XANTHINE OXIDASE INHIBITORS FROM SELECTED MALAYSIAN MEDICINAL PLANTS AS POTENTIAL REMEDIES FOR GOUT

BY

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ABSTRACT

Xanthine oxidase (XO) is an enzyme that catalyses the metabolism of hypoxanthine and xanthine to uric acid. It is accountable for the medical condition known as gout due to deposition of uric acid in the joints, causing painful inflammation. Inhibition of XO leads to remission in gout. Malaysia houses abundant of medicinal plants, which can be introduced as new natural sources of gout medication and as alternative to synthetic xanthine oxidase inhibitors (XOI), like allopurinol which brings many side effects. The preliminary screening study of different parts of plant from several plant species revealed that aqueous extract of *Carica papaya* L. leaves possess promising XO inhibitory activity at a concentration of 100 µg/ml. Thus, response surface methodology (RSM) from Design Expert® v.6.0.8 was used to study the effects of temperature (ºC), time (hour), agitation speed (rpm), and ratio of sample to the solvent (g/ml) on enhancement of XOI production. The analysis of variance (ANOVA) demonstrated that the F-value was 15.07, which implies that the model is significant with low probability value (p<0.0001); having the residuals distributed along a well randomized straight line. Coefficient of determination (R^2) was 93.36%. The p-value of 0.2349 for the lack of fit indicated the model has no lack of fit that further validates the model. Statistical optimization helped in developing the process conditions and the percentage of XO inhibition was enhanced to 88.68 ± 1.82%, obtained at 30ºC, 15 hours, 125 rpm and 1g/20ml. The inhibition was less by 5% as compared to allopurinol (93.69 ± 0.2%). Qualitative analysis on the optimized distilled water extract of *Carica papaya* L. leaves (ODEC) showed the presence of secondary metabolites such as flavonoids, alkaloids, xanthine alkaloids, saponins, anthranol glycosides and terpenoids. Quantitative analysis of ODEC showed the presence of 27.51-33.15% flavonoids, 14.43-16.72% alkaloids and 1.12-1.97% saponins. The ODEC was subjected to reversed-phase flash column chromatography and high performance thin layer chromatography for rapid separation and purification. Fraction EEA1 with R_f value of 0.767 was isolated and demonstrated 95.70 ± 2.57% of XO inhibition, much higher than allopurinol. Partial identification via HPLC on the ODEC and EEA1 against phenolic acids and flavonoids standards revealed the presence of three phenolic acids and four flavonoids. They were caffeic acid, *p*-coumaric acid, ferulic acid, myricetin, quercetin, kaempferol and apigenin whereas EEA1 has only quercetin (now denoted as purified compound, PC), which could explain the pharmacological properties of this plant and demonstrate its importance in daily intake especially for gout patient. Quercetin demonstrated competitive mode of inhibition against XO. Antioxidant activities were investigated with BHT, a renowned antioxidant used primarily as food additive as positive control. ODEC and PC scavenged 2, 2-diphenyl-1-picrylhydrazyl (DPPH) activity at an IC_{50} of 2.86 µg/ml and 3.14 µg/ml, respectively. ODEC inhibited β-carotene bleaching at an IC_{50} of 2.13 µg/ml whereas PC at 2.16 µg/ml. Ferric reducing antioxidant power (FRAP) of ODEC was 1146.89 ± 44.24 µmol $FeSO_4.7H_2O$/g dry, 1.8-fold higher than unoptimized extract (UODEC), 1.3-fold higher than PC and 1.5-fold higher than BHT. However, ODEC and PC exhibited minimal anti-diabetic activities. The data grants imperative breakthrough to discover natural XOI from plants and the findings are encouraging to plan clinical studies in hyperuricemic patients, and in the formulation of nutraceuticals or pharmaceuticals, or cosmeceuticals products incorporating natural-based XOI.

