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AN APPROACH FOR IDENTIFICATION AND VALIDATION OF *Eurycoma longifolia*, *Labisia pumila*, AND *Orthosiphon stamineus*

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ABSTRACT

This study is to establish a Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method and diagnostic PCR based on nuclear DNA internal transcribed spacer 2 (ITS2) for *E. longifolia, L. pumila,* and *O. stamineus*. PCR-RFLP was then used to validate the species identification for herbal products. The PCR-RFLP was developed for rapid identification using restriction enzymes *TaqI, BamHI, HinfI, EcoRI, EcoRV, Mbol*, and *Mspl*. The ITS2 sequences were identified and compared between plant specimens of *E. longifolia, L. pumila,* and *O. stamineus* with 106 samples of commercial herbal products. As a result, 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 the 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. Specific PCR combined with digestion using the restriction enzyme of *Mbol* allowed for identifying *O. stamineus* and *E. longifolia*. In contrast, *Mspl* allowed the identification of *L. pumila* by producing specific restriction patterns of plant samples. As a 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.

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PENDEKATAN UNTUK IDENTIFIKASI DAN PENGESAHIHAN *Eurycoma longifolia, Labisia pumila,* DAN *Orthosiphon stamineus*

ABSTRAK

Kajian ini bertujuan untuk membangunkan kaedah *Polymerase Chain Reaction-Restriction Fragment Length Polymorphism* (PCR-RFLP) dan *PCR* diagnostik berdasarkan *nuclear DNA internal transcribed spacer 2* (ITS2) untuk *E. longifolia, L. pumila* dan *O. stamineus. PCR-RFLP* digunakan untuk mengesahkan pengecaman spesies bagi produk herba. PCR-RFLP telah dibangunkan untuk pengecaman pantas menggunakan enzim pembatasan *TaqI, BamHI, HinfI, EcoRI, EcoRV, Mbol*, dan *Mspl*. Jujukan ITS2 telah dikenalpasti dan dibandingkan antara spesimen tumbuhan *E. longifolia, L. pumila* dan *O. stamineus* dengan 106 sampel produk herba komersial. Hasilnya, sampel tumbuhan E*. longifolia, L. pumila,* dan *O. stamineus* berjaya dikenal pasti dengan analisis persamaan yang tinggi masing-masing sebanyak 100%, 100% dan 99.33%, berdasarkan *National Center for Biotechnology Information (NCBI) GenBank*. Pemulihan jujukan DNA daripada produk herba adalah 60.4%, dimana 81.97% jujukan telah dikenal pasti dan 18.03% tidak menunjukkan jujukan melalui pengecaman *Basic Local Alignment Search Tool (BLAST)*. PCR khusus yang digabungkan dengan pencernaan menggunakan enzim pembatas *Mbol* membolehkan pengenalpastian *O. stamineus* dan *E. longifolia*. Sebaliknya, *Mspl* membolehkan pengenalpastian *L. pumila* dengan menghasilkan corak pembatas khusus pada sampel tumbuhan. Sebagai kesimpulan, pendekatan yang boleh dipercayai untuk mengenal pasti dan mengesahkan spesies tumbuhan dalam produk herba telah dicipta dengan menggunakan enzim pembatas. Pendekatan PCR-RFLP yang mudah dan tepat ini secara efisien mengenal pasti *E. longifolia, L. pumila* dan *O. stamineus* dengan menganalisis jujukan ITS2, memastikan Kesihatan dan keselamatan pengguna.

