UTS UNIVERSITY OF TECHNOLOGY SYDNEY

A FORENSIC INVESTIGATION INTO THE PRESENCE OF PHOSPHODIESTERASE 5 INHIBITORS AS ADULTERANTS IN HERBAL REMEDIES

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Thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy (Science)

under the supervision of

Professor Shanlin Fu and Doctor Linda Xiao

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September 2020

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Ahmad Yusri Mohd Yusop declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy (Science), in the Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by the Australian Government Research Training Program.

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ABSTRACT

The proliferation of herbal-based male sexual performance products, particularly those adulterated with phosphodiesterase 5 (PDE5) inhibitors, has sparked grave public health and food safety concerns. The advent of their unapproved analogues presents an additional challenge to forensic drug testing laboratories, as these adulterants may evade detection during routine screening. A comprehensive strategy is warranted to address these problems and protect consumers' health and well-being. This study investigated the presence of PDE5 inhibitors as adulterants in herbal remedies, using a two-tier screening strategy of rapid qualitative assay and confirmatory analytical analysis.

A bioactivity-based PDE5 inhibition assay was established using fluorescein-labelled cyclic-3',5'-guanosine monophosphate as substrates to PDE5 enzyme. The PDE5 inhibitions, measured using a fluorescence polarisation technique, was applied to 50 herbal-based food samples. The results were in agreement with the confirmatory analytical analysis for all food products, except for the instant coffee premix samples, postulated due to the presence of caffeine. The assay, nevertheless, exhibited a promising potential to rapidly screen PDE5 inhibitors in various types of food products, except those containing naturally-occurring phosphodiesterase inhibitors.

A confirmatory liquid chromatography-high-resolution mass spectrometry (LC-HRMS) analysis was developed using 23 target analytes; selected to represent different groups of PDE5 inhibitors, based on their structural similarities. The targeted analysis was primarily optimised to mitigate the matrix effect (ME), via chromatographic separation, sample extraction, and sample dilution. The insignificant ME percentages, within -9.2%–8.8% for all target analytes in food and pharmaceutical matrices, were evidenced with satisfactory validation results; notably, the accuracy was within 77.4%–124.7%. The development, optimisation, and validation of the targeted analysis provided a solid foundation for suspected-target and non-targeted screenings. The suspected-target screening employed a library comprising 95 PDE5 inhibitors, providing extended coverage of known analytes. Contrarily, the non-targeted screening adopted top-down and bottom-up approaches to flag novel PDE5 inhibitors analogues based on common fragmentation patterns of target analytes.

The confirmatory LC-HRMS analysis was applied to 50 herbal-based food samples and 52 herbal-based pharmaceutical samples. The targeted analysis and the suspected-target screening identified 11 target analytes and detected five suspected analytes, respectively, from 74 adulterated samples. The non-targeted screening returned insignificant signals, indicating the absence of potentially novel analogues. Some of these samples contained up to five different PDE5 inhibitors and quantified at supratherapeutic level, making them unsafe for consumption. The comprehensive strategies provide a superior approach to curb the widespread adulteration of herbal remedies, thus, safeguarding the public's health.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my principal supervisor, Professor Shanlin Fu. He has patiently guided me throughout my PhD journey and shared his knowledge while entrusting me to take charge of my research. I attribute the completion of this research to his guidance, support, and encouragement. He is my mentor, and I am forever in debt to him.

I also wish to express my sincere gratitude to my co-supervisor, Dr Linda Xiao, for her consistent support and guidance during the running of this research. She continuously encouraged me and was always willing and keen to assist in any way she could throughout the research project.

My sincere appreciation goes to all the academic and professional staff who have supported me throughout my time at UTS. Thanks to Dr Ronald Shimmon for helping me in the laboratories as well as the training on NMR spectroscopy, and to Dr Verena Taudte and Dr Dayanne Bordin for the technical support with LC-MS/MS instruments. I also very much valued the feedback I have received from the forensic science and toxicology research group to improve my research. To Professor Claude Roux and the Centre for Forensic Science, thank you for providing financial support, particularly with the attendance of conferences.

I extend my warmest thanks to my family and friends for their moral support, which made my journey endurable. Finally, I am grateful to the Ministry of Health Malaysia for the Study Leave with Full Pay and Federal Training Reward that helped me to achieve my dream.

