DNA BARCODING FOR AUTHENTICATION OF *Orthosiphon aristatus* HERBAL MEDICINAL PRODUCTS

JONATHAN GOH JIA EN

A dissertation submitted in fulfilment of the
requirements for the award of the degree of
Master of Science

Faculty of Science
Universiti Teknologi Malaysia

MAY 2021

DEDICATION

This dissertation is dedicated to my beloved family, supervisor, friends and colleagues who have supported and guided me throughout this entire journey while encouraging me to continue on amidst difficult times.

ACKNOWLEDGEMENT

First and foremost, I would like to express my heartfelt gratitude and appreciation towards my supervisor, Dr Faezah binti Mohd Salleh for her valuable guidance, warm encouragement and advices in this study. The constant feedbacks and support I received from our discussions had greatly helped in shaping the journey for completing this project. I would also like to express my appreciation to the staffs of Faculty of Science especially Puan Noor Hidayah binti Md Nooh and Encik Yusnizam bin Samsudin in the provision of conducive environment with equipped facilities that aided in this endeavour.

I would also like to extend my gratitude to all postgraduate students and my colleagues in Plant Biotechnology Laboratory who had enlightened me by providing valuable guidance and knowledge throughout this study. Their assistance had greatly aided me in various stages especially during circumstances filled with unknowns or uncertainties.

Finally, I would like to thank my family members and friends for their constant support and understanding. No amount of words and thanks are able to describe the help that I have received during the completion of this study

ABSTRACT

Orthosiphon aristatus (Misai Kucing) was traditionally known for its anti-diuretic properties and currently being studied for modern medicine applications had became part of high-value herbs targeted for production of commercialised herbal medicinal products (HMPs) in Malaysia. However, issues of adulterated HMPs rose as an unintended consequence of the booming herbal industry and current conventional methods including morphological identification and chemical analysis were unable to identify the source of contaminated herbs within adulterated HMPs. In this study, DNA barcoding was applied for authenticating six *Orthosiphon aristatus* HMPs that consisted of four monoherbal tea products and two polyherbal capsule products using chloroplastic (*rbcL*) and nuclear (*ITS2*) barcodes. Genomic DNA (gDNA) was extracted from *Orthosiphon aristatus* plant (MKP) and five HMPs but only *rbcL* and *ITS2* barcodes of MKP and two HMPs (MKT 1 and MKC 1) were able to be amplified, direct sequenced and verified. Verified *rbcL* and *ITS2* barcodes of MKP were included as reference barcodes for HMPs authentication and only *rbcL* barcode of MKP was deposited in GenBank with accession ID (MW492548.1). Bioinformatic analysis of HMPs barcodes using BLAST and Neighbour-Joining (NJ) method revealed that the monoherbal MKT 1 was authentic with both *rbcL* and *ITS2* barcodes being identified as *Orthosiphon aristatus.* As for the polyherbal MKC 1, the *rbcL* barcode was identified as *Astragalus membranaceus* whereas the *ITS2* barcode was identified as *Strobilanthes namkadingensis*. MKC 1 was considered contaminated as *Strobilanthes namkadingensis,* a species belonging to Acanthaceae family was an unlisted active ingredient within the labelled polyherbal composition. Overall, the findings from this study can served as a preliminary guidance for future authentication using DNA barcoding and potentially beneficial towards official regulatory bodies in standardising the quality and safety of HMPs.

ABSTRAK

Orthosiphon aristatus (Misai Kucing) dikenali secara tradisional untuk ciri-ciri anti-diuretik dan kini dikaji dalam kegunaan bidang perubatan moden telah menjadi sebahagian daripada herba bernilai tinggi yang disasar untuk pengeluaran komersial produk perubatan (HMPs) di Malaysia. Namun begitu, isu berkaitan penjejasan keaslian HMPs semakin meningkat akibat perkembangan industri herba yang pesat dan kaedah konvensional terkini yang merangkumi pengenalan morfologi dan analisis kimia tidak dapat mengenali sumber herba yang tercemar dalam HMPs yang tidak asli. Untuk kajian ini, kaedah barkod DNA digunakan untuk pengesahan keaslian enam *Orthosiphon aristatus* HMPs yang terdiri daripada empat monoherba produk teh dan dua poliherba produk kapsul dengan barkod kloroplastik (*rbcL*) dan nuklear (*ITS2*). Genomik DNA (gDNA) diekstrak daripada pokok *Orthosiphon aristatus* (MKP) dan lima HMPs tetapi hanya barkod *rbcL* dan *ITS2* untuk MKP dan dua HMPs (MKT 1 dan MKC 1) diamlifikasi, dijujukan dan disahkan. Penjujukan *rbcL* dan *ITS2* yang sah digunakan sebahagian daripada barkod rujukan untuk pengesahan HMPs dan hanya barkod *rbcL* untuk MKP disimpan di GenBank dengan aksesi ID (MW492548.1). Analisis bioinformatik untuk barkod HMPs dijalankan meggunakan BLAST dan kaedah 'Neighbour-Joining' (NJ). Keputusan analisis mendedahkan bahawa monoherba MKT 1 adalah asli kerana barkod *rbcL* dan *ITS2* untuk MKT 1 dikenalpasti sebagai *Orthosiphon aristatus*. Untuk analisis poliherba MKC 1, barkod *rbcL* dikenalpasti sebagai *Astragalus membranaceus* manakala barkod *ITS2* dikenalpasti sebagai *Strobilanthes namkadingensis*. MKC 1 didapati tidak asli kerana *Strobilanthes namkadingensis* yang merupakan spesis herba tergolong dalam keluarga Acanthaceae tidak disenaraikan dalam label bahan aktif MKC 1. Secara keseluruhan, hasil kajian ini boleh digunakan sebagai panduan awal untuk pengesahan HMPs pada masa hadapan menggunakan kaedah barkod DNA dan berpotensi dimanfaatkan badan pengawaln rasmi dalam menyeragamkan kualiti dan keselamatan HMPs.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