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LIST OF SYMBOLS AND ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Database
DNA	Deoxyribonucleic Acid
GenBank	An online publicly available sequence database maintained by NCBI
HP	Herbal product
ITS2	Internal transcribed spacer 2
Kg	Kilogram
MatK	Maturase K
Ml	Mililitre
Mg	Milligram
NCBI	National Center for Biotechnology Information
NJ	Neighbour Joining
PCR	Polymerase Chain Reaction
rbcL	Ribulose biphosphate carboxylase
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
Rpm	Revolution per minute
WHO	World Health Organization
µl	microliter

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µm	micromolar
w/v	weight/volume
v/v	volume/volume
ng/µl	nanogram/microlitre
°c	degree celcius
%	percentage

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CHAPTER 1

INTRODUCTION

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1.1 Introduction

Consumer interest in the food industry has increased recently due to the popularity of products made using botanical ingredients (Colombo et al., 2020). Botanical extracts are used as ingredients or flavorings in these herbal products, dietary supplements, herbal teas, traditional herbal medicines, and botanical medicinal products (Low et al., 2017). Herbal products and dietary supplements, formerly known as "botanical natural

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products," are composed of diverse compounds derived from various sources, including bacteria, fungi, marine organisms, and plants (Kellogg et al., 2019). The phrases may apply to complicated mixes and single isolated substances derived from such mixtures (Kellogg et al., 2019). The European Food Safety Authority (EFSA) has described "botanical" as "medicinal, cosmetic, and aromatic plants" that may be employed or altered to serve as "ingredients of foods, food supplements, cosmetic, drugs, medical devices, animal feed, household products, pesticides, and biocidal products" and therefore are governed by various law (Colombo et al., 2020). Various terminology associated with herbal products were based upon the legislation of the country in which they are sold, i.e., supplements, herbal products, natural health products, traditional medicine, herbal medications, herbs, botanical preparations, nutraceuticals, phytomedicines, and botanical medicines are used (Ramirez et al., 2022).

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The Dietary Supplement Health and Education Art classed herbs as dietary supplements, which include raw plant material such as leaves, flowers, fruit, seeds, stems, wood, bark, roots, rhizomes, or other plant components that may be whole, fragmented, or powdered by World Health Organization (WHO) (Yeong & Choong, 2017). According to the Food and Drug Administration (FDA), a dietary supplement is a non-drug product meant to add one or more of the following to your diet: vitamins, minerals, herbs, and amino acids (El Khoury et al., 2016). Moreover, food supplements are defined as "foodstuffs whose goal is to supplement the regular diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological impact" by the European Directive 2002/46/EC (Biesterbos et al., 2019). These food supplements, which can be made from a single ingredient or a combination of

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ingredients, are sold in dose form, including liquid and powder sachets, ampoules, drop dispensing bottles, capsules, pastilles, tablets, and other similar forms that are intended to be taken in precise, quantifiable portions (Biesterbos et al., 2019; Low et al., 2017).

Despite variances in botanical food supplement legislation, consumer demand for these products continues to increase (Low et al., 2017). Most people worldwide still use medicinal herbs to cure and prevent disease (Palabaş Uzun & Koca, 2020). Herbal products have been essential in preserving global health as safe, effective, low-cost therapy (Liu et al., 2018). It is believed that around 80% of the world population and up to 95% of the population in developing countries rely on herbal products to meet their essential healthcare requirements (Ganie et al., 2015; Ramirez et al., 2022), and roughly 30% of Malaysian utilise traditional herbal products for their health requirements (Ariffin et al., 2021). The global market's value for dietary supplements was assessed at USD 109.8 billion in 2013, and it is expected to rise to USD 18 billion by 2020. The largest market segment, with a 2013 market value estimated at USD 54.6 billion, is for botanical supplements (Low et al., 2017).

The forest in Malaysia is abundant with a wide variety of medicinal plants, offering significant potential for the herbal industry (Fadzil et al., 2018). Malaysia's herbal products (HPs) industry has grown steadily over the last decade, owing to rising community interest in using herbal-based products as medicine (Hamid & Sairah, 2020). The Malaysian herbal business benefited from this rise, and by 2020, the market value is predicted to reach MYR 32 billion, which would indicate an annual growth rate

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of 8–15% (Pauzi et al., 2022). According to a Technavio research analyst's report, the market for health supplements is anticipated to expand at a Compound Annual Growth Rate (CAGR) of 7% every year (Ismail et al., 2020). Notably, throughout 2021, more than 1700 new product registration applications were received, and more than 1400 products were registered (NPRA, 2022).

