Antidiabetic Activity of Rhinacanthins-rich Extract and Rhinacanthin-C

Muhammad Ajmal Shah

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Thesis Title	Antidiabetic Activity of Rhinacanthins-rich Extract and Rhinacanthin-C
Author	Mr. Muhammad Ajmal Shah
Major Program	Pharmaceutical Sciences

Major Advisor	**Examining Committee:**
....	....Chairperson
(Assoc. Prof. Dr. Pharkphoom Panichayupakaranant)	(Assist. Prof. Dr. Supreeya Yuenyongsawad)
	....Committee
Co-Advisor	(Assoc. Prof. Dr. Wantana Reanmongkol)
....	....Committee
(Assoc. Prof. Dr. Wantana Reanmongkol)	(Assoc. Prof. Dr. Pharkphoom Panichayupakaranant)
	....Committee
	(Assoc. Prof. Dr. Boonchoo Sritularak)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Pharmaceutical Sciences.

....

(Assoc. Prof. Dr. Teerapol Srichana)

Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

…….……………………………………….Signature
(Assoc. Prof. Dr. Pharkphoom Panichayupakaranant)
Major Advisor

...…..………………………………………. Signature
(Mr. Muhammad Ajmal Shah)
Candidate

I hereby certify that this work has not been accepted in substance for any other degree, and is not being currently submitted in candidature for any degree.

……………………………………. Signature

(Mr. Muhammad Ajmal Shah)

Candidate

ชื่อวิทยานิพนธ์ ฤทธิ์ลดน้ำตาลในเลือดของสารสกัดที่มีไรนาแคนธินปริมาณสูงและของไรนา-แคนธินซี

ผู้เขียน Mr. Muhammad Ajmal Shah

สาขาวิชา เภสัชศาสตร์

ปีการศึกษา 2559

บทคัดย่อ

สารสกัดที่มีไรนาแคนธินปริมาณสูงเป็นสารสกัดกึ่งบริสุทธิ์ ที่มีปริมาณไรนาแคนธินซีร้อยละ 60 โดยน้ำหนัก สารสกัดดังกล่าวเตรียมได้จากใบทองพันชั่ง ซึ่งเป็นพืชสมุนไพรชนิดหนึ่งที่นิยมนำมาใช้รักษาโรคในการแพทย์แผนไทย นอกจากนี้ในประเทศไต้หวันและจีนยังใช้เป็นเครื่องดื่มสมุนไพรด้วย การเตรียมสารสกัดที่มีไรนาแคนธินปริมาณสูงจะใช้วิธีการสกัดด้วยคลื่นไมโครเวฟ ซึ่งเป็นวิธีการสกัดสารที่ได้รับการยอมรับว่าเป็นวิธีที่เป็นมิตรต่อสิ่งแวดล้อม จากนั้นนำสารสกัดมาแยกสารด้วยคอลัมน์ชนิด Amberlite® ส่วนการแยกสารไรนาแคนธินซี, ไรนาแคนธินดี และ ไรนาแคนธินเอ็น จากสารสกัดที่มีไรนาแคนธินปริมาณสูง ใช้คอลัมน์ชนิด silica gel การศึกษานี้ได้ทดสอบศักยภาพในการรักษาโรคเบาหวานของสารสกัดที่มีไรนาแคนธินปริมาณสูง และ ไรนาแคนธินที่ใช้เป็นสารมาตรฐาน โดยการทดสอบฤทธิ์ยับยั้งเอนไซม์ α-glucosidase ในหลอดทดลอง และทดสอบฤทธิ์ superoxide scavenging, ฤทธิ์ต้านการเกิดไกลเคชัน, ฤทธิ์กระตุ้นการนำกลูโคสเข้าเซลล์ และ ฤทธิ์ยับยั้งการสร้างเซลล์ไขมัน นอกจากนี้ ยังทดสอบฤทธิ์ลดระดับน้ำตาลและไขมันในเลือด ของสารสกัดที่มีไรนาแคนธินปริมาณสูงและ ไรนาแคนธินซี ในหนูทดลองที่ถูกทำให้เป็นโรคเบาหวานด้วยการให้ nicotinamide และ streptozotocin และศึกษาเพื่อทำนายความสัมพันธ์ระหว่างโครสร้างทางเคมีของสารกับ รูปแบบของเภสัชจลนศาสตร์และความเป็นพิษของไรนาแคนธินโดยใช้โปรแกรมคอมพิวเตอร์

สารสกัดที่มีไรนาแคนธินปริมาณสูงมีฤทธิ์ยับยั้งเอนไซม์ α-glucosidase (IC_{50} เท่ากับ 25.0 μg/mL) ได้ใกล้เคียงกับไรนาแคนธินซี (IC_{50} เท่ากับ 22.6 μg/mL) และมีฤทธิ์ดีกว่าไรนา-แคนธินดี (IC_{50} เท่ากับ 71.5 μg/mL) และยามาตรฐาน acarbose (IC_{50} เท่ากับ 395.4 μg/mL) ในขณะที่ไรนาแคนธินเอ็นไม่มีฤทธิ์ดังกล่าว การศึกษาทางจลศาสตร์พบว่าทั้งสารสกัดที่มีไรนา-แคนธินปริมาณสูงและไรนาแคนธินซีมีฤทธิ์ยับยั้งเอนไซม์ α-glucosidase แบบ noncompetition และสารสกัดที่มีไรนาแคนธินปริมาณสูงและไรนาแคนธินซียังช่วยเสริมฤทธิ์ของ acarbose (competitive inhibitor) เมื่อให้ร่วมกันที่ระดับความเข้มข้นต่ำ (¼IC_{50}, ½IC_{50} and IC_{50}) และจากการศึกษาด้วยโปรแกรมคอมพิวเตอร์พบว่าไรนาแคนธินซีจับกับเอนไซม์ α-glucosidase โดยใช้ส่วนของโมเลกุลทั้งที่มีขั้วและไม่มีขั้ว

การทดสอบฤทธิ์ superoxide scavenging ใช้เทคนิค cyclic voltammetry และการทดสอบฤทธิ์ต้าน ไกลเคชันใช้ระบบ fructose mediated human serum albumin การศึกษาความสัมพันธ์ของโครงสร้างทางเคมีของไรนาแคนธินและฤทธิ์ใช้ molecular interaction studies พบว่าสารสกัดที่มีไรนาแคนธินปริมาณสูงมีฤทธิ์ต้านออกซิเดชันผ่านกลไก E_rC_i โดยมีค่า IC_{50} เท่ากับ 8.0 μg/mL มีค่า antioxidant capacity เท่ากับ 39439 M^{-1} และ มีค่า binding constant เท่ากับ 45709 M^{-1} การทดสอบฤทธิ์ต้านไกลเคชันพบว่าสารสกัดที่มีไรนาแคนธินปริมาณสูงมีฤทธิ์ดีเทียบเท่าไรนาแคนธินซี โดยมีค่า IC_{50} เท่ากับ 39.7 และ 37.3 μg/mL ตามลำดับ แต่มีฤทธิ์ดีกว่าไรนา-แคนธินดี (IC_{50} เท่ากับ 50.4 μg/mL), ไรนาแคนธินเอ็น (IC_{50} เท่ากับ 89.5 μg/mL) และสารมาตรฐาน rutin (IC_{50} เท่ากับ 41.5 μg/mL) การที่สารสกัดมีฤทธิ์ superoxide scavenging และฤทธิ์ต้านไกลเคชันได้ดีมาก แสดงให้เห็นว่าสารสกัดที่มีไรนาแคนธินปริมาณสูงมีศักยภาพในการนำไปใช้รักษาโรคเรื้อรังต่างๆ โดยเฉพาะโรคแทรกซ้อนที่มักจะเกิดในผู้ป่วยโรคเบาหวาน

โรคอ้วนเป็นเป็นอีกโรคหนึ่งที่มักจะพบในผู้ป่วยโรคเบาหวน โดยเฉพาะโรคเบาหวานชนิดที่ 2 มีรายงานว่าสารสกัดใบทองพันชั่งมีศักยภาพในการรักษา

โรคเบาหวานและโรคอ้วนได้ ในการศึกษานี้ได้ศึกษาฤทธิ์กระตุ้นการนำกลูโคสเข้าเซลล์ และ ฤทธิ์ต้านการสร้างเซลล์ไขมัน ของสารสกัดที่มีไรนาแคนธินปริมาณสูงและไรนาแคนธินมาตรฐาน ในเซลล์ชนิด 3T3-L1 adipocytes และ L6 myotubes การทดสอบฤทธิ์กระตุ้นการนำกลูโคสเข้าเซลล์ทำการทดลองในเซลล์ชนิด 3T3-L1 และ L6 ด้วยวิธีการวัดปริมาณกลูโคสที่เหลือในอาหารเพาะเลี้ยงโดยใช้ glucose oxidase kit ส่วนการทดสอบฤทธิ์ต้านการสร้างเซลล์ไขมันทำเฉพาะในเซลล์ไขมันชนิด 3T3-L1 ด้วยการวัดปริมาณไขมันในเซลล์โดยใช้ oil red O dye พบว่าที่ขนาดยาที่ให้ผลดีที่สุด สารสกัดที่มีไรนา-แคนธินปริมาณสูง (20 μg/mL) มีฤทธิ์กระตุ้นการนำกลูโคสเข้าเซลล์ชนิด 3T3-L1 ได้ดีเท่ากับไรนาแคนธินเอ็น (20 μg/mL) และอินซูลิน (0.58 μg/mL) และมีฤทธิ์ดีกว่าไรนาแคนธินซี (20 μg/mL) และไรนาแคนธินดี (20 μg/mL) ส่วนในเซลล์ชนิด L6 พบว่าสารสกัดที่มีไรนาแคนธินปริมาณสูง (2.5 μg/mL) มีฤทธิ์กระตุ้นการนำกลูโคสเข้าเซลล์ (>80%) ได้ดีเท่ากับไรนาแคนธินซี (2.5 μg/mL) และยามาตรฐานอินซูลิน (2.90 μg/mL) และ เม็ตฟอร์มิน (219.5 μg/mL) และมีมีฤทธิ์ดีกว่าไรนาแคนธินดี (2.5 μg/mL) และไรนาแคนธินเอ็น (2.5 μg/mL) นอกจากนี้ สารสกัดที่มี ไรนาแคนธินปริมาณสูง (20 μg/mL) ยังมีฤทธิ์ต้านการสร้างเซลล์ไขมันในเซลล์ชนิด 3T3-L1 ได้ดีมาก โดยออกฤทธิ์ได้ใกล้เคียงกับไรนาแคนธินซี (20 μg/mL) และมีฤทธิ์ดีกว่าไรนาแคนธินดี (20 μg/mL) และไรนาแคนธินอ็น (20 μg/mL)

การศึกษาในหนูทดลองที่ถูกทำให้เป็นเบาหวานด้วยการให้ nicotinamide และ streptozotocin เพื่อเปรียบเทียบผลในการลดระดับน้ำตาลและไขมันในเลือดของสารสกัดที่มีไรนา-แคนธินปริมาณสูง (24.11 mg/kg ซึ่งเป็นขนาดที่สมมูลกับไรนาแคนธินซี 15 mg/kg) ไรนาแคนธินซี (15 mg/kg) และยามาตรฐาน glibenclamide (600 μg/kg) โดยให้สารสกัดและยาติดต่อกันเป็นระยะเวลา 28 วัน พบว่าสารสกัดที่มีไรนาแคนธินปริมาณสูงและไรนาแคนธินซีสามารถลดระดับน้ำตาลในเลือด (fasting blood glucose), ระดับ HbA1c และการกินอาหารและน้ำ และเพิ่มระดับของอินซูลินและน้ำหนักตัวหนูทอลองได้ แต่ไม่มีผลเหล่านี้ในหนูปกติ และมีผลทำให้ระดับของไขมันในซีรั่มและระดับของ

biomarkers ในตับและไตกลับมาเป็นปกติในหนูทดลองที่เป็นเบาหวานทั้งในกลุ่มที่ได้รับสารสกัดที่มีไรนาแคนธินปริมาณสูงและกลุ่มที่ได้รับไรนาแคนธินซี แต่ไม่มีผลเหล่านี้ในหนูปกติ นอกจากนี้ในการศึกษาพยาธิวิทยาระดับเซลล์ของตับอ่อนพบว่า สารสกัดที่มีไรนาแคนธินปริมาณสูงและไรนาแคนธินซียังช่วยฟื้นฟูเซลล์ islets of Langerhans ในหนูทดลองที่เป็นเบาหวานด้วย จากผลการศึกษาทั้งหมดนี้แสดงให้เห็นว่า สารสกัดที่มีไรนาแคนธินปริมาณสูงมีศักยภาพในการรักษาโรคเบาหวานได้ทัดเทียมกับไรนา-แคนธินซี และจากการทำนายผลการวิเคราะห์ทางเภสัชจลนศาสตร์และความเป็นพิษด้วยโปรแกรมคอมพิวเตอร์พบว่า สามารถให้ไรนา-แคนธินซีโดยการรับประทานได้โดยไม่เกิดความเป็นพิษ ไม่เป็นสารก่อมะเร็งและสารก่อการกลายพันธุ์ และ มีชีวปริมาณออกฤทธิ์ (bioavailability) ที่เหมาะสม การศึกษานี้แสดงให้เห็นว่าสารสกัดที่มีไรนาแคนธินปริมาณสูง เป็นสารสกัดจากธรรมชาติที่มีศักยภาพในการนำมาใช้รักษาโรค เบาหวานได้อย่างมีประสิทธิภาพ

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Author Mr. Muhammad Ajmal Shah
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ABSTRACT

Rhinacanthins-rich extract (RRE) is a semi purified leaf extract that contains 60% w/w of rhinacanthin-C (RC) obtained from *Rhinacanthus nasutus* leaf, a popular medicinal plant used in Thai traditional medicine and an herbal drink in Taiwan and China. RRE was prepared by microwave assisted green extraction method along with a simple step of fractionation using Amberlite® column. RC, rhinacanthin-D (RD) and rhinacanthin-N (RN) were isolated from the RRE using silica gel column chromatography. In this study, RRE and its marker compounds were investigated for their antidiabetic potential using *in vitro* α-glucosidase inhibition, superoxide scavenging, antiglycation, glucose uptake stimulation and adipogenic inhibition assays. An *in vivo* experiment using nicotinamide-streptozotocin induced diabetic rats was also performed to validate and compare the hypoglycemic and hypolipidemic activity of RRE with its major constituent RC. Moreover, *in silico* studies were conducted to predict structure activity relationship, pharmacokinetic and toxicity profile of rhinacanthins.

RRE (IC_{50} value of 25.0 μg/mL) exhibited α-glucosidase inhibitory activity nearly equal to that of RC (IC_{50} value of 22.6 μg/mL) but stronger than that of RD (IC_{50} value of 71.5 μg/mL) and the standard drug, acarbose (IC_{50} value of 395.4 μg/mL), while RN was inactive. Kinetic studies revealed that both RRE and RC exhibited noncompetitive α-glucosidase inhibitory activity, while combinations of either RRE or rhinacanthin-C with acarbose (competitive inhibitor) at low concentrations (¼IC_{50}, ½IC_{50} and IC_{50}) showed a synergistic inhibitory effect. *In*

silico studies identified the binding mode of RC highlighting the formation of both polar and apolar contacts of ligand with α-glucosidase.

Superoxide scavenging activity was performed by cyclic voltammetry and fructose mediated human serum albumin glycation model was used for antiglycation activity. Molecular interaction studies were conducted to identify the structure activity relationships of rhinacanthins. On the basis of kinetic measurements, RRE exhibited the most potent antioxidant activity via E_rC_i mechanism, with an IC_{50} value of 8.0 µg/mL, antioxidant capacity of 39439 M^{-1} and binding constant of 45709 M^{-1}. Antiglycation assay showed that RRE exhibited almost same glycation inhibitory effect to that of RC, with IC_{50} values of 39.7, and 37.3 µg/mL, respectively, but higher than that of RD (IC_{50} of 50.4 µg/mL), RN (IC_{50} of 89.5 µg/mL) as well as the positive control, rutin (IC_{50} of 41.5 µg/mL). The potent superoxide scavenging and albumin glycation inhibitory effect of RRE rationalized its therapeutic application in various chronic diseases especially in the complications of diabetes.

Obesity is one of the imperative dynamic in the incidence and intensification of type 2 diabetes mellitus (T2DM). *R. nasutus* leaf extracts are previously reported for their antidiabetic and antiobesity potential. In the present study, RRE and its marker compounds have been evaluated for glucose uptake stimulatory and antiadipogenic activities in 3T3-L1 and L6 cells. Glucose uptake stimulation in both 3T3-L1 and L6 cells was performed by quantification of residual glucose in the media using glucose oxidase kit. Antiadipogenic activity in 3T3-L1 adipocytes was performed by intracellular lipids quantification using oil red O dye. At the highest effective dose, RRE (20 µg/mL) exhibited satisfactory glucose uptake stimulatory effect in 3T3-L1 adipocytes that is nearly equal to RN (20 µg/mL) and the positive control insulin (0.58 µg/mL), but higher than RC (20 µg/mL) and RD (20 µg/mL). In addition, treatments of L6 myotubes showed that RRE (2.5 µg/mL) exhibited potent and same glucose uptake stimulation (>80%) to RC (2.5 µg/mL) and the standard drugs, insulin (2.90 µg/mL) and metformin (219.5 µg/mL), but higher than RD (2.5 µg/mL) and RN (2.5 µg/mL). Furthermore, RRE (20 µg/mL) exhibited

potent antiadipogenic effect in 3T3-L1 adipocytes, which was equivalent to RC (20 μg/mL) but higher than RD (20 μg/mL) and RN (20 μg/mL).

