UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE DETECTION OF MITRAGYNINE AND APPLICATION IN ELECTROCHEMICAL IMMUNOSENSOR

RADHIAHTUL RAEHAN BINTI MUSTAFA

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By

RADHIAHTUL RAEHAN BINTI MUSTAFA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Chairman : Rashidah binti Sukor, PhD
Faculty : Food Science and Technology

Mitragyna speciosa Korth. (kratom) is a tropical plant which has been used since many centuries in traditional human remedies. It contains an alkaloid, i.e., mitragynine, that could render psychotropic effects and is often being misused in substitution for commercial drug. Nowadays, the growing popularity of kratom has led to development of a rapid and effective detection method. Chromatographic methods have been used for the mitragynine detection. The techniques are highly sensitive detection, but they are restricted due to the sophisticated instrument and long-time analysis which is not suitable for routine analysis. Immunoassay has become the standard method for rapid detection of target analyte. They are remarkable for their sensitivity and convenience in sample preparation. Therefore, the main goal of this study was to develop an immunoassay for the detection of mitragynine. To support the main objective, the specific objectives were carried out (1) to extract and purify mitragynine from *M. speciosa* Korth leaves using solvents with different polarities, (2) to determine reproducibility of mitragynine conjugates using different approach, (3) to develop and optimise enzyme-linked immunosorbent assay (ELISA) with high sensitivity dan specificity detection of mitragynine and (4) to develop and optimise the electrochemical immunosensor for mitragynine based on competitive indirect ELISA. For the first objective, mitragynine extract was obtained using sequential extraction process, whereby solvents with increasing polarities, i.e., hexane, chloroform and methanol were used. Retention factor (R_f) value of mitragynine was identified using thin layer chromatography (TLC) at 0.80 of chloroform and methanol extracts as compared to 0.82 of mitragynine standard. Gas chromatography-mass spectrometry (GC-MS) analysis confirmed the presence of mitragynine in chloroform and methanol extracts. The purity of mitragynine determined based on average intensity ratio of its carbon signals (^{13}C-NMR) to trace impurities which produced 0.075 (g/g) of pure mitragynine. For the second objective, mitragynine molecule was modified at the 16-$COOCH_3$ (methyl ester) and 9-OCH_3 (aromatic ether) positions and conjugated to cBSA and OVA for

immunogen and coating antigen, respectively. Successful of mitragynine-protein conjugates had shown by 2,4,6-Trinitrobenzenesulfonic acid (TNBS) which number of bound amino groups for C22-MG-cBSA and C9-MG-cBSA were 45 and 46, respectively. Fourier transform infrared spectroscopy (FTIR) showed the changes of the spectra at C22-hydroxymitragynine and C9-hydroxymitragynine as compared to the mitragynine, indicates a successful reduction and demethylation process, respectively. UV-Vis spectra showed successful conjugates with quantitative changes in the spectral region of 240–300 nm for conjugated mitragynine to cBSA and OVA. For the third objective, the immunogens were immunised into rabbits (n=2 for each immunogen) for polyclonal antibody (pAb) production. Binding affinity of anti-sera and purified IgG were examined using indirect ELISA. The affinity of purified IgGs from rabbits immunised with C22-MG-cBSA showed mean K_d of 7.965×10^{-6} µM, which was significantly higher affinity ($p < 0.05$) than those immunised with C9-MG-cBSA at K_d of 1.390×10^{-4} µM. The mitragynine immunoassay showed a limit of detection (LOD) and limit of quantification (LOQ) of 0.412 µg/mL and 1.25 µg/mL, respectively. The measurement range was between 0.01 to 100.0 µg/mL and minimal inhibition (IC_{50}) value of 0.152 µg/mL. For the final objective, optimum ELISA system was applied in electrochemical immunosensor to enhance sensitivity detection of mitragynine. Differential pulse voltammetry (DPV) analysis showed that, the detection potential immunosensor of mitragynine was confirmed at +0.25 ± 0.1 V. Non-linear calibration curve was in the range of 0-50 µg/mL. A 10-fold higher sensitivity was obtained using electrochemical immunosensor system with LOD and LOQ at 0.018 and 0.06 µg/mL, respectively and IC_{50} of 0.097 µg/mL. Electrochemical immunosensor also showed a good precision with reproducibility of 6.2%, repeatability of 9.5% and acceptable recovery range of 93 to 113%. In conclusion, an immunoassay was successfully developed with high sensitivity and specificity detection of mitragynine. The finding of this study can potentially be improved with increase the hapten numbers (i.e., 10-20 molar ratio) for optimal coupling rate, highly immunogenic carrier protein such as keyhole limpet hemocyanin (KLH) and suitable spacer arm with appropriate length (i.e, 3-6 carbon) which is not too short or long.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN ASAI IMUNOSERAP TERANGKAI ENZIM BAGI PENGESANAN MITRAGININ DAN APLIKASI DALAM SENSOR IMMUNO ELEKTROKIMIA