Ethnic groups like the Malays, Chinese, Indians, and Aborigines all practice traditional medicine in various ways in Malaysia due to the country's rich cultural diversity and abundant of indigenous healing practices (Aziz et al., 2015). With its vast biodiversity and multi-ethnic communities, Malaysia provides a rare mix for the growth of the herbal industry (Ahmad et al., 2015) Traditional Chinese Medicine (TCM), Ayurveda, Jammu, and other forms of alternative treatment are well-received by Malaysians (Ahmad et al., 2015). Herbal medicine is practiced according to traditions passed down through the ages and founded on scientific evidence, cultural norms, and religious dogma (Aziz et al., 2015; Tengku Mohamad et al., 2019). Traditional and herbal products have been a trendy and preferred treatment option from generation to generation since they are regarded as one of the most trustworthy and affordable medical solutions (Ismail et al., 2018).

Indeed, many medicines dependent on local herbs have long been commonly used in Malaysian households (Ahmad et al., 2015). The Economic Transformation Program (ETP), which began in early 2011, identified the herbal industry as a new source of growth and one of the Entry Point Projects (EPP) under the Agriculture New

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Main Economic Area (NKEA) (Ahmad et al., 2015). The local herbal industry and international endeavors have benefited from the EPP1 project's plan to improve product quality and marketing efforts to meet global demand for dietary, herbal, and botanical drugs (Ahmad et al., 2015). *Labisia pumila* (*L. pumila*) (Kacip Fatimah), *Eurycoma longifolia* (*E. longifolia*) Jack (Tongkat Ali), and *Orthosiphon stamineus* (*O. stamineus*) (Misai Kucing) are just some of the well-known herbs used and researched extensively in Malaysia (Nik Hussain & Kadir, 2013). Acknowledging the growth opportunities of herbal/medicinal plants' health value, Malaysia officially announced 11 essential herbs, including *E. longiflolia*, *L. pumila*, *O. stamineus*, and others to be commercialized (Fadzil et al., 2018). These plants share comparable antimicrobial phytochemical characteristics (Nadia et al., 2012). However, as demand for herbal products has increased, there has been concern over the safety and effectiveness of raw herbal materials, including just the required plant species or identified plant products (Santhosh Kumar et al., 2020).

1.2 Research Background

As herbal supplements become more commonplace in today's healthcare, they must be optimized for their intended use by giving relevant information (Liu et al., 2018). Consumers may still be exposed to botanicals and compounds derived from them through their intake of various product forms (Low et al., 2017). Botanicals and chemicals produced from them are used in food and as nutritional supplements; this

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includes herbal teas, traditional herbal products, and botanical medicinal products (Low et al., 2017).

National Pharmaceutical Regulatory Agency (NPRA) and Food Safety & Quality Division (FSQD) came under the spotlight when it comes to categorizing whether a product falls within the food or medicine categories (Aziz et al., 2020). By the regulations, the NPRA will regulate any product that contains more than 20% active substances and less than 80% food-based ingredients (Daud et al., 2017). An "active component" is any substance that plays a role in diagnosing, treating, mitigating, or preventing disease by altering the structure or function of the human or animal body (Daud et al., 2017). Meanwhile, the FSQD will regulate the product if it contains more than 80% food-based constituents and fewer than 20% active substances (Daud et al., 2017). When a product falls within the jurisdiction of FSQD, it must comply with and adhere to the Food Act of 1983 requirements (Daud et al., 2017). Since nutraceuticals fall under the food category, they were exempt from the Control of Drugs and Cosmetics Regulation of 1984's severe licencing requirements (Daud et al., 2017). It has become a primary reason that herbal products may be easily sold (Saleh et al., 2016).