In animal experiments, RRE (24.11 mg/kg equivalent to 15 mg/kg RC content), RC (15 mg/kg) and the standard drug glibenclamide (600 μg/kg) were comparatively assessed for their hypoglycemic and hypolipidemic activity in nicotinamide-streptozotocin induced diabetic rats for 28 days. RRE and RC significantly reduced the fasting blood glucose, HbA1c and food/water intake, while increased the insulin level and body weight in diabetic rats without affecting the normal rats. The serum lipid, liver and kidney biomarkers were markedly normalized by both RRE and RC in diabetic rats without affecting the normal rats. Moreover, the histopathology of pancreas revealed that RRE and RC evidently restored the islets of Langerhans in diabetic rats. The overall results indicated that RRE has significantly comparable antidiabetic potential to that of RC. Furthermore, the *in silico* pharmacokinetic and toxicity analysis predicts that RC is orally non-toxic, non-carcinogenic and non-mutagenic with a decent bioavailability. The undertaken study suggests that RRE could be used as an effective natural remedy in the treatment of diabetes.

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Muhammad Ajmal Shah

CONTENTS

	Page
List of Abbreviations and symbols	xiv
List of Publications	1
Acceptance letter of Paper I from the journal	2
Reprint of Paper II with permission from the publisher	3
Acceptance letter of Paper III from the journal	4
1. General Introduction	5
1.1 Background and rationale	5
1.2 Objectives	9
2. Significant results and general discussion	10
2.1. α-Glucosidase inhibition by RRE and its synergism with acarbose	10
2.2. Molecular docking studies on RC interaction with α-glucosidase	11
2.3. Antioxidant activity of RRE with *in silico* biopredictions	12
2.4. Antiglycation activity of RRE and interaction studies of rhinacanthins	13
2.5. Glucose uptake stimulatory effect of RRE in 3T3-L1 and L6 cells	14
2.6. Adipogenic inhibitory effect of RRE in 3T3-L1 adipocytes	15
2.7. Effect of RRE on different parameters in diabetic rats	16
2.8. Pharmacokinetic and toxicity properties of RC	18
3. Concluding remarks	20
4. References	22
5. Appendices	
Paper I	29
Paper II	55
Paper III	63
Paper IV	84
Paper V	103
Animal ethic certificate	129
6. Vitae	130

LIST OF ABBREVIATIONS AND SYMBOLS

ADMET	Absorption, distribution, metabolism, excretion and toxicity
AGEPs	Advanced glycation end products
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
BSA	Bovine serum albumin
BUN	Blood urea nitrogen
CBD	Conservation Biodiversity
CV	Cyclic voltammetry
DM	Diabetes mellitus
DMEM	Dulbecco’s modified Eagle’s medium
E_rC_i	Reversible electron transfer followed by an irreversible chemical reaction
FBG	Fasting blood glucose
FBS	Fetal bovine serum
g	Gram
Glb	Glibenclamide
GLUT2	Glucose transporter 2
GLUT4	Glucose transporter 4
GO	Glucose oxidase
HbA1c	Glycated hemoglobin
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
HS	Horse serum
HSA	Human serum albumin
IBMX	3-Isobutyl-1-methylxanthine
IC_{50}	50% Inhibitory concentration
Kao	Antioxidant activity coefficient
Kb	Binding constant

L	Liter
LDL	Low density lipoprotein
mg	Milligram
MD	Molecular docking
MOE	Molecular operating environment
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NA	Nicotinamide
nM	Nanomolar
NMR	Nuclear magnetic resonance
PASSonline	Online prediction of activity spectra for substances
*p*NPG	Para nitrophenyl α-D-glucopyranoside
RC	Rhinacanthin-C
RD	Rhinacanthin-D
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
RN	Rhinacanthin-N
RRE	Rhinacanthins-rich extract
STZ	Streptozotocin
T2DM	Type-2 diabetes mellitus
TC	Total cholesterol
TG	Triglyceride
w/w	Weight by weight
WHO	World Health Organization
α-MEM	α-Minimum essential medium
μg	Microgram
μg/mL	Microgram per milliliter
μL	Microliter
μM	Micromolar

LIST OF PUBLICATIONS

This thesis contains introduction, significant results and discussion, and concluding remarks, and follows by two accepted (Paper I and III), one published (Paper II) paper as indicated by roman number, and two submitted manuscripts (Paper IV and V).

Paper I Shah MA, Keach J, Panichayupakaranant P**.** Antidiabetic naphthoquinones and their plant resources in Thailand**.** Review.

(Accepted in Chemical and Pharmaceutical Bulletin)

Paper II Shah MA, Khalil R, Haq ZU, Panichayupakaranant P. α-Glucosidase inhibitory effect of rhinacanthins-rich extract from *Rhinacanthus nasutus* leaf and synergistic effect in combination with acarbose. Journal of Functional Foods, (2017) 36, 325-331

(Reprinted with permission of Elsevier)

Paper III Shah MA, Muhammad H, Mehmood Y, Khalil R, Haq ZU, Panichayupakaranant P. Superoxide scavenging and antiglycation activities of rhinacanthins-rich extract from *Rhinacanthus nasutus* leaves.

(Accepted in Pharmacognosy Magazine)

Paper IV Shah MA, Jakkawanpitak C, Decha S, Panichayupakaranant P. Rhinacanthins-rich extract enhance glucose uptake and inhibit adipogenesis in 3T3-L1 adipocytes and L6 myotubes.

(Submitted to Pharmacognosy Magazine)

Paper V Shah MA, Reanmongkol W, Radenahmad N, Khalil R, Haq ZU, Panichyupakaranant P. Hypoglycemic and hypolipidemic effect of rhinacanthins-rich extract in nicotinamide-streptozotocin induced diabetic rats.

(Under process for submission to Biomedicine and Pharmacotherapy)

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Acceptance letter from Chemical and Pharmaceutical Bulletin

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With kind regards

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PAPER II

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PAPER III

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Dear Dr. Panichayupakaranant,

We are pleased to inform that your manuscript "Superoxide Scavenging and Antiglycation Activity of Rhinacanthins-rich Extract Obtained from the Leaves of Rhinacanthus nasutus" is provisionally accepted. You would receive an edited version of article in about 8-10 weeks based on the publishing process and schedules from now for a final check and correction.

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GENERAL INTRODUCTION

1.1 Background and rationale

Blood glucose homeostasis is crucial to keep balance between glucose production and depletion in various body tissues. Numerous hormonal regulated metabolic pathways are involved in the maintenance of glucose homeostasis. Any abnormality in this homeostasis results in several fatal disorders including diabetes mellitus (Gupta et al. 2016).

Diabetes mellitus (DM) is a multifarious chronic hyperglycemic disorder, characterized by altered metabolism of carbohydrates, lipids and proteins. DM can be classified into two main types i.e. type-1 (insulin-dependent) and type-2 (noninsulin-dependent) differ in their pathogenesis, but both have a common symptom of hyperglycemia. Type-1 DM is due to the loss of insulin-secreting beta cells whereas type-2 DM (T2DM) is caused by impaired insulin secretion combined with either a reduction of insulin activity or impairment of maintained activity (Perez et al. 1998). According to WHO, T2DM is a major type of diabetes comprising 90% of total diabetic cases globally. The international prevalence of DM increased from 4.7-8.5% during the last three decades, and the number of diabetic patients was estimated as 422 million in 2014 with 1.5 million deaths each year (World Health Organization, 2016). In 2015, more than 4 million cases of DM have been reported in Thailand, while diabetic prevalence was 8% among the individuals having age between 20-79 years old (International Diabetes Federation, 2016).

Excess body weight and physical inactivity are the main causes of T2DM (Olokoba et al. 2012). Hyperglycemia and hyperlipidemia are prime characteristics in the progression of T2DM and chronic cardiovascular disorders (Ogden et al. 2006). Insulin resistance, the main cause of T2DM is linked with the release of free fatty acids and proinflammatory cytokines from adipose tissues in obesity or excessive adiposity, which stimulates beta cells for over secretion of insulin and reduces its receptors (Kahn et al. 2006). The global prevalence of obesity and overweight raised to almost double with the reported 600 million obese adults and 41 million obese children having high mortality than underweight (WHO, 2016).

Oxidative stress caused by an imbalance of free radicals is a common factor in the occurrence and progression of DM and its complications. The oxidative

mechanisms which intensify DM include triggering transcription factors, advanced glycation of protein and activation of protein kinase-C. Insulin resistance, a leading factor in the development and intensification of T2DM can be linked with the oxidative stress due to the impairment in enzymes action and high level of lipid peroxidation (Maritim et al. 2003). The overproduction of free radicals can be attenuated by intake of antioxidants (Asmat et al. 2016).

Chronic DM leads to serious complications including nephropathy, neuropathy, retinopathy and cardiovascular problems which ultimately increase the risk of diabetic mortality. Diabetic complications are instigated by 'macromolecule aging' phenomena involving non-enzymatic glycation reactions such as nucleophilic addition reactions between the amino groups of proteins and the carbonyl groups of reducing sugars in chronic hyperglycemic conditions (Xi et al. 2008; Abdallah et al. 2017) . Hyperglycemia and oxidative stress are the major factors in accelerating the formation of early glycation products that subsequently rearrange and dehydrate into more stable compounds known as advanced glycation end products (AGEPs) (Xi et al. 2008; Yamagishi et al. 2012). Formation of AGEPs act as positive feedback for oxidative stress that further damages cells and intensifies diabetic complications (Li et al. 2012). A number of compounds have been used to inhibit AGEPs formation such as aminoguanidine but toxicity and adverse effects limit use of these agents (Peng et al. 2008).

Clinically available oral antidiabetic drugs exert their therapeutic effect by various mechanisms including elevation of insulin secretion, glucose absorption and metabolism. α-Glucosidase is a key enzyme that converts disaccharides into simple absorbable monosaccharides within the gastrointestinal tract and α-glucosidase inhibition is therefore a prominent therapeutic strategy to control postprandial hyperglycemia in T2DM. The clinically available α-glucosidase inhibitors including acarbose, voglibose and miglitol are currently administered orally as monotherapeutics or in combination with other oral antidiabetic drugs. However, these compounds are high cost and are known to cause gastrointestinal side effects. In addition, long term use of the commercially available α-glucosidase inhibitors is also associated with cardiac hazards (Fisman et al. 2008). Moreover, both insulin and non-insulin therapy in T2DM promote weight gain probably via adipogenesis, the

foremost cause of T2DM (Carver, 2006; Phung et al. 2010). The therapeutic molecule that can effectively control hyperglycemia with antiadipogenic potential would be an ideal antidiabetic agent. Therefore, adipogenic inhibition and glucose uptake stimulation in adipose and muscle tissue also present the prominent strategies to control obesity associated T2DM (Klein et al. 2007).

Plants are rich sources of various therapeutic molecules. More than 400 medicinal plants have been reported for their antidiabetic potential but their mechanisms of action are known for only 109 plants (Prabhakar et al. 2008). In addition, particular phytochemicals have been identified from plants having antidiabetic potential, i.e. alkaloids, terpenoids, flavonoids, polysaccharides and naphthoquinones. Various plant extracts and phytochemicals have been reported to offer potential as antidiabetic drugs, which function via α-glucosidase inhibition, antioxidant and anti-AGEPs mechanisms (Kumar et al. 2011; Chinchansure et al. 2015; Suantawee et al. 2016). Some of them are effective to combat obesity associated diabetes *via* glucose uptake stimulatory and adipogenic inhibitory potential (Yun, 2010; Arulselvan et al. 2014).

Regarding the natural biodiversity resources, Thailand is among one of the World's top countries that have rich natural biodiversity resources due to its unique geographical location. Thai traditional medicines obtained from natural resources especially medicinal plants play an important role in public health as well as the economy of the country. In 2013, the export value of Thai traditional medicines and herbs was 8.06 million US$. Thai floras consist of almost 10, 250 plants, which are supposed to be 5% of the entire world (Convention on Biological Diversity Thailand, 2015). Many plants containing naphthoquinone compounds are found in Thailand and have been used as a single ingredient or in polyherbal formulations for the cure of various disorders including DM (Farnsworth and Bunyapraphatsara, 1992).

R. nasutus (L.) Kurz (Family Acanthaceae), a medicinal plant native to Thailand, has been traditionally used in the cure of several disorders including DM (Brimson and Tencomnao, 2014). In China and Taiwan, *R. nasutus* has been consumed as an herbal drink (Huang et al. 2015; Li et al. 2017). *R. nasutus* leaf extracts with a broad pharmacological potential, have been reported for various

biological activities including antioxidant, antiglycation and antiobesity (Thephinlap et al. 2013; Sompong and Adisakwattana, 2015; Sompong et al. 2016; Wannasiri et al. 2016).

Rao and Naidu (2010) extensively studied methanol extract (200 mg/kg) of *R. nasutus* leaf for various antidiabetic parameters in streptozotocin (STZ) induced diabetic rat model. The leaf extract markedly reduced the fasting sugar level, normalized the altered lipid profile and enhanced the level of antioxidant enzymes, i.e. superoxide dismutase, catalase, glutathione peroxidase in diabetic rats (Rao and Naidu, 2010; Rao et al. 2011; Rao et al. 2012). It was further reported that methanol extract (200 mg/kg) is also effective to normalize the levels of mitochondrial (succinate dehydrogenase, glutamate dehydrogenase and glucose-6-phosphate dehydrogenase) and liver (glucokinase, phosphofructokinase, and pyruvate kinase) enzymes, and glycogen level in diabetic rats (Rao et al. 2013a; Rao et al. 2013b). Moreover, the same dose of methanol extract significantly normalized the level of liver function markers and protein contents in the liver tissue of diabetic rats (Rao et al. 2013b).

The major phytoconstituents in *R. nasutus* leaf are 1,4-naphthoquinone esters, namely rhinacanthin-C (RC), rhinacanthin-D (RD) and rhinacanthin-N (RN). These compounds are the marker naphthoquinones of *R. nasutus* leaf (Panichayupakaranant et al. 2009). RC is a reddish yellow oily 1,4-naphthoquinone found as a major naphthoquinone in leaves and roots of *R. nasutus*. Recently, RC (5 mg/kg/day and 20 mg/kg/day) has been reported for its hypoglycemic, hypolipidemic and pancreatic protective effects in nicotinamide-streptozotocin induced diabetic rats (Adam et al. 2016). RC decreased blood glucose level and lipid profile as well as exerted pancreatic protective effect by reducing the level of inflammatory and apoptosis mediators i.e. TNFα, Ikkβ and caspase-3 in diabetic rats. An enhanced insulin level was also observed due to higher glucose transporter 2 (GLUT2) level in pancreas. It has been concluded that RC exerts its antidiabetic activity by different mechanisms, i.e. an increased glucose uptake in adipocytes, a pancreatic protection by enhanced antioxidative enzymes and suppressed cellular apoptotic mediators.

However, RC is not available commercially and its isolation involves a time-consuming, energy-intensive, multi-stage process that requires a large amount of

toxic organic solvents, ultimately increase its production costs. An alternative approach that presents the possibility of synergistic effect is to utilize rhinacanthins-rich extract (RRE), a semi purified *R. nasutus* leaf extract that features a total rhinacanthins content of not less than 70% w/w, with 60-70% w/w comprising RC as the major component (Panichayupakaranant et al. 2009). Previously, RRE prepared by conventional extraction methods has been reported for antimicrobial and anti-inflammatory activity (Panichayupakaranant et al. 2009; Bhusal et al. 2014; Puttarak et al. 2010).

1.2. Objectives

In the present study, RRE was prepared using microwave assisted extraction followed by a simple step of fractionation with Amberlite® column. Moreover, only the green solvents, ethanol and water, were used in the extraction and fractionation processes. The marker compounds (RC, RD and RN) were isolated from RRE using a silica gel column chromatography. To investigate the antidiabetic activity of RRE and its marker compounds, we define the following objectives:

1. To investigate *in vitro* α-glucosidase inhibition of RRE, RC, RD and RN, to evaluate their synergistic potential with the standard antidiabetic drug acarbose and to explain the structure activity relationship of potent rhinacanthin using molecular docking studies.
2. To determine free radical scavenging and human albumin glycation inhibitory activity of RRE, RC, RD and RN, and to predict rhinacanthins interaction with albumin using *in silico* predictions.
3. To determine the glucose uptake stimulatory and adipogenic inhibitory effects of RRE, RC, RD and RN in 3T3-L1 adipocytes and L6 myotubes.
4. To determine and compare the hypoglycemic and hypolipidemic properties of RRE and RC using nicotinamide-streptozotocin (NA-STZ) induced diabetic Wistar rats.

SIGNIFICANT RESULTS AND DISCUSSION

2.1. α-Glucosidase inhibition by RRE and its synergism with acarbose

The inhibitory effect of RRE and its marker compounds RC, RD and RN against α-glucosidase was assessed in order to assess their antidiabetic potential. Both RRE and RC exhibited satisfactory inhibitory activity against α-glucosidase with IC_{50} values of 25.0 µg/mL and 22.6 µg/mL, that was much higher activity than acarbose (IC_{50} value of 395 µg/mL) (**Paper II**). RRE and RC display almost equal α-glucosidase inhibitory activity that similar to previously been reported to possess almost equal antimicrobial and anti-inflammatory activity (Panichayupakaranant et al. 2009; Bhusal et al. 2014; Puttarak et al. 2010). RD, a minor naphthoquinone ester of RRE, also showed good inhibitory activity, with an IC_{50} value of 71.5 µg/mL, while RN was found to be inactive. This implies that an aromatic ring on substituted R group of rhinacanthins (**Paper I**) may reduce their α-glucosidase inhibitory effect by interference to the enzyme binding site.

