UNIVERSITI PUTRA MALAYSIA

EFFICACY OF HERBAL EXTRACT FORMULATIONS IN INHIBITING α-GLUCOSIDASE AND NITRIC OXIDE ACTIVITY USING METABOLOMICS APPROACH

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By

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Chairman : **Professor Faridah Abas, PhD**
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Development of poly-herbal products is at the pinnacle in today"s herbal market. The idea of mixing multiple herbs as a single product has shown promising results in fighting non-communicable diseases. Two commonly grown herbs in Malaysia, *Gynura procumbens* (GP) and *Cleome gynandra* (CG) have been previously reported to possess health benefiting activities especially in treating diabetes and inflammation. However, the idea of mixing both herbs as a single product and testing the formulation for the aforementioned illness has never been reported before. Thus, this study was proposed to investigate the anti-diabetic and anti-inflammation properties of herbal extract formulations between GP and CG using metabolomics approach. Prior to the preparation of the extract formulations, freeze dried GP and CG leaves were extracted with different ethanol concentrations (0%, 20%, 50%, 70% and 100%). The individual extracts were tested for their total phenolic content (TPC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging, α-Glucosidase and nitric oxide (NO) inhibitory activities. The results indicated that 100% ethanolic extract of GP had the highest TPC (5.91 mg GAE/100mgde) and the lowest IC_{50} values for DPPH scavenging (63.73 µg/mL) and NO inhibition (25.20 µg/mL) activity. For CG, the 20% and 100% ethanolic extracts had insignificant high TPC at 3.46 and 3.71 mg GAE/100mgde respectively. However, 20% ethanolic extract of CG has the lowest IC_{50} value (40.36 µg/mL) in DPPH scavenging activity and in returns, 100% ethanolic extract of CG has the lowest IC_{50} value (60.75 µg/mL) in NO inhibition activity. However, none of the extracts have any inhibition

activity against α-Glucosidase enzyme; and subsequently not tested for the extract formulations. Extracts with best activities overall (100% GP, 100% and 20% CG) were then analysed via UHPLC-ESI-Orbitrap-MS/MS. A total of 58 metabolites from the groups of hydroxycinnamic acids, hydroxybenzoic acids, and flavonoid derivatives were tentatively identified from the extracts in which 24 of them were assigned for the first time from both herb species. In the second part of the study, ^{1}H NMR based metabolomics approach was applied to all extracts from both herbs. Principal component analysis (PCA) and hierarchical cluster analysis (HCA) revealed a good separation between the extracts and the corresponding metabolites identified via ^{1}H NMR spectroscopy. Phenolic compounds such as flavonoids (kaempferol, quercetin), hydroxycinnamic acids (caffeoylquinic, dicaffeoylquinic acids), hydroxybenzoic acid (gallic acid), organic acids (mallic acid, citric acid) and amino acids (phenylalanine, choline) are among the metabolites that contributed to the correlation with the extract bioactivities. The PLS model for both herbs has an overall acceptable goodness of fit and predictive power, which further strengthens the validity of this study. In the final part of the study, six formulations were designed from the best extracts of both herbs in certain ratios and their ability to scavenge DPPH, NO and inhibiting NO were re-tested. The results showed that formulation of 100% GP + 20% CG at 3:1 ratio has the lowest IC_{50} at 76.72 µg/mL but not significant compared to 20% CG extract alone (71.12 µg/mL) in DPPH scavenging assay. In returns, NO scavenging assay indicated a significant inhibition ($p<0.05$) in 100% GP + 100% CG at 1:3 ratio with IC_{50} registered at 85.01 µg/mL. On contrary, formulation of 100% GP + 100% CG at 3:1 ratio has the lowest IC_{50} (94.56 µg/mL) in NO inhibition activity compared to other formulation extracts. The interaction patterns between the extract formulations were studied using the combination index method. In general, the formulations showed a synergistic>additive>antagonistic interaction from low to high fraction affect. The present study offers the idea on how metabolomics approach can be utilized in developing poly-herbal based products. Integrating the data obtained from bioactivities studies, UHPLC-ESI-Orbitrap-MS/MS and ^{1}H NMR spectroscopy, a proper and standardised extract formulations can be developed. In conclusion, this study provides a strong justification of GP/CG herbal extract formulations as a potential anti-inflammatory agent in functional food prospect.