The concern arose from the public's assumption that all products made with herbal components are secure and may be used without any warnings (Saleh et al., 2016). Unregistered and adulterated products exploit the people in this situation (Saleh et al., 2016). The component species of products are frequently offered in processed or

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modified forms, such as powders, dried material, pills, capsules, and tea bags, making species identification practically impossible (Singtonat & Osathanunkul, 2015). Consumer safety should be a concern as a result. A significant health risk to the consumers might result from adding unwanted, unrelated species due to incorrect identification of the component plants (Singtonat & Osathanunkul, 2015). Intentional or accidental ingredient substitutions can severely impact customers and manufacturers (Singtonat & Osathanunkul, 2015). Any approaches that might help identify herbal products would be helpful due to these identification problems (Singtonat & Osathanunkul, 2015).

Medical plants are natural resources meant to be used to produce high-quality herbal products (Steinhoff, 2019). Due to their pharmacological ability to treat illness or preserve health, plant is commonly employed for medical reasons throughout the world (Grazina et al., 2020). According to Rohman et al. (2014), herbal products may be presented as single herbs or as a combination containing several medicinal herbs in composite formulae. Herbal products and herbal product mixtures have traditionally been evaluated based on one or two pharmacologically active components or active biological markers (Rohman et al., 2014). The performance of herbal products depends on their number of active ingredients, which can differ considerably in their contents (Rohman et al., 2014).

As stated by Fatma et al. (2016), traditional medicine, supplementary and alternative medicine heavily rely on the use of locally produced medicines with plant origins. The lack of standardization and quality management profiles is one of the barriers to accepting herbal formulations. Because of the complex nature and intrinsic

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uncertainty of plant-based medicines and chemical constituents, it is challenging to set requirements for quality control. Consumer sample adulteration is still a severe issue in local and international marketplaces (Fatma et al., 2016).

Although herbal products are becoming more popular in society, customers have specific health issues and want to know that the items they purchase are always healthy and genuine (Shanmughanandhan et al., 2016). According to Shanmughanandhan et al. (2016), since plant extracts can interfere with pharmaceutical drugs, they may be naturally toxic. As a result, if herbal products are adulterated, allergic reactions may occur because the buyer is unaware that the substance is tainted with species that may interfere with those pharmaceuticals (Shanmughanandhan et al., 2016).

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Several genetic methods have been explored to enhance species recognition and move beyond the bounds of morphological identifications. 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 (Kinyanjui et al., 2016; Meyer et al., 1995). Following this, restriction enzymes are used to cleave the amplified DNA fragments at precise locations (Rasmussen, 2012). Gel electrophoresis is used to size-separate the resultant fragments, and the pattern of the fragments produced is compared with reference patterns to identify the species or find variants within the species (Rasmussen, 2012). PCR-RFLP has been used in several studies to identify herbal products and verify their authenticity. For example,

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several studies used PCR-RFLP to identify the species of *Ginkgo biloba*, *Panax ginseng*, and *Echinacea purpurea* in herbal products (Grazina et al., 2020).

In conclusion, the molecular verification and identity of herbal products can be accomplished effectively using the PCR-RFLP technique. The unique and specific patterns produced by this method can offer reliable identification of the species or genetic differences within a species, assisting in verifying the legitimacy of the herbal products. As an added measure, PCR-RFLP can detect impurities or adulterants in herbal products, facilitating quality assurance and safety monitoring.

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1.3 Problem Statement

Over countless generations, medicinal plants and herbal supplements have played an essential role in the health of human societies (Yu et al., 2021). The herbal products industry is one of the world's fastest developing industries and is forecasted to grow yearly (Unnikrishnan et al., 2020; Urumarudappa et al., 2020; Yu et al., 2021). Malaysia is no exception as the demand and consumption of medicinal plants and herbal products have risen over time, notably *L. pumila* (Tarmizi et al., 2021)*, O. stamineus* (Liow et al., 2021), and *E. longifolia* (Abubakar et al., 2018).