To determine the mechanism of inhibition, RRE and RC were used in three different concentrations i.e. 12.5, 25 and 50 µg/mL as inhibitors in kinetic experiment to elucidate the type of inhibition. Possible interference by RRE and RC was examined at five different concentrations of para nitrophenyl α-D-glucopyranoside (*p*NPG) i.e. 0.16 to 2.65 mM. The absorbance was first plotted against time to obtain velocities of reactions and the velocities were subsequently plotted against the reciprocal of substrate concentration to construct Linearweaver-Burk plots. The Lineweaver–Burk plots for α-glucosidase inhibition by RRE and RC generated straight lines, which intersected at the same point on X-axis in the second quadrant, indicating noncompetitive inhibition (**Paper II**). Acarbose is a competitive α-glucosidase inhibitor (Ag, 1994), thus it was of interest to establish whether RRE and RC, as noncompetitive inhibitors, might interact synergistically with acarbose in inhibiting α-glucosidase. The experiment was performed at three different concentrations at $\frac{1}{4}IC_{50}$, $\frac{1}{2}IC_{50}$ and IC_{50}. It was found that lower concentrations of acarbose combined with RRE or RC at $\frac{1}{4}IC_{50}$ and $\frac{1}{2}IC_{50}$ resulted in significant inhibition compared with the individual compounds at the same concentration (**Paper II**) indicating synergistic inhibitory activity against α-glucosidase. These findings suggest that the combination of acarbose with RRE or RC having different inhibitory

mechanisms could inhibit α-glucosidase activity more effectively at low doses compared with the single compounds, resulting in a reduction of postprandial blood glucose in T2DM and avoiding adverse effects due to acarbose.

2.2. Molecular docking studies on RC interaction with α-glucosidase

The mechanism of α-glucosidase inhibition by RC was elucidated by molecular docking (MD) studies according to the protocol previously described study (Barakat et al. 2016). Molecular Operating Environment-dock was used to generate 100 conformations of RC. The resultant poses were clustered and the pose presenting the highest score for the largest cluster was selected for further analysis. The binding mode and interaction profile of RC with yeast α-glucosidase is represented in (**Paper II**). The molecular surface model of the protein suggested that the presence of basic residues around the naphthoquinone ring of the ligand complements binding of the relatively more electronegative part of the ligand. The tail of the ligand containing an aliphatic chain has folded to accommodate itself in the hydrophobic groove of the protein.

Ligand-protein interaction profiling confirmed the formation of various hydrogen and hydrophobic bonds between RC and α-glucosidase (Salentin et al. 2015). As depicted in **Paper II**, the core of RC has anchored to the protein via special bidentate interactions with LYS232 and LYS414. Another hydrogen bond has also been observed between the backbone N atom of SER157 and RC. The hydrophobic core that surrounds the tail of the ligand includes residues LYS143, PRO144, THR160 and PHE161. The observed affinity in molecular modeling studies between the ligand and enzyme that helps to explain the experimental findings of high inhibition of α-glucosidase activity by RC.

In order to investigate the stability of the proposed ligand-protein model, a short production run (10 ns) of all atom MD simulation was performed using AMBER14. MD simulation has emerged as a major technique in the array of tools to design bioactive molecule and investigate their mode of action. Apart from extraction of information regarding the distances and interactions between ligand and residues of interest, MD trajectories allow estimation of overall stability of complex. We have measured different characteristics of the system to measure the dynamic differences induced in system upon ligand binding. Root Mean Square Deviation (RMSD) is a

measure of stability of complex, the lower the RMSD the higher the stability. The average RMSD values of the two systems; apo and complex, were 1.5 and 2.0 Å, respectively. The plot shows more fluctuation in complex as compared to apo-structure, though the overall system remains unstable (**Paper II**). Further, to investigate the exact nature of this deviation, Root Mean Square Fluctuation (RMSF) was calculated (**Paper II**). We have observed higher fluctuations in case of α-glucosidase-RC complex with notably higher deviations in case of residues LYS229, ASP278, and GLU548. Visual inspection of these residues annotated by secondary structure suggests that these residues are present in exposed loops which are highly dynamic in nature. The visual analysis of MD trajectories revealed that the ligand anchored the protein via hydrogen bonds during first 2 ns of the simulation. The ligand presented significant displacement and there is a difference of 2.46 Å in the coordinates before and after simulation. Moreover, it was observed that the contacts were later stabilized by hydrophobic interactions displayed by ligand.

2.3. Antioxidant activity of RRE with *in silico* biopredictions

The free radical scavenging ability of RRE and its marker compounds was assessed by adding increasing volumes of RRE (10 to 25 µL), RC (12 to 40 µL), RD (50 to 350 µL) and RN (20 to 200 µL) solutions to react with the electrochemically generated superoxide (**Paper III**). RRE exhibited the highest superoxide scavenging activity with IC_{50} values of 8.0 µg/mL. These results are significantly higher than the marker naphthoquinone esters, RC (IC_{50} value 9.6 µg/mL) RD (IC_{50} value 91.4 µg/mL) and RN (IC_{50} value 45.1 µg/mL) (**Paper III**). These *in vitro* findings support a previous *in vivo* study on enhanced antioxidative enzymes in liver and pancreas of diabetic rats by *R. nasutus* leaf methanol extract and RC (Rao et al., 2012; Adam et al., 2016).

Various kinetic parameters such as antioxidant activity coefficient (Kao), binding constant (Kb) and spontaneity of the interaction (ΔG) were studied for RRE, RC, RD and RN (**Paper III**). Based on the irreversible scavenging of superoxide and the values of kinetic parameters, RRE may be classed as a potent superoxide scavenger, operating *via* E_rC_i mechanism and probably involving a synergistic effect due to the combination of rhinacanthins (**Paper III**).

PASSonline was used to predict the potential targets and pharmacological effect of the marker compounds of RRE based on structural information. The analysis presents the ratio of probability of being active (Pa) or inactive (Pi) with regards to a particular biological effect (Kadir et al. 2013). The antioxidant potential and selection of predicted biological activities for the marker compounds of RRE presented (**Paper III**), provides additional strong support for the superoxide scavenging activity of RRE.

2.4. Antiglycation activity of RRE and interaction studies of rhinacanthins

The fructose mediated HSA glycation inhibitory activity of RRE and its marker compounds was evaluated to explore their potential role in treating diabetic complications. Previous reports rationalized the anti-AGEPs activity of RRE, RC, RD and RN on the basis of their 1,4-naphthoquinone skeletal structure (Sompong and Adisakwattana, 2015; Jung et al. 2005). In the present study, both RRE and RC were found to exhibit significant glycation inhibitory activity with IC_{50} values of 39.7 and 37.3 µg/mL, respectively that were slightly higher than that of the positive control, rutin (41.5 µg/mL) (**Paper III**). RRE and RC showed nearly equal antiglycation activity, similar to previously reported anti-inflammatory and antimicrobial activities (Bhusal et al. 2014; Puttarak et al. 2010). Furthermore, the antiglycation activity of RC supports the findings of a study by Adam et al. (2016) in which RC caused a reduction of glycated hemoglobin levels in diabetic rats. RD and RN, the minor naphthoquinone compounds of RRE also showed impressive antiglycation activity with IC_{50} values of 50.4 and 89.5 µg/mL, respectively (**Paper III**). Diabetes and age-related diseases including neurotoxic disorders are mainly caused by the unusual protein aggregation (Li et al. 2012). Thus, the potent anti-AGEPs activity of RRE, measured in this study recommends further evaluation of RRE as a therapeutic agent for treatment of a range of conditions of major clinical and global significance.

We applied molecular docking protocols to explore binding between human serum albumin (HSA), the major transport protein in the circulatory and lymphatic system (Nicholson et al. 2000) and RRE and its marker compounds (RC, RN and RD) and thereby help to explain their observed anti-glycation activity. The non-enzymatic glycation of lysine and arginine residues in HSA; in the case of diabetes, impairs the transport of several moieties leading to detrimental physiological

effects (Iqbal et al. 2016). Masking of the lysine and arginine residues has therefore been proposed as an effective strategy to inhibit non-enzymatic glycation of HSA. There are two main sites in the HSA structure which offer opportunities for drug action; Sudlow's site I and site II. Docking simulations were performed using both sites to investigate ligand binding. Sudlow's site I was identified using the coordinates of warfarin from the PDB: 2BXD while, ibuprofen from PDB: 2BXG identified Sudlow's site II (Ghuman et al. 2005). The docking scores of each compound calculated for the Sudlow's site I and II of HSA are presented in **Paper III.**

The binding mode of RC, RD and RN in each site is presented in **Paper III**. The compounds establish polar contacts with surrounding arginine and lysine residues. In Sudlow's site I, RC forms hydrogen bonds with Lys195, and Arg222, RD binds to Lys199 and Arg257 and RN interacts with Lys195, Lys199, Arg218 and Arg222. The rhinacanthins are also involved in the formation of salt bridges. In the case of Sudlow's site II, RC, RD and RN were found to interact with Arg410 and Lys 414. The complexes are also stabilized by Van der Waal's forces between the ligands and other amino acid residues at Sudlow's site I, namely Tyr150, Leu238, and Leu260. Residues Ile388, Asn391 and Phe403 lining Sudlow's site II provided anchorage for the ligands *via* formation of hydrophobic and aromatic contacts. Interestingly, the binding pattern of RC, RD an RN in this analysis is consistent with docking studies of cinnamic acid reported earlier (Qais et al. 2016).

2.5. Glucose uptake stimulatory effect of RRE in 3T3-L1 and L6 cells

Insulin resistance is the major cause of T2DM, the search of small molecules with insulin like glucose uptake stimulation potential is an effective approach in diabetic treatment. Based on the previous glucose uptake report of RC (Adam et al. 2016), RRE and its naphthoquinone constituents, RC, RD and RN were evaluated for their glucose uptake stimulation effect in differentiated 3T3-L1 adipocytes using glucose oxidase method. The results showed that RRE and RN exhibited higher glucose uptake stimulation effect than RC and RD, and in a dose dependent manner (5, 10 and 20 μg/mL). The activity at concentration of 20 μg/mL was nearly equal to the positive control, insulin (0.58 μg/mL) (**Paper IV**). The mechanism of glucose uptake enhancement by 1,4-naphthoquinones of RRE may be *via* an insulin independent tyrosine kinase pathway, which is previously reported for

shikonin, a 1,4-naphthoquinone of *Lithospermum erythrorhizon* (Kamei et al. 2002). This is a preliminary study (**Paper IV**); however it provides an interesting research insight to elucidate in depth and exact glucose enhancement mechanism of rhinacanthins in 3T3-L1 adipocytes. Furthermore, the glucose uptake stimulation along with adipogenic inhibitory potential of RRE provides an interesting strategy to control obesity associated T2DM and other related complications.

Regarding the body mass, skeletal muscles are the major body part which utilizes 80% of blood glucose, presenting a prominent therapeutic target for diabetic treatment. Based on the previous reports on muscular glucose uptake stimulatory potential of 1,4 naphthoquinone (Oberg et al. 2011; Sunil 2012), RRE and its naphthoquinone compounds (RC, RD and RN) were determined for their glucose uptake enhancement potential in L6 myotubes. RRE possessed higher glucose uptake enhancing activity than RC, RD and RN in a dose dependent manner (0.63, 1.25 and 2.5 μg/mL) (**Paper IV**). RRE at a dose of 2.5 μg/mL showed nearly equal glucose uptake stimulating activity (>80%) to insulin (2.90 μg/mL) and almost 87-folds higher than that of metformin (219.5 μg/mL). The strong glucose uptake stimulatory potential of RRE might be due to the possible synergism among the component rhinacanthins as previously reported in antimicrobial and anti-inflammatory activities (Bhusal et al. 2014; Puttarak et al. 2010). These results provide a strong base for further detail mechanistic study of glucose uptake stimulation by rhinacanthins in L6 myotubes that could be insulin dependent via glucose transporter 4 (GLUT4) or insulin independent calcium dependent pathway, as previously reported for other natural 1,4 naphthoquinones (Oberg et al. 2011; Sunil et al. 2012).

2.6. Adipogenic inhibitory effect of RRE in 3T3-L1 adipocytes

Adipogenesis or excess intracellular lipid accumulation is the main factor behind obesity and insulin resistance that leads to T2DM. Adipogenic inhibitory property is therefore an effective strategy to control these pathological disorders (Zeng et al. 2012). RRE and its naphthoquinone compounds (RC, RD and RN) showed potent and comparable dose dependent adipogenic inhibitory activity in 3T3-L1 adipocytes (**Paper IV**). At the highest effective dose (20 μg/mL), the antiadipogenic activity of RRE (<20% intracellular lipids) was significantly equal to RC but higher than RD (20.5% intracellular lipids) and RN (39% intracellular lipids).

The microscopic images of stained lipid droplets in various treated cells further confirmed the consistent dose dependent adipogenic inhibition by RRE and its marker compounds (**Paper IV**). The antiadipogenic potential of RRE correlated with the previous report of shikonin that inhibited adipogenesis *via* inhibiting FABP4 and LPL genes expression (Le et al. 2010). 1,4-Naphthoquinones exert their antiadipogenic activity by both upstream (SREBP1C) and downstream (PPARγ and C/EBPα) regulations (Lee et al. 2010). Rhinacanthins should be therefore subjected to further study on antiadipogenic molecular mechanism. Apart from diabetes, obesity has been reported to be linked with atheromas, cardiovascular disorders and malignant tumors (Park et al. 2007). The epidemiological reports interlinked obesity with metabolic disorders which is further associated with the increased circulation of inflammatory adipocytokines such as leptin, interleukin-6 and tumor necrotic factor, which resulting in malignant growth enhancement (Hsing et al. 2007). Adipocytes are supposed to be responsible for the release of tumor enhancing adipocytokines (Percik et al. 2009). The antiadipogenic effect of rhinacanthins could protect against malignancy *via* reduction in tumor enhancing and inflammatory adipocytokines, which can be correlated with the previous anti-inflammatory activity of rhinacanthins (Bhusal et al. 2014).

2.7. Effect of RRE on different parameters in diabetic rats

In the present study (**Paper V**), oral administration of RRE (24.11 mg/kg equivalent to 15 mg/kg RC content), RC (15 mg/kg) and the standard drug glibenclamide (600 µg/kg) were comparatively evaluated for their hypoglycemic and hypolipidemic activities in NA-STZ induced diabetic rats for 28 days. Diabetes was confirmed by hyperglycemia along with characteristic symptoms i.e. polydipsia (thirst), hyperphagia (appetite) and weight loss in NA-STZ induced diabetic rats**.** The initial water and food intake and body weight of nondiabetic and diabetic rats were comparable. After four weeks, a significant rise in water and food intake with reduction in body weight was observed in diabetic rats in comparison with the normal rats. Oral administration of RRE (24.11 mg/kg) and RC (15 mg/kg) significantly normalized the water and food intake, and body weight of diabetic rats without any significant effect on the normal rats (**Paper V**)**.**

In the current study, NA along with STZ has been used for partial destruction of pancreas to develop T2DM model which was confirmed by higher fasting blood glucose (FBG) and lower serum insulin levels in the diabetic rats (**Paper V**). In normal control and nondiabetic rats receiving 24.11 mg/kg of RRE and 15 mg/kg of RC, the FBG and insulin levels were consistently normal throughout the 28 days (**Paper V**). Diabetic rats treated with RRE (24.11 mg/kg), RC (15 mg/kg) or the standard drug Glb (600 μg/kg) gradually decreased the hyperglycemia and markedly increased the serum insulin throughout the experimental period of 28 days (**Paper V**). Furthermore, it is evident from the histopathological images of pancreas (**Paper V**) that RRE markedly decreased beta cells destruction in diabetic rats. Pancreatic protective effect of RRE can be correlated to the previous study on its marker compound RC (Adam et al. 2016). During hyperglycemia, the excess sugar reacts with the protien resulting in glycated hemoglobin (HbA1c) which is a laboratory marker of diabetes and the associated risk of diabetic complications due to the formation of advanced glycation products (**Paper III**). In the present study, the increased HbA1c level in diabetic rats was significantly reduced by RRE (24.11 mg/kg) and RC (15 mg/kg) comparatively with the standard drug Glb at a dose of 600 μg/kg (**Paper V**). However, there was no significant effect on the HbA1c level of normal treated rats. The results can be correlated with our previous *in vitro* study on the antiglycation potential of RRE and RC (**Paper III**).

Insulin deficiency instigates the hormone sensitive lipase which stimulates fatty acid release from adipocytes (Al-Shamaony et al. 1994). The extra fatty acid enhances the production of phospholipids and cholesterol in hepatocytes. The elevated levels of phospholipids and cholesterol along with triglycerides in serum are the biomarkers of hyperlipidemia (Rajasekaran et al. 2006). In the present study, the serum levels of TC, TG, HDL and LDL were significantly normalized by RRE (24.11 mg/kg), RC (15 mg/kg) and Glb (600 μg/kg) (**Paper V**). However, there was no significant lipid profile alteration of normal rats treated with RRE and RC at same dose. The hypolipidemic results of RRE are consistent with previous report on hypolipidemic activity of crude methanol extract (Rao et al. 2010) and RC (Adam et al. 2016).

