 2% agarose gels stained with GelRed nucleic acid gel stain.

2.6 Application of the PCR–RFLP on herbal products

To confirm the existing methodologies, 106 bought herbal products were verified using the recognised PCR–RFLP and diagnostic PCR systems, utilising the methods outlined above. The ITS2 barcode region of these samples was sequenced and analysed with the barcode of life data system (BOLD) identification search engine. The BOLD retrieval findings were used to validate the efficiency of the developed approaches. Furthermore, for the identification of these samples, DNA barcoding was used.

3 Results

3.1 Species sequence identification for plant species

DNA barcoding was employed successfully to achieve accurate identification (100%), with the high percentage sequence identification obtained for *O. stamineus*, *L. pumila*, and *E. longifolia* using ITS2 (Table 2), a single PCR product about 316, 315, and 315 bp was amplified (Fig. 1).

For certain plants, like *E. longifolia, L. pumila,* and *O. stamineus*, their ITS2 reference sequence database was found to be insufficient as only a handful of sequences were based upon ITS2. BLAST query top scores show the number of sequences that were of high similarity with studied sample sequence, i.e. four samples for *E.*

Table 1 Herbal products used in this study

No.	Code	Front of pack name	Product form	Ingredient target species
1	HMP01	Premix coffee	Powder	Tongkat Ali
2	HMP02	Kopi pracampur	Powder	Tongkat Ali
3	HMP03	Energy coffee	Powder	Tongkat Ali (*Eurycoma longifolia*)
4	HMP04	Minuman Raja Herba	Powder	Tongkat Ali (*Eurycoma longifolia*)
5	HMP05	Health drink	Tea bags	Tongkat Ali (*Eurycoma longifolia*)
6	HMP06	Minuman campuran Jus Mangga dengan madu	Liquid	Kacip Fatimah
7	HMP07	Kacip Fatimah Capsule	Capsule	Kacip Fatimah (*Labisia pumila*)
8	HMP08	Per'l	Powder	Kacip Fatimah
9	HMP09	Kacip Fatimah tea	Tea bags	Kacip Fatimah (*Labisia pumila*)
10	HMP10	Misai Kucing tea	Tea bags	Misai Kucing (*Orthosiphon stamineus*)
11	HMP11	The Misai Kucing	Tea bags	Misai Kucing (*Orthosiphon stamineus*)
12	HMP12	Misai Kucing tea	Tea bags	Misai Kucing (*Orthosiphon stamineus*)
13	HMP13	Kopi cintaku	Powder	Kacip Fatimah (*Labisia pumila*)
14	HMP14	Kopi tongkat	Powder	Tongkat Ali, Misai Kucing
15	HMP15	White coffee	Powder	Tongkat Ali
16	HMP16	Kopi pracampur	Powder	Tongkat Ali
17	HMP17	Gamat mengkudu	Paste	*Eurycoma longifolia*
18	HMP18	Bawang putih	Pill	*Eurycoma longifolia*
19	HMP19	Manjakani	Capsule	*Labisia Pathoina*
20	HMP20	Minuman kesihatan	Liquid	Tongkat Ali
21	HMP21	Kopi plus	Powder	Kacip Fatimah
22	HMP22	Teh tarik	Powder	Tongkat Ali
23	HMP23	Kopi pracampuran	Powder	Tongkat Ali
24	HMP24	Kopi herba	Powder	Misai Kucing
25	HMP25	Kopi tok guru	Powder	Misai Kucing
26	HMP26	Kopi tongkat ali	Powder	Tongkat Ali
27	HMP27	Tongkat ali coffee	Powder	Tongkat Ali
28	HMP28	Coffee gadis	Powder	Kacip Fatimah
29	HMP31	Kapsul plus	Capsule	Labisia Pathoina
30	HMP32	Candy	Paste	Kacip Fatimah
31	HMP33	Anggun bistari	Paste	Labisia pumila
32	HMP37	the herba	Tea bags	Misai Kucing
33	HMP38	Kopi tok	Powder	Tongkat Ali, Misai Kucing
34	HMP39	Kopi badang kacip fatimah	Powder	Kacip Fatimah
35	HMP40	Kopi pracampuran Garam bukit	Powder	Tongkat Ali
36	HMP41	Kopi Badang Tongkat ali	Powder	Tongkat Ali
37	HMP42	Kopi putery	Powder	Kacip fatimah (*Labisia pumila*)
38	HMP43	Kopi pracampur tongkat	Powder	Tongkat Ali (*Eurycoma longifolia*)
39	HMP44	Coffee tongkat ali	Powder	Tongkat Ali
40	HMP45	Pejuang	Powder	Tongkat Ali
41	HMP46	Kopi Pracampuran 5 dalam 1	Powder	Kacip Fatimah
42	HMP49	Jus tongkat ali	Liquid	Tongkat Ali
43	HMP50	Beauties	Powder	Kacip Fatimah
44	HMP51	Glucouric Tea	Tea bags	Misai Kucing
45	HMP52	Herbal bevergae mix	Powder	Misai Kucing
46	HMP53	B-up	Powder	Kacip Fatimah (*Labisia pumila*)
47	HMP54	herbal beverage	Powder	Misai Kucing
48	HMP55	Guco herbal	Powder	Misai Kucing
49	HMP56	Jus qasih	Liquid	Kacip Fatimah

Table 1 (continued)

