MECHANISM AND SPECIFICITY OF ANGIOTENSIN CONVERTING ENZYME INHIBITION ACTIVITY BY *SYZYGIUM POLYANTHUM* WIGHT (WALP.) LEAVES

BY

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ABSTRACT

Syzygium polyanthum is an ethnomedicinal plant used for the treatment of hypertension. This study investigates the anti-hypertensive property of this plant using angiotensin-converting enzyme (ACE) inhibition assay. The ability to inhibit this enzyme *in vitro* indicates the ability to decrease blood pressure. This study aims to determine the ACE inhibitory activity of *S. polyanthum* leaves, its inhibition specificity, mechanism and the possible bioactive compound. *S. polyanthum* leaves were dried, ground, and then macerated in a bath-sonicator with four different solvents; water, methanol, ethyl acetate, and hexane producing four types of extracts, the aqueous (ASP), methanolic (MSP), ethyl acetate (EASP) and hexane (HSP) extracts. Each extract (100 µg/ml) was initially screened using ACE inhibition assay according to Cushman and Cheung method and then compared with the standard drug, captopril (2.06 ng/ml). The most active extract was further tested at a range of concentrations (1 to 1000 µg/ml) to determine its potency and then tested for the mechanism and inhibition specificity using zinc chloride, bovine serum albumin (BSA), α-chymotrypsin, and trypsin. The phytochemical composition in the most active extract was then analysed using Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry (LC-QTOF/MS). *In silico* docking analysis was then performed between the major identified compound in the most active extract with the ACE. ASP at 100 µg/ml exhibited the highest inhibition activity (69.43 ± 0.60%) compared to MSP (41.63 ± 0.15%), EASP (9.62 ± 1.60%) and also HSP (45.40 ± 0.15%). From the dose-response curve for ACE inhibition activity of ASP, the inhibitory concentration of ASP that causes 50% of ACE inhibition activity (IC_{50}) was 41 µg/ml. ACE inhibition activity by ASP was significantly reduced by the presence of BSA, indicative of interaction of ASP with albumin. ACE inhibition activity by ASP was not significantly affected with the presence of zinc chloride, indicating that its inhibitory activity on ACE was non-dependent of zinc at the ACE active site. There was no significant inhibition by ASP on other enzymes such as α-chymotrypsin and trypsin. There were 26 compounds identified in ASP with the following major compounds; *1-galloyl-glucose* (21%), *feroxin A* (12%), *nilocitin* (10 %), *2,6-di-O-galloyl-β-D-glucose* (9%), *1-O-galloylpedun-culagin* (7%), *5-desgalloylstachyurin* (6%), and *yakuchinone A* (6%). Molecular docking analysis showed that 1-galloyl-glucose has lower binding energy (-7.7 kcal/mol) with the ACE, as compared to the standard drug, captopril (-5.6 kcal/mol); indicative of good interaction between 1-galloyl-glucose and the ACE. In conclusion, this study showed that the enzyme inhibition activity by *S. polyanthum* leaves extract was specific towards ACE. This inhibition possibly occurs *via* protein precipitation which was non-dependent to the chelation with zinc at the ACE active site with 1-galloyl-glucose, suggested as the potential bioactive compound.