HMP	-	Herbal Medicinal Products
WHO	-	World Health Organization
ETP	-	Economic Transformation Programme
EPP	-	Entry Point Project
DNA	-	Deoxyribonucleic Acid
USD	-	United States Dollar
GACP	-	Good Agricultural and Collection Procedures
GAP	-	Good Manufacturing Practice
GLP	-	Good Laboratory Practices
GCP	-	Good Clinical Practices
HPLC-MS	-	High Performance Liquid Chromatographic-mass spectrometry
NIRS	-	Liquid chromatography-mass spectrometry
NIRS	-	Near-infrared spectroscopy
RFLP	-	Restriction Fragment Length Polymorphism
AFLP	-	Amplified Fragments Length Polymorphisms
RAPD	-	Random Amplification of Polymorphic DNA
NGS	-	Next Generation Sequencing
ITS2	-	Internal Transcribed Spacer 2
rbcL	-	Ribulose bisphosphate carboxylase
NJ	-	Neighbour-Joining
NAP	-	National Agro-Food Policy
NKEA	-	National Key Economic Areas
ECER	-	East Cost Economic Region
TKPM	-	Permanent Food Production Park
IRDA	-	Iskandar Regional Development Authority
ETP	-	Economic Transformation Programme
KESEDAR	-	South Kelantan Development Authority
KETENGAH	-	Central Terengganu Developemtn Authority
DCA	-	Drug Control Authority

NPRA	-	National Pharmaceutical Regulatory Agency
UV	-	Ultraviolet
ELS	-	Evaporative Light Scattering
GC	-	Gas Chromatography
SSR	-	Simple Sequence Repeats
SCAR	-	Sequence Characterisation of Amplified Regions
PCR	-	Polymerase Chain Reaction
COI	-	cytochrome *c* oxidase I
matK	-	maturase K
bp	-	base pairs
ITS	-	Internal transcribed spacer
cpDNA	-	Chloroplast DNA
NCBI	-	National Center for Biotechnology Information
EMBL-EBI	-	EBI European Nucleotide Archive
DDBJ	-	DNA Data Bank of Japan
INSDC	-	International Nucleotide Sequence Database Collection
BLAST	-	Basic Local Alignment Search Tool
CTAB	-	Cetyltrimethylammnonium bromide
SDS	-	Sodium dodecyl sulphate
mg	-	Milligram
µl	-	Microliter
mg	-	Milligram
w/v	-	Weight per volume
nm	-	nanometer
TAE	-	Tris-acetate-EDTA
A	-	Ampere
K2P	-	Kimura-2-Pararmeter
T	-	Temperature

LIST OF SYMBOLS

%	-	Percentage
℃	-	Degree Celsius

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Herbal medicinal product, from a humble beginning in traditional usage to being internationally recognised by World Health Organization (WHO) and other health governing bodies for its health benefits. According to European Union (2004), herbal medicinal products (HMP) are formally categorised as 'any medicinal product, exclusively containing as active ingredients one or more herbal substances, one or more herbal preparations, or a combination of the two' (Tan *et al.*, 2020; European Union, 2004). Global herbal trade generates multibillion dollars in revenues which attracts various parties to participate in herbal industries. For example, in 2012, US$12.8 billion was spent on herbal products in the United States whereas China, being one of the major global producers, manufactured approximately US$83.1 billion worth of herbal products in the same year (Tnah *et al.*, 2019; Nahin *et al.*, 2016; WHO, 2013). This industry also represents an economic opportunity to Malaysia which houses to one of the world oldest rainforest that contained around 12,500 seed plants. From this pool, it is estimated that approximately 1200 species with medicinal values can be found in this rainforest (Tan *et al.*, 2020; Ramlan, 2003). To exhibit the value of this opportunity, it is recorded that the revenue generated from traditional medicine trade in Malaysia rose from US$385 million in 2000 to US$1.29 billion in 2005 (Abubakar *et al.*, 2018; Aziz and Tey, 2009; Mohammad Azmin *et al.*, 2016). Furthermore, the import and export value of herbal trade in Malaysia were worth around US$2077 million and US$ 441.7 million

respectively for the year 2014 (Tnah *et al.,* 2019; Zakaria, 2015). This event is contributed by the development of the herbal industry in Malaysia under the implementation of Economic Transformation Programme (ETP) in September 2009 which includes Entry Point Project (EPP) High-Value Herbal Products that contributed towards herbal products development for high value botanical drugs and nutraceuticals purpose (Tan *et al.,* 2020; PEMANDU, 2012)

However, the rise of global herbal industry creates a growing widespread of cases that involved fraudulent practices that are based on herbal products adulteration. These cases range from misidentification of herbs to substitution of herbal materials with cheaper materials or synthetic drugs that will lead to health hazards or reduced herbal efficacy (Tnah *et al.,* 2019; China Plant BOL Group, 2011). A study by Ichim (2019) found that out of 5,957 herbal products sold in global market, 27% of them contained undeclared contaminant, substitute, filler species or none of the labelled species. The same study also found that the distribution of adulterated herbal products also varied by countries with the highest amount found in Brazil (68%) to the lowest in China (19%) using DNA-based authentication method. 24% of the adulterated herbal products were found in Malaysia. Furthermore, non-standardised regulatory policies between countries caused major difficulties in standardising quality control, safety and efficacy of herbal products due to differences in acceptance criteria such as limits for presence of heavy metals, microbial contamination and pesticide (Ramadoss & Koumaravelou, 2019; Tnah *et al.,* 2019). These issues will only exacerbate in the future as it is projected that the global market for herbal products will continue to grow as high as 7% per annum towards USD 5 trillion by the year 2050 (Tan *et al.,* 2020; Ahmad and Othman, 2013).