No.	Code	Front of pack name	Product form	Ingredient target species
50	HMP57	Jus wanita	Liquid	Kacip Fatimah
51	HMP58	Maxx herbs	Liquid	Tongkat Ali
52	HMP60	Maxx herbs	Powder	Tongkat Ali
53	HMP61	Coffee + male	Powder	Tongkat Ali (*Eurycoma longifolia*)
54	HMP62	Coffee + female	Powder	Kacip Fatimah
55	HMP64	D coffee	Powder	Kacip Fatimah
56	HMP67	Maajun fatimah plus	Paste	*Labisia Pathoina*
57	HMP68	Capsule	Capsule	Kacip fatimah (*Labisia pumila*)
58	HMP69	Bidan	Capsule	Kacip fatimah (*Labisia pumila*)
59	HMP70	Femina plus	Capsule	Labisia pumila
60	HMP71	Kopi extra	Liquid	Tongkat Ali
61	HMP72	Ajaib plus	Pill	Tongkat Ali
62	HMP75	Qhita	Capsule	Kacip Fatimah (*Labisia pumila*)
63	HMP77	Kapsul plus	Capsule	Kacip Fatimah (*Labisia pumila*)
64	HMP78	Super	Powder	Tongkat ali, Misai Kucing
65	HMP79	Super	Powder	Kacip Fatimah
66	HMP80	KF minuman herba	Liquid	Kacip Fatimah, Manjakani
67	HMP81	longjack	Liquid	Tongkat Ali
68	HMP82	Manja fatimah	liquid	Kacip Fatimah
69	HMP84	JSW Jamu Surga Wanita	Pill	Kacip Fatimah, Manjakani
70	HMP87	White coffee	Powder	Tongkat Ali
71	HMP88	Herbs coffee	Powder	Tongkat Ali
72	HMP89	Teh asli	Tea bags	Misai Kucing (*Orthosiphon stamineus*)
73	HMP90	Angkasawan coffee	Powder	Tongkat Ali
74	HMP91	Herbanika	Powder	Misai Kucing (*Orthosiphon stamineus*)
75	HMP92	Air Jamu Pak Tani	Powder	Tongkat Ali
76	HMP95	Perawan Gold	Liquid	Kacip Fatimah
77	HMP96	Air Jamu Pak Tani	Liquid	Tongkat Ali
78	HMP98	Herbal drink mixes	Powder	Tongkat ali (*Eurycoma longifolia*), Misai Kucing (*Orthosiphon stamineus*)
79	HMP101	Pracampuran kopi	Powder	Kacip Fatimah
80	HMP105	Jus herbs	Liquid	Kacip Fatimah
81	HMP106	Kopi Mustajab	Powder	Tongkat Ali
82	HMP108	Kopi emas	Powder	Tongkat Ali
83	HMP109	mummy hot	Powder	Kacip Fatimah
84	HMP116	Kapsul plus	Capsule	Kacip Fatimah (*Labisia pumila*)
85	HMP118	Tongkat ali	Pill	Tongkat Ali (*Eurycoma longifolia*)
86	HMP122	LIVITA Tongkat Ali	Liquid	Tongkat Ali
87	HMP123	KF minuman herba Kacip Fatimah	Liquid	Kacip Fatimah
88	HMP124	Instant coffee	powder	Kacip Fatimah
89	HMP125	Tongkat ali plus	Powder	Tongkat Ali
90	HMP126	Detox	Liquid	Kacip Fatimah
91	HMP127	Kopi bidadari	Powder	Kacip Fatimah
92	HMP128	Kopi segera	Powder	Tongkat Ali (*Eurycoma longifolia*)
93	HMP129	Pracampuran sawda	Powder	Tongkat Ali
94	HMP130	Instant coffee	Powder	Tongkat Ali
95	HMP131	Kopi pracampuran collagen	Powder	Kacip Fatimah
96	HMP132	teh misai kucing	Tea bags	Misai Kucing (*Orthosiphon stamineus*)
97	HMP133	teh belalai gajah	Tea bags	Misai Kucing
98	HMP134	Tea with tongkat ali	Tea bags	Tongkat Ali

Table 1 (continued)

No.	Code	Front of pack name	Product form	Ingredient target species
99	HMP135	Stamina maxx	Powder	Tongkat Ali
100	HMP136	Kopi pracampuran	Powder	Tongkat Ali
101	HMP137	Coffee ali	Powder	Tongkat Ali
102	HMP138	Kopi pracampuran dewa	Powder	Tongkat Ali
103	HMP139	Kopi pracampuran	Powder	Tongkat Ali
104	HMP140	UP café	Powder	Kacip Fatimah
105	HMP142	Kembali dara	Powder	Kacip Fatimah
106	HMP143	Kopi pracampuran tongkat ali	Powder	Tongkat Ali

Table 2 Species sequences identification using GenBank

Code	Common name claim species	Species identification Scientific name	Percentage identification (%)	Reference GenBank accession
FP_PL_001	*Orthosiphon stamineus* (Misai kucing)	*Orthosiphon stamineus*	100.00	OR264480
FP_PL_003	*Labisia pumila* (Kacip Fatimah)	*Labisia pumila* var *alata*	100.00	OR264132
FP_PL_002	*Eurycoma longifolia* (Tongkat ali)	*Eurycoma longifolia*	99.33	OR263275

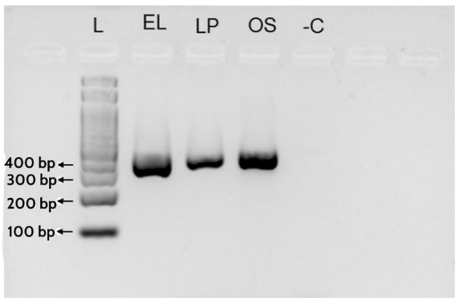

Fig. 1 Agarose gel electrophoresis of PCR results from leaves of *E. longifolia* (EL), *L. pumila* and *O. stamineus* (OS), negative control (–C), and 100 bp DNA ladder (L)

longifolia, five samples for *L. pumila*, and six samples for *O. stamineus* (Table 3).

3.2 Identification results of herbal products

DNA extraction only successfully extracted from 60out of 106 tested herbal products (56.6%). Successful PCR product shows a relatively higher chance of using ITS2 region which amplified 50 herbal products (47.16%). The following Fig. 2 shows a successful PCR product using ITS2 DNA barcoding.

The herbal product sequences were queried in GenBank using BLAST, with the highest sequence similarity amongst samples, and the most likely related species are presented in Table 4. Under optimised conditions,

Table 3 Result of GenBank ITS2 reference sequence that deposited for identification of *E. longifolia, L. pumila* and *O. stamineus*

Plant species	*E. longifolia*	*L. pumila*	*O. stamineus*
Sequences from GenBank	MN715379.1 (ITS1-5.8S-ITS2)	MW414685.1 (ITS1-5.85-ITS2)	AY 506663.1 (ITS1-5.8S-ITS2)
	MG643109.1 (ITS1-5.8S-ITS2)	MH838010.1 (ITS2)	MT251295.1 (ITS2)
	KY264053.1 (ITS2-28S)	MH838008.1 (ITS2)	JF301407.1 (ITS1-5.8S-ITS2)
	KY553292.1 (ITS2)	MH828448.1 (ITS2)	EF421427.1 (ITS1-5.8S-ITS2-26S)
		MH766971.1 (5.8S-ITS2)0	MW3155930.1 (ITS1-5.8S-ITS2)
			FJ593403.1 (ITS1-5.8S-ITS2)
			LC456390.1 (5.8S-ITS2-28S)