خلاصة البحث

يستخدم نبات الطب العرقي *Syzygium polyanthum* للتخفيف من ارتفاع ضغط الدم. باستخدام مقايسة تثبيط الإنزيم المحول للأنجيوتنسين (ACE)، تبحث هذه الدراسة في خصائص النبات المضادة لارتفاع ضغط الدم. يشير تثبيط هذا الإنزيم في المختبر إلى القدرة على خفض ضغط الدم. الهدف من هذا البحث هو تحديد النشاط المثبط للإنزيم المحول للأنجيوتنسين لأوراق *S. polyanthum*، بالإضافة إلى خصوصية التثبيط، والآلية، والمكون النشط بيولوجيًا المحتمل. تم تجفيف أوراق *S. polyanthum* وسحقها ثم نقعها في حمام سونيك بأربعة مذيبات متميزة: الماء والميثانول وخلات الإيثيل والهكسان، مما ينتج عنه أربعة أنواع مختلفة من المستخلصات: المائية (ASP) والميثانولية (MSP)، وخلات الإيثيل (EASP)، والهكسان (HSP). تم تقييم كل مستخلص (100 جم / مل) باستخدام طريقة Cushman وCheung لفحص تثبيط الإنزيم المحول للأنجيوتنسين ثم مقارنته مع الدواء القياسي كابتوبريل (2.06 نانوغرام / مل). تم تحديد فاعلية المستخلص الأكثر نشاطًا باستخدام كلوريد الزنك وألبومين مصل الأبقار (BSA) و α-chymotrypsin و trypsin بمدى تركيزات (1 إلى 1000 جم / مل). تم تحديد التركيب الكيميائي النباتي للمستخلص الأكثر نشاطًا باستخدام الكروماتوغرافيا السائلة (LC-QTOF/MS). تم بعد ذلك إرساء المكون الرئيسي المكتشف في المستخلص الأكثر نشاطًا مع ACE في تحليل رسو السيليكو *silico*. كان نشاط التثبيط أعلى بالنسبة لـ ASP عند 100 جم / مل (69.43 ± 0.60٪) يليه MSP (41.63 ± 0.15%)،HSP (45.40 ± 0.15%) , EASP (9.62 ± 1.60%) كان التركيز المثبط لـ ASP الذي يسبب 50 ٪ من نشاط تثبيط الإنزيم المحول للأنجيوتنسين (ICE)41 ميكروغرام / مل ، وفقًا لمنحنى الاستجابة للجرعة لنشاط تثبيط ACE الخاص بـ ASP.أدى وجود BSA إلى انخفاض كبير في نشاط ASP في تثبيط ACE ، مما يشير إلى أن ASP تفاعل مع الألبومين. لم يكن لإضافة كلوريد الزنك أي تأثير على العمل المثبط لـ ASP على الإنزيم المحول للأنجيوتنسين، مما يدل على أن نشاطه المثبط على الإنزيم المحول للأنجيوتنسين لا يعتمد على الزنك في موقع ACE النشط. لم يتم تثبيط الإنزيمات الأخرى، مثل α-chymotrypsin وtrypsin، بشكل كبير بواسطة ASP. تم العثور على 26 مادة كيميائية في ASP، بما في ذلك المركبات الهامة التالية: *1-galloyl-glucose* (21%), *feroxin A* (12%), *nilocitin* (10 %), *2,6-di-O-galloyl-β-D-glucose* (9%), *1-O-galloylpedun-culagin* (7%), *5-. desgalloylstachyurin* (6%), and *yakuchinone A* (6%). كشفت دراسات الالتحام الجزيئي أن galloyl-glucose-1 يحتوي على طاقة ارتباط أقل (-7.7 kcal / mol) مع ACE مقارنة بالدواء النموذجي Captopril (-5.6 kcal / mol)، مما يشير إلى أن العقارين يتفاعلان جيدًا. في الختام ، وجد أن النشاط المثبط للإنزيم لمستخلص أوراق *S. polyanthum* خاص بـ ACE في هذه الدراسة. يمكن التوسط في هذا التثبيط عن طريق ترسيب البروتين ، والذي لا يعتمد على عملية إزالة معدن ثقيل الزنك في موقع ACE النشط مع galloyl-glucose-1 ، والذي تم اقتراحه كمركب نشط بيولوجيًا محتمل.

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APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion; it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Chemistry).