Currently, production of standard herbal products are determined by following good agricultural and collection procedures (GACP), good manufacturing practice (GMP, good laboratory practices (GLP) and good clinical practices (GCP) (Ramadoss &

Koumaravelou, 2019). However, there is still a need for fast with precise and accurate methods to authenticate herbal products as to match with the rapid pace of output. Conventional authentication methods such as macroscopic and microscopic identification and chemical analysis which includes high performance liquid chromatographic-mass spectrometry (HPLC-MS), liquid chromatography-mass spectrometry (NIRS) and near-infrared spectroscopy (NIRS) are currently implemented in commercial productions. On the other hand, recent development on DNA sequencing technology provides an alternative authentication method known as DNA barcoding which utilises biomarkers or 'barcodes' that consist of short conserved regions within the genome (Ichim, 2019; Herbert *et al.,* 2003). It complements with current methods as it is able to bypass the limitations of conventional methods via determining the source of herbs or differentiate highly related herb species that share morphological characteristics or chemical profiles (Tnah *et al.,* 2019)

Orthosiphon aristatus which is also commonly known as Misai Kucing remains as among the widely used herbs for herbal products. It is traditionally popular in Southeast Asia due to its long history for its diuretic properties. Currently, numerous scientific studies are carried out to investigate its pharmacological properties (Silalahi, 2019) and applications in treating diabetes-related illness (Abdullah *et al.*, 2020). In Malaysia, *Orthosiphon aristatus* is one of the five herbs that was focused for commercial production of higher-value herbal products under EPP 1 (PEMANDU, 2012). Thus, the need for fast and reliable authentication method for quality control can be fulfilled via DNA barcoding as to ensure safety and efficacy of *Orthosiphon aristatus* herbal products amid the rapid output in current times.

1.2 Problem Statement

Currently, there are few notable issues faced by herbal industry in dealing with the growing cases of herbal product adulteration. First, multiple limitations arose within

conventional authentication methods that utilised techniques of morphology identification, botanical taxonomy and chemical analysis. For example, the applications of morphology identification and botanical taxonomy are applicable if the distinct physical features of the herbs remain intact. However, it is not applicable for HMP as most of the herbs used as active ingredients had undergone processes which caused to lose its distinct physical features (Abubakar *et al.,* 2018). On the other hand, chemical analysis is applicable in detecting contaminants and toxins but not capable to identify the source of contamination (Ichim, 2019) or differentiating closely related herb species that share high similarities in morphological characteristics or chemical profiles (Tnah *et al.,* 2019).

In response towards limitations of conventional methods, introduction of new methods that are DNA-based technology provides a common advantage of requiring small amount of samples regardless of their sources or forms (Drouet *et al.,* 2018). These methods range from restriction fragment length polymorphisms (RFLP), amplified fragments length polymorphisms (AFLP), random amplification of polymorphic DNA (RAPD) to recent techniques such as DNA barcoding, microsatellite-based markers and Next Generation Sequencing (NGS)-based markers. Although these methods are effective in detecting the source of biological materials, most of them except DNA barcoding are not applicable for authentication of large scale commercial products due to high cost, labour intensive or requiring specific criteria that complicate the authentication process (Drouet *et al.,* 2018). Thus, DNA barcoding has been determined to be a fast and reliable species discriminator with remarkable accuracy and cost effective for authentication of herbal products while addressing the limitations of previous methods.

There are previous studies that successfully determined the authenticity *Orthosiphon aristatus* HMP based on detection of bioactive compounds using chemical or spectroscopy fingerprinting analysis (Hernadi *et al.,* 2019; Yuliantini *et al.,* 2020).

However, both methods were only able to identify by comparing the chemical compositions with known substituted herbs whereas it cannot determine the source of adulterants if new or novel substituted herb species are used. A DNA barcoding study was carried out using loci combination of *rbcL* and *trnH-psbA* by Tnah *et al.,* (2019) which found out that a HMP which claimed to use *Orthosiphon aristasus* as its active ingredient was substituted with *Clinacanthus nutans*. However, there are no studies that utilised other loci combination such as *ITS2* and *rbcL* for authentication of *Orthosiphon aristatus* HMP although various studies have shown that *ITS2* provides better species resolution in comparison with other loci for identifying plants taxa. Furthermore, the study by Tnah *et al.* (2019) only tested one HMP of *Orthosiphon stamineus* (synonym: *Orthosiphon aristatus*), which could not represent the current adulteration and authentication status of this species in Malaysian market.

1.3 Objectives of Study

The objectives of this study were as follow:-

i. To generate nuclear (*ITS2*) and chloroplast (*rbcL*) reference barcodes for *Orthosiphon aristatus* plant.

ii. To verify the authenticity of *Orthosiphon aristatus* HMP via DNA barcoding method.