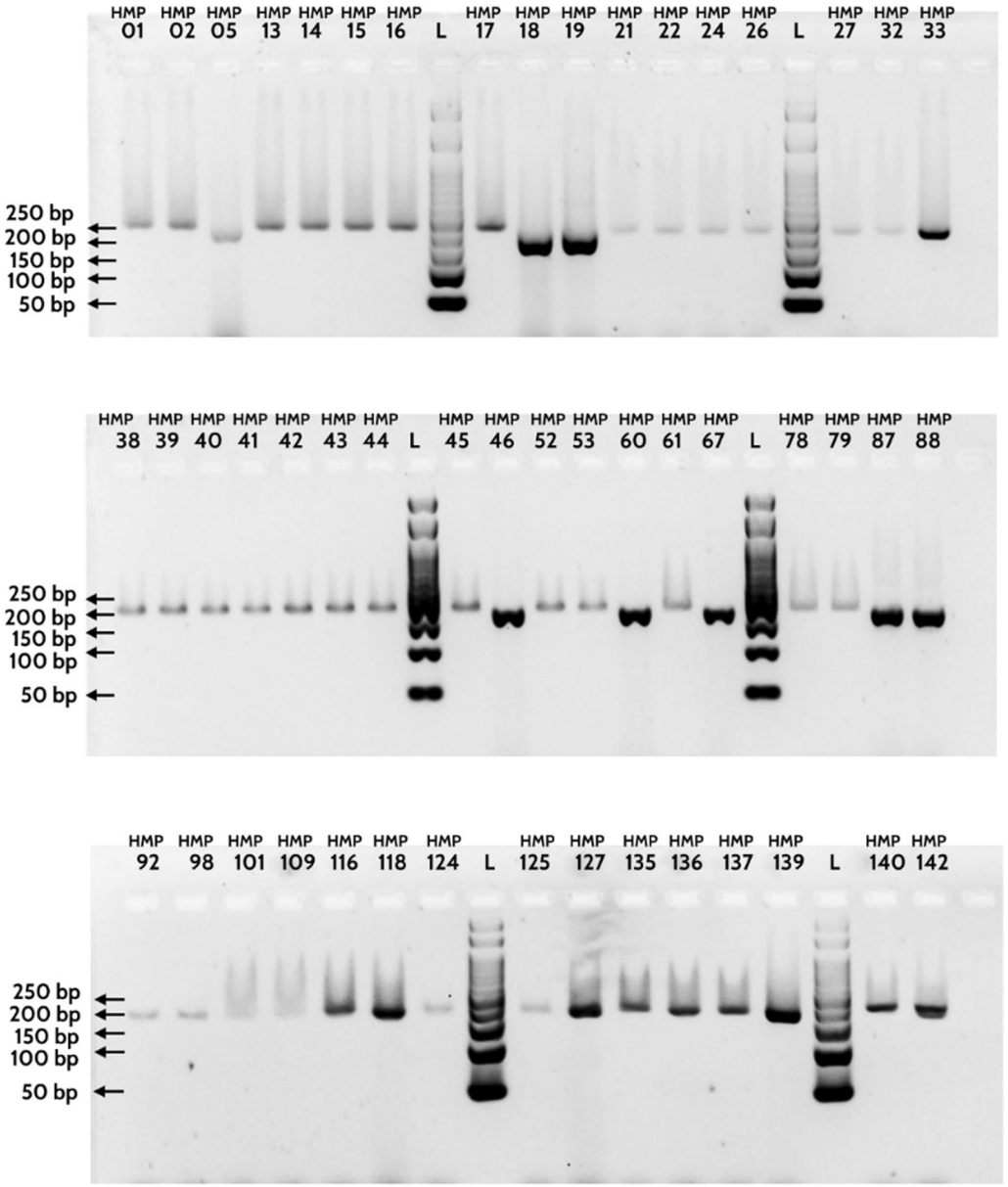

Fig. 2 Agarose gel electrophoresis of successful PCR results from the herbal product and 50 bp DNA ladder (L)

a single, distinct, and brightly resolved band of range with minimum read length was 177 bp with a maximum of 479 bp for 50 of the tested herbal products (Table 4), remaining 10 samples of amplifiable DNA of samples were designated as "No sequence" and were not considered for further analysis.

Forty-six out of 106 (43.4%) of the tested herbal products did not produce amplicons even after repeated attempts. The recovery of genomic DNA from degraded samples of herbal products was insufficient, making it challenging to amplify DNA barcodes from samples.

3.3 Authentication of the herbal products

The species identification and their authenticity are summarised in Table 5, which result shows 15.09% of the products were considered as authentic, whereas 39.62% of the herbal products were considered substituted, and 52.83% of the herbal products were unable to be identified..

The identity of sequencing recovered from the products was determined according to Shanmughanandhan [25], Tnah [29]. If a sequence matched the species and was present on the label of the products, these herbal

Table 4 Identification results of herbal product based on ITS2 GenBank BLAST

No.	Code	Common name claim species	Species identification	Percentage identification (%)	Reference GenBank accession
1	HMP01	Tongkat Ali	*Orthosiphon stamineus*	100.00	MT251295.1
2	HMP02	Tongkat Ali	*Orthosiphon stamineus*	100.00	MT251295.1
3	HMP05	Tongkat Ali (*Eurycoma longifolia*)	*Pedicularis lachnoglossa*	98.78	MZ198677.1
4	HMP13	Kacip fatimah (*Labisia pumila*)	*Orthosiphon stamineus*	100	MT251295.1
5	HMP14	Tongkat Ali, Misai Kucing	*Orthosiphon stamineus*	100.00	MT251295.1
6	HMP15	Tongkat Ali	*Orthosiphon stamineus*	100.00	MT251295.1
7	HMP16	Tongkat Ali	*Orthosiphon stamineus*	100.00	MT251295.1
8	HMP17	*Eurycoma longifolia*	*Orthosiphon stamineus*	100.00	MT251295.1
9	HMP18	*Eurycoma longifolia*	*Coriandrum sativum*	100.00	ON685496.1
10	HMP19	*Labisia Pathoina*	*Coriandrum sativum*	93.13	ON685496.1
11	HMP21	Kacip Fatimah	*Orthosiphon stamineus*	99.29	MT251295.1
12	HMP22	Tongkat Ali	*Orthosiphon stamineus*	100.00	MT251295.1
13	HMP24	Misai Kucing	*Orthosiphon stamineus*	99	MT251295.1
14	HMP26	Tongkat Ali	*Orthosiphon stamineus*	99.64	MT251295.1
15	HMP27	Tongkat Ali	*Orthosiphon stamineus*	99.64	MT251295.1
16	HMP32	Kacip Fatimah	*Orthosiphon stamineus*	100.00	MT251295.1
17	HMP33	Labisia pumila	*Orthosiphon stamineus*	96.09	MT251295.1
18	HMP38	Tongkat Ali, Misai Kucing	*Orthosiphon stamineus*	100.00	MT251295.1
19	HMP39	Kacip Fatimah	*Orthosiphon stamineus*	100.00	MT251295.1
20	HMP40	Tongkat Ali	*Orthosiphon stamineus*	99.64	MT251295.1
21	HMP41	Tongkat Ali	*Orthosiphon stamineus*	100	MT251295.1
22	HMP42	Kacip fatimah (Labisia pumila)	*Orthosiphon stamineus*	99.64	MT251295.1
23	HMP43	Tongkat Ali (Eurycoma longifolia)	*Orthosiphon stamineus*	100	MT251295.1
24	HMP44	Tongkat Ali	*Orthosiphon stamineus*	100	MT251295.1
25	HMP45	Tongkat Ali	*Orthosiphon stamineus*	100	MT251295.1
26	HMP46	Kacip Fatimah	*Coriandrum sativum*	99.33	ON685496.1
27	HMP52	Misai Kucing	*Orthosiphon stamineus*	100	MT251295.1
28	HMP53	Kacip Fatimah (Labisia pumila)	*Orthosiphon stamineus*	93.55	MT251295.1
29	HMP60	Tongkat Ali	*Coriandrum sativum*	87.34	ON685496.1
30	HMP61	Tongkat Ali (*Eurycoma longifolia*)	*Orthosiphon stamineus*	96.35	MT251295.1
31	HMP67	Labisia Pathoina	*Coriandrum sativum*	89.35	ON685496.1
32	HMP78	Tongkat ali, Misai Kucing	*Orthosiphon stamineus*	89.96	MT251295.1
33	HMP79	Kacip Fatimah	*Orthosiphon stamineus*	100	MT251295.1
34	HMP87	Tongkat Ali	*Coriandrum sativum*	82.83	ON685496.1
35	HMP88	Tongkat Ali	*Coriandrum sativum*	86.54	ON685496.1
36	HMP92	Tongkat Ali	*Anethum foeniculum*	96.01	ON685481.1
37	HMP98	Tongkat ali (*Eurycoma longifolia*), Misai Kucing (*Orthosiphon stamineus*)	*Anethum foeniculum*	98.67	ON685481.1
38	HMP101	Kacip Fatimah	*Hordeum vulgare*	79.31	XR_006625164.1
39	HMP109	Kacip Fatimah	*Deverra tortuosa*	85.27	KJ473888.1
40	HMP116	Kacip Fatimah (*Labisia pumila*)	*Orthosiphon stamineus*	92.65	MT251295.1
41	HMP118	Tongkat Ali (*Eurycoma longifolia*)	*Anethum graveolens*	99.32	ON685466.1
42	HMP124	Kacip Fatimah	*Anethum foeniculum*	91.83	MZ779166.1
43	HMP125	Tongkat Ali	*Anethum foeniculum*	93.82	KP406140.1
44	HMP127	Kacip Fatimah	*Anethum graveolens*	85.71	MN257763.1
45	HMP135	Tongkat Ali	*Orthosiphon stamineus*	100	MT251295.1
46	HMP136	Tongkat Ali	*Orthosiphon stamineus*	89.29	MT251295.1
47	HMP137	Tongkat Ali	*Orthosiphon stamineus*	100	MT251295.1