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Herbal products are often mixed with various plant materials that go through several processing procedures to be created and will appear in a variety of forms, most of which are processed or modified, making it challenging to differentiate authentic medicinal plants (Anthoons et al., 2021; Tarmizi et al., 2021). In addition, they may contain substitutes or adulterants that are unrelated to the targeted species, which is unethical and endangers consumers health and safety (Fadzil et al., 2018; Urumarudappa et al., 2020).

Admixture can occur through deliberate and accidental practices, such as blatant and obvious adulteration, typically done for benefit due inadequate quality control steps (Urumarudappa et al., 2020). These practices are standard in plant species with low supply but great demand (Anantha Narayana & Johnson, 2019; Malik et al., 2019). If left uncontrolled, adulteration can severely impacts the reputation of the place of origin and the export trade of medicinal plants in issue (Anantha Narayana & Johnson, 2019). However, adulteration is only sometimes purposeful, and herbal products may be tainted due to unintentional adulteration, misidentification, and misunderstanding caused by vernacular names (Raclariu et al., 2017). Misidentification of plant species is relatively common as macroscopic in the form of morphological identification of plant species needs to be clarified and requires the expertise of a qualified specialist (Fatma et al., 2016; Osathanunkul et al., 2016). Furthermore, it may be challenging to identify extracted natural product materials to the species level using morphology as the products are processed (Kellogg et al., 2019; Noh et al., 2021).

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Herbal products must meet the same quality, safety, and effectiveness standards as conventional pharmaceuticals; therefore, it is essential that the plants used in these products be appropriately identified and authenticated (Malik et al., 2019; Steinhoff, 2019). As consumers frequently rely on product packaging labels to learn about the contents, accurate identification of such species, and their validity is critical in ensuring their quality and making them safe for consumption (Tarmizi et al., 2021).

The lack of defined quality evaluation methodologies, and the highly competitive market for herbal products has increased the motivation to utilise replacements and unlabelled fillers (Raclariu et al., 2017). DNA molecular diagnostics has proven to be an effective tool for ongoing market studies (Shanmughanandhan et al., 2016). Therefore, the purpose of this research is to develop a simple, reliable, and accurate PCR-RFLP DNA molecular method for *E. longifolia, L. pumila,* and *O. stamineus* herbal product supervision to guarantee the security and effectiveness of the herbal product for consumer health without subjecting them to a time-consuming and expensive DNA sequencing process. Eventually, the ultimate goal of this PCR-RFLP authentication method is to verify herbal products containing *E. longifolia, L. pumila* and *O. stamineus*.

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1.4 Objectives of the Study

The study's aims were as follows:

1. To identify suitable primers between ITS2, *rbcL, matK,* and *psbA-trnH* used to identify *Eurycoma longifolia*, *Labisia pumila,* and *Orthosiphon stamineus.*
2. To verify the presence of *Eurycoma longifolia*, *Labisia pumila*, and *Orthosiphon stamineus* in the herbal products using ITS2-RFLP.

1.5 Research Questions

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The following research questions were applied:

1. Which suitable primer between ITS2, *rbcL, matK,* and *psbA-trnH* can be used to provide identification for *Eurycoma longifolia*, *Labisia pumila*, and *Orthosiphon stamineus*?
2. Does ITS2-RFLP help to verify the presence of *Eurycoma longifolia*, *Labisia pumila*, and *Orthosiphon stamineus* in the herbal products?

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1.6 Scope of Study

This study's primary objective is to conduct a comprehensive molecular analysis of herbal products containing *E. longifolia* (Tongkat Ali), *L. pumila* (Kacip Fatimah), and *O. stamineus* (Misai Kucing). The application of Internal Transcribed Spacer 2-Restriction Fragment Length Polymorphism (ITS2-RFLP) markers will be utilised for this analysis. The research will involve a methodical gathering of authentic plant specimens of *E. longifolia, L. pumila,* and *O. stamineus*, as well as herbal products comprised of these particular plant species. The specimens will be subjected to a thorough authentication procedure employing a variety of morphological, anatomical, and molecular tests to confirm their genetic purity and plant integrity.