1.4 Scope of Study

In this study, a purple variety of *Orthosiphon aristatus* plant and six herbal medicinal products (HMP) related to *Orthosiphon aristatus* (four tea products and two capsules products) were chosen. *Orthosiphon aristatus* leaves and HMP were subjected to DNA extraction and purification using NucleoSpin® Plant II (Macherey-Nagel) kit

while the quality and quantity of extracted genomic DNA (gDNA) were determined using Nanodrop spectrophotometer and agarose gel electrophoresis. The chloroplast *(rbcL)* and nuclear *(ITS2)* barcode regions of the gDNA amplified using universal primers via polymerase chain reaction (PCR) were later sent for Sanger sequencing. The generated sequences were analysed and verified using bioinformatic tools. The verified *ITS2* and *rbcL* sequences of *Orthosiphon aristatus* plant were used as internal reference barcodes whereas the authentication of HMP were determined by comparing *ITS2* and *rbcL* barcodes with the internal reference barcodes of *Orthosiphon aristatus* and other reference barcodes from GenBank via BLASTn and neighbour joining (NJ) tree analysis.

1.5 Significance of Study

With the concern of growing number of cases related to adulteration of HMP on global trade, this study can be used to highlight the benefits of DNA barcoding as a reliable alternative tool in authenticating HMP in comparison to conventional authentication methods. It also highlights the effectiveness of chloroplastic *(rbcL)* and nuclear *(ITS2)* genes as barcodes for identifying plant materials at species level that were used in HMP. Overall, the findings of this study will contribute to a better understanding in authentication of *Orthosiphon aristatus* HMP using *ITS2* and *rbcL* markers and may help future works related in authenticating HMP that claimed having *Orthosiphon aristatus* as their active ingredient. This will also indirectly create improvement in quality control of herbal products.

REFERENCES

Abdullah, F. I., Chua, L. S., Mohd Bohari, S. P., & Sari, E. (2020). Rationale of *Orthosiphon aristatus* for Healing Diabetic Foot Ulcer. *Natural Product Communications*, *15*(9), 1–13.

Abdullah, F. I., Chua, L. S., Rahmat, Z., Samad, A. A. & Wagiran, A. (2016). Plant genomic DNA extraction for selected herbs and sequencing their internal transcribed spacer regions amplified by specific primers. *Natural Product Communications, 11*(10), 1491-1496.

Aboul-Maaty, N. A.-F., & Oraby, H. A.-S. (2019). Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. *Bulletin of the National Research Centre*, *43*(1), 1–10.

Abubakar, B., Wagiran, A. and Salleh, F. (2016). Assessing product adulteration in selected Malaysian herbal product using DNA barcoding. *International Conference on Agricultural and Food Engineering*, 98–104.

Abubakar, B. M., Salleh, F. M., Shahir, M., Omar, S., & Wagiran, A. (2017). Review : DNA Barcoding and Chromatography Fingerprints for the Authentication of Botanicals in Herbal Medicinal Products. *Evidence-Based Complementary and Alternative Medicine*, *2017*, 1–28.

Abubakar, B. M., Salleh, F. M., Shamsir, M. S., & Wagiran, A. (2018). Assessing product adulteration of *Eurycoma longifolia* (Tongkat Ali) herbal medicinal product using DNA barcoding and HPLC analysis. *Pharmaceutical Biology*, *56*(1), 368–377.

Abubakar, B. M., Salleh, F. M., Wagiran, A., & Abba, M. (2021). Comparative Evaluation of Different DNA Extraction Methods from *E. longifolia* Herbal Medicinal Product. *eFood*, 1–6.

Adnyana, I. K., Setiawan, F., & Insanu, M. (2013). From Ethnopharmacology to Clinical Study of *Orthosiphon stamineus* Benth. *International Journal of Pharmacy and Pharmaceutical Sciences*, *5*(3).

Ahmad, S., Othman, N., (2013). Strategic Planning, issues, prospects and the future of

the Malaysian herbal industry. *IJARAFMS*, 3(4), 91-102.

Alam, A., Ferdosh, S., Ghafoor, K., Hakim, A., Juraimi, A.S., Khatib, A., *et al.* (2016). *Clinacanthus nutans*: A review of the medicinal uses, pharmacology and phytochemistry. *Asian Pacific Journal of Tropical Medicine*, 9, 402-409.

Ali, N., Rampazzo, R. D. C. P., Costa, A. Di. T., & Krieger, M. A. (2017). Current Nucleic Acid Extraction Methods and Their Implications to Point-of-Care Diagnostics. *BioMed Research International*, *2017*.

Almatar, M., Rahmat, Z., & Salleh, F. M. (2013). Preliminary morphological and anatomical study of *Orthosiphon stamineus*. *Indian J.Pharm.Biol.Res*, *1*(4), 1–6.

Artanti, D. (2017). Efektivitas Perasan Daun Keji Beling (Sericocalyx crispus Linn) Dalam Menghambat Pertumbuhan *Staphylococcus aureus*. *The Journal of Muhammadiyah Medical Laboratory Technologist*, *1*(1), 78.

Aziz, Z., Tey, N. (2009). Herbal medicines: prevalence and predictors of use among Malaysian adults. *Complement Ther. Med.* 17, 44-50.

Bhardwaj, S., Verma, R., & Gupta, J. (2018). Challenges and future prospects of herbal medicine. *International Research in Medical and Health Science*, *1*(1), 12–15.

Boiso, L., & Hedman, J. (2017). Overcoming sodium dodecyl sulfate induced PCR inhibition. *Forensic Science International: Genetics*, *29*, e16–e18.

CBOL Plant Working Group. (2009). A DNA barcode for land plants. *PNAS*, *106*(31), 12794–12797.