Table 4 (continued)

No.	Code	Common name claim species	Species identification	Percentage identification (%)	Reference GenBank accession
48	HMP139	Tongkat Ali	*Salacia menglaensis*	97.24	MZ568402.1
49	HMP140	Kacip Fatimah	*Orthosiphon aristatus*	93.93	MW315930.1
50	HMP142	Kacip Fatimah	*Orthosiphon aristatus*	93.93	MW315930.1

products were declared as “Authentic". The herbal products were declared "Substituted" if additional identification species other than those listed on the herbal products were discovered with species identified for the primary component. Finally, the herbal product is labelled as “no sequence” (NS) if the sequence could not be extracted from the examined herbal products [25, 30].

Identification analysis shows that out of 42 herbal products are substitution by other plant species and no detection of contamination of plant species (Table 5). The identity of other plant taxa in substitution of the product’s primary listed ingredient on the label, which was not detected in the sample, was the criteria utilised to determine product substitution. Nehal [31] has highlighted that labels alone may not be sufficient to be aware of the precise components or content of a product because of the risk of illegal substitutions, which can harm not only the image of the manufacturer but also the well-being of the consumer.

Sequence identification from HMP124, HMP125, HMP92, and HMP98 was revealed to contain *Anethum foeniculum* (*A. Foeniculum*), confirming substitution. *A. foeniculum* (synonym as *Foeniculum vulgare*) is used for managing female sterility and for its antiseptic, palliative, and anti-inflammatory properties in traditional medicine. The content of HMP127 and HMP118 is believed to have been substituted with *Anethum graveolens* (*A. graveolens*). *A. graveolens* is suggested for the management of diabetic patients [32]. HMP101 was revealed to contain substituted *Hordeum vulgare*. *H. vulgare* is a species of cereal plant, and is used for medicinal and therapeutic purposes.

Sequence identification of HMP18, HMP19, and HMP67 revealed to contain *Coriandrum sativum* (*C. sativum*), which is identified as authentic as presented on the labelling. Meanwhile, HMP46, HMP60, HMP87, and HMP88 are thought to have been replaced by *C. sativum*. *C. sativum* is most widely used for seasoning, but it is also well recognised for its antioxidant, antidiabetic, antimutagenic, antianxiety, and antibacterial action, which promotes a variety of health advantages [33].

Furthermore, HMP109 was revealed to contain substituted *Deverra tortuosa* (*D. tortuosa*). *D. tortuosa* is popularly used to treat conditions that are prevalent in Saudi Arabia, such as fever, hepatitis, diabetes, stomachache, rheumatism, and asthma. [34]. HMP05 was revealed to contain substituted *Pedicularis lachnoglossa* (*P. lachnoglossa*). *Pedicularis* species are appealing as ornamental and melliferous plants, and they also have great therapeutic potential, displaying antiphlogistic, antipyretic, detoxication, diuretic, choleretic, antibacterial, and antioxidant properties [35]. Next, the ingredient of HMP139 is believed to have been substituted with *Salacia menglaensis* (*S. menglaensis*). *S. menglaensis* is known as new species from China [36], but a number of Salacia species’ roots and stems have been used in traditional medicine to cure conditions such as diabetes, arthritic conditions diarrhoea, and skin diseases [37].

Sequences identification for HMP01, HMP02, HMP13, HMP15, HMP16, HMP17, HMP21, HMP22, HMP26, HMP27, HMP32, HMP33, HMP39, HMP40, HMP41, HMP42, HMP43, HMP44, HMP45, HMP53, HMP61, HMP79, HMP116, HMP135, HMP136, and HMP137 was revealed to contain with substituted *Orthosiphon stamineus*. Sequence analysis from HMP14, HMP24, HMP38, HMP78, and HMP52 was revealed to contain *Orthosiphon stamineus*, confirming authenticity as it presented on the labelling. *O. stamineus* has been known used as traditional medicine for diuretics and treating catarrh of the bladder [38]. Lastly, HMP140 and HMP142 contents were substituted with *Orthosiphon aristatus*. *O. aristatus* is a synonym to *O. stamineus*.

From this identification of the species, findings imply that manufacturers committed misleading and deceptive conduct by adding inferior species. There are several possible explanations for the presence of non-listed species but not restricted to intentional adulteration and accidental substitution, which can occur at any point in the medicinal plant supply chain, from initial cultivation and storage to final product packaging and distribution [40]. The substitution of those plant species may have contributed to medicinal value but may pose serious health risks, as the efficiency of the substituted plant species compound with the other species cannot be determined. Moreover, the finding shows that a parasite was found in the herbal product, thus indicating the need to follow a quality standard protocol during the manufacturing process.