Liver is the insulin dependent organ for production of carbohydrate metabolizing enzymes essential for accumulation and consumption of glycogen. Low insulin level particularly in DM markedly reduced the expression of these enzymes and other important proteins (Hikino et al. 1989). Further, the insulin deficiency severely disturbs the carbohydrate and fat metabolism (Rao et al. 2013b). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are the key enzymes which are known to be the liver function marker (Williams et al. 1988). It is indicated (**Paper V**) that diabetic rats have elevated level of AST and ALT which is significantly reduced by RRE (24.11 mg/kg), RC (15 mg/kg) and Glb (600 μg/kg). However, there is no effect of RRE and RC administration on liver markers of normal control rats (**Paper V**). These results show the hepatic safety of RRE (24.11 mg/kg) and RC (15 mg/kg).

DM is a metabolic disorder which affects all the vital organs including kidney, diabetic nephropathy is a one of the major diabetic complication. Oxidative stress, protien glycation and hyperlipidemia are the prominent diabetic factors which are responsible in the occurrence of diabetic nephropathy (El Barky et al. 2016). The serum BUN and creatinine levels are the important markers to evaluate the kidney function in nephropathic condition (Ronco et al. 2010). In the current study, the elevated level of BUN and creatinine were markedly reduced by RRE (24.11 mg/kg), RC (15 mg/kg) and the standard drug Glb (600 μg/kg) in diabetic rats (**Paper V**). However, there was no significant effect of RRE and RC administration on BUN and creatinine level of normal rats which indicate the renal safety of the selected dose of RRE and RC. The results can be correlated with previous *in vitro* reports on the antioxidant and antiglycation potential of RRE and RC (**Paper III**).

2.8. Pharmacokinetic and toxicity properties of RC

In order to identify the pharmacokinetic and toxicity properties of RC, various *in silico* calculations were performed. The RC complies well with the Lipinski's rule of drug-likeliness with a violation count value of 0 (**Paper V**). RC was found to be BBB+ (0.44) that suggesting that it can penetrate in the blood brain barrier which can be correlated with a recent report on the modulatory effect of RC through high-mobility group box 1 related pathway to attenuate brain apoptosis in the pathogenesis of subarachnoid hemorrhage (Chang et al. 2016). In this study (**Paper**

V), ADMET profile of RC is presented as established by using admetSAR, a free web based tool for evaluation of ADMET properties.

As mentioned in **Paper V**, RC can be absorbed by both human intestine and brain. It was also predicted to display Caco-2 monolayer permeability. The Caco-2 serves as *in vitro* model of human intestinal mucosa for prediction of the oral bioavailability of a drug (Fricker and Millar, 2002). The observation is consistent with the fact that the compound was found to comply with Lipinksii rules which define properties of orally available drugs (Leeson, 2012). Though, the drug was found to be both substrate and inhibitor of P-glycoprotein (**Paper V**), the probability of being an inhibitor is greater. The P-glycoprotein plays a significant role in drug absorption and deposition by actively transporting a drug from cell cytoplasm to the intestine, thus limiting the oral bioavailability of the drug (Fricker et al. 2002; Fromm, 2000). As an inhibitor of P-glycoprotein, RC may present good bioavailability and the drug concentration shall remain stable.

Human Ether-A-Go-Go-Related gene (hERG) has emerged as an important anti-target in the drug development. Therefore, the hERG inhibitory potential of a drug is assessed at the preclinical stages during pharmaceutical testing (Nogawa and Kawai 2014). The hERG inhibitory potential of the RC was evaluated using Pre-ADMET and admetSAR web servers. RC presented medium to low risk of being hERG inhibitor. The compounds having LD_{50} between 500-5000 mg/kg are included in category III of acute oral toxins. However, as suggested by the experimental results, RC was found to be effective at a dose of 15 mg/kg which suggest the therapeutic window of the drug dose is many folds lower than the lower LD_{50}.

Cytochrome P450 is a family of isozymes responsible for the bio-transformation of several drugs (Ogu and Maxa 2000). This study (**Paper V)** shows the tendency of RC as CYP450 3A4 substrate. The presence of this isoform in liver and intestine pronounce these organs as sites of clearance of RC. Server Xenosite was used to identify the potential sites of metabolic-oxidation carried out by CYP450 3A4 (**Paper V**). The terminal methyl group seems to be most vulnerable to oxidation based degradation followed by the benzene ring.

CONCLUDING REMARKS

This thesis was focused to investigate the antidiabetic potential of RRE from *R. nasutus* leaves using a green extraction method in comparison to its marker compounds and standard antidiabetic drugs. For this purpose, various antidiabetic experimental models such as *in vitro* enzyme (α-glucosidase) inhibition and non-enzyme inhibition (antioxidant and antiglycation) assays, cell-based (glucose uptake and antiadipogenic) assays and *in vivo* study were used. Moreover, *in silico* studies were also performed to predict the structure activity relationship, pharmacokinetic and toxicity profiles. From the results of undertaken study the following conclusions can be drawn;

1. RRE containing RC at a level of 62.2% w/w was obtained from *R. nasutus* leaves using a green extraction method. RRE exhibited a non-competitive inhibitory effect against α-glucosidase. RRE displayed synergistic inhibition of α-glucosidase in combination with acarbose, suggesting its clinical use to reduce the dose and adverse effects related to acarbose and may be considered as an alternative natural antidiabetic agent to control postprandial blood glucose levels. This is the first report on α-glucosidase inhibitory activity of *R. nasutus* leaf extract and its marker compounds.
2. RRE and its marker compounds showed potent superoxide scavenging and antiglycation effects. The docking studies determined the binding mode of rhinacanthins with respect to human serum albumin. Rhinacanthins exhibited antiglycation activity by masking different residues of albumin. This is the first report on antioxidant and antiglycation potential of RRE and its marker compounds. The potent superoxide scavenging and remarkable protein glycation inhibitory effects of RRE further rationalized its therapeutic application in various chronic diseases especially in the complications of diabetes.
3. RRE showed potent glucose uptake stimulatory and antiadipogenic effects in 3T3-L1 adipocytes and L6 myotubes. These results provide a strong base for further detail mechanistic study of glucose uptake stimulation by rhinacanthins in L6 myotubes that could be insulin dependent via GLUT4 or insulin independent calcium dependent pathway, as previously reported for other natural 1,4 naphthoquinones. RRE may be used as a potential candidate for antidiabetic and

antiobesity drug development. Further mechanistic *in vivo* antiobesity studies of RRE and safety assessment are recommended.

4. RRE showed significant hypoglycemic and hypolipidemic effects comparable to RC. In term of green processing, low production cost and remarkable antidiabetic potential, RRE is more suitable candidate for antidiabetic drug development.

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PAPER I

Review: Antidiabetic naphthoquinones and their plant resources in Thailand

Muhammad Ajmal Shah[a], James Keach[b], Pharkphoom Panichyupakaranant[a, c,*]

[a]Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

[b]Research and Horticulture Department, Gardens by the Bay, 018953, Singapore

[c]Phytomedicine and Pharmaceutical Biotechnology Excellence Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

*Corresponding author. Pharkphoom Panichyupakaranant. Tel. /fax: +66 74 428220.

E-mail address: pharkphoom.p@psu.ac.th (P. Panichayupakaranant).

Abstract

Diabetes mellitus is the seventh leading cause of death globally. Ninety percent of the diabetic population suffers from type-2 diabetes, which still needs an effective, safe and economical oral hypoglycemic therapy. Plants are rich sources of various therapeutic molecules. More than 400 medicinal plants that contain interesting phytochemical diversity have been reported for their antidiabetic potential. Naphthoquinones are a group of phytochemicals which have a wide range of pharmacological potential, including antidiabetic activity. Naphthoquinones exert their antidiabetic effects through various mechanisms such as inhibition of α-glucosidase and protein tyrosine phosphatase 1B, increased glucose uptake in myocytes and adipocytes *via* GLUT4 and GLUT2 translocations, enhanced PPARγ ligand activity, and normalizing carbohydrate metabolizing enzymes in the liver. Moreover, naphthoquinones inhibit adipogenesis by both upstream and downstream regulation to control obesity, which is one of the important risk factors for diabetes. Naturally occurring naphthoquinones, as well as their plant sources, are therefore of interest for exploring their antidiabetic potential. The present review aims to overview the antidiabetic potential of naphthoquinones and their plant resources in Thailand.

Key words: hypoglycemic, medicinal plant, naphthoquinone, adipogenesis, obesity

Introduction

Diabetes mellitus (DM) is a chronic metabolic hyperglycemic disease, characterized by altered metabolism of carbohydrates, lipids, and proteins due to a defect of insulin secretion or body tissue resistance to insulin**.** Two forms of diabetes (type-1 and type-2) differ in their pathogenesis, but both have hyperglycemia as a common symptom.[1)] Type-1 DM is due to loss of the insulin-secreting beta cells whereas type-2 DM is due to impairment in insulin secretion combined with or without impairment of insulin action. In 2015, more than 4 million cases of DM have been reported in Thailand, while DM prevalence was 8% for people between the ages of 20 and 79 years old.[2)] The only treatment for type-1 DM is insulin administration whereas type-2 DM can be treated with commercially available oral antidiabetic drugs. However, many problems are associated with the long-term use of the currently available hypoglycemic drugs, such as their cost and cardiac hazards. It is therefore a challenging task to search a molecule which is devoid of the undesirable adverse effects in existing drugs.[3)]

Plants are rich sources of various therapeutic molecules. More than 400 medicinal plants have been reported for their antidiabetic potential but the mechanisms of action are known for only 109 of these plants.[4)] In addition, particular phytochemicals have been identified from plants having antidiabetic potential, i.e. alkaloids, terpenoids, flavonoids, polysaccharides, and naphthoquinones. Thailand is among one of the world's top countries in terms of natural biodiversity resources, due to its unique geographical location.[5)] Thai traditional medicines are obtained from natural biological resources, especially medicinal plants, and play an important role in

public health as well as the economy of the country. In 2013, the export value of Thai traditional medicines and herbs was $8.06 million USD. Thai flora consist of almost 10,250 described species, which makes up approximately 5% of the diversity in the entire world.[5] Many plants containing naphthoquinone compounds are found in Thailand and have been used both as single ingredient preparations or in polyherbal formulations for the treatment of various disorders including DM.[6] In this paper, the authors put emphasis on summarizing the antidiabetic potential of naphthoquinones and their plant resources, as well as mechanistic insight of naphthoquinones in diabetes amelioration.

Naphthoquinones containing plants in Thailand

Naphthoquinones are biologically active naturally occurring compounds found in various plant families, including Avicenniaceae,[7] Acanthaceae,[8,9,10,11] Balsaminaceae, Bignoniaceae,[12,13] Boraginaceae,[14] Droseraceae,[15,16] Ebenaceae,[17,18] Juglandaceae,[19] Lythraceae, Nepenthaceae[20] and Plumbagnaceae.[21,22] The common naphthoquinones found in these plant families are shown in Figures 1-6. They are biosynthesized via various biosynthetic pathways, such as the acetate and malonovate pathways for plumbagin and the shikimate pathway for lawsone.[23] Known naphthoquinone-containing plants are summarized in Table 1. Some of these exhibited antidiabetic activity in previous studies. In addition, they have also been reported to possess other medicinal applications, such as antimicrobial,[24,25] antiviral,[26] anticancer,[27] anti-inflammatory,[28,29] antipyretic,[29] analgesic, [29] antioxidant,[30] antihemolytic,[31] immunomodulatory,[32] antiallergic,[33] neuroprotective,[34,35] antiglycation,[36] and antiobesity[37] activities (Table 2).

Table 1

Table 2

Antidiabetic potential of naphthoquinone containing plants in Thailand

Due to their unique phytochemical diversity, plants or natural medicines are considered to be more effective in treating chronic diseases, including DM. *Rhinacanthus nasutus* (L.) Kurz (Family Acanthaceae), a medicinal plant native to Thailand, has been traditionally used in the cure of several disorders including DM.[30)] *R. nasutus* has been reported to express various biological activities, as shown in Table 2. Rao et al. (2010) extensively studied a methanol extract of *R. nasutus* leaf (200 mg/kg) for various antidiabetic parameters in a streptozotocin (STZ) induced diabetic rat model. The leaf extract markedly reduced the fasting sugar level and normalized the altered lipid profile and the level of antioxidant enzymes, i.e. superoxide dismutase, catalase, and glutathione peroxidase in diabetic rats.[125,126)] The methanol extract (200 mg/kg) was also effective for normalizing the levels of mitochondrial (succinate dehydrogenase, glutamate dehydrogenase and glucose-6-phosphate dehydrogenase) and liver (glucokinase, phosphofructokinase, and pyruvate kinase) enzymes, as well as glycogen level in diabetic rats.[127,128)] Moreover, the same dose of methanol extract significantly normalized the level of liver function markers and protein contents in the liver tissue of diabetic rats.[128)]

The major phytoconstituents in *R. nasutus* are 1,4-naphthoquinone esters (Fig 1), namely rhinacanthin-C **(3)**, rhinacanthin-D **(4)** and rhinacanthin-N **(12).** These compounds are the marker naphthoquinones in *R. nasutus* leaves.[24)] Rhinacanthin-C is a reddish-yellow, oily 1,4-naphthoquinone found as a major

naphthoquinone in leaves and roots of *R. nasutus*. Recently, rhinacanthin-C (5 mg/kg/day and 20 mg/kg/day) has been reported for its hypoglycemic, hypolipidemic, and pancreatic protective effects in STZ nicotinamide induced diabetic rats.[129) Rhinacanthin-C normalized the blood glucose level and lipid profile as well as exerting a pancreatic protective effect by reducing the level of inflammatory and apoptosis mediators i.e. TNFα, Ikkβ, and caspase-3 in diabetic rats. An increased insulin level was also observed due to higher GLUT2 levels in the pancreas. It has been concluded that rhinacanthin-C exerts its antidiabetic activity by different mechanisms, i.e. an increased glucose uptake in adipocytes, pancreatic protection by enhanced antioxidative enzymes, and cellular apoptotic mediators. Currently, our group is working on the antidiabetic potential of a rhinacanthin-rich extract and its marker compounds, i.e. rhinacanthin-C, rhinacanthin-D and rhinacanthin-N.[24) The rhinacanthins-rich extract exhibited α-glucosidase inhibitory activity (IC_{50} value of 25.0 µg/mL) almost equivalent to that of purified rhinacanthin-C (IC_{50} value of 22.6 µg/mL) but stronger than that of the standard drug, acarbose (IC_{50} value of 395.4 µg/mL). Furthermore, the rhinacanthins-rich extract and rhinacanthin-C, being non-competitive α-glucosidase inhibitors, showed synergistic activity with acarbose (competitive inhibitor).[130) In an antiglycation assay, the rhinacanthins-rich extract (IC_{50} value of 39.7 µg/mL) and rhinacanthin-C (IC_{50} value of 37.3 µg/mL) showed a higher inhibitory effect than that of the positive control, rutin (IC_{50} value of 41.5 µg/mL).[131) Furthermore, the rhinacanthins-rich extract and rhinacanthin-C were also studied for their glucose uptake stimulatory effect in 3T3-L1 and L6 cells.[132) The results indicated that the rhinacanthins-rich extract (20 µg/mL) and rhinacanthin-C (20 µg/mL) enhanced glucose uptake in 3T3-L1 adipocytes, which was comparable to

the standard drug treatment with insulin (0.58 µg/mL).[132] In contrast, rhinacanthins-rich extract (2.5 µg/mL) and rhinacanthin-C (2.5 µg/mL) exhibited stronger activity for enhanced glucose uptake in L6 myotubes than that induced by the standard drugs insulin (2.9 µg/mL) and metformin (219.5 µg/mL).[132] Moreover, the rhinacanthins-rich extract and rhinacanthin-C were also evaluated for adipogenic inhibition in 3T3-L1 adipocytes.[132] The results indicated that both the rhinacanthins-rich extract and rhinacanthin-C at 5, 10 and 20 µg/mL showed satisfactory dose dependent adipogenic inhibition.[132]

Kigelia africana (Lam.) Benth. (Family Bignoniaceae), a medicinal and ornamental tree native to Thailand and Africa, has been traditionally used in the treatment of several disorders, including DM.[55] The phytochemical profile of *K. africana* contains over 145 phytochemicals, which have been purified from its various parts. One of the major chemical constituent groups of *K. africana* are naphthoquinones, which are shown in Figure 2. Due to its unique phytochemical diversity, *K. africana* has been reported to possess various biological activities (Table 2). A methanol extract (250 and 500 mg/kg) of *K. africana* flower has been reported as showing antidiabetic potential in STZ induced diabetic rats.[133] During an experimental period of 21 days *K. africana* extract successfully normalized the glycemic level and lipid profile of the test subjects. In addition, *K. africana* leaf extract (100-400 mg/kg) has been tested for its antidiabetic potential in an alloxan induced diabetic rat model. The results indicated that the extract markedly reduced the blood glucose level in diabetic rats.[134] In a separate study, extracts of *K. africana* leaves, using acetone, ethanol, chloroform, and water, were tested for α-amylase

inhibition;[135] the ethanol extract (500 μg/mL) showed the highest α-amylase inhibition.