Chen, F., Chan, H.Y.E., Wong, K.L., Wang, J., Yu, M.T., But, P.P.H., Shaw, P.C. (2008). Authentication of *Saussurea lappa*, an endangered medical material, by ITS DNA and 5S rRNA sequencing. *Planta Med*., *74*, 889-892.

Chen, S., Yao, H., Han, J., Liu, C., Song, J., Shi, L., & Zhu, Y. (2010). Validation of the ITS2 Region as a Novel DNA Barcode for Identifying Medicinal Plant Species. *PLoS ONE*, *5*(1), 1–8.

China Plant BOL Group. (2011). Comparative analysis of a large dataset indicates that internal transcribed spacer (ITS) should be incorporated into the core barcode for seed plants. *Proceedings of the National Academy of Sciences of the United States of America*, 108. 19641 - 19646.

Cline, E. (2012). Marketplace substitution of Atlantic salmon for Pacific salmon in

Washington State detected by DNA barcoding. *Food Res. Int.*, 45, 388-393.

Costa, R., Pereira, G., Garrido, I., Tavares-De-Sousa, M. M., & Espinosa, F. (2016). Comparison of RAPD, ISSR, and AFLP Molecular Markers to Reveal and Classify Orchardgrass (*Dactylis glomerata* L.) Germplasm Variations. *PLoS ONE*, *11*(4), 1–15.

Cui, X., Li, W., Wei, J., Qi, Y., Li, R., Yang, Y., Shi, Y., Meng, X., Mi, Y., Huot, T, Sun, W. & Zheng, X. (2020). Assessing the Identity of Commercial Herbs From a Cambodian Market Using DNA Barcoding. *Frontiers in Pharmacology*, *11*(March), 1–10.

Cui, X., Sun, W., Xiong, C., Meng, X., Shi, Y., Wu, L., *et al.* (2019). Identification of 23 unknown Li medicinal plants based on DNA barcoding. *China J. Chin. Mater. Med.*, 44, 357-366.

de Boer, H.J., Ichim, M.C., Newmaster, S.G. (2015). DNA barcoding and pharmacovigilance of herbal medicines. *Drug Saf.*, 38, 611-620.

De Pinto, A., Mottola, A., Marchetti, P., Bottaro, M., Terio, V., Bozzo, G., Bonerba, E., Ceci, E., Tantillo, G. (2016). Packaged frozen fishery products: Species identification, mislabelling occurrence and legislative implications. *Food Chem.*, 194, 279-283.

Dechbumroong, P., Aumnouypol, S., Denduangboripant, J., & Sukrong, S. (2018). DNA barcoding of *Aristolochia* plants and development of species-specific multiplex PCR to aid HPTLC in ascertainment of *Aristolochia* herbal materials. *PLoS ONE*, *13*(8), 1–16.

Drouet, S., Garros, L., Hano, C., Tungmunnithum, D., Renouard, S., Hagège, D., Maunit, B., Lainé, É. (2018). A critical view of different botanical, molecular, and chemical techniques used in authentication of plant materials for cosmetic applications. *Cosmetics*, *5*(2).

Edwards, K., Johnstone, C. & Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research*, *19*(6), 1349.

Eurlings, M., Lens, F., Pakusza, C., Peelen, T., Wieringa, J.J., Gravendeel. B. (2013). Forensic identification of Indian snakeroot (*Rauvolia serpentine* Benth. ex. Kurz)

using DNA barcoding. *J. Forensic Sci.*, 58, 822-830.

European Union. (2004). Directive 2004/24/EC of the European Parliament and of the Council. *European Union*, 85-89.

Faller, A. C., Ragupathy, S., Shanmughanandhan, D., Zhang, Y., Lu, Z., Chang, P., Swanson, G. & Newmaster, S. G. (2019). DNA quality and quantity analysis of *Camellia sinensis* through processing from fresh leaves to a green tea extract. *Journal of AOAC International*, *102*(6), 1798–1807.

Febjislami, S., Kurniawati, A. N. I., Melati, M., & Wahyu, Y. (2019). Morphological characters, flowering and seed germination of the indonesian medicinal plant Orthosiphon aristatus. *Biodiversitas*, *20*(2), 328–337.

Fu, J., Wang, Z., Huang, L., Zheng, S., Wang, D., Chen, S., Zhang, H. & Yang, S. (2014). Review of the Botanical Characteristics, Phytochemistry, and Pharmacology of *Astagalus membranaceus* (Huangqi). *Phytotherapy Research*, 1–9.

Ganthier, G., van der Niet, T., Peelen, T., van Vugt, R.R., Eurlings, M.C.M., Gravendeel, B. (2013). Forensic identification of CITES preotected slimming Cactus (Hoodia) using DNA barcoding. *J. Forensic Sci.*, 58, 1467-1471.

Gao, Z., Liu, Y., Wang, X., Wei, X., & Han, J. (2019). DNA Mini-Barcoding: A Derived Barcoding Method for Herbal Molecular Identification. *Frontiers in Plant Science*, *10*(August), 1–11.

Ghorbani, A., Saeedi, Y., de Boer, H.J. (2015). DNA barcoding in ethnobotany and ethnopharmacology: Identifying medicinal plants traded in local markets. *Genome*, 58, 220-220.

Goldenberger, D., Perschil, I., Ritzler, M., & Altwegg, M. (1995). A simple "universal" DNA extraction procedure using SDS and proteinase K is compatible with direct PCR amplification. *Genome Research*, *4*(6), 368–370.

Grazina, L., Amaral, J. S., & Mafra, I. (2020). Botanical origin authentication of dietary supplements by DNA-based approaches. *Comprehensive Reviews in Food Science and Food Safety*, *19*(3), 1080–1109.