Oleh

RADHIAHTUL RAEHAN BINTI MUSTAFA

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Mitragyna speciosa Korth (ketum) adalah tumbuhan tropika yang telah digunakan sejak berabad lalu dalam rawatan tradisional. Ia sering ditemui di rantau Asia Tenggara termasuk Malaysia, Thailand dan Myanmar. *M. speciosa* Korth mengandungi alkaloid (mitraginin) yang menyebabkan kesan psikotropik yang sering disalah guna sebagai penggantian dadah komersial. Pada masa kini, kratom yang semakin popular telah menyebabkan pembangunan satu kaedah yang pantas dan efektif. Kaedah kromatografi telah digunakan bagi pengesanan mitraginin. Tekniknya merupakan pengesanan kepekaan yang tinggi, tetapi terhad disebabkan oleh alat yang canggih dan masa analisis yang lama yang mana ianya tidak sesuai bagi analisis rutin. Immunoasai telah menjadi kaedah standard bagi mengesan analit sasaran dengan pantas. Mereka luar biasa dengan kepekaan dan penyediaan sampel yang mudah. Oleh tu, matlamat utama bagi kajian ini adalah membangunkan imunoasai bagi pengesanan mitraginin. Bagi menyokong matlamat utama, objektif-objektif khusus telah dijalankan, (1) untuk mengekstrak dan menulen mitraginin daripada daun *Mitragyna speciosa* Korth menggunakan pelarut dengan polariti berbeza, (2) untuk menentukan tahap keboleh hasilan-semula konjugat mitraginin menggunakan pendekatan berbeza, (3) untuk membangun dan mengoptima asai imunoserap terangkai enzim (ELISA) bagi mengesan mitraginin dengan kekhususan dan kepekaan yang tinggi dan (4) untuk membangun dan mengoptima sensor imuno elektrokimia bagi mitraginin berdasarkan ELISA kompetitif tidak langsung. Bagi objektif pertama, ekstrak mitraginin diperolehi menggunakan proses pengekstrakan berurutan, di mana pelarut dengan peningkatan polariti, iaitu heksana, kloroform dan metanol telah digunakan. Nilai faktor pengekalan (R_f) mitraginin ditentukan dengan menggunakan kromatografi lapis tipis (TLC) pada 0.08 ekstrak kloroform dan metanol sebagai perbandingan kepada 0.82, piawai mitraginin. Analisis kromatografi gas-spektrometri jisim (GC-MS) mengesahkan kehadiran mitraginin dalam ekstrak kloroform dan metanol. Ketulenan mitraginin ditentukan berdasarkan nisbah purata intensiti