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KEBERKESANAN FORMULASI EKSTRAK HERBA DALAM MERENCAT AKTIVITI α-GLUCOSIDASE DAN NITRIK OKSIDA DENGAN MENGGUNAKAN PENDEKATAN METABOLOMIKS

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Pembangunan produk berasaskan poli-herba semakin mendapat perhatian di pasaran kini. Idea mencampurkan perlbagai herba sebagai produk tunggal telah menunjukkan hasil yang memberangsangkan dalam menangani penyakit tidak berjangkit. Dua herba yang biasa didapati di Malaysia, *Gynura procumbens* (GP) dan *Cleome gynandra* (CG) telah dilaporkan mempunyai aktiviti kesihatan terutamanya dalam merawat diabetik dan keradangan. Walaubagaimanapun, idea untuk mencampurkan kedua-dua herba tersebut sebagai produk tunggal dan kemudiannya diuji keberkesanan dalam merawat diabetik dan keradangan belum pernah dilaporkan lagi. Maka, satu kajian menggunakan formulasi ekstrak herba antara GP dan CG dalam menentukan aktiviti anti-diabetik dan anti-keradangan melalui pendekatan metabolomiks telah dicadangkan. Sebelum formulasi ekstrak dibuat, daun GP dan CG yang telah disejuk beku telah diekstrak menggunakan etanol pada beberapa kepekatan berbeza (0%, 20%, 50% dan 100%). Ekstrak individu disaring untuk mendapatkan jumlah kandungan asid fenolik (TPC), aktiviti pemerangkap radikal bebas 2,2-difenil-1-pikrilhidrazil (DPPH), perencatan enzim α-glucosidase dan nitrik oksida (NO). Analisis menunjukkan ekstrak 100% etanol GP mempunyai kandungan asid fenolik tertinggi (5.91 mg GAE/100mgde) dan nilai IC_{50} terendah untuk aktiviti pemerangkap radikal bebas DPPH (63.73 µg/mL) dan perencatan NO (25.20 µg/mL). Untuk CG, didapati ekstrak etanol pada 20% dan 100% mempunyai kandungan asid fenolik tertinggi tetapi tidak signifikan pada bacaan 3.46 and 3.71 mg GAE/100mgde masing-masing. Walaubagaimanapun, ekstrak 20% etanol CG mempunyai nilai