Table 5 DNA barcoding for the authenticity of 50 amplified herbal products

No.	Code	Common name claim species	Species identification	Species identity
1	HMP01	Tongkat Ali	*Orthosiphon stamineus*	Substituted
2	HMP02	Tongkat Ali	*Orthosiphon stamineus*	Substituted
3	HMP05	Tongkat Ali (*Eurycoma longifolia*)	*Pedicularis lachnoglossa*	Substituted
4	HMP13	Kacip fatimah (*Labisia pumila*)	*Orthosiphon stamineus*	Substituted
5	HMP14	Tongkat Ali, Misai Kucing	*Orthosiphon stamineus*	Authentic
6	HMP15	Tongkat Ali	*Orthosiphon stamineus*	Substituted
7	HMP16	Tongkat Ali	*Orthosiphon stamineus*	Substituted
8	HMP17	*Eurycoma longifolia*	*Orthosiphon stamineus*	Substituted
9	HMP18	*Eurycoma longifolia*	*Coriandrum sativum*	Authentic
10	HMP19	*Labisia Pathoina*	*Coriandrum sativum*	Authentic
11	HMP21	Kacip Fatimah	*Orthosiphon stamineus*	Substituted
12	HMP22	Tongkat Ali	*Orthosiphon stamineus*	Substituted
13	HMP24	Misai Kucing	*Orthosiphon stamineus*	Authentic
14	HMP26	Tongkat Ali	*Orthosiphon stamineus*	Substituted
15	HMP27	Tongkat Ali	*Orthosiphon stamineus*	Substituted
16	HMP32	Kacip Fatimah	*Orthosiphon stamineus*	Substituted
17	HMP33	Labisia pumila	*Orthosiphon stamineus*	Substituted
18	HMP38	Tongkat Ali, Misai Kucing	*Orthosiphon stamineus*	Authentic
19	HMP39	Kacip Fatimah	*Orthosiphon stamineus*	Substituted
20	HMP40	Tongkat Ali	*Orthosiphon stamineus*	Substituted
21	HMP41	Tongkat Ali	*Orthosiphon stamineus*	Substituted
22	HMP42	Kacip fatimah (*Labisia pumila*)	*Orthosiphon stamineus*	Substituted
23	HMP43	Tongkat Ali (*Eurycoma longifolia*)	*Orthosiphon stamineus*	Substituted
24	HMP44	Tongkat Ali	*Orthosiphon stamineus*	Substituted
25	HMP45	Tongkat Ali	*Orthosiphon stamineus*	Substituted
26	HMP46	Kacip Fatimah	*Coriandrum sativum*	Substituted
27	HMP52	Misai Kucing	*Orthosiphon stamineus*	Authentic
28	HMP53	Kacip Fatimah (*Labisia pumila*)	*Orthosiphon stamineus*	Substituted
29	HMP60	Tongkat Ali	*Coriandrum sativum*	Substituted
30	HMP61	Tongkat Ali (*Eurycoma longifolia*)	*Orthosiphon stamineus*	Substituted
31	HMP67	Labisia Pathoina	*Coriandrum sativum*	Authentic
32	HMP78	Tongkat ali, Misai Kucing	*Orthosiphon stamineus*	Authentic
33	HMP79	Kacip Fatimah	*Orthosiphon stamineus*	Substituted
34	HMP87	Tongkat Ali	*Coriandrum sativum*	Substituted
35	HMP88	Tongkat Ali	*Coriandrum sativum*	Substituted
36	HMP92	Tongkat Ali	*Anethum foeniculum*	Substituted
37	HMP98	Tongkat ali (*Eurycoma longifolia*), Misai Kucing (*Orthosiphon stamineus*)	*Anethum foeniculum*	Substituted
38	HMP101	Kacip Fatimah	*Hordeum vulgare*	Substituted
39	HMP109	Kacip Fatimah	*Deverra tortuosa*	Substituted
40	HMP116	Kacip Fatimah (*Labisia pumila*)	*Orthosiphon stamineus*	Substituted
41	HMP118	Tongkat Ali (*Eurycoma longifolia*)	*Anethum graveolens*	Substituted
42	HMP124	Kacip Fatimah	*Anethum foeniculum*	Substituted
43	HMP125	Tongkat Ali	*Anethum foeniculum*	Substituted
44	HMP127	Kacip Fatimah	*Anethum graveolens*	Substituted
45	HMP135	Tongkat Ali	*Orthosiphon stamineus*	Substituted
46	HMP136	Tongkat Ali	*Orthosiphon stamineus*	Substituted
47	HMP137	Tongkat Ali	*Orthosiphon stamineus*	Substituted
48	HMP139	Tongkat Ali	*Salacia menglaensis*	Substituted
49	HMP140	Kacip Fatimah	*Orthosiphon aristatus*	Substituted

Table 5 (continued)

No.	Code	Common name claim species	Species identification	Species identity
50	HMP142	Kacip Fatimah	*Orthosiphon aristatus*	Substituted

3.4 Restriction enzyme digestion

Seven restriction enzymes were used to digest the PCR-amplified products of the ITS2 region: *TaqI, BamH I, HinfI, EcoRI, EcoRV, MboI,* and *MspI.* Restriction fragments after digestion of the ITS2 region are shown in Fig. 3. Amplified fragment was subjected to restriction digests and the products ranged from 50 to 300 bp. Restriction profiles for each plant species, *E. longifolia*, *O. stamineus*, and *L. pumila* exhibited by enzyme *EcoRI, EcoRV,* and *BamHI,* failed to yield fragments; hence, this restriction enzyme has been ruled out from further analysis. *MboI, TaqI, MspI,* and *HinfI* digestion profiles and in silico prediction are summarised in Table 6.

The resultant fragment after digestion is equal in quantity; hence, the band brightness is lesser as the shorter the fragment is. It should be noted that the fragment's weak bands are compatible with the expectations. Thus, the result shows certain restriction enzymes with PCR product has resulted in incomplete digestion and may be due to unsuitability.

3.5 Application of the PCR–RFLP assay for identification of herbal products derived from *O. stamineus, E. longifolia*, and *L pumila*

To determine the plant origins of the herbal products, the established PCR–RFLP assay was used. Based on the species identification of herbal products, only 32 herbal products have a high identification of *O. stamineus*. Figure 4 shows the agarose gel profile on a 2% agarose gel for fragments of PCR product of 32 samples corresponding to fragments of *O. stamineus*. The PCR–RFLP assay results of all samples show restriction profiles for herbal products using *MboI* digestion (Fig. 5). The resulting fragments are in a faint band since the herbal product was highly degraded DNA.