Diospyros kaki L. (Ebenaceae), a popular, fruit-producing plant native to China, has traditionally been used for the treatment of various diseases in many regions of the world, including Thailand.[65] Various biological activities have been reported for *D. kaki,* as shown in Table 2. Its phytochemical profile contains a number of naphthoquinones, which have been isolated from different parts of the plant (Fig. 3). Deng et al. (2011) investigated the antidiabetic activity of various extracts (ethanol, ethyl acetate, butanol and water) of *D. kaki* and found that the extracts markedly reduced hyperglycemia and increased insulin sensitivity index in STZ induced diabetic rats.[136] Jung et al. (2012) studied the effect of 5% w/w *D. kaki* leaf powder in mice for a period of 35 days and found that the leaf powder normalized the blood sugar and lipid profile of the mice. The hypoglycemic effect was additionally related to reducing the level of gluconeogenesis enzymes and increasing the level of glycogen. It also decreased lipogenesis by decreasing peroxisome proliferator-activated receptor gamma (PPARγ): inhibiting the gene expression and reducing the lipogenic enzymes activity. These experiments show that *D. kaki* leaf powder can successfully reduce the risk of obesity-associated type 2 DM.[137]

Plumbagnaceae is a diverse family of 24 genera and 775 species, many of which are naphthoquinone containing.[23] *Plumbago zeylanica* L. and *Plumbago indica* L. are two well-known species found in Thailand. Naphthoquinones (Fig. 4) are the principal chemical constituents responsible for the pharmaceutical potential of *P. zeylanica* and *P. indica* (Table 1). Plumbago extracts and purified naphthoquinones

have been reported to exhibit various pharmacological activities (Table 2), including against DM. Published reports evaluating antidiabetic effects are available only for *P. zeylanica*. Olagunju et al. (1999) demonstrated the antidiabetic activity of an ethanol extract of *P. zeylanica* root (400 mg/kg) in STZ induced diabetic rats. This extract controlled the glycemia by reducing glycolytic enzyme levels (hexokinase, phosphofructokinase, pyruvate kinase and lactate dehydrogenase) and increasing the synthesis of muscular protein.[138)] Zarmouh et al. (2010) studied the effect of an ethanol extract of *P. zeylanica* root (100, 200 mg/kg) on hyperglycemia and liver enzyme levels in STZ induced diabetic rats.[139)] Their results indicated that the ethanol extract normalized the levels of liver enzymes (hexokinase, decreased hepatic glucose-6-phosphatase, serum acid phosphatase, alkaline phosphatase and lactate dehydrogenase) in diabetic rats.[139)] Furthermore, it was confirmed that the antidiabetic potential of extracts from *P. zeylanica* roots is due to their principal naphthoquinone i.e. plumbagin (**26**).[140)] The study supporting this was performed in STZ induced diabetic rats. Plumbagin (15 and 30 mg/kg) exerted antidiabetic effects by enhancing insulin secretion from β-cells. Along with alteration of other biochemical parameters, plumbagin significantly normalized the levels of carbohydrate metabolism enzymes i.e. glucose-6-phosphatase, fructose-1, 6-bisphosphatase, and hexokinase. The strong antidiabetic mechanism of plumbagin was explained by restoring the expression and translocations of GLUT4 in the skeletal muscle of plumbagin treated diabetic rats.[140)] Plumbagin (2 mg/kg) also markedly improved diabetic nephropathy in STZ induced diabetic mice by inhibiting the TGFβ1via Nox4 pathway.[141)]

Impatiens balsamina L. (Family Balsaminaceae) is a naphthoquinone containing plant native to Thailand. In Thai traditional medicine, *I. balsamina* is

commonly used as a topical remedy for wound healing, antimicrobial, and antiallergic purposes.[6)] Naphthoquinones are the major identified medicinal constituents of *I. balsamina* (Table 1). *I. balsamina* extracts and isolated naphthoquinones (Fig. 5) have been reported to exhibit various pharmacological activities, as shown in Table 2. In terms of antidiabetic potential, there has been one report demonstrating α-amylase inhibitory activity. An ethanol extract (200-400 µg/mL) of *I. balsamina* seed was tested for *in vitro* α-amylase inhibitory activity. The extract showed moderate α-amylase inhibitory activity in comparison to the standard drug acarbose.[142)]

Lawsonia inermis L. (Family Lythraceae), commonly known as henna, has tremendous pharmaceutical and cosmeceutical applications, which are associated with its historical folk uses in various traditions, including those in Thailand.[143)] *L. inermis* possesses multiple forms of biological activity (Table 2) due to the presence of a number of naphthoquinones (Table 1) (Fig. 5). In the context of antidiabetic potential, *L. inermis* leaf extracts have been studied extensively for different diabetic parameters. Neeli et al. (2007) and Syamsudin et al. (2008) evaluated a methanol extract of *L. inermis* leaves in diabetic rats (200 mg/kg) and mice (800 mg/kg), respectively. The results of both experiments showed that the extracts markedly decreased blood sugar levels and normalized the lipid profile in the diabetic animals.[144,145)] Another *in vivo* experiment was performed by Chauhan et al. (2011) in alloxan induced diabetic rats to study the effect of a methanol leaf extract (600 mg/kg) of *L. inermis.*[146)] The results demonstrated that the methanol extract markedly normalized blood sugar levels and the lipid profile in diabetic rats. Furthermore, an *in vitro* α-amylase inhibition study indicated that a methanol extract of *L. inermis* had equivalent inhibitory activity to a standard drug, acarbose.[147)]

Antidiabetic mechanisms of naphthoquinones

DM is a multifactorial chronic disease which needs multidimensional therapeutic strategies. Therefore a molecule which can act on more than one mechanism is considered to be an ideal candidate when being considered in antidiabetic drug development. Naphthoquinone containing extracts and purified naphthoquinones exert their antidiabetic activity through several mechanisms of action, as summarized in Figure 7. Rhinacanthin-C expresess antidiabetic activity by inhibition of intestinal α-glucosidase, stimulating glucose uptake in muscles and adipocytes, probably *via* GLUT4 translocation.[130,132)] TNFα, Ikkβ, and caspase-3 reduction in the pancreas of diabetic rats interlink anti-inflammatory activity to antidiabetic potential, while antioxidant and GLUT2 enhancement in the pancreas sensitize insulin secretion to control glycemia.[129)] Plumbagin exerts an antidiabetic effect *via* GLUT4 translocation and normalizing serum and hepatic carbohydrate metabolizing enzymes.[141)] Shikonin **(47)** is a naturally occurring 1,4-naphthoquinone which contributes to the purple color of *Lithospermum erythrorhizon* Siebold & Zucc. (Family Boraginaceae), a native plant of China. This compound is a potent antidiabetic molecule which has been investigated by different researchers for a range of antidiabetic mechanisms. Kamei et al. (2002) studied the glucose uptake enhancing effects of shikonin in adipocytes and cardiomyocytes. Their results indicated that shikonin (60 μM) significantly stimulated glucose uptake in adipocytes and cardiomyocytes through a tyrosine kinase-dependent pathway by inducing Thr-308 and Ser-473 phosphorylation of Akt.[148)] Oberg et al. (2011) studied the glucose uptake stimulation potential of shikonin in L6 myotubes and also its hypoglycemic activity in diabetic rats. Shikonin (1 μM) enhanced glucose uptake in L6 myotubes *via* calcium

influx and a GLUT4 translocation mechanism. Furthermore, in the same study shikonin (10 mg/kg intraperitoneally for 4 days) markedly normalized blood sugar level in diabetic rats.[149)] Another interesting antidiabetic mechanism reported for shikonin is its insulin-like action through inhibition of the phosphatidylinositol 3,4,5-triphosphate (Pt-3,4,5-P3) phosphatase pathway, which involved deletion of a tensin homolog on chromosome 10. It also caused accumulation of Pt-3, 4, and 5-P3, activation of protein kinase B, and inhibited several protein phosphatases in different cell systems.[150)]

Hyperglycemia and hyperlipidemia are prime identifying characteristics in the progression of type-2 DM and chronic cardiovascular disorders.[132)] Insulin resistance, the main cause of type-2 DM, is linked with the release of free fatty acids and proinflammatory cytokines from adipose tissues in subjects with obesity or excessive adiposity, which stimulates beta cells to over-secrete insulin and reduce its receptors.[132)] Rhinacanthin-C was recently reported to inhibit adipogenesis in 3T3-L1 adipocytes.[132)] Shikonin (IC_{50} value of 1.1 mM) has been documented as having an anti-obesity effect in 3T3-L1 by inhibiting accumulation of triglycerides *via* inhibition of the FABP4 and LPL genes expression, both of which are involved in lipid metabolism. It has also been reported that shikonin inhibits adipogenesis by both up- (SREBP1C) and downstream regulation (PPARγ and C/EBPα).[151)]

Various synthetic naphthoquinones have also been reported upon due to their antidiabetic potential. For example, 5,8-diacetyloxy-2,3-dichloro-1,4-naphthoquinone **(48)** was obtained through a screening of numerous chemical libraries, consisting of almost 4500 natural and synthetic molecules.[152)] This

compound (10 μM) was found to be a potent insulin receptor activator and glucose uptake enhancer in adipocytes. It has a strong affinity to bind with a receptor kinase and trigger its activity by Akt and ERK phosphorylations.[152)] The hypoglycemic mechanisms were further validated through an *in vivo* experiment in the same study.

Protein tyrosine phosphatase 1B (PTP1B) is an important enzyme in insulin signaling and resistance, which relates to type-2 diabetes. 1,2-Naphthoquinone (IC_{50} of 1.64 mM) was identified in the course of a high throughput screening, and a group of 1,2-naphthoquinone **(49)** derivatives were synthesized from 1,2-naphthoquinone, and were found to be potential inhibitors of PTPB1 at very low concentrations.[153)] Later derivatization of the molecule showed that molecules having phenyl or indole functional groups were more potent as compared to those having nitrogen or oxygen functional groups.[153)]

Peroxisome proliferator-activated receptor gamma (PPARγ) ligand activity also provides an interesting target for metabolic disorders such as diabetes, obesity, inflammatory diseases, and cancer. Recently, a series of compounds with 2-hydroxy-1,4-naphthoquinone **(50)** as an acidic group were predicted to express PPARγ ligand activity; a hypothesis which was later validated experimentally.[154)] Furthermore, it was concluded that the hydrogen bonding of the compound with the receptor is important for its activation.[154)]

Future prospects

The current literature review has summarized that most naphthoquinone-containing plant extracts have been reported to exhibit antidiabetic effects. However, many plants with these compounds need to be explored further. In this context, there

are some reports on antidiabetic activity from two plant species in the family *Nepenthaceae*, i.e. *Nepenthes bicalcarata* and *N. khasiana*, which are not available in Thailand. Thai native species of *Nepenthes*, including, *N. ampullaria*, *N. gracilis, N. mirabilis, N. smilessi*, and *N. thorelii*, currently lack investigation into their potential antidiabetic activity. There is therefore a need to determine the possible naphthoquinone-based antidiabetic and antiobesity activity of these species. Previously isolated naphthoquinones, such as the major naphthoquinones from *I. balsamina* and *L. inermis*, would also be good targets to evaluate for their mechanism of antidiabetic action. In conclusion, naphthoquinones are ideal candidates for antidiabetic drug development due to their diverse therapeutic potential and multimechanism antidiabetic activity.

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Table 1 Plants containing naphthoquinones in Thailand

Plant name	Part(s)	Naphthoquinone(s)
Rhinacanthus nasutus (L.) Kurz. (syn. *R. communis* Nees) Thai name: Thong phan chang Family Acanthaceae	root, leaf	rhinacanthin-A (**1**), rhinacanthin-B (**2**), rhinacanthin-C (**3**), rhinacanthin-D (**4**), rhinacanthin-G (**5**), rhinacanthin-H (**6**), rhinacanthin-I (**7**), rhinacanthin-J (**8**), rhinacanthin-K (**9**), rhinacanthin-L (**10**), rhinacanthin-M (**11**), rhinacanthin-N (**12**), rhinacanthin-O (**13**), rhinacanthin-P (**14**), rhinacanthin-Q (**15**)[8-11, 38)]
Kigelia africana (Lam.) Benth. (syn. *K. pinnata* DC.) Thai name: Sai krok apfrika Family Bignoniaceae	root, wood, fruit	kigelinol (**16**), isokigelinol (**17**), kigelinone (**18**), 2-(1-hydroxyethyl)naphtho(2, 3-b) furan-4,9-dione (**19**), pinnatal (**20**), isopinnatal (**21**), lapachol (**22**), dehydro-α-lapachone (**23**) and 2-acetylnaptho [2, 3-b] furan-4,9-quinone (**24**), tecomaquinone (**25**)[39-45)]
Diospyros kaki L. Thai name: Phlap chin Family Ebenaceae	root and wood	plumbagin (**26**), naphthazarin (**27**), dichlon (**28**), 2-bromo-1,4-naphthoquinone (**29**), 2,3-dibromo-1,4-naphthoquinone (**30**), methyl juglone (**31**), isodiospyrin (**32**), mamegakinone (**33**), shinanolone (**34**)[46, 47)]
Plumbago zeylanica L. Thai name: Chettamun phloeng khao *P. indica* L. (syn. *P. rosea* L.) Thai name: Chettamun phloeng daeng Family Plumbagnaceae	whole plant	plumbagin (**26**), 6-hydroxyplumbagin (**35**), 3,3-biplumbagin (**36**), elliptinone (**37**), 3,8-dihydroxy-6-methoxy-2-isopropyl-1,4-naphthoquinone (**38**), 5,7-dihydroxy-8-methoxy-2-methyl-1,4-naphthoquinone (**39**)[48-52)]
Impatiens balsamina L. Thai name: Thain-dok Family Balsaminaceae	root	lawsone (**40**), methylene-3,3'-bilawsone (**41**), 2-methoxy-l,4 naphthoquinone (**42**), impatienol (**43**)[53)]
Lawsonia inermis L. Thai name: Thian king Family Lythraceae	leaf, seed, fruit	lawsone (**40**), 2-methoxy-l,4 naphthoquinone (**42**), isoplumbagin (**44**), lawsonadeem (**45**), 3-amino-2-methoxycarbonyl-1,4-naphthoquinone (**46**)[54)]

Table 2 Biological activities of plants containing naphthoquinones in Thailand

Plant name	Part(s)	Biological activities
R. nasutus	root, leaf whole plant	antimicrobial, antiviral, anticancer, anti-inflammatory, antipyretic, analgesic, antioxidant, anti-hemolytic, immunomodulatory, anti-allergic, neuroprotective, antiglycation, antiobesity activities[24-37)]
K. africana	root, wood, fruit	antioxidant, wound healing, antimicrobial activity, antiviral, antimalarial, anti-inflammatory, hepatoprotective, analgesic, anti-ulcerogenic, anti-diarrheal, anticancer activities[55-64)]
D. kaki	root, fruit, wood	cardiopretotective, antioxidant, anti-anaphylactic, antimicrobial, anticancer, immunological, neuroprotective, thrombolytic, and tyrosinase inhibitory, antihypertensive activities[65-67)]
P. zeylanica *P. indica*	root, leaf, whole plant	anticancer, acaricidal, anti-arthritic, anti-candidal, anti-convulsant, anti-allergic, antimicrobial, antifertility, antiviral, hyperlipidaemic, anti-plasmodial, cardiotonic activities[68-85)]
I. balsamina	root, leaf, seed, hole plant	antipruritic, anti-anaphylactic, antioxidant, antimicrobial, anticancer activities[86-91)]
L. inermis	leaf, seed, fruit	antioxidant, immunomodulatory, hepatoprotective, antimicrobial, anti-trypanosomal, anti-parasitic, molluscicidal, anti-dermatophytic, antifertility, analgesic, anti-inflammatory, anti-sickling, abortifacient, antitrypsin, nematicidal, wound healing, anti-glycation activities[92-124)]