IC_{50} terendah untuk aktiviti pemerangkap radikal bebas DPPH (40.36 µg/mL) dan sebaliknya, ekstrak 100% etanol mencatatkan nilai IC_{50} terendah untuk perencatan NO (60.75 µg/mL). Keputusan juga menunjukkan tiada satu ekstrak daripada kedua-kedua herba dapat merencatkan tindakbals enzim α-glucosidase, maka asai ini tidak diuji kepada formulasi ekstrak. Seterusnya, ekstrak yang mempunyai aktiviti terbaik keseluruhannya (100% GP, 100% and 20% CG) dianalisa menggunakan UHPLC-ESI-Orbitrap-MS/MS. Sejumlah 58 metabolit daripada kumpulan asid hidroksisinamik, asid hidroksibenzoik dan flavanoid terbitan telah dikenalpasti secara tentatif dan 24 daripadanya kenalpasti buat pertama kali dalam ekstrak kedua-dua herba tersebut. Pada bahagian kedua kajian, pendekatan metabolomiks menggunakan ^{1}H NMR telah dilaksanakan pada ekstrak kedua-dua herba. Analisis komponen principal (PCA) dan analisis kluster hierarki (HCA) menunjukkan pemisahan yang bagus antara extrak dan metabolit sepadan yang dikenalpasti melalui ^{1}H NMR. Asid fenolik seperti flavonoid (kaempferol, kuersetin), asid hidroksisinamik (asid kafeolkuinik, asid dikafeolkuinik), asid hidroksibenzoik (asid galik), asid organik (asid malik, asid sitrik) dan asid amino (fenilalanin, kolin) adalah antara metabolit yang dikenalpasti menyumbang kepada bioaktiviti ekstrak. Modal PLS untuk kedua-dua herba juga mempunyai uji kesesuaian dan kuasa jangkaan yang sesuai, dimana ianya mengukuhkan lagi validiti kajian ini. Enam formulasi herba telah direka daripada ekstrak terbaik GP dan CG pada nisbah tertentu di bahagian terakhir kajian ini. Kebolehan formulasi ekstrak untuk memerangkap radikal bebas DPPH dan merencat NO telah diuji semula. Analisis menunjukkan formulasi 100% GP + 20% CG pada nisbah 3:1 mempunyai IC_{50} 76.72 µg/mL tetapi tidak signifikan berbanding ekstrak 20% CG sahaja (71.12 µg/mL) untuk asai pemerangkap radikal bebas DPPH. Manakala, dalam asai pemerangkap radikal bebas NO, formulasi 100% GP + 100% CG pada nisbah 1:3 mempunyai nilai IC_{50} terendah yakni 85.01 µg/mL ($p<0.05$). Sebaliknya, formulasi 100% GP + 100% CG pada nisbah 3:1 mempunyai nilai IC_{50} terendah iaitu 94.56 µg/mL untuk aktiviti perencatan NO berbanding formulasi lain. Corak interaksi antara ekstrak di dalam formulasi juga dikaji menggunakan kaedah indeks kombinasi. Secara am, formulasi-formulasi tersebut menunjukkan interaksi sinergi> penambahan> antagonistik dari kesan fraksi rendah ke tinggi. Kajian ini memberikan idea bagaimana pendekatan metabolomiks dapat digunakan dalam pembangunan produk poli-herba. Satu formulasi ekstrak yang piawai dapat dibangunkan apabila maklumat daripada kajian bioaktiviti, UHPLC-ESI-Orbitrap-MS/MS dan spektroskopi ^{1}H NMR diintegrasikan. Secara kesimpulannya, kajian ini menunjukkan potensi formulasi ekstrak GP/CG sebagai agen anti-keradangan dalam prospek makanan berfungsi.

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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 Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{1}H NMR	Protonated nuclear magnetic resonance
ACD	Advanced chemistry development
ANOVA	Analysis of variance
ATCC	American type culture collection
C18	Carbon 18
CD_3OD	Deuterated methanol
CG	*Cleome gynandra*
CI	Combination index
CMC	Carboxymethyl cellulose
D_2O	Deuterated oxide
de	Dried extract
DMEM	Dulbecco"s modified eagle"s medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
eNOS	Epithelial nitric oxide synthase
ETOH	Ethanol
Fa	Fraction affect
Fasn	Fatty acid synthase
FBS	Fetal bovine serum
GAE	Gallic acid equivalent
GCMS	Gas chromatography mass spectrometry
GLP-1	Glucagon-like peptide-1
GP	*Gynura procumbens*

HCA	Hierarchical clustering analysis
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
iNOS	Inducible nitric oxide synthase
IFN-γ	Interferon gamma
KH_2PO_4	Potassium dihydrogen phosphate
LCMS	Liquid chromatography mass spectrometry
LDL	Low density lipoprotein
LPO	Lipid peroxide
LPS	Lipopolysaccharide
MARDI	Malaysia Agricultural, Research and Development Institute
MOLGEN	Molecular structure generation
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MVDA	Multivariate data analysis
$NaNO_2$	Sodium nitrite
NHMS	National health and Morbidity Survey
NaOD	Sodium deuterium oxide
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NSAIDs	Non-steroidal anti-inflammatory drugs
PBS	Phosphate-buffered saline
PCA	Principal component analysis