isyarat karbon (^{13}C-NMR) kepada bendasing, menghasilkan 0.075 (g/g) mitraginin tulen. Bagi objektif kedua, molekul mitraginin diubahsuai pada kedudukan 16-$COOCH_3$ (ester metil) dan 9-OCH_3 (eter aromatik) dan dikonjugat kepada cBSA dan OVA masing-masing untuk imunogen dan lekatan antigen. Kejayaan konjugat mitraginin protein ditunjukkan oleh *2,4,6-Trinitrobenzenesulfonic acid (TNBS*) di mana, bilangan kumpulan amino terikat bagi C22-MG-cBSA and C9-MG-cBSA masing-masing adalah 45 dan 46. Spektroskopi inframerah transformasi fourier (FTIR) menunjukkan perubahan spektra pada C22-hydroxymitragynine dan C9-hydroxymitragynine berbanding mitraginin, masing-masing menunjukkan kejayaan proses penurunan dan pendemetilan. Ultraungu-nampak (UV-Vis) menujukkan kejayaan konjugat dengan perubahan kuantitatif dalam kawasan spektrum pada 240-300 bagi konjugat mitraginin kepada cBSA dan OVA. Bagi objektif ketiga, imunogen disuntik ke dalam arnab (n=2; bagi setiap imunogen) bagi penghasilan antibodi poliklonal (pAb). Ikatan afiniti IgG yang ditulenkan dari arnab yang disuntik C22-MG-cBSA, menunjukkan nilai purata K_d 7.965×10^{-6} µM adalah bererti (P<0.05) lebih rendah (ikatan tinggi) berbanding C9-MG-cBSA pada K_d, 1.390×10^{-4} µM. Imunoasai mitraginin menunjukkan had pengesanan (LOD) dan had kuantifikasi (LOQ) masing-masing pada 0.412 µg/mL and 1.25 µg/mL. Julat pengukuran adalah di antara 0.01 hingga 100.0 µg/mL dan nilai perencatan minima (IC_{50}) pada 0.152 µg/mL. Bagi objektif terakhir, sistem ELISA yang optima diaplikasikan ke dalam sensor imuno elektrokimia bagi meningkatkan tahap kepekaan pengesanan mitraginin. *Differential pulse voltammetry (DPV)* menunjukkan potensi pengesanan sensor immuno bagi mitraginin dipastikan pada +0.25 V ± 0.1. Lengkung tidak linear keluk tentukuran dihasilkan dengan julat 0-50 µg/mL. Kepekaan yang tinggi diperoleh dengan LOD dan LOQ masing-masing pada 0.018 dan 0.06 µg/mL dan IC_{50} pada 0.097 µg/mL. Sensor imuno elektrokimia menunjukkan ketepatan dengan kebolehasilan semula sebanyak 6.2%, kebolehulangan semula 9.5% dan perolehan baik dengan julat 93 hingga 113%. Kesimpulannya, satu imunoasai telah berjaya dibangunkan dengan kepekaan dan kekhususan yang tinggi bagi pengesanan mitraginin. Dapatan kajian ini berpotensi ditambah baik dengan meningkatkan bilangan hapten (iaitu, 10-20 nisbah molar) untuk kadar gandingan yang optima, protin pembawa imunogenik tinggi seperti keyhole limpet hemocyanin (KLH) dan lengan penghubung yang sesuai (iaitu, 3-6 karbon) yang mana tidak terlalu pendek atau panjang.

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"In the name of Allah, the Most Gracious, the Most Merciful"

'Whoever treads a path, seeking in that path knowledge, Allah will make easy for him the path to paradise' (the Prophet said inciting the seeking of knowledge for the sake of Allah); collected by Muslim 3699.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

Ab	Antibody
ACN	Acetonitrile
Ag	Antigen
AgCl	Silver/silver chloride
Anti-IgG-HRP	Anti-antibody IgG labeled with hydrogen peroxidase
ANOVA	Analysis of variance
BBD	Box Behnken Design
BSA	Bovine Serum Albumin
cBSA	Cationic Bovine Serum Albumin
CCD	Central composite design
C.E	Counter electrode
CI-ELISA	Competitive indirect Competitive Indirect Enzyme-Linked Immunosorbent Assay
COOH	Carboxyl group
CS	Chitosan
CV	Cyclic voltammetry
CV (%)	Coefficient of variation
DPV	Differential pulse voltammetry
EDC	N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide
ELISA	Enzyme-Linked Immunosorbent Assay
EtBr	Ethidium bromide
FTIR	Fourier Transform Infrared Spectroscopy
GC-MS	Gas chromatography–mass spectrometry
GPES	General Purpose Electrochemical Software
H_2O_2	Hydrogen peroxide

HRP	Hydrogen peroxidase
IC_{50}	Half maximal inhibitory concentration
$K^3[Fe(CN)]^6$	Potassium Ferricyanide
kg	Kilogram
KLH	Keyhole limpet hemocyanin
LC-MS	Liquid chromatography–mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MG	Mitragynine
mL	Millilitre
ng	Nanogram
MWCNTs	Multi-walled carbon nanotubes
NHS	N-hydroxysuccinimide
$O\text{-}CH_3$	Methoxy group
OVA	Ovalbumin
Ox	Oxidised
PBS	Phosphate-buffered saline
PBST	PBS with 0.05% Tween-20 (v/v)
PVDF	Polyvinylidene fluoride
R_{ct}	Charge transfer resistance
R. E	Reference electrode
Red	Reduced
RSD	Relative standard deviation
RSE	Residual standard errors
SA	Succinic anhydride

SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPCE	Screen-printed carbon electrode
TEMED	Tetramethylethylenediamine
TLC	Thin layer chromatography
TMB	3,3′,5,5′-Tetramethylbenzidine
Tris HCl	Tris(hydroxymethyl)aminomethane hydrochloride
W.E	Working electrode
µL	Microlitre

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Herbs are the source of essential bioactive compounds which provides nutrients and supplement to mankind. *Mitragyna speciosa* Korth. or known as kratom or ketum is an ethnomedicinal that is commonly found in the Southeast Asian region including Malaysia, Thailand, and Myanmar. Over the past decades, it has been used for as a traditional medicine for nausea, constipation, fever and chronic pain (Veltri & Grundmann 2019; Hassan et al., 2013). Nevertheless, *M. speciosa* leaves active alkaloids, i.e., mitragynine and 7-hydroxymitragynine that could render psychotropic and toxic effects that are often being misused as substitute for commercial drug.

Previous study has shown the juice of *M. speciosa* leaves was consumed with the addition of other ingredients such as cola beverages, codeine- or diphenhydramine-containing cough syrup, mosquito coils, herbicide, and other toxic substances to enhance the cocktail's effect (Hassan et al., 2013). The use of the leaves can cause strong addiction potential and adverse health effect such as insomnia, anorexia, constipation and darkening of skin (Ancuceanu et al., 2010; Bergen-Cico & MacClurg, 2016). The effect of the leaves varied according to the dose, whereby at lower dose it may cause stimulation or dopaminergic-dominant, while at higher dose, it may cause μ-opioid receptor-dominant effect (Fu et al., 2015). Besides, the higher dosage can cause addiction to the user which leads to prolonged sleep and other withdrawal symptoms, for instance tearfulness, muscle pain, aggression, tremor, convulsion and hostility (Ancuceanu et al., 2010; Chan et al., 2005; Veltri & Grundmann, 2019).

Today, *M. speciosa* leaves were banned by most countries including Malaysia, Thailand, Vietnam, Myanmmar, Australia, New Zealand and UK. The rampant abuse in Thailand and Malaysia as well as the hazardous health potential, had forced both governments to schedule the leaves under Schedule 5 Narcotic Act Level in Thailand and Poison Act 1952 in Malaysia. The use of the leaves is also considered illegal to European countries like Sweden, Denmark, Romania, Poland, Latvia and Lithuania (Bergen-Cico & MacClurg, 2016; Hassan et al., 2013). Hence, it is crucial to not only control misuse of the leaves, but also to detect and monitor the users. Therefore, there is a need for a rapid and sensitive method to be developed for the detection of the main alkaloid (mitragynine) in the biological sample.

Development of analytical methods such as gas chromatography-mass spectrometry (GC-MS) (Philipp et al., 2011a), high performance liquid chromatography-supercritical fluid extraction (HPLC-SF) (Wang et al., 2014), liquid chromatography-mass spectrometry (LC-MS) (Chan, 2005; Lu et al., 2009a; Philipp et al., 2010; Philipp et al., 2011b) and ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) (Vuppala et al., 2011) for the detection of mitragynine detection have been reported. These methods produced high specificity and sensitivity of the detection. However, they are restricted due to the sophisticated instrument, high technical skill and cost required as well as long-time analysis.

Over many decades, immunoassay has been widely used and plays an important role in clinical laboratory analyses for detection of small target analyte including hormones, proteins, nucleic acid and drugs (Ahn et al., 2016). This method offers highly advantageous such as inherent high sensitivity and specificity, high throughput, robust and high sensitivity for the analysis of wide range of analytes in biological samples. For drug detection or analysis, some common biological samples are normally being used like tears, sweat, saliva, blood and urine. Urine is the most preferable specimen due to the easy collection, reduce the risk of adulterant, higher concentrations of parent drug and high stability for up to few weeks (Hadland & Levy, 2016). Previous study reported that, mitragynine and other metabolites were found about 50% eliminate from the body after a day consumption. Its metabolites could still be found in urine after 10-14 days (Meireles et al., 2019). Thus, urine is the common biological specimen that use for detection of main active compound of *M. speciosa* leaves. Previous findings were reported on immunoassay development for detection of mitragynine and its metabolites in urine (Lee et al., 2020; Benchikh et al., 2018; Limsuwanchote et al., 2014). Therefore, immunoassay was developed in this study for the detection of mitragynine in human urine.