4 Discussion

This study revealed successful DNA barcoding identification for *E. longifolia, L. pumila,* and *O. stamineus* using ITS2 barcode region. The ITS2 region may be amongst the most promising standard DNA barcodes for identifying medicinal plants, according to an earlier study by Chen et al. [41]. The highest success rates for species identification were 78% and 100% at the species and genus levels, respectively, according to colleagues [34] who looked at the ITS2 locus for closely related species

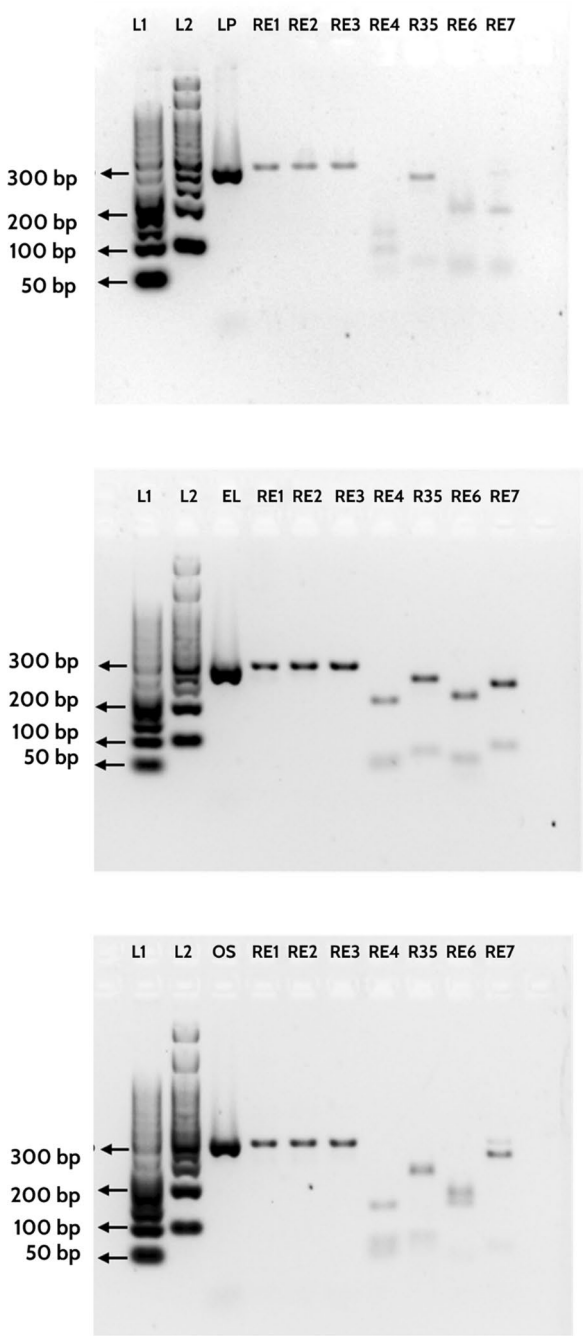

Fig. 3 Restriction fragment patterns of the PCR-amplified ITS2 region of *E. longifolia* (EL), *L. pumila*, and *O. stamineus* (OS) species on a 2% agarose gel. L1: 100 bp, L2: 100 bp DNA ladder, RE1: *EcoRI,* RE2: *EcoRV,* RE3: *BamHI,* RE4: *HinfI,* RE5: *MspI,* RE6: *TaqI,*and RE7: *MboI*

Table 6 The fragment size (bps) by in silico restrictions predictions and restriction profiles exhibited by these enzymes *MboI, TaqI, MspI, HinfI, EcoRI, EcoRV,* and *BamHI* on plant species specimen

Plant species	***E. longifolia***		***O. stamineus***		***L. pumila***	
Amplified fragment	315		316		315	
Restriction enzyme	In silico restriction	Restriction profiles	In silico restriction	Restriction profiles	In silico restriction	Restriction profiles
EcoRI	NA	NA	NA	NA	NA	NA
EcoRV	NA	NA	NA	NA	NA	NA
BamHI	NA	NA	NA	NA	NA	NA
HinfI	21 + 45 + 46 + 203	50 + 265	31 + 45 + 45 + 59 + 136	76 + 104 + 136	21 + 81 + 98 + 115	102 + 213
MspI	6 + 66 + 243	66 + 249	26 + 65 + 225	91 + 225	73 + 242	73 + 242
TaqI	19 + 26 + 51 + 219	41 + 51 + 219	39 + 108 + 169	39 + 108 + 169	33 + 54 + 55 + 173	109 + 206
MboI	46 + 269	46 + 269	20 + 296	20 + 296	30 + 51 + 58 + 269	51 + 88 + 269

in several Rosaceae species [42]. The study also demonstrated that the use of ITS2 is particularly effective in the detection of adulterants or substitutes including species that are difficult to tell apart based on their morphological and/or phytochemical similarities.

Highlighting the importance of a good barcode pattern, Hou [43] emphasised that effective barcodes should exhibit high interspecific divergence and low intraspecific variability. Hence, the effectiveness of a barcode depends on its ability to differentiate between closely related species, an accomplishment that can only be accomplished when the genetic dissimilarity between species is significantly greater than the genetic similarity within a single species [55]. Research showed that the rhubarb species' intraspecific variation in the ITS2 region was surprisingly minimal, measuring just 0.0036, both within and between the species. This discovery offers a substantial benefit for precisely identifying herbal products and plant species, verifying their validity, and separating them from adulterants. A significant downside is the high interspecific divergence of 0.0970 found between officinal rhubarb and its adulterants, which may make the identification and authentication procedure more difficult. [44]. The results of this study have demonstrated that there is genetic differentiation between the three species of *E. longifolia*, *L. pumila*, and *O. stamineus*, with the intraspecific genetic distance between each species being only 0.00 and the interspecific genetic distance between the three species being 0.32. This outcome has established ITS2 as a useful barcode. A previous study has pointed out that prominent factors for characterising a suitable barcode include the intraspecific and interspecific divergence that has been explicitly mentioned. The finding has summaries that when comparing averages across species, interspecific variation in barcodes was found to be greater than intraspecific variation. Furthermore, the ITS region had the highest rate of correct identifications using the closest distance approach, followed by the ITS2 area, *psbA-trnH*, *matK*, and *rbcL* regions [45]. A BLAST search was performed against GenBank databases which have been employed in identifying all query sequences. Samples acquired from the raw market were authenticated using the best-match method. The BOLD and NCBI nucleotide databases were searched using BLAST to analyse the unmatched specimens. This study made it possible to identify accurate matches for both unambiguous and ambiguous. One of the most prevalent causes of incorrect identifications was that the species in question was either not included in the reference library, or there was insufficient variation in the sequences being compared [46].

Species identifications are crucial in these situations, but the analysis is frequently hampered by an absence of taxonomic specialists. As a result, there is growing interest in using DNA-based methods to identify species [47]. Our study proposes that it would be beneficial to create a Barcode REF library with a carefully curated set of verified entries for each species to improve BOLD's capacity to offer accurate identifications. To effectively match unidentified query sequences and facilitate accurate species-level identification, it is imperative to develop a thorough and reliable reference sequence database [48].