PLS	Partial east Square
PNPG	4-nitrophenyl α-D-glucopyranoside
Rf	Retention factor
SGLT2	Sodium glucose cotransporter 2
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TLC	Thin layer chromatography
TLRs	Toll-like receptors
TPC	Total phenolic content
TSP	Trimethylsilylpropanoic acid
UHPLC-ESI-Orbitrap-MS/MS	Ultra high performance liquid chromatography-electron spray ionisation- orbitrap-mass spectrometer/mass spectrometer
UV	Ultraviolet
VIP	Variable importance in projections

CHAPTER 1

GENERAL INTRODUCTION

The rising incidence of non-communicable diseases is in the spotlight. The occurrence of diabetes, cardiovascular disease, cancer and some diseases has sparked the development of modern medicines. However, what is at stake is that these modern medicines exhibit some unwanted side effects on internal organs after a prolonged usage. As such, finding a cure for these diseases has always been a challenging task. Natural products, in turn, have been stealing the limelight as an alternative solution in combating against these diseases.

Inflammation in the cells has been associated with the development of diabetes in past studies (Wellen and Hotamisligil, 2005; Sjoholm and Nystrom, 2006). The accumulation of certain hormones and anti-inflammatory factors in damaged cells somehow inhibits the insulin signaling pathway, which in turn increases blood glucose levels. Previous studies using *Gynura procumbens* extract have shown that it has anti-diabetic and anti-inflammatory effects (Iskander et al., 2002; Siti Pauliena, 2006; Hassan et al., 2010, Kaewseejan et al., 2012, Wong et al., 2015). In addition, *Cleome gynandra* has been studied extensively for its anti-inflammation and various health properties (Sivanesan & Hazeena, 2007, Mishra et al., 2011, Ethadi et al., 2013, Msika et al., 2013). In-vivo studies also have indicated that none of the extracts possessed toxicity when they were tested at 5000 mg/kg and 3000 mg/kg for *G. procumbens* and *C. gynandra* respectively (Narendhirakannan et al., 2005, Zahra et al., 2011). However, none of the past studies have shown the efficacy of these herbs to act as anti-diabetic or anti-inflammatory agents when they are formulated as herbal mixtures.

The emergence of products based on herbal formulation has had a major impact on the development of the herbal industry in Malaysia. As the saying goes, „two is better than one", herbal practitioners and users tend to believe that mixed herbals have a more synergistic effect in fighting against non-communicable diseases rather than their individual herbs. Their dogma is in line with previously reported data. Kaur and Velecha (2014) in their review paper reported the efficacy of various herbal formulations products such as *Dihar*, *Diabet*, *Diasol*, and *Dianex* in treating diabetes. Herbal formulation between *Zingiber officinalis*, *Piper nigrum* and *Terminalia belerica* were found to reduce serum triglyceride, total cholesterol and systolic blood pressure in high fat-diet fed rats compred to the individual herbal extract (Aziz et al., 2013).

To date, studies on the efficacy of herbal formulations consisting of *G. procumbens* and *C. gynandra* in treating diabetes and inflammation have not been reported. It was hypothesized that the formulated herbal extract between

G. procumbens and *C. gynandra* will have better efficacy in treating diabetes and inflammation compared to their individual extracts. The hypothesis was postulated based on the fact that both herbs have been studied extensively for their anti-diabetic and anti-inflammatory activities. A significant activity from the herbal extract mixture will definitely boost the herbal industry in Malaysia. As such, the present study was proposed to investigate the efficacy of *G. procumbens* and *C. gynandra*, outlining a few objectives that reflect in-depth studies:

1. To determine the effect of solvent extraction on the biological activities of *G. procumbens* and *C. gynandra* extracts,

2. To identify the secondary metabolites in the active extracts by means of the UHPLC-Orbitrap-MS/MS approach,

3. To differentiate the *G. procumbens* and *C. gynandra* extracts and correlate them with their biological activities by using ^{1}H NMR-based metabolomics, and

4. To derive herbal extract formulations from active extracts of *G. procumbens* and *C. gynandra* and re-analyse their bioactivity in comparison to the individual extracts.

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