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DECLARATION

I hereby declare that this thesis is the result of my investigation, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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LIST OF SYMBOLS

±	Plus minus
%	Percentage
°C	Degree celcius
α	Alpha
β	Beta
δ	Delta
µg/ml	Microgram per mililitre
µm	Microgram per meter
µl	Microgram per litre
λ	Lambda
ACE	Angiotensin-converting enzyme
ACEi	Angiotensin-converting enzyme inhibitors
Ang I	Angiotensin I
Ang II	Angiotensin II
ANOVA	Analysis of Variance
ASP	Aqueous extract of S*yzygium polyanthum*
AT_1	Angiotensin II receptor
cm	Centimetre
DBP	Diastolic blood pressure
DCM	Dichloromethane
E.A	Ethyl acetate
EASP	Ethyl acetate extract of *Syzygium polyanthum*
et al.	And others
g	Gram
gACE	Germinal cell of ACE
HCl	Hydrochloric acid
HSP	Hexane extract of *Syzygium polyanthum*
IC_{50}	Concentration that causes 50% inhibition of maximal
IIUM	International Islamic University Malaysia
LC-MS	Liquid chromatography mass spectrometre
LC-QTOF/MS	Liquid chromatography quadrupole-time-of-flight mass spectrometre
MeOH	Methanol
mg	Milligram
mg/ml	Milligram per millilitre
min	Minute
ml	Millilitre
ml/min	Millilitre per minute

mM	Millimolar
mm	Millimetre
mm Hg	Millimetre mercury
MSP	Methanol extract of *Syzygium polyanthum*
m/z	Mass per charge ratio
NaOH	Sodium hydroxide
n	Number of sample size
ng/ml	Nanogram per millilitre
nm	Nanometre
n.s	Non-significant
no.	Number
pH	Logarithmic scale used to specify the acidity or basicity of an aqueous solution
ppm	Part per million
RAAS	Renin-angiotensin-aldosterone-system
HPLC	High performance liquid chromatography
RT	Retention time
SD	Standard deviation
sACE	Somatic cell of ACE
SBP	Systolic blood pressure
UAE	Ultrasound assisted extraction
UMP	Universiti Malaysia Pahang
UV	Ultraviolet
VIS	Visible
WHO	World Health Organization
w/v	Weight per volume
vs	*Versus*

CHAPTER ONE:

INTRODUCTION

1.1 BACKGROUND OF STUDY

Hypertension is an alarming disease condition involving chronic elevation of blood pressure especially in Malaysia. It was found out that nearly two out of three adults in Malaysia are suffering from hypertension with numbers up to 11 million of adults. There was an alarming increase in the overall prevalence of high blood pressure in Malaysia according to the latest National Health and Morbidity Survey 2015. This nationwide prevalence of hypertension among 15,738 adults in Malaysia was 66.8%; 45.8% had prehypertension, 15.1% were diagnosed with stage 1 hypertension and 5.9% were diagnosed with stage 2 hypertension, out of 15 million adults (Mahadir Naidu et al., 2019).

There are various classes of antihypertensive drugs that target different mechanisms or pathways available in the market. One of the mechanisms of blood pressure reduction is by inhibiting the renin-angiotensin-aldosterone system (RAAS). One of RAAS inhibitors is the angiotensin-converting enzyme inhibitor group, such as captopril and lisinopril. ACE inhibitors reduce peripheral vascular resistance by inhibiting the formation of angiotensin II, a potent vasoconstrictor. ACE inhibitors are widely utilised for the management of hypertension, but some has reported side-effects such as angioedema, fetal toxicity, and persistent dry cough with the use of these drugs (Hanafi et al., 2018). Therefore, research is still ongoing to search for drugs with better efficacy yet with fewer side effects.

Plant is one of the rich natural resources for drug discovery. *Syzygium polyanthum* or locally known as “serai kayu” or “salam” is one of the ethnomedicinal

plants which are unique to Malay and has been traditionally used for treating hypertension. *S. polyanthum* leaves are used in cooking to enhance flavor and also consumed fresh as "ulam". Apart from the routine usage of this plant, *S. polyanthum* also possess several bioactivities such as antioxidant, antidiabetic, antimicrobial, anti-cancer, anti-tumour, dental plaque inhibition properties, lipid lowering activity, acetylcholine-esterase inhibitory activity, antidiarrheal, and also anti-hypertensive (Ismail & Wan Ahmad, 2019).