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Following the collection and verification of plant samples and medicinal products, known techniques will be used to extract high-quality genomic DNA. The first critical stage before conducting any molecular research would be extracting high-quality genomic DNA. In this study, extraction was performed on fresh plant samples using commercially available kits, specifically the Nucleospin Plant II kit. Meanwhile, samples of herbal products were extracted using a Nucleospin Food kit with different sample preparation techniques. The herbal products come in various forms, including powder, liquid, paste, tablets, capsules, and tea. Consequently, identifying the specific species present in the constituents and detecting the potential presence of degraded DNA can be difficult. Eliminating excipients and other PCR inhibitors from processed

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foods and degraded products is one of the obstacles that must be overcome to obtain high-quality DNA.

Therefore, this study amplified genomic DNA extracted from plant specimens and herbal products of *E. longifolia, L. pumila,* and *O. stamineus* using internal transcribed spacer 2 (ITS2), ensuring specificity and accuracy. The sequences generated from these were analyses and identified from NCBI Genbank. The generated DNA fragments will be subjected to precise digestion with restriction enzymes to generate specific restriction patterns. Gel electrophoresis will be used to analyze these patterns, allowing the identification and documentation of distinct restriction profiles associated with each species. Additionally, the acquisition of herbal products will be conducted using the same methodology. The obtained DNA will be analyzed and compared to verified plant samples, with a particular concentration on ITS2 RFLP profiles, to accurately identify these commercial products' botanical composition.

The findings will be analyzed to determine whether commercially available herbal products contain contaminants, misrepresentations, or errors in their botanical composition. Nonetheless, it is essential to note that this investigation will focus solely on *E. longifolia, L. pumila,* and *O. stamineus*, excluding any study of other plant species. In addition, the research will not investigate these botanical compounds' biological activity or therapeutic efficacy, focusing instead on their species-level identification.

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1.7 Significance of Study

The Malaysian government is aware of the various advantages of the herbal industry, and the increasing demand for high-value products (Tan et al., 2020). Through the Entry Point Project (EPP), the NKEA focuses on developing and marketing herbal products with high-value claims. As the herbal industry expands, it is critical to ensure the quality of herbal products. Quality control might be complicated owing to variances in plant ingredients (particularly their chemical components) for identification and the fact that natural material quality and safety are not adequately controlled (Tan et al., 2020).

As a result, it is crucial to have more reliable objective and scientific techniques for confirming the identity of each plant species used in herbal products. This has allowed for the testing and monitoring of plant product purity. This research proposes to establish the most reliable and more accessible ways of molecular analysis approaches that will provide usable resources that can be used immediately to identify plant products and aid in guaranteeing their safe use. ITS2-RFLP markers will provide a more rapid, cost-effective, and potentially more sensitive screening and monitoring tool. This ITS2-RFLP is potentially helpful as a regulation instrument to verify the authenticity of multi-ingredient herbal formulations. Hence, local authorities can prioritize this approach for intensive quality surveys to identify plant species in complex multi-ingredient herbal products. Distributing these approaches across most of Malaysia will help with quality assessment of plant products and pharmacovigilance.

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The Food Safety and Quality Division (FSQD) and the National Pharmaceutical Regulatory Agency (NPRA) in Malaysia may benefit from using the ITS2 PCR-RFLP method for identifying medicinal plants in herbal products. To a large extent, this aids in preventing the inappropriate substitution or contamination of herbal products with non-medicinal substances. A standardized food labeling system helped ensure that customers had accurate and up-to-date information about the origins and preparation methods of the food products they were buying.

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