Hafizudin, M., Roslina, Z., Nor Amna A'liah, M. N., & Nik Rahimah, N. O. (2019). Transformation of Herbal Industry in Malaysia. *FFTC Agricultural Policy Articles*,

1–8.

Hajibabaei, M., Singer, G.A., Hebert, P.D., Hickey, D.A. (2007). DNA barcoding: How it complements taxonomy, molecular phylogenetics and population genetics. *Trends Genet., 23*, 167-172.

Hall, T. (2011). BioEdit: An important software for molecular biology. *GERF Bulletin of Biosciences*, *2*(1), 60–61.

Han, J., Pang, X., Liao, B., Yao, H., Song, J., & Chen, S. (2016). An authenticity survey of herbal medicines from markets in China using DNA barcoding. *Nature Publishing Group*, 1–9.

Hanner, R., Becker, S., Ivanova, N.V., Steinke, D. (2011). FISH-BOL and seafood identification: Geographically dispersed case studies reveal systemic market substitution across Canada. *Mitochondrial DNA*, 22, 106-122.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & Jeremy, R. (2003). Biological identifications through DNA barcodes. *The Royal Society*, (September 2002), 313–321.

Hebert, P.D.N., Gregory, T.R., (2005). The promise of DNA barcoding for Taxonomy. *Syst. Biol.* 54, 852-859.

Hernadi, E., Rohaeti, E., Rafi, M., Wahyuni, W. T., Putri, S. P., & Fukusaki, E. (2019). HPLC fingerprinting coupled with linear discriminant analysis for the detection of adulteration in *Orthosiphon aristatus*. *Journal of Liquid Chromatography and Related Technologies*, *42*(15–16), 513–520.

Heubl, G. (2010). New aspects of DNA-based authentication of Chinese medicinal plants by molecular biological techniques. *Planta Medica*, 76(17), 1963-1974.

Hite, J. M., Eckert, K. A., & Cheng, K. C. (1996). Factors affecting fidelity of DNA synthesis during PCR amplification of d(C-A)n·d(G-T)n microsatellite repeats. *Nucleic Acids Research*, *24*(12), 2429–2434.

Hollingsworth, P. M., Graham, S. W., & Little, D. P. (2011). Choosing and using a plant DNA barcode. *PLoS ONE*, *6*(5), 1–13.

IBOL. (2021). DNA Barcoding: A Tool for Specimen Identification and Species Discovery. *International Barcode of Life*. Retrieved from https://ibol.org/about/dna-barcoding/ .

Ichim, M. C. (2019). The DNA-based authentication of commercial herbal products reveals their globally widespread adulteration. *Frontiers in Pharmacology*, *10*, 1–9.

Id, K. A. M., Damaso, N., & Robertson, J. M. (2019). Assessment of BOLD and GenBank – Their accuracy and reliability for the identification of biological materials. *PLoS ONE*, *14*(6), 1–14.

Ismail, S. F., Azmi, I. M. A. G., Daud, M., Jalil, J. A., & Safuan, S. (2020). Regulatory Control of Herbal and Traditional Medicines in Malaysia: Issues and Concerns. *International Journal of Business and Society*, *21*(S1), 192.

Izzany, F., Bakar, A., Fadzelly, M., Bakar, A., Abdullah, N., Endrini, S., & Rahmat, A. (2020). A Review of Malaysian Medicinal Plants with Potential Anti-Inflammatory Activity. *Advances in Pharmacological Sciences*, *2018*, 1–13.

Janjua, S., Fakhar I Abbas, W.K., Malik, I.U., Mehr, J. (2016). DNA Mini-barcoding for wildlife trade control: A case study on identification of highly processed animal materials. *Mitochondrial DNA Part A,* 28, 444-456.

Keng, C.L., Siong, L.P. (2006). Morphological similarities and differences between the two varieties of cat's whiskers (*Orthosiphon stamineus* Benth.) grown in Malaysia. *Int. J. Bot.*, 2, 1-6.

Kress, W. J., & Erickson, D. L. (2007). A Two-Locus Global DNA Barcode for Land Plants: The Coding rbcL Gene Complements the Non-Coding *trnH-psbA* Spacer Region. *PLoS ONE*, *2*(6).

Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*, *35*(6), 1547–1549.

Lee, W.L. (2004). Micropropagation and cell culture of Misai Kucing (*Orthosiphon aristatus* Benth) and detection of rosmarinic acid in vitro cultures. *MSc. Thesis, Universiti Sains Malaysia.*

Lee, P. Y., Costumbrado, J., Hsu, C. Y., & Kim, Y. H. (2012). Agarose gel electrophoresis for the separation of DNA fragments. *Journal of Visualized Experiments*, (62), 1–5.

Li, M., Cao, H., But, P.P.H., and Shaw, P.C. (2011). Identification of herbal medicinal materials using DNA barcodes. *Journal of Systematic and Evolution*. 49(3), 271-

283.

Li, X., Yang, Y., Henry, R. J., Rossetto, M., Wang, Y., & Chen, S. (2015). Plant DNA barcoding: from gene to genome. *Biological Reviews of the Cambridge Philosophical Society*, *90*(1), 157–166.

Liu, P., Zhao, H., & Luo, Y. (2017). Anti-aging implications of *Astragalus Membranaceus* (Huangqi): A well-known Chinese tonic. *Aging and Disease*, *8*(6), 868–886.

Llongueras, J. P., Nair, S., Salas-Leiva, D., & Schwarzbach, A. E. (2013). Comparing DNA extraction methods for analysis of botanical materials found in anti-diabetic supplements. *Molecular Biotechnology*, *53*(3), 249–256.