Antibodies are valuable tools in fundamental immunological research, therapeutics, diagnostic infectious diseases, and the application for targeted drug delivery systems (Leenaars & Hendriksen, 2005) and are important for the immunoassay development. In this study, the production of polyclonal antibodies was obtained by immunisation of rabbit with immunogenic compound of target analyte. An application of immunoassay requires antibodies with high specificity and sensitivity towards the target antigen, in this case, mitragynine.

Basically, small molecule or hapten including mitragynine with molecular weight of 10, 000 Da or less are unable to illicit an immune response in animals. It is due to the lack of epitopes, an important site for the binding of antigens to the antibodies. Therefore, conjugation of mitragynine with carrier protein such as bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH) is a crucial step for antibody production against mitragynine. Development of ELISA with optimal conditions (i.e., pH, temperature, solvent, incubation time, ionic strength and detergent) is important to increase ELISA performance for highly and sensitivity detection towards mitragynine. Thus, the developed immunoassay is

important for the polyclonal antibody to recognise mitragynine with high specificity and sensitivity detection.

In order to enhance specificity and sensitivity of detection, optimised ELISA condition was furthered applied in adaptation of the developed immunoassay into an electrochemical immunosensor. Previous finding reported the enhanced sensitivity detection of immunosensor over immunoassay (Azri et al., 2018; Sánchez-Tirado et al., 2016). Immunosensor is one type of biosensor which offers high advantageous over immunoassay (ELISA) such as high sensitivity, rapid detection of target analyte (in minutes), small amount of reagent and less time. These advantages have made an electrochemical immunosensor as an exciting approach for analyte detection over conventional ELISA. Other than that, performance of electrochemical immunosensor influence by the high specificity and sensitivity of the antibody produce which enhance electrochemical immunosensor performance.

1.2 Statement of problem

Globally, about 750, 000 deaths of illicit drug, 585, 000 premature deaths and over 166, 000 die from drug overdose each year until 2017 (Ritchie & Roser, 2018). In Malaysia, a statistic of drug addicts was reported by the National Anti-Drugs Agency (NADA) revealed that, a total of 130,478 cases and 116,204 drug users from 2014-2018. Mostly, young people aged between 18 to 39 listed in drug of abuse. Mitragynine (from *M. speciosa* leaves) is one of the drugs that has gained more popularity amongst young people over the last few years. The leaves are now widely use as drug of abuse and known as new psycoactive substance (NPS). The mitragynine is treated as altenative to opioids due to cheaper price and does not require for medical prescription (Meireles et al., 2019).

Previous studies have been reported on detecting mitragynine using chromatography methods (Wang et al., 2014; Philipp et al., 2010; Vuppala et al., 2011). Although chromatography methods are sensitive, they require extensive sample preparation, time-consuming, costly, and must be operated by trained personnel. Therefore, a simple and rapid method such as ELISA is crucial to detect mitragynine at sensitive detection to reduce the need for more costly chromatography methods.

Immunoassays for detection of mitragynine had been developed and previously reported in urine with LOD at 15 ng/mL (Lee et al., 2020), IC_{50} at 1.19 ng/mL (Benchikh et al., 2018) and plant samples with LOD at 32.47 mg/mL (Limsuwanchote et al., 2014). Limsuwanchote et al. (2014) reported that, the ELISA was developed using monoclonal antibodies (mAb) for the detection of mitragynine. The production of mAb also necessitates the use of tissue culture laboratory settings and competent technical staff on mammalian cell

environment. The study also reported an LOD of 32.47 µg/mL, whereby it is important for the detection of mitragynine at lowest level.

The use of monoclonal antibody is more sufficient and robust as compared to polyclonal antibody. However, the production of monoclonal antibody is limited due the high cost to operate, labour-intensive and long-time analysis required. Apart from that, the rise in kratom abuse in Malaysia is causing significant concern among the public and government. Therefore, this study was conducted using polyclonal antibody for mitragynine immunoassay, which can produce a kit to monitor the usage of ketum and help the government to control the misuse of ketum. Lee et al. (2020) reported the advantage of the use of polyclonal antibody which able to detect multiple epitopes and has greater sensitivity in the antibody detection. In addition, application of electrochemical immunosensor enhanced sensitivity mitragynine detection was explored at the end of the thesis. The immunosensor offers small amount of reagent used as well as rapid analysis time.