Table of contents

List of tables

List of figures

List of equations

Eq. 7.4.7B $$\%\ of\ PDE5\ activity = \frac{FP_{SPL} - FP_{SUB}}{FP_{POS} - FP_{SUB}} \times 100\%$$ 279

Eq. 7.4.7C $$\%\ of\ PDE5\ inhibition = 100 - \%\ of\ PDE5\ activity$$ 279

Eq. 7.4.7D $$T_{inhibition} = \mu + 3\sigma$$ 279

Eq. 7.4.7E $$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(Log\ IC_{50} - X) \times Hill\ Slope}}$$ 279

List of abbreviations and symbols

%	percentage
%RSD	percentage of relative standard deviation
-	minus
+	plus
$[M+H]^+$	protonated molecule
<	less than
>	more than
±	plus, or minus
×	times
°C	degree Celsius
μ	average
μg	microgram
μL	microlitre
μm	micrometre
μM	micromolar
^{13}C	carbon-13
^{1}H	hydrogen-1 or proton
AMP	5’-adenosine monophosphate
AOAC	The Association of Official Agricultural Chemists
BPC	base peak chromatogram
brs	broad singlet
Ca^{2+}	calcium
cAMP	3',5'-cyclic adenosine monophosphate

Cap	capsule
$CDCl_3$	deuterated chloroform
CE	collision energy
cGMP	3’,5’-cyclic guanosine monophosphate
CI	chemical ionisation
CID	collision-induced dissociation
COOH	carboxyl
CRM	certified reference material
CWG	chewing gum
CYP3A4	cytochrome P3A4
d	doublet
D&S	dilute and shoot
Da	Dalton
DAD	diode array detector
dd	doublet of doublet
DDA	data-dependent acquisition
DMSO	dimethyl sulfoxide
ED	erectile dysfunction
EFS	electrical field stimulation
EIC	extracted ion chromatogram
Eq.	Equation
ESI	electrospray ionisation
eV	electronvolt
FAM-cGMP	fluorescein-labelled cyclic-3’,5’-guanosine monophosphate
FAM-GMP	fluorescein-labelled 5’-guanosine monophosphate

Fig.	Figure
FP	fluorescence polarisation
FP_{POS}	fluorescence polarisation (positive control)
FP_{SPL}	fluorescence polarisation (sample)
FP_{SUB}	fluorescence polarisation (substrate control)
FT-ICR	Fourier transform-ion cyclotron resonance
FTIR	Fourier transform infrared
g	gravity
g	gram
GC	guanylyl cyclase
GC-MS	gas chromatography-mass spectrometry
GC-QQQ-MS	gas chromatography-triple quadrupole mass spectrometry
GMP	5’-guanosine monophosphate
GTP	guanosine triphosphate
HCD	hard candy
HNY	honey
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin-layer chromatography
HRMS	high-resolution mass spectrometry
Hz	hertz
$I_{\parallel}$	parallel emission light intensities
$I_{\perp}$	perpendicular emission light intensities
IC_{50}	half-maximal inhibitory concentration
ICH	The International Conference on Harmonisation
ICP	instant coffee premix

IR	infrared
IS	internal standard
IT	ion trap
IUPAC	The International Union of Pure and Applied Chemistry
J	coupling constants
JLY	jelly
L	litre
LC	liquid chromatography
LC-DAD	liquid chromatography-diode array detection
LC-HRMS	liquid chromatography-high-resolution mass spectrometry
LC-MS	liquid chromatography-mass spectrometry
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LC-QTOF-MS	liquid chromatography-quadrupole time-of-flight mass spectrometry
LC-UV	liquid chromatography-ultraviolet
LLE	liquid-liquid extraction
LOD	limit of detection
Log P	logarithm of the partition coefficient
Log_{10}	logarithm with base 10
LOQ	limit of quantification
m	multiplet
M	molar mass
m/z	mass-to-charge ratio
MDMA	3,4-methylenedioxymethamphetamine
ME	matrix effect

mg	milligram
MHz	megahertz
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
mP	millipolarisation
MRM	multiple reaction monitoring
MS	mass spectrometry
ms	millisecond
MS/MS	tandem mass spectrometry
MΩ-cm	megaohm-centimetre
NA	not applicable
ND	not detected
ng	nanogram
NH_2	amino
NIR	near-infrared
nM	nanomolar
nm	nanometre
NMR	nuclear magnetic resonance
NO	nitric oxide
P	phosphorylation
PCDL	personal compound database and library
PDA	photodiode array
PDE	phosphodiesterase