The development of DNA-based identification systems would benefit from the availability of sequence information that is readily available in public databases. The GenBank entries in this study need to be submitted and to be used as supplemental material for publication. However, many medicinal plant species are still absent from the BOLD index, and many of the medicinal species do not have full coverage of numerous barcode regions, especially ITS2 [49]. A study by Bell [50] has found that since they have successfully established a repository for ITS2 and *rbcL* in combination, most species in the mixtures could be distinguished. [50].

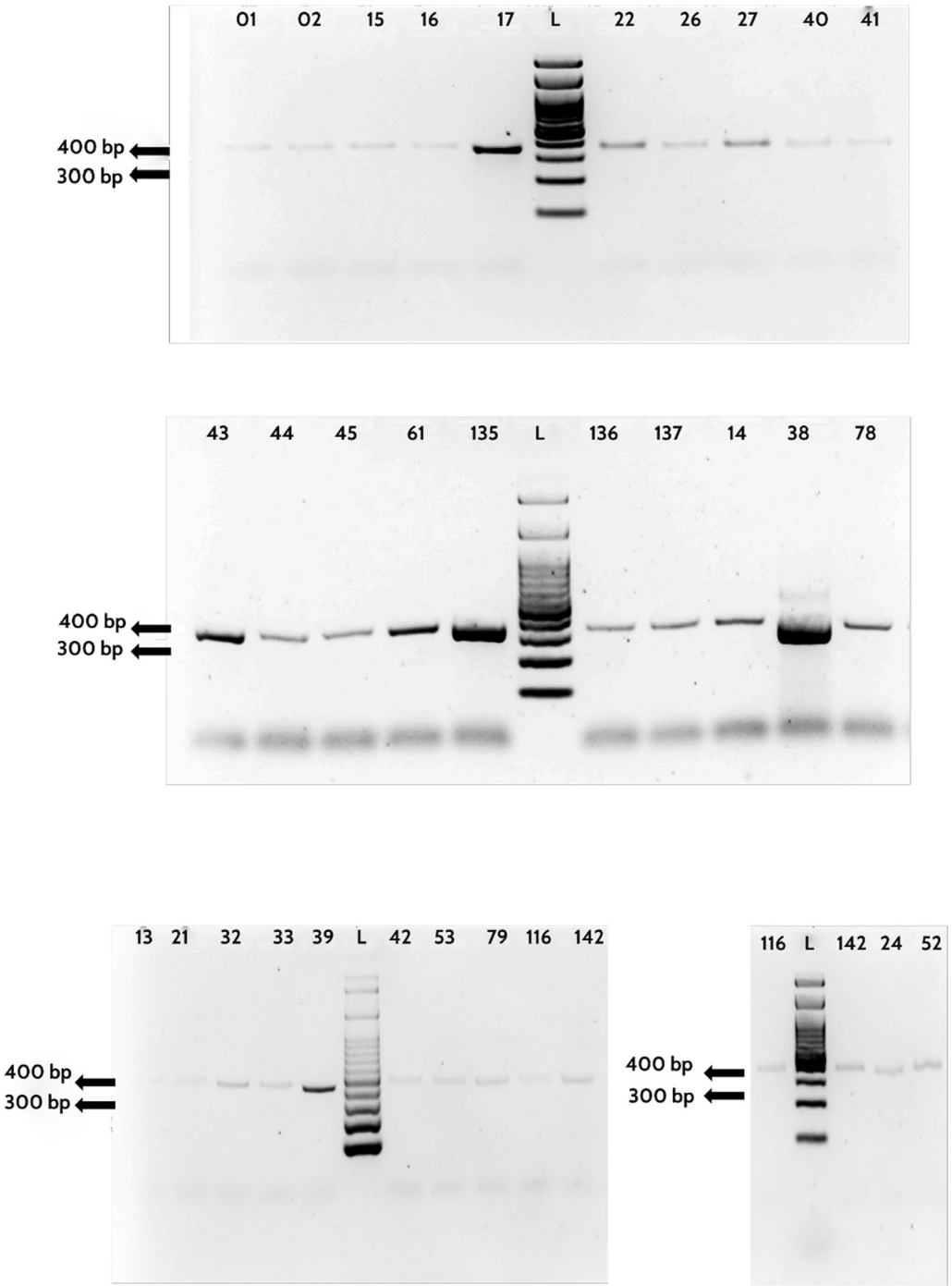

Fig. 4 Agarose gel profile on a 2% agarose gel of PCR products herbal products identification to be *O. stamineus* (codes listed in material and method), showing the resulting fragments. L: 50 bp DNA ladder

The study's findings show that manufacturers' mislabelling of herbal products and incorrectly labelled sequences submitted to GenBank are realistic variables to examine. Howard [49] recognised the occurrence of mislabelled sequences and emphasised the disadvantages associated with the amount of publicly available sequence information, which impedes the improvement of DNA-based identification systems. As a result, relying merely on the top BLAST result for species verification is no longer sufficient; users need to develop particular criteria for

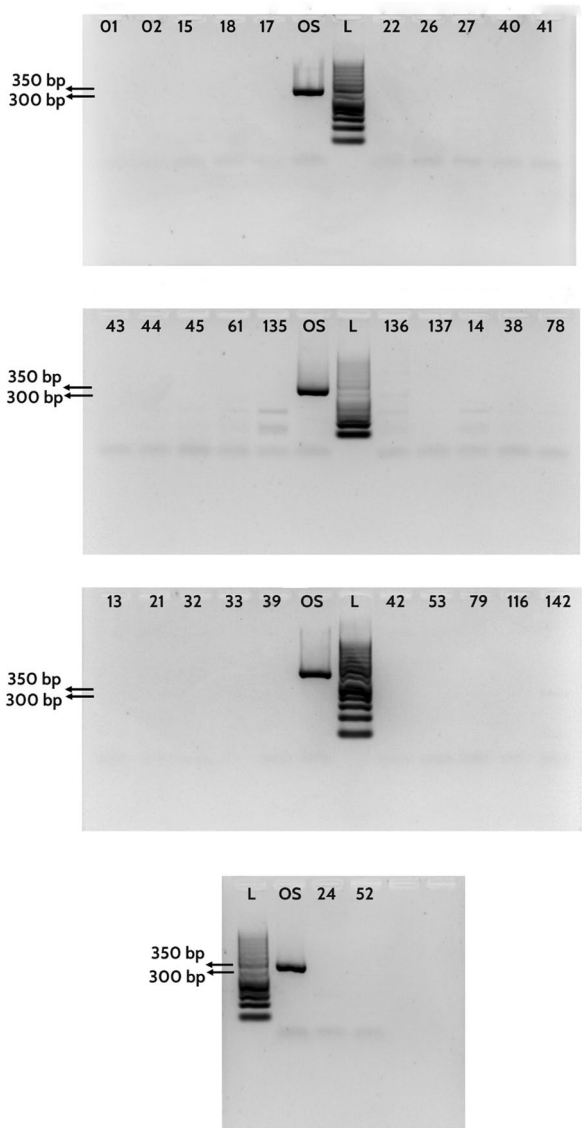

Fig. 5 Agarose gel profile on a 2% agarose gel of PCR products herbal products identification to be *O. stamineus* (codes listed in material and method), after digestion with MboI, showing the resulting fragments. L: 50 bp DNA ladder

accepting sequences obtained from databases to assure their legitimacy. As a result, it is critical to have the original data in order to evaluate and replicate the analysis described in the paper. To address comparable concerns, the current study emphasises the importance of increasing data contributions to these database systems [49].