This research will contribute to rapid method for the detection of mitragynine in human urine. This will allow rapid toxicological screening for users of kratom abuse by the enforcement and agency task force. To the best of authors knowledge, there are several commercial rapid test kits for mitragynine available from Randox Toxicology (Ireland, UK), Premier Biotech (Minneapolis, USA), Safecare Biotech (Hangzhou, China) and CLIA Waived (San Diego, CA). However, the kits are costly and not commercially available in the local market.

1.3 Significance of the study

Mitragynine is an illegal psychcoactive substance that is banned in many countries including Malaysia, Vietnam, Myanmmar, Thailand, New Zealand Australia, and UK due to the addiction and health concern. However, it can be found easily in various forms (i.e., powder, cocktail, pill and tablet) via online purchase and low price. This is because the possession of kratom is legal in other countries including Austria, Belgium, Hungary, New York, Pennsylvania, and Nertherland (Bergen-Cico & MacClurg, 2016). Due to this easy availability, it has attracted many young people to seek the leaves as other alternatives to replace opioid which is pricier. The increasing of kratom abuse leads to urgency for a rapid and sensitive method for mitragynine detection. Therefore, a rapid analytical method for detection and quantification of mitragynine from user's specimen is needed. The finding of this study would help the agencies i.e., government agencies and agency task force to control the kratom abuse.

1.4 Objectives of the study

The main objective of this study is to develop an immunoassay for the detection of mitragynine. To achieve the main objective, this study is divided into sub-objectives as follows:

i. To extract and purify mitragynine from *M. speciosa* Korth. leaves using solvents with different solvent polarities

ii. To determine reproducibility of mitragynine conjugates using different approaches (i.e., methyl ester and aromatic ether modification)

iii. To develop and optimise competitive indirect ELISA with high sensitivity dan specificity detection of mitragynine

iv. To develop and optimise the electrochemical immunosensor for mitragynine based on competitive indirect ELISA.

1.5 Hypothesis of the study

i. Extraction of Mitragyna speciosa leaves using solvents with different polarities (i.e., hexane, chloroform and methanol) produce high yield and purity of mitragynine extract. Solvents with different polarities reduce the loss of targeted compound which lead to higher recovery of mitragynine.

ii. Different approaches of conjugation of mitragynine to carrier protein influence the reproducibility of the conjugates. Hapten designs, whereby methyl ester vs. aromatic ether position on mitragynine will compare to determine sensitivity of the antibody produce. Conjugation of mitragynine to protein at methyl ester position will produce high sensitivity of the antibody. The position maintains the immune characteristic of the antibody. Coupling method (carbodiimide method) and its condition (solubility of the reaction in organic solvent) and characteristic of the mitragynine conjugates (verify using analytical methods) will affect the reproducibility of the conjugates.

iii. The optimum conditions for mitragynine immunoassay affect the sensitivity and specificity detection of mitragynine. The factors including low acid (pH<4) and high alkaline (pH>9) will reduce mitragynine immunoassay performance. Salt concentration will affect the ionic strength, where at 0.17M (1x PBS) will produce high assay sensitivity. Inhibition step in immunoassay will increase assay sensitivity. The use of inhibition step and PBS as antibody diluent will enhance sensitivity of the assay.

iv. The optimum condition of ELISA in electrochemical immunosensor improves the sensitivity of mitragynine detection. For the electrochemical immunosensor, this method will give higher sensitivity as compared to ELISA which hypothesised to be at least 10-fold higher or more.