ملخص البحث

أوكسيديز الزانثين(XO) هو إنزيم يحفز عملية التمثيل الغذائي للهيبوزانتين والزانثين إلى حمض اليوريك. وهو المسؤول عن حالة طبية تعرف باسم مرض النقرس بسبب ترسب حمض البوليك في المفاصل ، مما يسبب التهاباً مؤلماً. تثبيط أوكسيديز الزانثين (XO) يؤدي الى الهوادة في مرض النقرس. تزخر معظم المنازل الماليزية بمختلف النباتات الطبية التي يعتبر بعضاً منها قادراً على تثبيط أنزيم أوكسيديز الزانثين, الذي يمكن تقديمه كمصدر طبيعي جديد لأدوية مرض النقرس و كبديلاً لمثبطات أوكسيديز الزانثيم (XO) الإصطناعية, مثل ألوبيورينول التي لها آثاراً جانبية عديدة . كشفت دراسة الفرز الأولي لمائتي جزء نباتي من ثلاثين نوعاً مختلفاً من الأصناف النباتية أن المستخلص المائي لأوراق نبات (كاريكا البابايا) يمتلك نشاطاً مثبطاً لإنزيم أوكسيديز الزانثين عند تركيز ١٠٠ ميكروجرام/ مل, لذلك تم استخدام منهجية الإستجابة السطحية (RSM) لدراسة تأثير كلاً من درجة الحرارة (درجة مئوية), الوقت (ساعة), سرعة التهيج (دورة في الدقيقة), و نسبة العينة إلى المذيب (جرام/ مل) على تعزيز أنتاج إنزيم أوكسيديز الزانثين (XO). أظهرت نتائج تحليل التباين (ANOVA) أن قيمة F-value تساوي ١٥,٠٧ والتي تشير إلى أن النموذج ذو أهمية (significant) مع احتمال ضعيف بحوالي(p <٠,١٠٠٠) , ومخلفات موزعة على طول خط مستقيم بطريقة عشوائية. وكان معامل التحديد (R^2) يساوي القيمة ٣٦,٩٣٪. قيمة عدم وجود التناسب (p--٠,٩٤٣٢) تشير إلى أن النموذج يُظهر عدم وجود أي تناسب عند التأكد من صحة النموذج. ساعدت عملية التحسين الإحصائية في تطوير ظروف العملية وتعززت نسبة تثبيط أوكسيديز الزانثين (XO) إلى ١,٨٢ ±٨٨,٦٨٪, عند ٣٠ درجة مئوية , ١٥ ساعة, ١٢٥ دورة في الدقيقة, و ١ جرام/ ٢٠ مل. وكان النشاط أقل بنسبة ٥ ٪ بالمقارنة مع نشاط ألوبيورينول (٠,٢ ± ٩٣,٦٩٪). وأظهر التحليل النوعي لمستخلص الماء المقطر الأمثل من أوراق كاريكا البابايا (ODEC) وجود عدة عمليات أيض ثانوية, وبصورة رئيسية ، الفلافونويد ، قلويدات ، قلويدات الزانثين ، الصابونين ، جليكوسيدات اللأنثانول و التيربينويدس. وأظهر التحليل الكمي لل ODEC وجود مركبات الفلافونويد (٥١,٢٧ ١٥,٣٣٪)، قلويدات(٤٣,١٤ ,٢٧ ١٦٪) والصابونين(١٢,١ -٩٧,١ ٪). تعرضت خلاصة الماء المقطر لأوراق نبات كاريكا البابايا (ODEC) إلى عملية كروماتوغرافيا عكس المرحلة لفلاش العمود و كروماتوغرافيا الورقية عالية الأداء للحصول على عملية فصل و تنقية سريعة. الجزء رقم (١) من ايثانول: ايثايل اسيتيت مع قيمة الترددات اللاسلكية R_f= ٠,٧٧٦ عُزل و أثبت أن ٥٧,٢ ± ٧٠,٩٥٪ من تثبيط أنزيم اوكسيديز الزانثين (XO) أعلى بكثير من اللوبيورينول. من خلال استخدام تقنية (HPLC) على ODEC و EEA1 ضد معايير الأحماض الفينولية و الفلافونيدات تبين وجود ثلاثة أحماض فينولية و أربعة فلافونيدات. وهي حمض الكافيك , حمض بي-كوماريك, حمض الفيروليك, مايريسيتين, كويرسيتين, كيمبفيرول, و ابيجينين بينما يحتوي EEA1 على كويريسيتين فقط (تدل على المركب النقي, PC), والتي يمكن أن تفسر الخصائص الدوائية لهذا النبات وتظهر أهميته في الجرعة اليومية وخاصة بالنسبة لمريض النقرس. و قد تم دراسة نشاطات مضادات الأكسدة مع بوتيل هيدروكسي تولوين (BHT) , ومضادات الأكسدة المعروفة تستخدم في المقام الأول كا مضافات غذائية ومراقبات إيجابية. تم كسح و تثبيط نشاط ٢, ٢ ديفينايل ١ بيكرايل هيدرازيل (DPPH) باستخدام (ODEC) و (PC) عند IC=٥٠ و القيم الناتجة كانت (٨٦,٢ و ١٤,٣) ميكروجرام/مل على التوالي. (ODEC) ثبطت عملية التبييض الناتجة عن مادة بيتا كاروتين عند IC=٥٠ و القيمة الناتجة كانت (١٣,٢) ميكروجرام/ مل , بينما المركب النقي (PC) أعطى قيمة تبلغ (١٦,٢) ميكروجرام/ مل. كانت القوة الحديدية لخفض الأكسدة (FRAP) لخلاصة الماء المقطر غير المحسن لأوراق كاريكا البابايا (UODEC) تساوي ٨٩,١١٤٦٤±٢٤,٤٤ميكروجرام من $FeSO_4.7H_2O$/جرام جاف, والتي تعتبر ٨,١ أضعاف أعلى من مستخلص كاريكا البابايا غير المحسن, و ٣,١ أضعاف أعلى من (PC) , و ٥,١ أضعاف أعلى من (BHT) . و يملك كلاً من (UODEC) و المركبات النقية (PC) تأثيراً متفوقاً لتثبيط أنزيم أوكسيديز الزانثين (XO). إن البيانات التي تم الحصول عليها من خلال هذه الدراسة , توفر معلومات هامة لاكتشاف (XOI) جديدة من النباتات الطبيعية و هذه الأكتشافات تشجع على أنتاج منتج دوائي لمكافحة مرض النقرس, فضلاً عن خطة الدراسات السريرية للمرضى الذين يعانون من فرط حمض اليوريك في الدم.