Previous researches have reported the efficacy of *S. polyanthum* leaves as anti-hypertensive agent in normal and hypertensive rats model (Ismail, Mohamed, Sulaiman, & Wan Ahmad, 2013; Ismail & Wan Ahmad, 2017; Ramli, Muhammad, Safuan, & Noordin, 2017; Wan Ahmad et al., 2017). However, there are still scanty information on the mechanism of its antihypertensive action. In addition, Ismail et al. (2020) recently reported the presence of few potential compounds that can possibly be responsible for the antihypertensive effect by S. polyanthum (must be in italic) leaves extract such as 1-galloyl-glucose; however, these suggestions still requires further testing.

1.2 PROBLEM STATEMENT

Previous study has generally shown ACE inhibition activity by *S. polyanthum* leaves extract, however, the mechanism and specificity of ACE inhibition are not yet explored. Besides, there is no scientific report on the chemical constituents in *S. polyanthum* that exhibit the inhibition of ACE activity.

1.3 RESEARCH QUESTIONS

1. Which *S. polyanthum* leaves extracts have the most ACE inhibition activity?
2. Does *S. polyanthum* leaves extract inhibit ACE *via* protein precipitation?
3. Does ACE inhibition exhibited by *S. polyanthum* leaves extract depend on chelation with zinc at the ACE active site?
4. How specific is ACE inhibition exhibited by *S. polyanthum* leaves extract?
5. What are the chemical constituents in the most active *S. polyanthum* leaves extract?
6. Is there any interaction between ACE with the probable bioactive compound in the most active extract?

1.4 RESEARCH OBJECTIVES

1. To determine which *S. polyanthum* leaves extracts have the most ACE inhibition activity.
2. To determine whether the ACE inhibition by *S. polyanthum* leaves extract involves protein precipitation.
3. To determine whether the ACE inhibition by *S. polyanthum* leaves extract depends on chelation with zinc at the ACE active site.
4. To determine the specificity of ACE inhibition exhibited by *S. polyanthum* leaves extract.
5. To identify chemical constituents in the most active *S. polyanthum* extract through LC-MS analysis.
6. To determine the interaction between ACE and probable bioactive compound in the most active extract.

1.5 HYPOTHESIS

Angiotensin-converting enzyme (ACE) inhibition activity by *S. polyanthum* leaves extracts has its specific mode of inhibition through the presence of a particular chemical constituent interacting with the ACE.

1.6 SCOPE OF STUDY

This study explored the ACE inhibition properties of *S. polyanthum* leaves extracts, particularly its specificity, mode of ACE inhibition and the possible bioactive compound. The plant material was collected, authenticated, and extracted using the ultra-sound assisted extraction method producing four different types of extracts; aqueous extract of *S. polyanthum* (ASP), methanol extract of *S. polyanthum* (MSP), ethyl acetate extract of *S. polyanthum* (EASP) and n-hexane extract of *S. polyanthum* (HSP).

The extracts were then screened for ACE inhibition activity using Cushman and Cheung method (Cushman & Cheung, 1971) to identify the most active extract and further tested to find the effective concentration that causes 50% ACE inhibition (IC_{50}). To determine the specificity of enzyme inhibition by *S. polyanthum* leaves extract, the extract was tested against few more enzymes like trypsin and chymotrypsin. The ability of *S. polyanthum* leaves extract to exhibit protein precipitation property was also tested using bovine serum albumin. This test determines the plant extract's inhibition mechanism; either the inhibition occurs *via* precipitation towards the ACE enzyme or otherwise. The plant extract's mode of ACE inhibition was also tested using zinc chloride to identify the zinc metal ions' involvement in inhibiting the enzyme.

The most active crude extract was then proceeded with phytochemical identification using liquid chromatography-mass spectrometry (LC-MS) to determine

its phytochemical constituents. Finally, the major compound present in the extract was then proceeded with docking simulation study in order to study its interaction with ACE.