The amplification of the expected length ITS2 region was effective in comparison with fresh plant DNA due to the mostly intact genomic DNA. This was not the case with herbal products. Only 61 (60.4%) of the 101 herbal products examined produced amplifiable DNA. Despite several tries, the remaining 40 products (39.6%) failed to yield amplifiable DNA. This lack of amplification could be linked to DNA degradation during manufacturing procedures. According to Abubakar and colleagues [2], in processed goods, DNA is subjected to heat, physical, and chemical treatments, which frequently results in DNA disintegration, in order to extract bioactive components from herbal plants. Market herbs' harvesting, processing, and storage all contributed to DNA degradation and made it difficult to extract intact DNA. These factors are all probable sources of DNA degradation, according to Han and colleagues [16]. On the other hand, using conserved primers, the ITS2 region may be amplified and sequenced quickly [51].

The decreased extraction success rates seen in this study for market samples are not surprising, given that herbalists often do not prioritise approaches that improve DNA preservation in their plant product handling. It has been claimed that the material containing cells containing DNA is frequently destroyed during the extraction procedure, leaving only the phytochemical components. As a result, any remaining DNA in plant extracts is typically of poor quality and quantity. This is because of the high concentration of secondary metabolites such as polysaccharides, phenolic compounds, and other proteins, which may interfere with DNA extraction and preservation [52, 53], this has hindered high-quality DNA extraction suggested that the only possible.

In order to solve this problem, our study's findings suggest that the presence of many short DNA fragments may be a factor in certain samples' inability to be amplified, possibly as a result of the primer used in this study's use not being uniformly effective for amplifying all species. Sequences must be able to distinguish between species at the genus and species levels and be simple to amplify in order to function as DNA barcodes [54]. As suitable DNA barcodes for amplification, we used *ITS2, rbcL-1, rbcL-2, matK*, and *psbA-trnH*. However, the *rbcL* sequence is longer than the ITS2 sequence, and recovering full DNA from herbal products is difficult owing to DNA loss during processing and storage. As a result, this study concentrated on using the ITS2 region to differentiate between *E. longifolia*, *L. pumila*, and *O. stamineus*, as well as validating species identification in both fresh specimens and herbal products. Previous research has shown that the ITS2 region is suitable for the amplification and identification of these species [26, 30, 55].

Using the ITS2 sequence information of *E. longifolia*, *O. stamineus*, and *L. pumila*, a simple PCR–RFLP approach was used to develop a quick, simple, and dependable method for identifying these plant species [21]. Because it only takes a single unique site between the primers to identify the target species from others, PCR–RFLP is a

powerful method of testing. This process is straightforward and affordable, making it a good option for identifying different species.

According to Diguta and colleagues [56], the PCR–RFLP approach is effective in differentiating various species of genera like *Alternaria*, *Cladosporium*, *Botrytis*, *Fusarium*, *Pilidiella*, *Epicoccum*, and *Thanatephorus*. The researchers distinguished these fungi effectively by using the endonucleases *SduI* and *HaeIII*. In addition, the researchers discovered that this method may be used with a variety of samples and provides routine, sensitive, and reliable identification in contrast to conventional morphological identification techniques. In this work, it was discovered that using just one restriction enzyme was enough to identify the species and produce a molecular diagnostic profile. This strategy is consistent with a prior work by Sarin and colleagues that made use of a related technique and demonstrated the capacity to accurately identify three *Phyllanthus* species (*P. amarus*, *P. fraternus*, and *P. urinaria*) that had comparable morphological characteristics [57]. Additionally, this method was used to successfully identify a subset of 32 herbal items as *O. stamineus*. It is important to note that all digested fragments produced during the PCR–RFLP experiment have equimolar ratios. Shorter fragments have lesser band intensity; hence, the brightness of the bands is proportional to fragment length [37]. Fragments that were very faintly evident in the findings showed that the herbal items had degraded. These barely discernible bands were notable because they agreed with the theoretical predictions, demonstrating the accuracy of the PCR–RFLP approach in identifying *O. stamineus* [58].

5 Conclusion

In the current work, we have shown that the in vitro digestion of PCR amplicons using digestive enzymes and passing the samples via gel electrophoresis make up the entirety of the sample processing time in the developed ITS2 barcode-based PCR–RFLP technique. While the majority of enzymes take 60 min to digest, others, like *HinfI*, only need 30. In this investigation, we prolonged the incubation period by an extra day (about 16 h), as per the manufacturer's recommendations. It is important to keep in mind that PCR–RFLP may appear to be a slower procedure in some circumstances due to its prolonged incubation duration with endonucleases. Because various recombinant DNA manufacturing methods are used by different producers, there can be substantial variance in the kind and quality of synthesised enzymes. Therefore, although having comparable recognition sequences, restriction enzymes from two distinct providers may vary in terms of cost, shelf life, transit conditions, digestive efficiency, incubation length, and other aspects. To authenticate *O. stamineus*, *L. pumila*, and *E. longifolia*, we have created a reliable ITS2 barcode-based PCR–RFLP approach. These methods create a thorough system for the authentication and identification of these plant species when paired with DNA barcoding technology and macroscopic identification.

Abbreviations

PCR	Polymerase chain reaction
PCR–RFLP	Polymerase chain reaction–restriction fragment length polymorphism
ITS2	Internal transcribed spacer 2
BSA	Bovine serum albumin
BOLD	Barcode of life data system
BLAST	Basic Local Alignment Search Tool

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Author contributions
AAJ was involved in conceptualization; SNAMA, AAB, SS, BRN, and AAJ helped in methodology, AAJ and SNAMA contributed to formal analysis; AAJ and SNAMA and JRA-O helped in writing—original draft preparation. The published version of the work has been reviewed and approved by all authors.

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Data availability
Data available within the manuscript.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
Authors declare that they have no conflict of interest.

Author details
[1]Department of Biology, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, 35900 Tanjong Malim, Perak, Malaysia. [2]Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21300 Kuala Nerus, Terengganu, Malaysia. [3]Institute of Tropical Biodiversity and Sustainable Development, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia. [4]Center of Biodiversity and Conservation, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, 35900 Tanjung Malim, Perak, Malaysia.

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