Figure 1 Naphthoquinones in *Rhinacanthus nasutus*

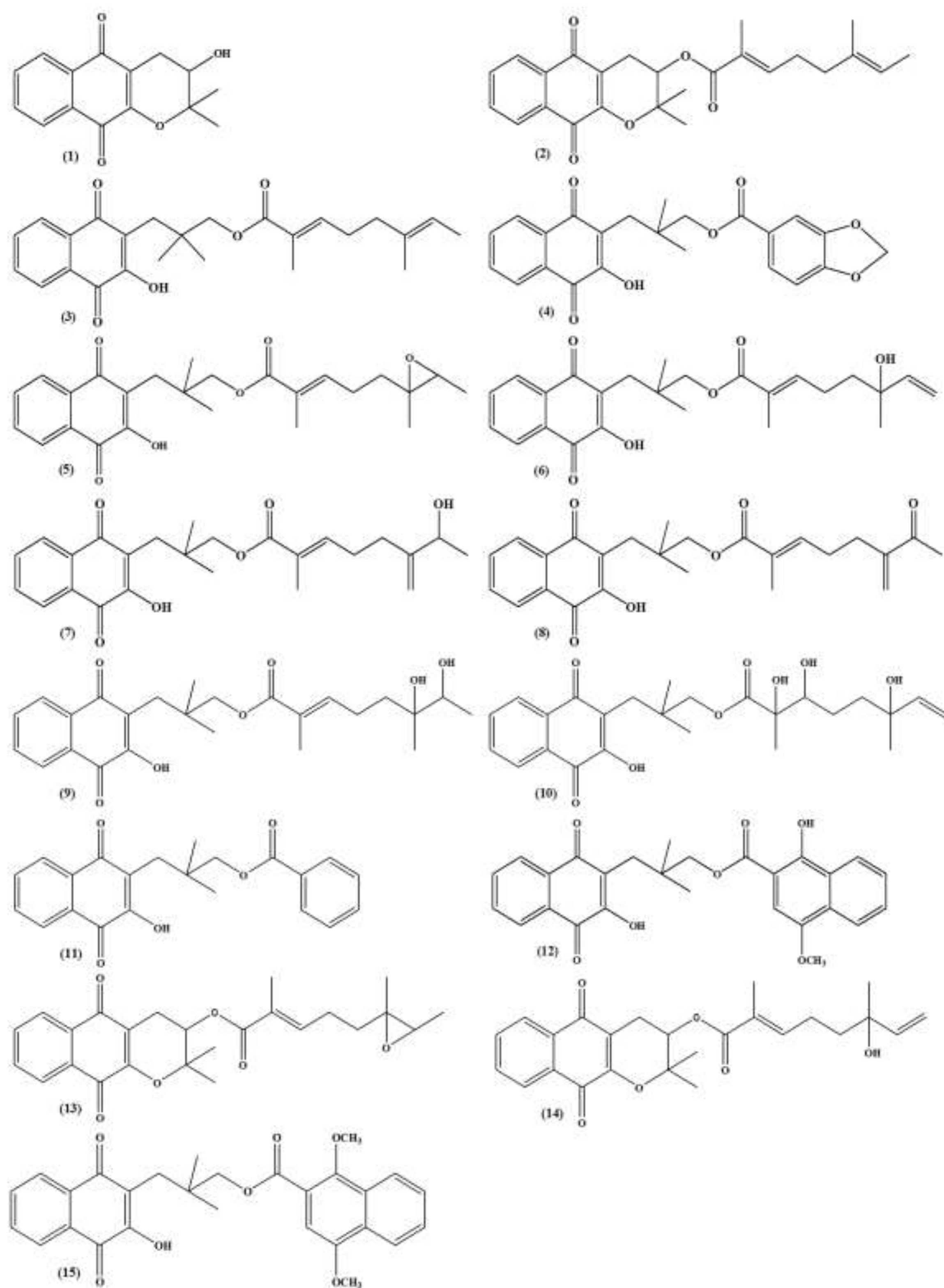


Figure 2 Naphthoquinones in *Kigelia africana*

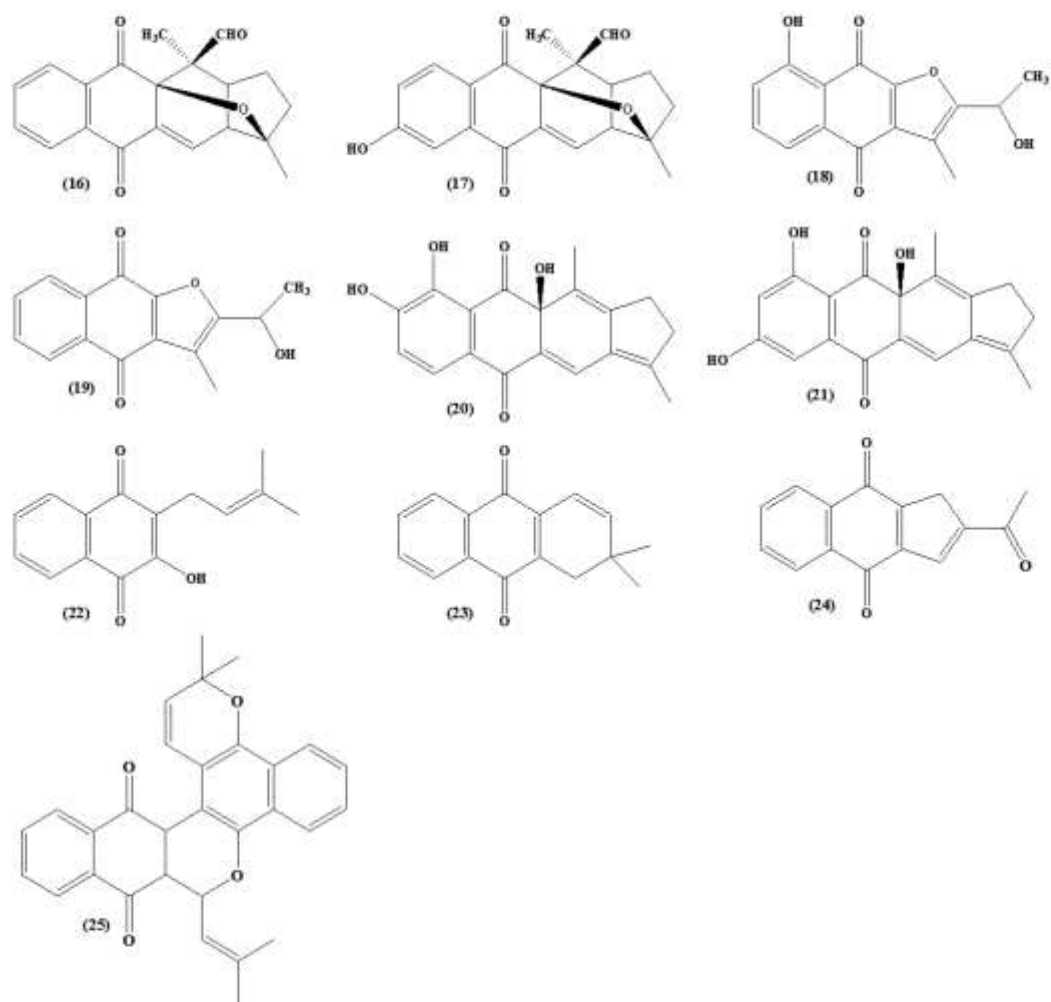


Figure 3 Naphthoquinones in *Diospyros kaki*

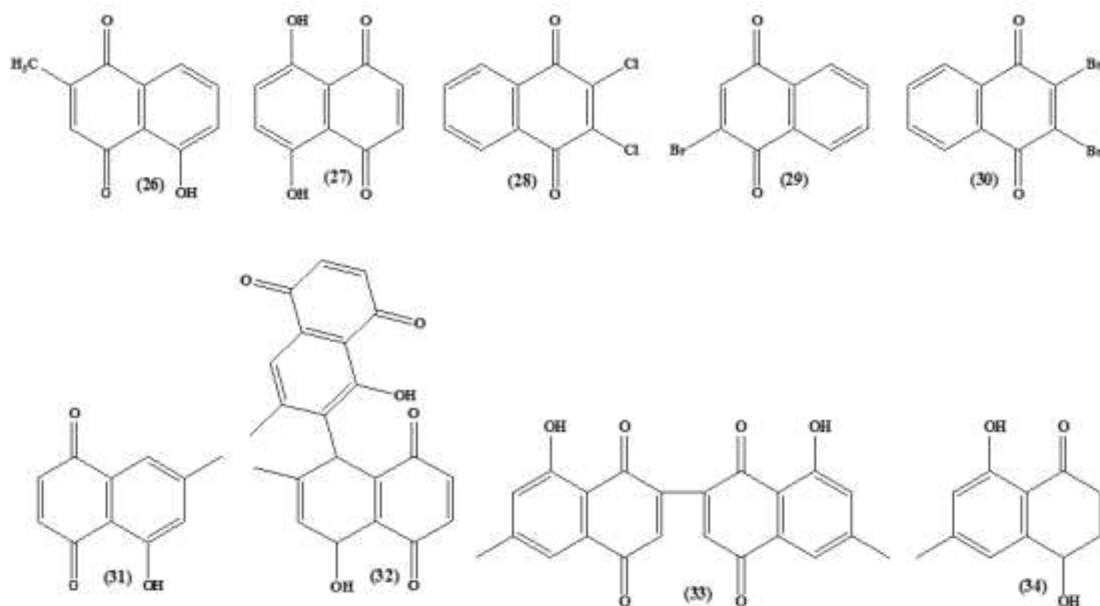


Figure 4 Naphthoquinones in *Plumbago zeylanica* and *P. indica*

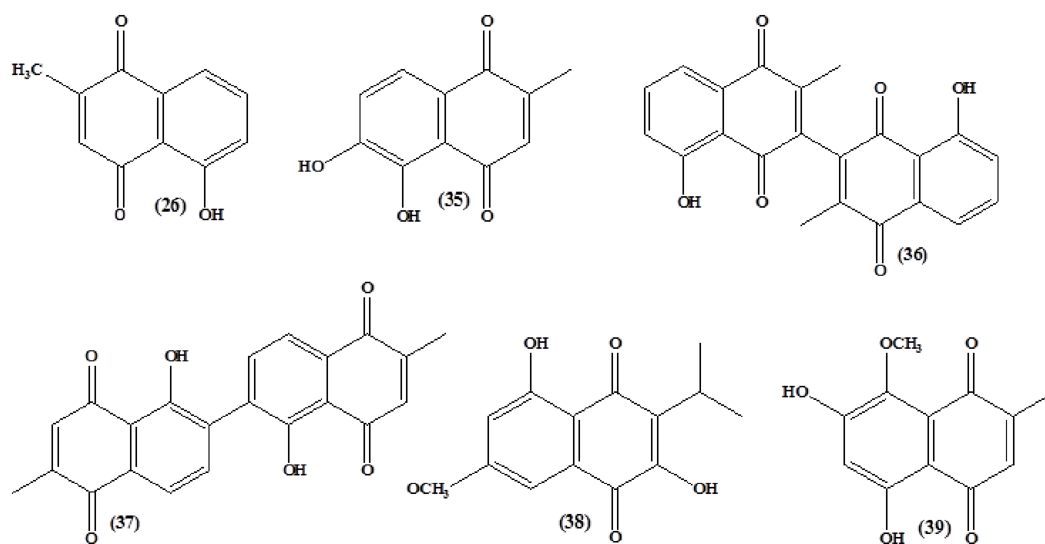


Figure 5 Naphthoquinones in *Impatiens balsamina* and *Lawsonia inermis*

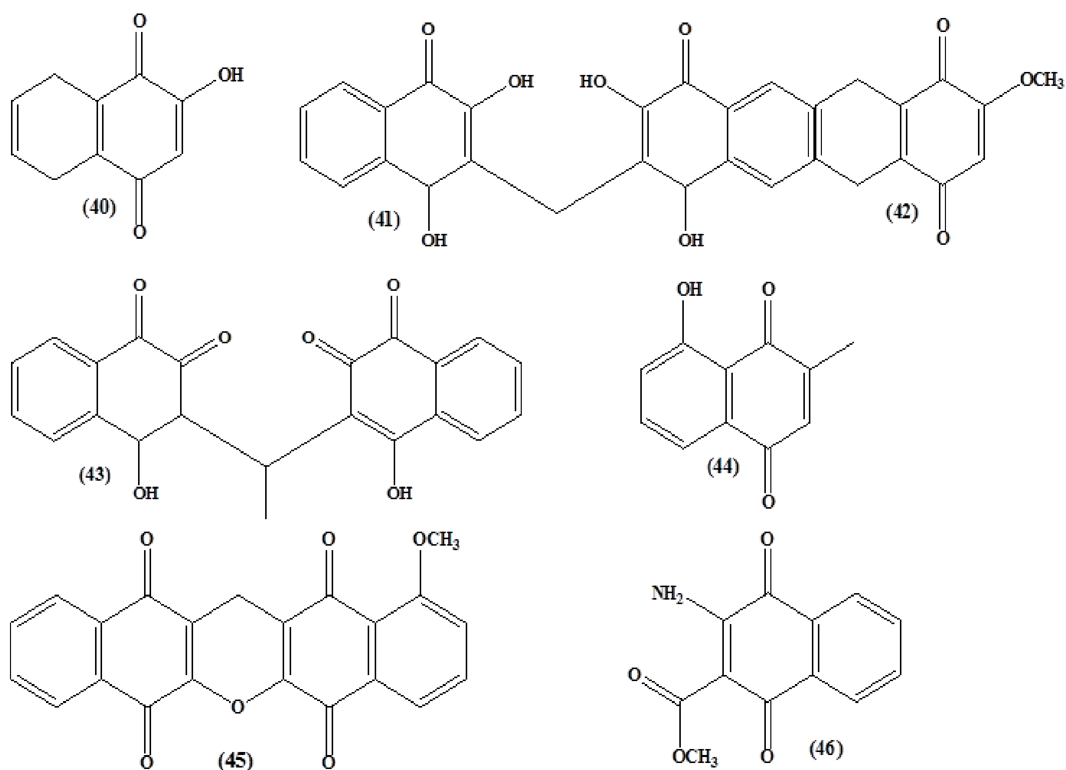


Figure 6 Miscellaneous antidiabetic naphthoquinones

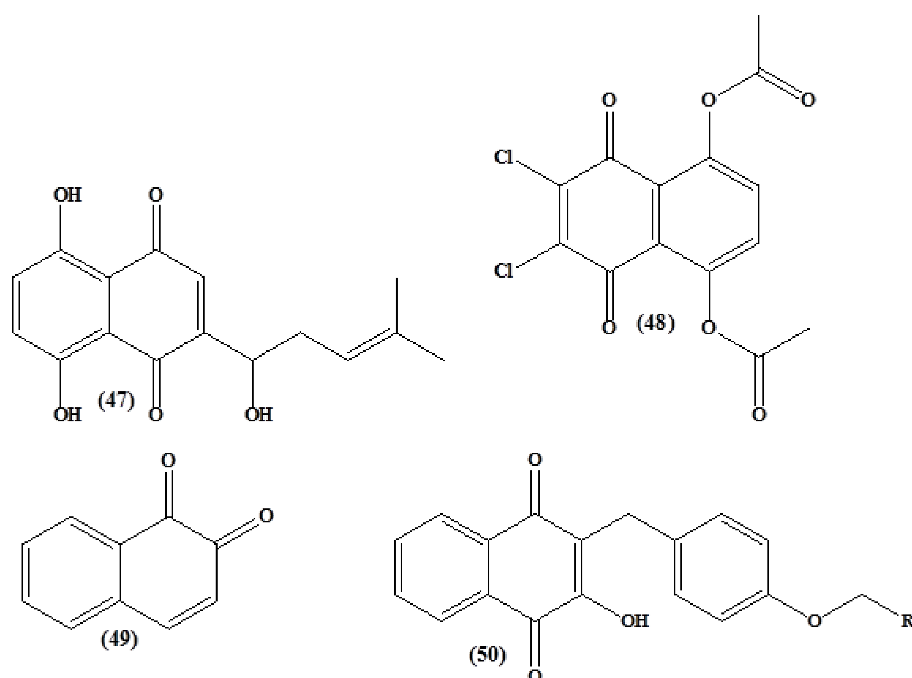
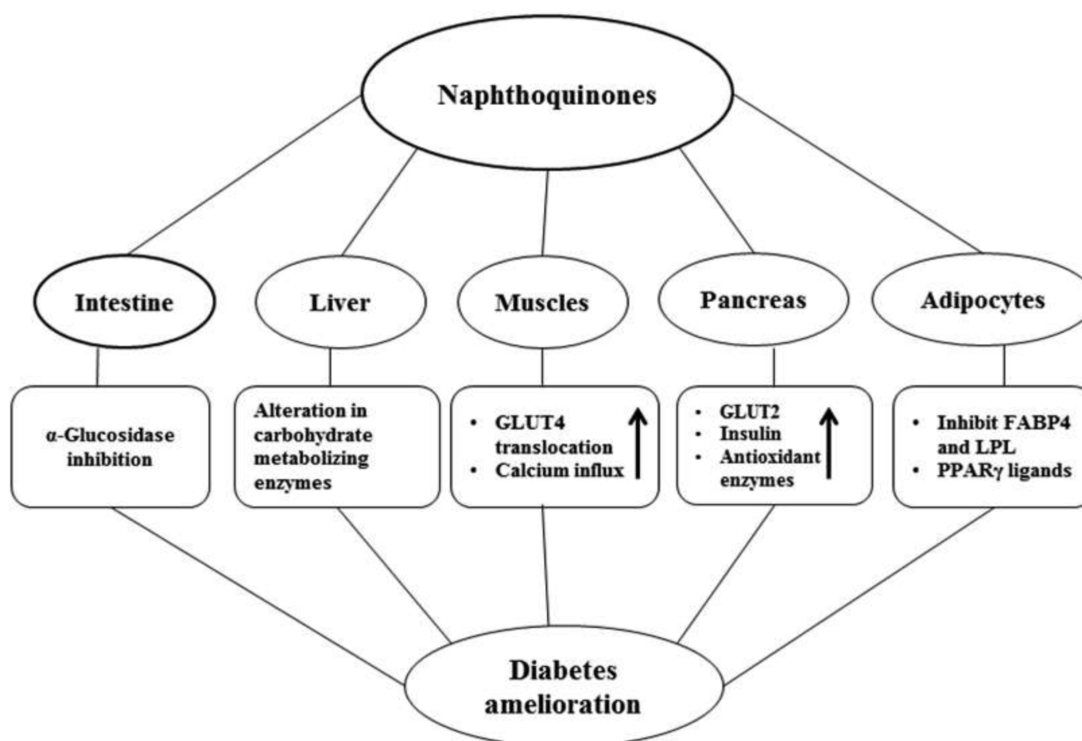


Figure 7 Mechanistic picture of diabetes amelioration by naphthoquinones in various target organs.