1.6 Research approach

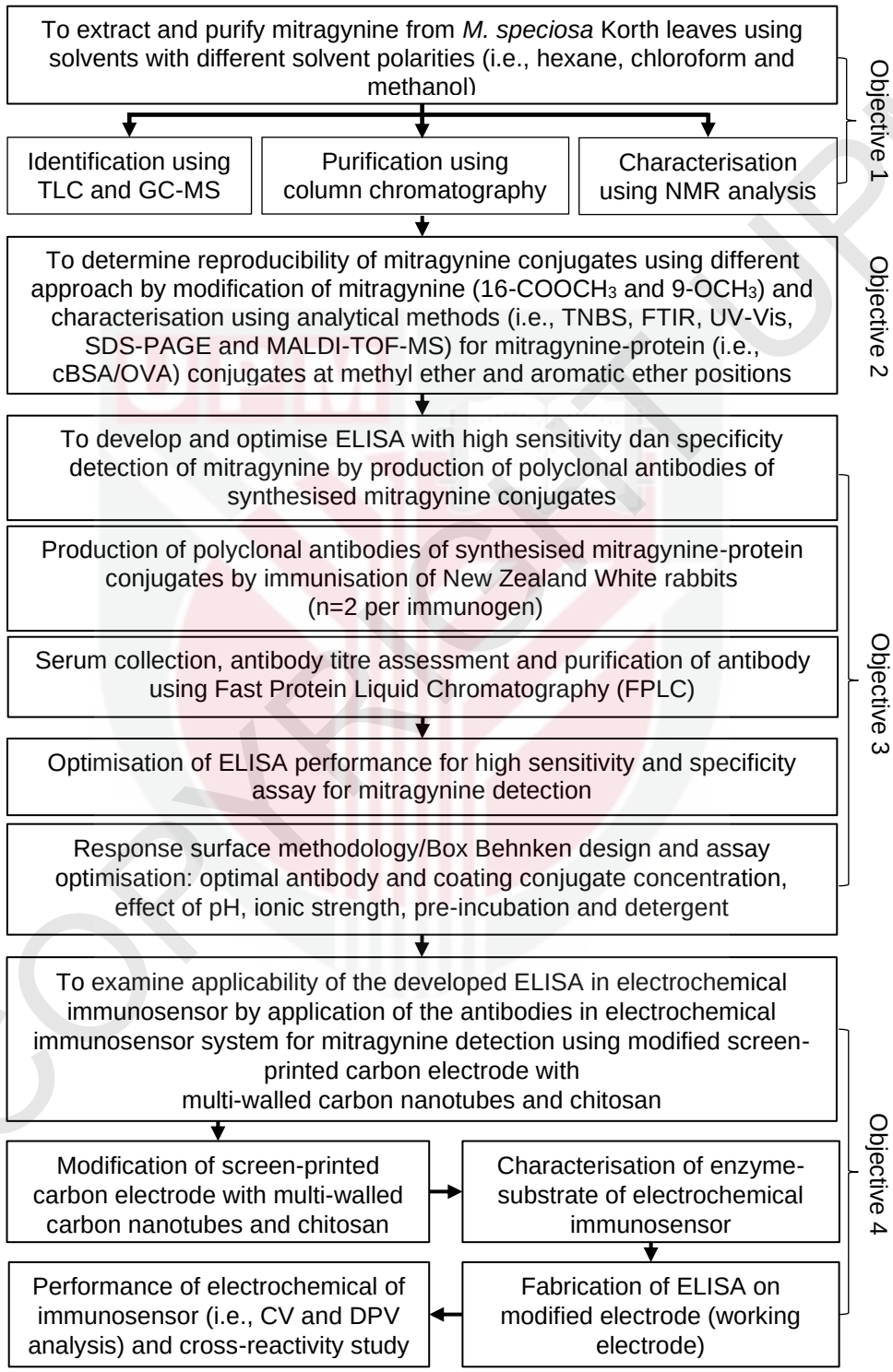

Figure 1.1 : Flow chart of the study

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LIST OF PUBLICATIONS

Mustafa, R.R., Sukor, R., Mohd Nor, S.M., Nazamid, S. & Azri, A.A. (2020). Enhancing extraction yield and purity of mitragynine from *Mitragyna speciosa* through sequential solvent extraction and characterisation using NMR technique. *International Journal of Scientific and Technology Research*, 9(3), 3846-3854.

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Mustafa, R.R., Sukor, R., Mohd Nor, S.M., Nazamid, S. & Mohsin, A.Z. Methyl Ester and Aromatic Ether Modification of Mitragynine for Generation of Mitragynine-Specific Polyclonal Antibodies. *Journal of Immunological Methods (Under review)*

Conferences

Mustafa, R.R., Sukor, R., Md.-Nor, S.M., Nazamid, S. (2019) Electrochemical determination of mitragynine using carbon electrode modified with multi-walled carbon nanotube and chitosan, Abstract/ oral presented in 4 th International Conference on Molecular Diagnostics and Biomarker, Discovery, 25-26 September, Penang, Malaysia.

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