Mohammad Azmin, S. N. H., Abdul Manan, Z., Wan Alwi, S. R., Chua, L. S., Mustaffa, A. A. & Yunus, N. A. (2016). Herbal processing and extraction technologies. *Sep Puriff Rev*, 45, 305 - 320.

Moyo, M., Amoo, S. O., Bairu, M. W., Finnie, J. F., & Van Staden, J. (2008). Optimising DNA isolation for medicinal plants. *South African Journal of Botany*, *74*(4), 771–775.

MyBIS. (2021). *Orthosiphon aristatus*. *Malaysia Biodiversity Information Sysytem (MyBIS)*. Retrieved from https://www.mybis.gov.my/sp/7911

Nahin, R. L., Barnes, P. M., & Stussman, B. J. (2016). Expenditures on complementary health approaches: United States. *National Health Statistics Report*, 95, 1 -11.

Naim, D. M., & Mahboob, S. (2019). Molecular identification of herbal species belonging to genus Piper within family Piperaceae from northern Peninsular Malaysia. *Journal of King Saud University - Science*, *32*(2), 1417–1426.

Newmaster, S.G., Grguric, M., Shanmughanandhan, D., Ramalingam, S., and Ragupathy, S. (2013). DNA barcoding detects contamination and substitution in North American herbal products, *BMC Medicine*, 11(222), 1-13.

National Parks. (2021). *Orthosiphon aristatus*. *Flora and Fauna Web*. Retrieved from https://www.nparks.gov.sg/florafaunaweb/flora/2/2/2285

NPRA. (2021). QUEST 3+ Product Search. *National Pharmaceutical Regulatory Agency*. Retrieved from https://npra.gov.my/index.php/en/consumers/information/products-search.html

Parveen, I., Gafner, S., Techen, N., Murch, S. J., & Khan, I. A. (2016). DNA barcoding for the identification of botanicals in herbal medicine and dietary supplements: Strengths and limitations. *Planta Medica*, *82*(14), 1225–1235.

Pawar, R. S., Handy, S. M., Cheng, R., Shyong, N., & Grundel, E. (2017). Assessment of the Authenticity of Herbal Dietary Supplements: Comparison of Chemical and DNA Barcoding Methods. *Planta Medica*, *83*(11), 921–936.

PEMANDU. (2012). *ETP Annual Report 2012*.

Purty, R., & Chatterjee, S. (2016). DNA Barcoding: An Effective Technique in Molecular Taxonomy. *Asutin Journal of Biotechnology & Bioengineering*, *3*(1), 1059–1.

Rahmawati, N., Widiyastuti, Y., Purwanto, R., Lestari, S. S., Sene, I. H. A., & Bakari, Y. (2020). Medicinal Plants Used by Traditional Healers for the Treatment of Various Diseases in Ondae Sub-ethnic of Poso District in Indonesia. *Advances in Health Sciences Research*, *22*(4th International Symposium on Health Research 2019), 460–468.

Ramadoss, M. S. K., & Koumaravelou, K. (2019). Regulatory compliance of herbal medicines – a review. *International Journal of Research in Pharmaceutical Sciences*, 10(4), 3127–3135.

Ramlan, A. B. (2003). *Turning Malaysian into a Global Herbal Producer: A Personal Perspective*. UTM.

Sayers, E. W., Beck, J., Brister, J. R., Bolton, E. E., Canese, K., Comeau, D. C., Funk, K., Ketter, A., Kim, S., Kimchi, A., Kitts, P. A., Kuznetsov, A., Lathrop, S., Lu, Z., McGArvey, K., Madden, T. L., Murphy, T. D., O'Leary, N., Phan, L., Schneider, V. A., Thibaud-Nissen, F., Trawick, B. W., Pruitt, K. D. & Ostell, J. (2020). Database resources of the National Center for Biotechnology Information. *Nucleic Acids Research*, *48*(D1), D9–D16.

Sayers, E. W., Cavanaugh, M., Clark, K., Ostell, J., Pruitt, K. D., & Karsch-Mizrachi, I. (2019). GenBank. *Nucleic Acids Research*, *48*(D1), D84–D86.

Secretariat, G. (2019). *Orthosiphon stamineus* Benth in GBIF Secretariat (Publication no. 10.15468/39omei). Retrieved from https://www.gbif.org/species/3891871

Seethapathy, G. S., Raclariu-Manolica, A. C., Anmarkrud, J. A., Wangensteen, H., & de

Boer, H. J. (2019). DNA metabarcoding authentication of ayurvedic herbal products on the european market raises concerns of quality and fidelity. *Frontiers in Plant Science*, *10*, 1–11.

Shanmughanandhan, D., Ragupathy, S., & Newmaster, S. G. (2016). Estimating Herbal Product Authentication and Adulteration in India Using a Vouchered , DNA-Based Biological Reference Material Library. *Drug Safety*, *39*(12), 1211–1227.

Silalahi, M. (2019). Orthosiphon stamineus Benth (Uses and Bioactivities). *Indonesian Journal of Science and Education*, *3*(1), 26. https://doi.org/10.31002/ijose.v3i1.729

Singh, M. K., Gidwani, B., Gupta, A., & Dhongade, H. (2015). A Review of the Medicinal Plants of Genus *Orthosiphon* (Lamiaceae). *International Journal of Biological Chemistry*, *9*(6), 318–331. https://doi.org/10.3923/ijbc.2015.318.331

Song, Y., Chen, Y., Lv, J., Xu, J., Zhu, S., Li, M. F., & Chen, N. (2017). Development of chloroplast genomic resources for *Oryza* species discrimination. *Frontiers in Plant Science*, *8*, 1–10.