PAPER II

α-Glucosidase inhibitory effect of rhinacanthins-rich extract from *Rhinacanthus nasutus* leaf and synergistic effect in combination with acarbose

Shah MA, Khalil R, Haq ZU, Panichyupakaranant P

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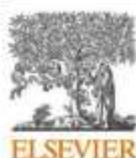

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α-Glucosidase inhibitory effect of rhinacanthins-rich extract from *Rhinacanthus nasutus* leaf and synergistic effect in combination with acarbose

Muhammad Ajmal Shah [a], Ruqaiya Khalil [b], Zaheer Ul-Haq [b], Pharkphoom Panichayupakaranant [a,c,*]

[a] Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand
[b] Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan
[c] Phytomedicine and Pharmaceutical Biotechnology Excellence Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

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ABSTRACT

Rhinacanthins-rich extract (RRE) from *Rhinacanthus nasutus* leaf and its marker compounds namely rhinacanthin-C, rhinacanthin-D and rhinacanthin-N were evaluated for α-glucosidase inhibitory activity. RRE (IC_{50} value of 25.0 μg/mL) exhibited α-glucosidase inhibitory activity almost equivalent to that of rhinacanthin-C (IC_{50} value of 22.6 μg/mL) but stronger than that of rhinacanthin-D (IC_{50} value of 71.5 μg/mL) and the standard drug, acarbose (IC_{50} value of 395.4 μg/mL), while rhinacanthin-N was inactive. Kinetic studies revealed that both RRE and rhinacanthin-C exhibited noncompetitive α-glucosidase inhibitory activity, while combinations of either RRE or rhinacanthin-C with acarbose at low concentrations (¼IC_{50}, ½IC_{50} and IC_{50}) showed a synergistic inhibitory effect. *In silico* studies identified the binding mode of rhinacanthin-C highlighting the formation of both polar and apolar contacts of ligand with α-glucosidase. The present study provides the first evidence that RRE containing rhinacanthin-C as the major compound, could find application as an α-glucosidase inhibitor.

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1. Introduction

The number of diabetic patients was reported to be around 382 million globally in 2013 and this number was predicted to rise to 592 million within the next two decades (Guariguata et al., 2014). Around 90% of diabetic patients suffer from type-2 diabetes mellitus (DM), a major type of DM that caused by impaired insulin secretion combined with either a reduction of insulin activity or maintained activity. Excess body weight and physical inactivity are the main causes of type-2 DM (Olokoba, Obateru, & Olokoba, 2012; Sui, Zhang, & Zhou, 2016). Clinically available oral antidiabetic drugs exert their therapeutic effect by various mechanisms including elevation of insulin secretion, and glucose absorption and metabolism. α-Glucosidase is a key enzyme that converts disaccharides into simple absorbable monosaccharides within the gastrointestinal tract and α-glucosidase inhibition is therefore a prominent therapeutic strategy to control postprandial hyperglycemia in type-2 DM. The clinically available α-glucosidase inhibitors including acarbose, voglibose and miglitol are currently administered orally as monotherapeutics or in combination with other oral antidiabetic drugs. However, these compounds are high cost and are known to cause gastrointestinal side effects. In addition, long term use of the commercially available α-glucosidase inhibitors is also associated with cardiac hazards (Fisman, Michael, & Tenenbaum, 2008).

A number of plant extracts and isolated phytochemicals have been reported to exhibit α-glucosidase inhibitory activity as their main antidiabetic mechanism (Kumar, Narwal, Kumar, & Prakash, 2011). *Rhinacanthus nasutus* (L.) Kurz (Family Acanthaceae) is a medicinal plant native to Thailand and Southeast Asia. *R. nasutus* is a well-known source of traditional medicines for the treatment of various diseases including DM (Brimson & Tencomnao, 2014). In China and Taiwan, it is often taken as an herbal tea (Huang, Lu, Inbaraj, & Chen, 2015). Rhinacanthin-C (RC), a major active constituent of *R. nasutus* leaf has recently been reported for to exhibit hypoglycemic, hypolipidemic and pancreatic protective effects in streptozotocin-nicotinamide induced diabetic rats (Adam et al., 2016). However, RC is not available commercially and its isolation involves a time-consuming, energy-intensive, multi-stage process that requires a large amount of toxic organic solvents, ultimately increase its production costs. An alternative approach that presents

* Corresponding author at: Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand.
E-mail address: pharkphoom.p@psu.ac.th (P. Panichayupakaranant).

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the possibility of synergistic effect is to utilize rhinacanthins-rich extract (RRE), a semi purified *R. nasutus* leaf extract that features a total rhinacanthins content of not less than 70% w/w, with 60–70% w/w comprising RC as the major component (Panichayupakaranant, Charoonratana, & Sirikatitham, 2009). In the present study, RRE was obtained using a simple environmentally friendly, 'green' extraction and fractionation method, and used to investigate α-glucosidase inhibitory activity *in vitro* as well as in combination with acarbose, an antidiabetic drug. *In silico* studies were also performed to determine the binding mechanism of RC with the target enzyme, and to explain the structure activity relationship of RC.

2. Materials and methods

2.1. Chemicals

α-Glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), *p*-nitrophenyl-α-D-glucopyranoside (pNPG) and acarbose were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). All others chemicals used were of analytical grade.

2.2. Plant material, extraction and isolation

The fresh leaves of *R. nasutus* were collected from the Botanical Garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai Campus, Thailand, an a voucher specimen (No. 0011814) was kept at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Thailand. Leaves were washed with tap water and dried at 60 °C for 24 h in a hot air oven and reduced to powders using a grinder, and the powders were passed through sieve No. 45.

RRE was prepared using ethanol by previously described methods (Panichayupakaranant et al., 2009) with some modifications. The present method used a microwave assisted extraction followed by a simple step of fractionation with Amberlite® column. Moreover, only the green solvents, ethanol and water, were used in the extraction and fractionation processes. Rhinacanthin-C (RC), rhinacanthin-D (RD) and rhinacanthin-N (RN) were isolated from the RRE using a silica gel column eluted by hexane and ethyl acetate (99:1, v/v). The structures of all three compounds (Fig. 1) were confirmed by comparing the ^{1}H and ^{13}C NMR spectral data with those from the literature (Sendl et al., 1996; Wu et al., 1998).

2.3. HPLC analysis of RRE

HPLC analysis of RRE was performed as previously reported (Panichayupakaranant et al., 2009) using a UFLC Shimadzu system incorporating a Discovery® C18 (5 μm, 4.6 × 150 mm) column (Supelco, PA, USA) equipped with a photodiode-array detector and autosampler (Shimadzu Corp. Kyoto, Japan).

2.4. α-Glucosidase inhibition assay

The α-glucosidase inhibitory activity was determined using the method described by Rengasamy, Aderogba, Amoo, Stirk, and Van Staden (2013). In samples preparation, the final concentration of DMSO was not more than 7%. The percentage inhibition was calculated by using the following equation.

$$\% \text{ Inhibition} = (Ac - As)/Ac$$

Whereas; Ac = Absorbance of control, As = Absorbance of sample.

2.5. Determination of the mechanism of α-glucosidase inhibition

An enzyme kinetic analysis was performed based on the α-glucosidase inhibition assay described above. The concentration of α-glucosidase was kept constant at 0.1 unit/mL and the pNPG concentrations varied from 0.16 to 2.65 mM in the absence and presence of RRE and RC (12.5, 25 and 50 μg/mL). The type of inhibition was determined by Lineweaver-Burk plot obtained by plotting velocities of reaction (vertical axis) and substrate concentrations (horizontal axis) reciprocally (Gu et al., 2009).

2.6. α-Glucosidase inhibition of RRE and RC in combinations with acarbose

On the basis of IC_{50} data, a series of three concentrations (¼IC_{50}, ½IC_{50} and IC_{50}) of RRE and RC as well as their combined mixtures with acarbose were prepared to investigate the combined

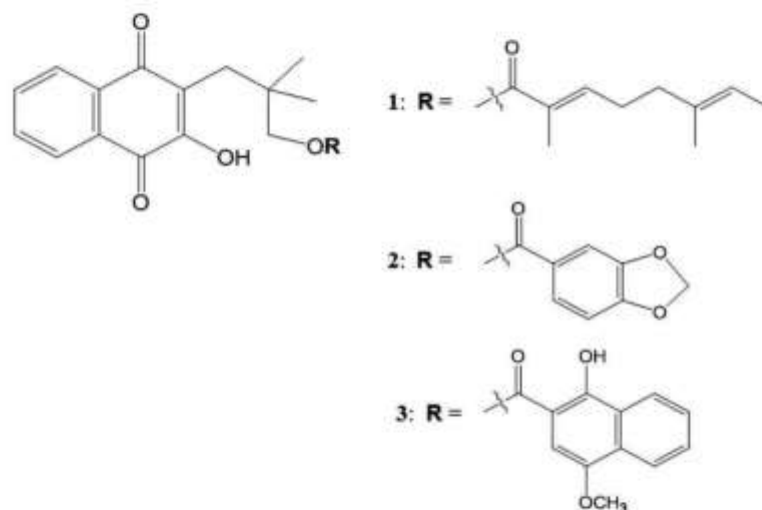

Fig. 1. Chemical structures of rhinacanthin-C (1), rhinacanthin-D (2) and rhinacanthin-N (3).

Table 1
Total rhinacanthins content in crude ethanol and rhinacanthins-rich extract.

Compounds	Rhinacanthin content (% w/w; Mean ± SD)	
	Crude ethanol extract	Rhinacanthins-rich extract
Rhinacanthin-C	6.6 ± 0.10	62.2 ± 2.3
Rhinacanthin-D	1.1 ± 0.02	7.9 ± 0.1
Rhinacanthin-N	0.5 ± 0.01	3.6 ± 0.2
Total rhinacanthins	8.2	73.7

Table 2
α-Glucosidase inhibitory activity of rhinacanthins-rich extract and rhinacanthin-C.

Compound	IC_{50} (μg/mL)
RRE	25.0 ± 0.8[a]
Rhinacanthin-C	22.6 ± 0.6[a]
Rhinacanthin-D	71.5 ± 1.0[b]
Rhinacanthin-N	n.a.[*]
Acarbose	395.4 ± 12.1[c]

Inhibitions concentrations are expressed as the mean ± SEM (n = 3). Mean values followed by different letters are significantly different ($P \leq 0.05$).
[*] Not active.

inhibitory effect of the samples with the control drug on α-glucosidase (Gao, Xu, Wang, Wang, & Hochstetter, 2013). Reaction was performed according to above α-glucosidase inhibition assay.

2.7. In silico studies

Molecular modeling studies were carried out to investigate the α-glucosidase inhibitory mechanism of RC. The FASTA sequence for *S. cerevisiae* α-glucosidase was retrieved from Uniprot (accession code P53051.1). The model was developed using SWISS MODEL (Schwede, Kopp, Guex, & Peitsch, 2003). The MOE 2015.1001 software suite (Wagner, Inceoglu, Gill, & Hammock, 2010) was used to construct Ramachandran and rotamer energy plots (threshold = −1 kcal/mol). The developed model was subjected to energy minimization by using default AMBER10 force field in MOE.

The builder module in MOE-2015.1001 was used to generate the chemical structure of RC. Molecular docking studies were performed by using a protocol we developed earlier (Barakat

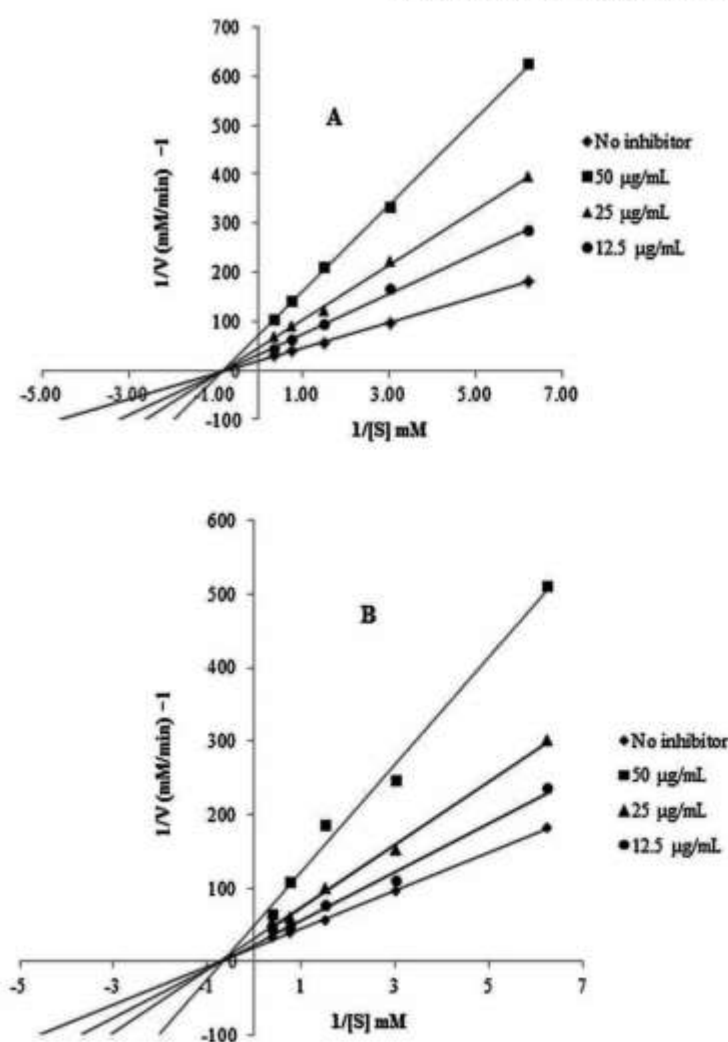

Fig. 2. Lineweaver-Burk plot of rhinacanthin-C (A) and rhinacanthins-rich extract (B) against α-glucosidase at different concentrations of *p*NPG.

et al., 2016). A 10 ns Molecular Dynamics (MD) Simulation was performed using AMBER14 (Case et al., 2014) to analyze the stability of protein-ligand complexes and the interactions observed during docking. Protein and ligand parameters were established using AMBER99SB and generalized AMBER force field (GAFF), respectively. A 10 Å water box was generated under periodic boundary conditions, which was solvated using the '3-point water model' (TIP3 P). To neutralize the protein surface charges, 16 Na^+ ions were added, which replaced water molecules at potentially favorable positions. In order to remove any steric clashes, the systems were subjected to a brief minimization. A series of restrained minimization were performed by gradually decreasing the strain from 25 to 5 kcal/mol.$Å^2$. A comprehensive unrestrained minimization was then performed.

The temperature was increased gradually from 0 to 300 K over 500 ps and the system was further equilibrated for a further 500 ps at constant pressure (1 bar) and temperature (300 K). Bond lengths involving hydrogen atoms were constrained using SHAKE algorithm (Kräutler, Van Gunsteren, & Hünenberger, 2001) with harmonic restraints of 25 kcal/mol.Å. Finally, a production run of 10 ns was performed with a 2 fs time step at constant pressure (1 bar) and temperature of 300 K. The resulting trajectories were analyzed using the CPPTRAJ module (Roe & Cheatham, 2013) implemented in AMBERTOOLS15 and VMD (Humphrey, Dalke, & Schulten, 1996).

3. Results and discussion

3.1. RRE extraction and standardization

RRE was prepared by modification of our earlier approach (Panichayupakaranant et al., 2009). RRE contained a high content of rhinacanthins (73.7% w/w) equal to that previously reported (Table 1) and RC was found to be a major constituent (62.2% w/w). The developed green processes in preparation of RRE and the obtained RRE is highly suitable for nutraceutical and industrial applications in term of safety and low cost.

3.2. α-Glucosidase inhibition activity of RRE and its marker compounds

The inhibitory effect of RRE and its marker compounds RC, RD and RN against α-glucosidase was assessed in order to assess their antidiabetic potential. Both RC and RRE exhibited satisfactory inhibitory activity against α-glucosidase with IC_{50} values of 22.6 μg/mL and 25.0 μg/mL, that was much higher activity than acarbose (IC_{50} value of 395 μg/mL) (Table 2). RRE and RC display almost equal α-glucosidase inhibitory activity that similar to previously been reported to possess almost equivalent antimicrobial and anti-inflammatory activity (Bhusal, Panichayupakaranant, & Reanmongkol, 2014; Panichayupakaranant et al., 2009; Puttarak, Charoonratana, & Panichayupakaranant, 2010). RD, a minor naphthoquinone ester of RRE, also showed good inhibitory activity, with an IC_{50} value of 71.5 μg/mL, while RN was found to be inactive. This implies that an aromatic ring on substituted R group of rhinacanthins (Fig. 1) may reduce their α-glucosidase inhibitory effect by interference to the enzyme binding site. More recently, RC has been shown to exhibit antidiabetic potential in streptozotocin–nicotinamide induced diabetic rats. Increased glucose uptake by adipocytes, expression of pancreatic GLUT2 and pancreatic protective effects due to lowering of inflammatory and cellular apoptosis mediators have been proposed to explain the antidiabetic activity of RC (Adam et al., 2016). The present study is the first report of α-glucosidase inhibition as an antidiabetic mechanism for RRE and its marker compounds.