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 dissertation for the degree of Master of Science (Biotechnology Engineering).

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, 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.

Saiful Mohammad Azmi bin Azmi

Signature ……..……. Date …….

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Affirmed by Saiful Mohammad Nizam bin Azmi.

…….. ……
Signature Date

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LIST OF ABBREVIATIONS

2Fe - 2S	Two distinct iron-sulphur
4CL	Coumaroyl-CoA-ligase
AC	Acetic acid in chloroform
ACN	Acetonitrile
$AlCl_3$	Alcoholic ferric chloride
AMP	Adenosine monophosphates
ANOVA	Analysis of variance
Aq-A	Aqueous acetic acid
Arg	Arginine
BAN	Anthocyanidin reductase
BAW	n-butanol : acetic acid : water
BEW	n-butanol : ethanol : water
BHT	Butylated hydroxytoluene
BMA	Benzene : methanol : acetic acid
BN	n-butanol : ammonium hydroxide
C4H	Cinnamate-4-hydroxylase
CBG	Cytosolic β-glucosidase
CH_2Cl_2	Dichloromethane
CHI	Chalcone isomerase
CHR	Chalcone reductase
CHS	Chalcone synthase
CCD	Central composite design
CPa-1	*Carica papaya* L. leaves
CPa-5	*Carica papaya* L. unripe fruit peels
CSa-1	*Cucumis sativus* L. ripe fruit peels
CSa-2	*Cucumis sativus* L. seeds
CV	Coefficient of variation
DAD	Diode array detection
DFR	Dihydroglavonol-4-reductase
dH_2O	Distilled water
DiEt	Diethyl ether
DMID	7,2′-dihydroxy-4′-methoxyisoflavonol dehydratase
DMSO	Dimethylsulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EAB	Ethyl acetate in benzene
Eq.	Equation
ESI	Electrospray ionization
EtAc	Ethyl acetate
EtOH	Ethanol
F3′H	Flavonoid 3′-hydroxylase
F3′5′H	Flavonoid 3′5′-hydroxylase
FAD	Flavin adenine dinucleotide
Fe $(TPTZ)_2$ (II)	Ferrous tripyridyltriazine
Fe $(TPTZ)_2$ (III)	Ferric tripyridyltriazine