Souladeth, P., Tagane, S., Zhang, M., Okabe, N., & Yahara, T. (2017). Flora of Nam Kading National Protected Area I: A new species of yellow-flowered *Strobilanthes* (Acanthaceae), *S. namkadingensis*. *PhytoKeys*, (81), 11–17.

Srivastava, S., Bhargava, M., & Sharma, A. (2016). DNA Barcoding of Medicinal Plants. Singapore: Springer.

Sudarmono, Kim, S. Y., & Paik, J. H. (2020). Contradictory between morphology and phylogenetic trees of *Orthosiphon* spp. (Lamiaceae) from Indonesia. *IOP Conference Series: Earth and Environmental Science*, *457*(1), 1-9.

Tan, T. Y. C., Lee, J. C., Mohd Yusof, N. A., Teh, B. P., & Syed Mohamed, A. F. (2020). Malaysian herbal monograph development and challenges. *Journal of Herbal Medicine*, *23*, 1–6.

Tarmizi, A. A. A., Wagiran, A., Salleh, F., M., Chua, L. S., Abdullah, F. I., Hasham, R. & Mostafiz, S. (2021). Integrated approach for species identification and quality analysis for *Labisia pumila* using DNA barcoding and HPLC. *Plants*, 10, 717, 1-17.

Techen, N., Crockett, S., Khan, I., & Scheffler, B. (2012). Authentication of Medicinal Plants Using Molecular Biology Techniques to Compliment Conventional Methods. *Current Medicinal Chemistry*, *11*(11), 1391–1401.

Techen, N., Parveen, I., Pan, Z., Khan, I.A. (2014). DNA barcoding of medicinal plant material for identification. *Curr. Opin. Biotechnol.*, 25, 103-110.

Tnah, L. H., Lee, S. L., Tan, A. L., Lee, C. T., Ng, K. K. S., Ng, C. H., & Nurul Farhanah, Z. (2019). DNA barcode database of common herbal plants in the tropics: a resource for herbal product authentication. *Food Control*, *95*, 318–326.

Urumarudappa, S. K. J., Tungphatthong, C., Prombutara, P., & Sukrong, S. (2020). DNA metabarcoding to unravel plant species composition in selected herbal medicines on the National List of Essential Medicines (NLEM) of Thailand. *Scientific Reports*, *10*(1), 1–11.

Valentine, A., Pompanon, F., Taberlet, P. (2009). DNA barcoding for ecologists. *Trends Ecol. Evol.,* 24, 110-117.

Wallace, L. J., Boilard, S. M. A. L., Eagle, S. H. C., Spall, J. L., Shokralla, S., & Hajibabaei, M. (2012). DNA barcodes for everyday life : Routine authentication of Natural Health Products. *Food Research International*, *49*(1), 446–452.

Wang, J., Sasse, A., & Sheridan, H. (2019). Tradtional Chinese Medicine: From Aqueous Extracts to Therapeutic Formulae. *IntechOpen*, (Plant Extracts), 1–15. Retrieved from https://www.intechopen.com/books/advanced-biometric-technologies/liveness-detection-in-biometrics

Wegener, T. (2017). Patterns and Trends in the Use of Herbal Products, Herbal Medicine and Herbal Medicinal Products. *International Journal of Complementary & Alternative Medicine*, *9*(6), 1-4.

WHO. (2013). *WHO traditional medicine strategy 2014-2023.* Geneva, Switzerland: World Health Organization

Wong, E.H.K., Hanner, R.H. (2008). DNA barcoding detects market substitution in North American seafood. *Food Res. Int.*, 41, 828-8237.

Wu, Y., Li, M., yang, Y., Jiang, L., Liu, M., Wang, B., *et al.* (2018). Authentication of small berry fruit in fruit products by DNA barcoding method. *J. Food Sci.*, 83, 1494-1504.

Xia, Y., Chen, F., Du, Y., Liu, C., Bu, G., Xin, Y & Liu, B. (2019). A modified SDS-based DNA extraction method from raw soybean. *Bioscience Reports*, 1-10.

Yancy, H.F., Zemlak, T.S., Mason, J.A., Washington, J.D., Tenge, B.J., Nguyen, N.L.T.,

Barnett, J. D., Savary, W.E., Hill, W.E., Moore, M.M., Fry, F.S., Randolph, S.C., Rogers, P.L., Gebert, P.D. (2008). Potential use of DNA barcodes in regulatory science: Applications of the regulatory fish encyclopedia. *J. Food Prot.*, 71, 210-217.

Yu, D., Zhang, J., Tan, G., Yu, N., Wang, Q., Duan, Q., Qi, X., Cheng, M., Yan, C., Wei, Z., Yu, Z., Huang, W. & Li, C. (2019). An easily-performed high-throughput method for plant genomic DNA extraction. *Analytical Biochemistry*, *569*, 28–30.

Yu, J., Wu, X., Liu, C., Newmaster, S., Ragupathy, S., & Kress, W. J. (2021). Progress in the use of DNA barcodes in the identification and classification of medicinal plants. *Ecotoxicology and Environmental Safety*, *208*.

Yuliantini, A., Rizkiya, A., & Muttaqin, F. Z. (2020). Fingerprint Analysis of Adulterant in Traditional Medicine of *Orthosiphon aristatus* Leaves Using FTIR Method, *26*, 36–38.

Zakaria, M. H. (2015). A Review of policies and issues in the Malaysian herbal industry. *FFTC Agricultural Policy Platform*, 1-6.