PDE5	phosphodiesterase 5
PDM	powdered drink mix
pg	picogram
pH	power of hydrogen
pK_a	negative log of acid dissociation constant
PKG	3’,5’-cyclic guanosine monophosphate-dependent protein kinase
ppm	parts per million
psi	pounds per square inch
psig	pounds per square inch gauge
PTFE	polytetrafluoroethylene
Pty Ltd.	Proprietary Limited
Q	single quadrupole
q	quartet
QC	quality control
QQQ	triple quadrupole
QTOF	quadrupole time-of-flight
QTOF-MS	quadrupole time-of-flight mass spectrometry
QuEChERS	quick, easy, cheap, effective, rugged, and safe
R^2	coefficient of determination (curve)
r^2	coefficient of determination (linear)
RE	extraction recovery
RT	retention time
s	singlet
SD	standard deviation

sec	second
SERS	surface-enhanced Raman spectroscopy
SPE	solid-phase extraction
SPR	supratherapeutic
SUB	subtherapeutic
t	triplet
Tab	tablet
THE	therapeutic
$T_{inhibition}$	threshold value of phosphodiesterase 5 inhibition
TLC	thin-layer chromatography
T_{max}	the amount of time that a drug is present at the maximum concentration in serum
TOF	time-of-flight
TRC	trace
U	unit
USFDA	The United States Food and Drug Administration
UV	ultraviolet
UV-Vis	ultraviolet-visible
V	volt
v/v	volume/volume
WHO	The World Health Organization
Zn^{2+}	zinc
α	alpha
δ	chemical shifts
σ	standard deviation

List of peer-reviewed journal publications

1. Mohd Yusop AY, Xiao L, Fu S. Determination of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes using liquid chromatography-high-resolution mass spectrometry (LC-HRMS). *Talanta* 2019;204:36-43. doi:https://doi.org/10.1016/j.talanta.2019.05.078 — Chapter 4
2. Mohd Yusop AY, Xiao L, Fu S. Data on the optimisation and validation of a liquid chromatography-high-resolution mass spectrometry (LC-HRMS) to establish the presence of phosphodiesterase 5 (PDE5) inhibitors in instant coffee premixes. *Data Brief* 2019;25:104234. doi:https://doi.org/10.1016/j.dib.2019.104234 — Chapter 4
3. Mohd Yusop AY, Xiao L, Fu S. Suspected-target and non-targeted screenings of phosphodiesterase 5 inhibitors in herbal remedies by liquid chromatography-quadrupole time-of-flight mass spectrometry. *Drug Test Anal* 2020. doi:https://doi.org/10.1002/dta.2861 — Chapter 6
4. Mohd Yusop AY, Xiao L, Fu S. Fluorescence polarisation for high-throughput screening of adulterated food products via phosphodiesterase 5 (PDE5) inhibition assay. *Drug Test Anal* 2020. doi: https://doi.org/10.1002/dta.2926 — Chapter 7

List of manuscripts submitted to peer-reviewed journal publications

1. Mohd Yusop AY, Xiao L, Fu S. Comparison of sample extraction techniques for the determination of erectile dysfunction drugs as adulterants in selected food products. *Manuscript submitted for publication* 2020.	Chapter 3
2. Mohd Yusop AY, Xiao L, Fu S. Isolation and identification of an isomeric sildenafil analogue as an adulterant in an instant coffee premix. *Manuscript submitted for publication* 2020.	Chapter 5

List of peer-reviewed conference proceedings

(presenting author underlined)

1. Mohd Yusop AY, Xiao L, Fu S. Mitigating matrix effects: an evaluation of sample extraction techniques for forensic investigations of synthetic aphrodisiacs illegally added into consumable products. *The 56th Annual Meeting of The International Association of Forensic Toxicologists.* Ghent, Belgium. 26–30 August 2018.
2. Mohd Yusop AY, Xiao L, Fu S. Strategies to overcome matrix effect for reliable determination of sexual enhancing drugs found as adulterants in premixed coffee. *The Australian and New Zealand Forensic Science Society 24th International Symposium.* Perth, Australia. 9–13 September 2018.
3. Mohd Yusop AY, Xiao L, Fu S. Adulterant detection in herbal dietary supplements marketed to enhance male sexual performance. *The Forensic and Clinical Toxicology Association Inc. 10th Conference.* Adelaide, Australia. 16–19 June 2019.
4. Mohd Yusop AY, Xiao L, Fu S. Application of liquid chromatography-high resolution mass spectrometry (LC-HRMS) to determine male sexual stimulant in selected food matrices. *The 57th Annual Meeting of The International Association of Forensic Toxicologists.* Birmingham, United Kingdom. 2–6 September 2019.

5. Mohd Yusop AY, Xiao L, Fu S. Safeguarding food safety: rapid screening of phosphodiesterase 5 (PDE5) inhibitors as adulterants in selected food matrices using enzyme assay. *The 55th Congress of the European Societies of Toxicology (EUROTOX 2019).* Helsinki, Finland. 8–11 September 2019.