$FeCl_3$	Ferric chloride
FHT	Flavonone 3â-hydroxylase
FLS	Flavonols synthase
FNS I/II	Flavone synthase
FRAP	Ferric reducing antioxidant power
Gln	Glutamine
Glu	Glutamic acid
GMa-1	*Garcinia mangostana* L. leaves
GMa-2	*Garcinia mangostana* L. ripe fruit peels
GMP	Guanosine monophosphates
H_2SO_4	Sulphuric acid
HCl	Hydrochloric acid
HGPRT	Hypoxanthine – guanine phosphoribosyltransferase
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
HPTLC	High Performance thin layer chromatography
Hun-2	*Hylocereus undatus* (Haw.) Britton & Rose ripe fruit peels
I2′H	Isoflavone 2-hydroxylase
IFR	Isoflavone reductase
IFS	Isoflavone synthase
IIUM	International Islamic University Malaysia
IMP	Inosine 5'-monophosphates
IOMT	Isoflavone *O*-methyltransferase
K_2HPO_4	Di-potassium hydrogen phosphates
$K_3Fe(CN)_6$	Potassium ferricyanide
KH_2PO_4	Potassium di-hydrogen phosphates
KOH	Potassium hydroxide
LAR	Leucoanthocyanidin reductase
LC	Liquid chromatography
LDOX (ANS)	Leucoanthocyanidin dioxygenase (anthocyanidin synthase)
LPH	Lactase - phlorizin hydrolase
LPO	Lipid peroxidation
MCi-4	*Morinda citrifolia* L. leaves
MeOH	Methanol
Mo	Molybdenum
Mo-pt	Molybdopterin cofactor
MS	Mass spectrometry
MSp-1	*Mentha spicata* L. leaves
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NAP3	National Agriculture Policy 3
NSAIDs	Non-steroidal anti-inflammatory drugs
ODEC	Distilled water extract of *Carica papaya* L. leaves
OMT	*O*-methyltransferase
PAL	Phenylalanine ammonialyase
PC	Purified compound, quercetin
PDB	Protein Data Bank
Phe	Phenylalanine

PhOH	Phosphoric acid : water
PRPP	5-phosphoribosyl-α-pyrophosphates
R_f	Retardation factor
ROS	Reactive oxygen species
RPFCC	Reversed-phase flash column chromatography
rpm	Revolution per minute
RSM	Response surface methodology
RT	Rhamnosyl transferase
SD	Standard deviation
SGTL1	Sodium-dependent glucose transporter
SJS	Steven-Johnsons syndrome
STS	Stilbene synthase
TENS	Toxic epidermal necrolysis syndrome
TLC	Thin layer chromatography
TPTZ	2, 4, 6-tripyridyl-S-triazine
UGT	UDP-glucose : flavonoid glycosyl transferase
UK	United Kingdom
UODEC	Unoptimized distilled water extract of *Carica papaya* L. leaves
USA	United States of America
UV/VIS	Ultra violet/visible
VAD	Valoneic acid dilactone
VR	Vestitone reductase
WHO	World Health Organization
XO	Xanthine oxidase
XOI	Xanthine oxidase inhibitor

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Uric acid is a product of purine metabolism generated during the enzymatic degradation of hypoxanthine and xanthine, catalyzes by an enzyme named xanthine oxidase (XO) (Ramallo et al., 2006; Wang et al., 2008). Elevated concentrations of uric acid in the blood stream created a metabolic arthritis disease called gout. Gout affects a substantial proportion of the adult population. It is the most common form of arthritis in men over the age of 40. Women are less commonly affected but the prevalence may be increased among postmenopausal women (Choi and Curhan, 2007). Gout is common in prosperous and affluent societies due to a diet rich in proteins, fat and alcohol (Choi and Curhan, 2004a; Trivieri et al., 1999). Factors like inherited enzyme deficiencies, obesity, decrease renal function and hypertension also contribute to the elevated concentrations of uric acid (Wright and Pinto, 2003). It is believed that either by increasing the excretion of uric acid or reducing the uric acid production helps to reduce the risk of gout (Umamaheswari et al., 2007).