CHAPTER TWO:

LITERATURE REVIEW

2.1 HYPERTENSION, RAAS AND ACE

Hypertension is a condition of sustained high blood pressure which involves raised arteriolar resistance and decreased venous system capacitance (Whalen et al., 2015). Only 2 to 5% of patients suffer hypertension, secondary to other conditions like renal or adrenal diseases, pregnancy, thyroid dysfunction, and medications such as birth control pills, and sympathetic drugs. Meanwhile other 95% of hypertension cases are classified as essential hypertension when there is an unclear identifiable cause of hypertension. Various interrelated factors such as genetics, sympathetic nervous system overactivity, endothelial cell dysfunction, renin-angiotensin system, excessive salt intake, obesity, insulin resistance, and many other factors are involved in the pathophysiology of hypertension; however, these factors might have different influences depending on individuals (Saxena et al., 2018).

Hypertension can be classified into several stages; normal, elevated, stage 1 and stage 2 hypertension. Normal blood pressure refers to systolic blood pressure (SBP) of lower than 120 mmHg and diastolic blood pressure (DBP) of less than 80 mmHg. Elevated blood pressure, meanwhile refers to the condition of SBP range of 120 to 129 mm Hg and DBP lower than 80 mm Hg. As for stage 1 hypertension, SBP ranges between 130 to 139 mm Hg and DBP ranges from 80 to 89 mm Hg, while Stage 2 refers to SBP of equals or higher than 140 mm Hg or SBP equals to or higher than 90 mm Hg (Whelton et al., 2018). Systematic clinical review on hypertension cases already demonstrated the association between hypertension with increased cardiovascular

disease risk which could lead to death. This disease includes angina, myocardial infarction, heart failure, stroke, peripheral artery disease, and abdominal aortic aneurysm (Whelton et al., 2018).

The renin-angiotensin-aldosterone system (RAAS) is a hormonal cascade that plays an integral function in the homeostatic control of arterial pressure, tissue perfusion, and extracellular volume. Cardiovascular and renal disorders are due to dysregulation of the RAAS. RAAS becomes the endocrine axis in which the active hormone, angiotensin II (Ang II), is formed in the extracellular space by sequential proteolytic cleavage of its precursors. This pathway is initiated by the regulated secretion of renin, the rate-limiting enzyme. Although renin was discovered more than a century ago, the significance of this system in the pathogenesis of cardiovascular and renal disorders has gained wide acceptance only during the past three decades, in large part because of the availability of specific pharmacologic agents that can block the system (Martini et al., 2015).

Angiotensin Converting Enzyme (ACE) enzyme is a vital component of the RAAS that controls blood pressure by regulating the fluid volume and the sodium-potassium balance in the body. The ACE enzyme can be found in the lungs' capillaries and is also present in endothelial and kidney epithelial cells (Sentandreu & Toldrá, 2006). ACE converts the inactive decapeptide hormone angiotensin I (Ang I) into the potent vasoconstrictor octapeptide Ang II by removing the dipeptide His-Leu from the C-terminus. Ang I is formed by renin's proteolytic action on circulating angiotensinogen released by the liver. Ang I is then converted into Ang II by ACE, which increases blood pressure by narrowing arterioles following binding to type 1 Ang II receptor (AT_1) on the smooth muscle cells, and by stimulating the secretion of aldosterone from the adrenal cortex, thus increasing the salt and water retention. ACE

also converts the nine-amino acid vasodilator peptide, bradykinin into inactive compounds, which further contributes to the increase in the blood pressure (Figure 2.1)(Sentandreu & Toldrá, 2006).

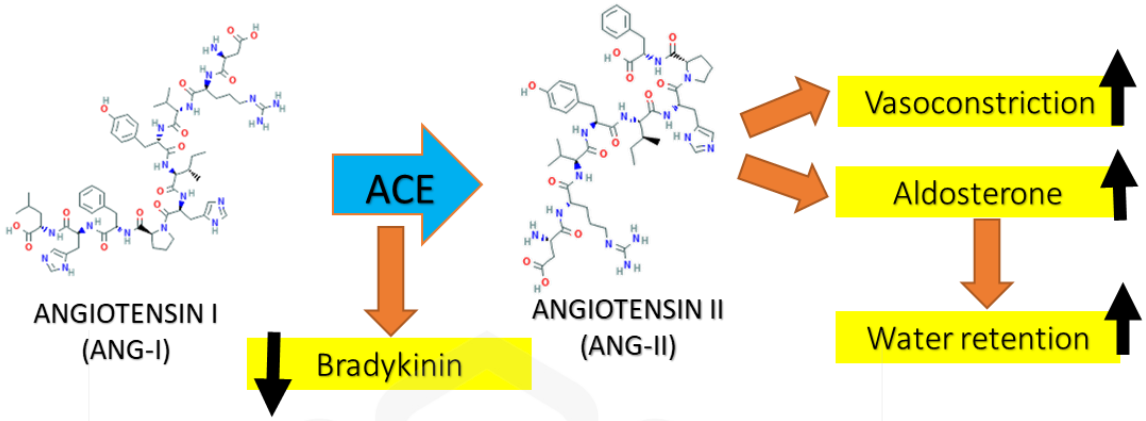

Figure 2.1 Angiotensin Converting Enzyme (ACE) mechanism of action

Human ACE is specifically produced from duplicated structure cDNA of germinal cell (gACE) and somatic cell (sACE), forming an enzyme with two domains, N-domain and C-domain. The M2-type zinc metallopeptidase motif is the primary functional unit in each domain. For peptidase catalytic activity to happen, it requires two histidines and glutamate to act as ligands for the zinc cofactor. These domains of ACE are functional with two different biochemical properties (Coates, 2003). C-domain exclusively hydrolyzes angiotensin-1, meanwhile N-domain involves anti-inflammatory peptide N-acetyl-SDKP, although both domains involve the hydrolyzation of bradykinins (Caballero, 2020).

2.2 ACE INHIBITOR DRUGS

One of the most common therapeutic agents used for hypertension and chronic heart failure is angiotensin–converting enzyme inhibitors (ACEIs). ACEIs are preferred to be prescribed when there are conditions, including contraindication or ineffectiveness of diuretics or β-blockers and if the patients are diagnosed with diabetes mellitus. ACEIs

produce vasodilation through inhibiting the ACE from converting the Ang I to Ang II, a potent vasoconstrictor (Whalen et al., 2015). Ang II constricts arteries and veins by binding to AT_1 receptors located on the smooth muscle, which are coupled to a Gq-protein and the IP_3 signal transduction pathway. Ang II also facilitates the release of norepinephrine from sympathetic adrenergic nerves and inhibits norepinephrine reuptake by these nerves. This effect of Ang II augments sympathetic activity on the heart and blood vessels (Sabroe & Black, 1997).

ACE inhibitor was first developed from the discovery of 'bradykinin potentiating factor' from viper's venom. This factor leads to inhibition of kininase II, enzyme that hydrolyze bradykinin, which was also known as ACE, the enzyme responsible for converting Ang I to Ang II. From this discovery, chemists successfully derived the first orally active ACE inhibitor, captopril. Captopril then was further reformulated to overcome its adverse side effect including loss of taste and skin rash, which lead to the development of new drugs such as enalapril and lisinopril (Raia Jr et al., 1990). Several ACE inhibitors are available to be used as anti-hypertensive medications such as enalapril, trandolapril, ramipril and also lisonopril, Each drug has specific implication and usage towards different groups of patients (Sun et al., 2016).

The therapeutic benefit of ACE inhibitors relies on their ability to inhibit the RAAS, which leads to lowering of the blood pressure. By inhibiting the ACE, this will not only blocks the conversion of Ang I and affects the bradykinin breakdown, leading to the promotion of vasodilation *via* the kinin-kallikrein-bradykinin system. However, elevating bradykinin would also lead to its adverse side effect of coughing and angioedema. There were reported incidences among patients receiving ACE inhibitors to develop dry cough at a generally-accepted range of 5 to 20%, while for angioedema, the incidence was only 0.3% (Turner & Kodali, 2020). Although there are several