To date, the treatment of gout entails the use of therapeutic agents such as xanthine oxidase inhibitors (XOI) by blocking the biosynthesis of uric acid from purine in the body (Unno et al., 2004). In the process of blocking the uric acid production, inhibition of XO causes an increase in hypoxanthine and xanthine, which are converted to purine ribotides adenosine and guanosine monophosphates. The increased level of these ribotides causes feedback inhibition of amidophosphoribosyl transferase, the first and rate-limiting enzyme of purine biosynthesis (Borges et al.,

2002). Allopurinol (naturally occurring purine in the body) is one of the few clinically used XOIs (Bieber and Terkeltaub, 2004; Wortmann, 2005). It is reported that allopurinol (i.e. Lopurin, Zurinol, and Zyloprim) inhibits XO and slows the uric acid production rate in the blood and urine (Fields et al., 1996). Allopurinol does not alleviate acute attacks of gout but is useful in preventing recurrence. It is the drug of choice for long-term prophylaxis and is the best medicine for people who have kidney problems caused by uric acid (Fuchs et al., 1999).

However, there are many adverse reactions associated with allopurinol (Kong et al., 2001; Wallach, 1998), ranging from mild skin allergy (i.e. skin rashes, stomach upsets, hives, itching, fever, nausea and muscle pain) to a concerted allopurinol hypersensitivity syndrome, which can cause death (Kong et al., 2000; Pacher et al., 2006; Umpie´rrez et al., 1998). Thus, the problems of using allopurinol imperatively required to be solved by any suitable alternative means such as medicinal plants.

Malaysia is a tropical country, which houses more than 12,000 species of flowering plants. Thus, it is expected that they have well diverse chemical structures from their secondary metabolite and chemical diversity. Some plant-based bioactive compounds such as polyphenols (Costantino et al., 1992), flavonoids (Chang et al., 1993; Cotelle et al., 1996; Selloum et al., 2001), coumarins (Chang and Chiang, 1995), ellagic acid, valoneic acid dilactone (VAD) (Unno et al., 2004), xanthones and beta carbolines have been reported to be potent XOI. In 2005, Malaysian government spent an estimated of RM 8 billion on herbal products and is expected to increase by 20% annually (BiotechCorp, 2010). Thus, a search for substitutes from the plant origin as alternatives to allopurinol for the treatment of gout is highly warranted.

There are numerous studies on XOI compound or compound that have anti-gout properties using medicinal plants. Many of these bioactive compounds were

initially examined for other medicinal benefits such as antioxidant, anticancer, antimicrobial, anti-diabetic, anti-inflammatory and anti-analgesic (Foyet et al., 2011; Goyal et al., 2010; Jung et al., 2006; Kaur et al., 2010; Singh et al., 2010; Soncini et al., 2011; Sulaiman et al., 2011; Wan Norhana et al., 2009). Different methods of extraction and process conditions were used to extract the desired compound. Therefore, optimization study by means of statistical experimental design is vital to determine the optimum conditions needed to yield high production of XOI compound, especially important for scaling-up. Isolation, purification and identification of bioactive compounds allow the formulation, development and commercialization of natural-based products (i.e. cosmeceuticals, nutraceuticals and pharmaceuticals).

This study aims to discover scientific basis for the reputed efficacy of Malaysian plants to inhibit XO. We expect to obtain natural XOI, which will be an alternative to the available treatment of gout and other inflammatory-related diseases.

1.2 PROBLEM STATEMENT AND ITS SIGNIFICANCE

XO plays a key physiological role in the hydroxylation of hypoxanthine and xanthine to uric acid (Ramallo et al., 2006; Wang et al., 2008). Increase in uric acid or decrease in its excretion is normally referred as hyperuricemia (Vázquez-Mellado et al., 2004), which is identified when uric acid concentration is $\geq$ 7 mg/dl for men and $\geq$ 6 mg/dl for women (Nakagawa et al., 2006; Schlesinger and Schumacher, 2002). XO also plays an important role in various forms of ischemic injuries, tissue and vascular injuries, inflammatory diseases and chronic heart failure (Pacher et al., 2006).

The prototypical of allopurinol (4-hydroxypyrazolo [3, 4-d] pyrimidine) as XOI has been the cornerstone of the clinical management of gout and conditions