3.3. Inhibitory mechanism of RRE and its synergistic activity with acarbose

To determine the mechanism of inhibition, RRE and RC were used in three different concentrations i.e. 12.5, 25 and 50 μg/mL as inhibitors in kinetic experiment to elucidate the type of inhibition. Possible interference by RRE and RC was examined at five different pNPG concentrations i.e. 0.16–2.65 mM. The absorbance was first plotted against time to obtain velocities of reactions and the velocities were subsequently plotted against the reciprocal of substrate concentration to construct Lineweaver-Burk plots. The Lineweaver–Burk plots for α-glucosidase inhibition by RC and RRE generated straight lines, which intersected at the same point on X-axis in the second quadrant, indicating noncompetitive inhibition (Fig. 2). Acarbose is a competitive α-glucosidase inhibitor (Ag, 1994), thus it was of interest to establish whether RRE and RC, as noncompetitive inhibitors, might interact synergistically with acarbose in inhibiting α-glucosidase. The experiment was performed at three different concentrations at ¼IC_{50}, ½IC_{50} and IC_{50}. It was found that lower concentrations of acarbose combined with RRE and RC at ¼IC_{50} and ½IC_{50} resulted in significant inhibition compared with the individual compounds at the same concentration (Fig. 3) indicating synergistic inhibitory activity against α-glucosidase. These finding suggest that the combination of acarbose with RRE or RC having different inhibitory mechanisms could inhibit α-glucosidase activity more effectively at low doses compared with the single compounds, resulting in a reduction of postprandial blood glucose in type-2 DM and avoiding adverse effects due to acarbose.

3.4. In silico studies

The crystallographic structure of yeast α-glucosidase has not yet been fully resolved. The SWISS-MODEL (Schwede et al., 2003) was applied instead of a number of related studies (Imran et al., 2015, 2016; Xu, 2010) to obtain three dimensional coordinates of *S. cerevisiae* using the FASTA sequence of α-glucosidase, under the accession code P53051.1 as a query string in BLAST search. The crystal structure of oligo-1,6-glucosidase of yeast in complex with its competitive inhibitor maltose (PDB 3AJ7) (Yamamoto, Miyake, Kusunoki, & Osaki, 2010) was identified as the most suitable template with sequence identity and similarity values of 72.68

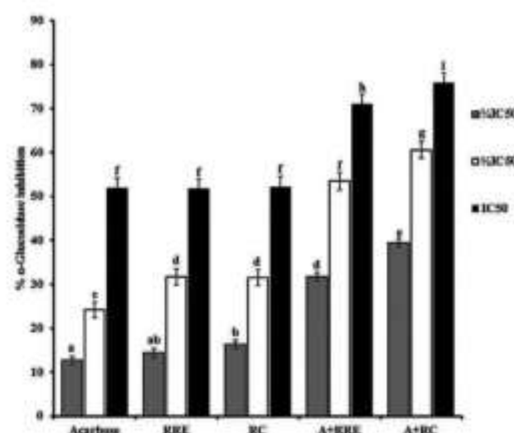

Fig. 3. Percentage inhibition of α-glucosidase by acarbose, RRE and RC, and combined acarbose with RRE (A+RRE) and RC (A+RC) at different concentration on the basis of IC_{50}. Results are expressed as mean ± SEM (n = 3). Mean values followed by different letter are significantly different ($P \le 0.05$).

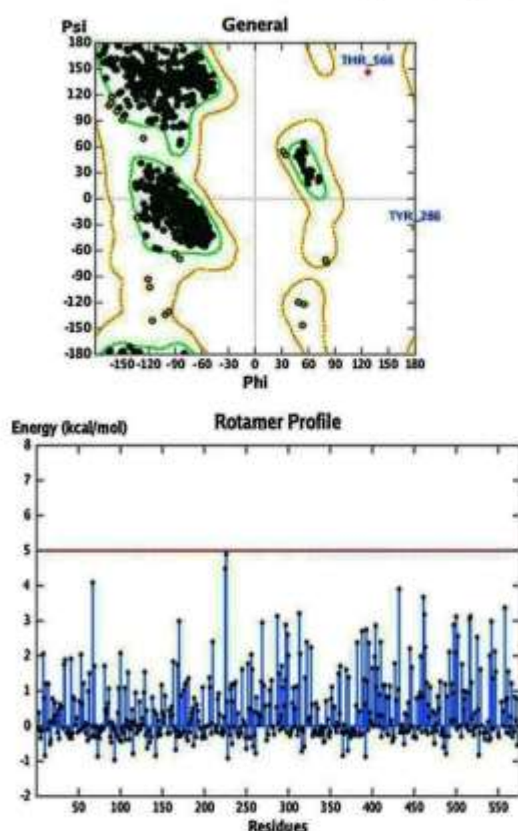

Fig. 4. The visuals showing the results of model assessment. The graph above shows the Ramachandran contours for newly developed model. Green dots donate the position of residues in core region. While, (+) sign highlights the outliers. The rotamer profile of the newly developed model highlights the side chain energy of each amino-acid. The energies are well below the threshold which pronounces the good quality of model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

and 0.54, respectively. The Qmean score of the model was found to be 0.729, which signifies the reliability of model. The Root Mean Square Deviation (RMSD) between the model and template was 0.19 Å. The quality of model was assessed using Ramachandran and rotamer plots. As evident from Fig. 4, most of the residues lie in the core or allowed region except TYR286 and THR566; the outliers. Ramachandran contours are useful for evaluation of backbone dihedral angles (Fisinger, Serrano, & Lacroix, 2001), while Rotamer plots display discrete sets of favorable conformations adopted by amino acid side chains depending on the relative strain energy of the side chains (Renfrew, Butterfoss, & Kuhlman, 2008). The rotamer energy plot of the developed model (Fig. 4) shows rotamer energies are generally well below the threshold level of 5 kcal/mol, providing further support for the reliability of our model.

The mechanism of α-glucosidase inhibition by RC was elucidated by molecular docking studies according to the protocol applied described in our previous study (Barakat et al., 2016). MOE-dock was used to generate 100 conformations of RC. The resultant poses were clustered and the pose presenting the highest score for the largest cluster was selected for further analysis. The binding mode and interaction profile of RC with yeast α-glucosidase is represented in Fig. 5. The MSMS surface model of the protein suggested that the presence of basic residues around the naphthoquinone ring of the ligand complements binding of the relatively more electronegative part of the ligand. The tail of the ligand containing an aliphatic chain has folded to accommodate itself in the hydrophobic groove of the protein.

Ligand-protein interaction profiling (Salentin, Schreiber, Haupt, Adasme, & Schroeder, 2015) confirmed the formation of various hydrogen and hydrophobic bonds between RC and α-glucosidase. As depicted in Fig. 5, the core of RC has anchored to the protein via special bidentate interactions with LYS232 and 414. Another hydrogen bond has also been observed between the backbone N atom of SER157 and RC. The hydrophobic core that surrounds the tail of the ligand includes residues LYS143, PRO144, THR160 and PHE161. The observed affinity in molecular modeling studies between the ligand and enzyme helps explains the experimental findings of high inhibition of α-glucosidase activity by RC.

In order to investigate the stability of the proposed ligand-protein model, a short production run (10 ns) of all atom MD simulation was performed using AMBER14. MD simulation has emerged as a major technique in the array of tools to design bioactive molecule and investigate their mode of action. Apart from extraction of information regarding the distances and interactions between ligand and residues of interest, MD trajectories allow estimation of overall stability of complex. We have measured different characteristics of the system to measure the dynamic differences induced in system upon ligand binding. Root Mean Square

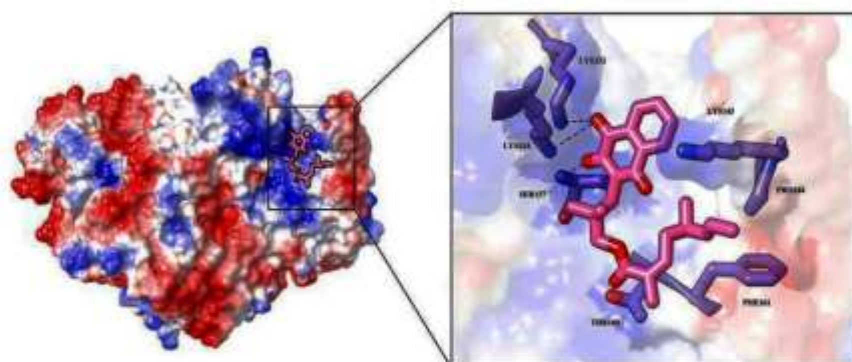

Fig. 5. The MSMS model of the *S. cerevisiae* α-glucosidase as rendered by columbic distribution. The electronegativity is depicted by Red, hydrophobicity is depicted by white while blue color highlight electropositive regions. The boxed picture depicts the interactions mediated by RC with protein. Also notable in the picture as residues lining the hydrophobic groove in cavity which help in establishment of apolar contacts with the tail of ligand. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

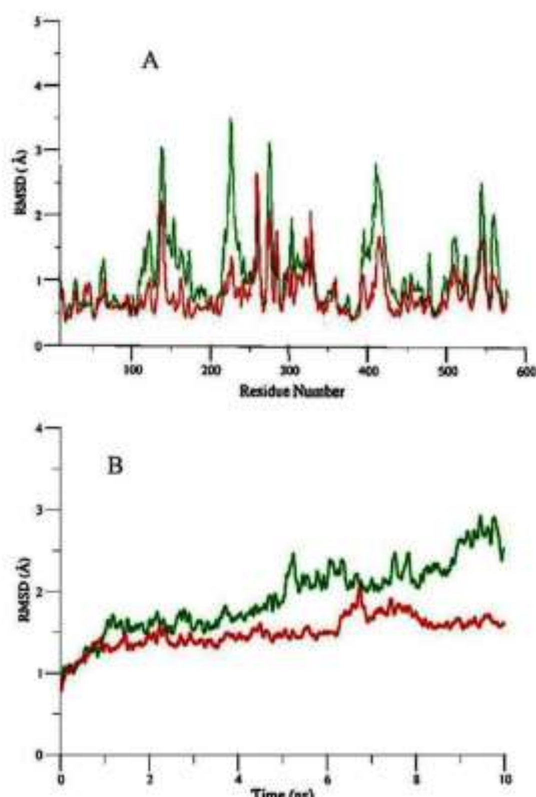

Fig. 6. The graphs showing root mean square fluctuation as function of residue number (A) and root mean square deviation as a function of time (B) of protein backbone.

Deviation (RMSD) is a measure of stability of complex, the lower the RMSD the higher the stability. The average RMSD values of the two systems; apo and complex, were 1.5 and 2.0 Å, respectively. The plot shows more fluctuation in complex as compared to apo-structure, though the overall system remains unstable (Fig. 6A). Further, to investigate the exact nature of this deviation, Root Mean Square Fluctuation (RMSF) was calculated (Fig. 6B). We have observed higher fluctuations in case of α-glucosidase-RC complex with notably higher deviations in case of residues LYS229, ASP278, and GLU548. Visual inspection of these residues annotated by secondary structure suggests that these residues are present in exposed loops which are highly dynamic in nature. The visual analysis of MD trajectories revealed that the ligand anchored the protein via hydrogen bonds during first 2 ns of the simulation. The ligand presented significant displacement and there is a difference of 2.46 Å in the coordinates before and after simulation. Moreover, it was observed that the contacts were later stabilized by hydrophobic interactions displayed by ligand.

4. Conclusion

RRE containing RC at a level of 62.2% was obtained from *R. nasutus* leaves using a green extraction method. RRE exhibited a non-competitive inhibitory effect against α-glucosidase and may be considered as an alternative natural antidiabetic agent to control postprandial blood glucose levels. RRE displayed synergistic inhibition of α-glucosidase in combination with acarbose, suggesting its clinical use to reduce the dose and adverse effects related to acarbose.

Author contribution

MAS and PP conceived and designed the research study. MAS conducted the experiments. RK and ZU designed and carried out *in silico* calculations. MAS, RK, ZU and PP analyzed the data, discussed the findings and prepared the manuscript.

Conflict of interest

The authors declared that they have no conflicts of interest.

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PAPER III

Superoxide Scavenging and Antiglycation Activity of Rhinacanthins-rich Extract Obtained from the Leaves of *Rhinacanthus nasutus*

Muhammad Ajmal Shah[1], Haji Muhammad[2], Yasir Mehmood[3], Ruqaiya Khalil[4], Zaheer Ul-Haq[4], Pharkphoom Panichayupakaranant[1, 5*]

[1]Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

[2]Department of Chemistry, Federal Urdu University of Arts, Science & Technology, Gulshan-e- Iqbal, Campus, Karachi-75300, Pakistan

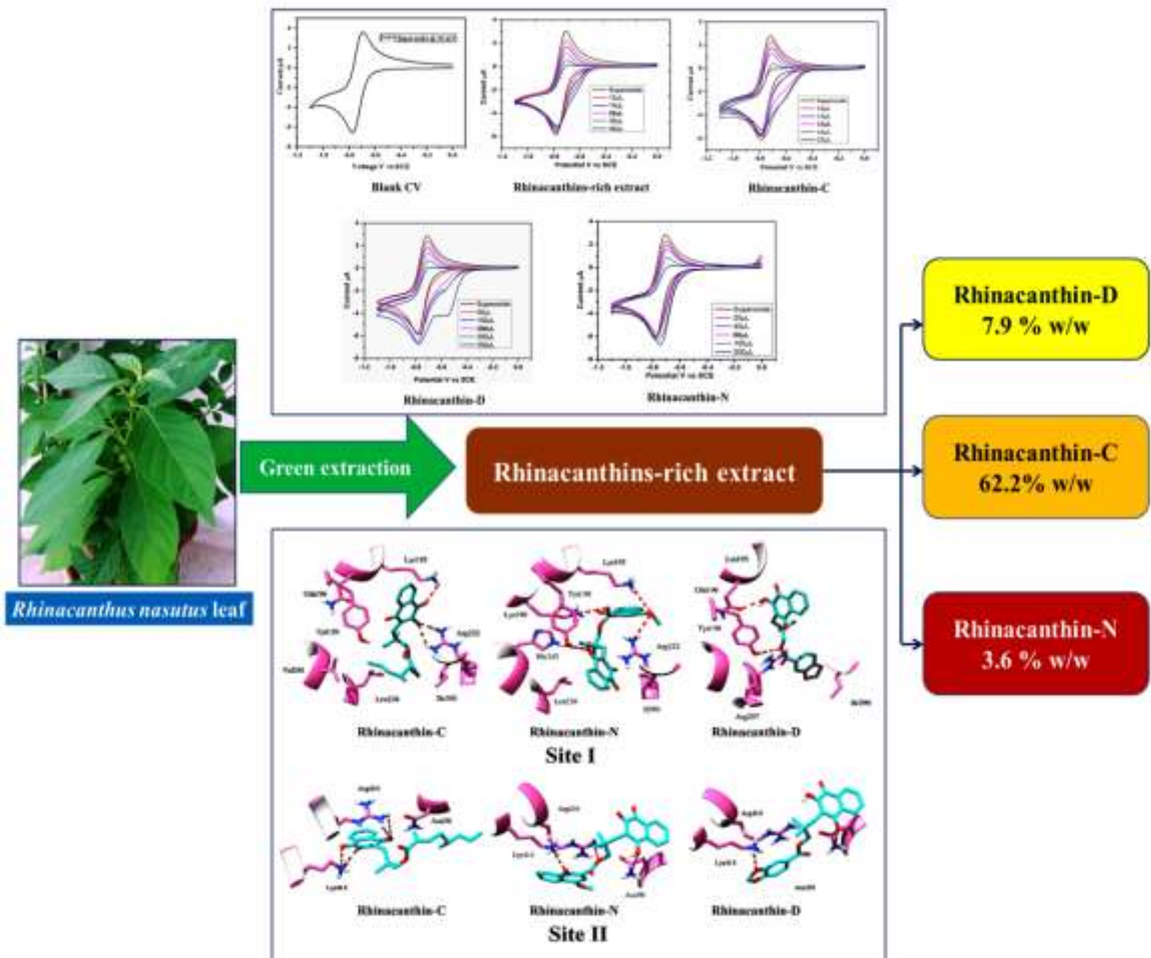
[3]Department of Chemistry, Quaid-i-Azam University, Islamabad, Pakistan

[4]Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

[5]Phytomedicine and Pharmaceutical Biotechnology Excellence Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

*Corresponding author: Pharkphoom Panichayupakaranant Tel. /fax: +66 74 428220.
E-mail address: pharkphoom.p@psu.ac.th (P. Panichayupakaranant)

GRAPHICAL ABSTRACT



ABSTRACT

Background: Oxidative stress and non-enzymatic protein glycation lead to serious diabetic complications that increase the risk of mortality. *Rhinacanthus nasutus* leaf crude extracts are previously reported for their antidiabetic, antiglycation and antioxidant potential. **Objective:** The present study was performed to prepare a standardized rhinacanthins-rich extract (RRE) and evaluate its superoxide scavenging and antiglycation effects compared to its marker compounds namely rhinacanthin-C (RC), rhinacanthin-D (RD) and rhinacanthin-N (RN). **Materials and methods:** RRE was obtained by microwave assisted green extraction along with a simple step of fractionation using Amberlite® column. RC, RD and RN were isolated from the RRE using silica gel column chromatography. Superoxide scavenging activity was performed by cyclic voltammetry and fructose mediated human serum albumin glycation model was used for antiglycation activity. *In silico* studies were conducted to identify the structure activity relationships of rhinacanthins. **Results:** On the basis of kinetic measurements, RRE exhibited the most potent antioxidant activity via E_rC_i mechanism, with an IC_{50} value of 8.0 µg/mL, antioxidant capacity of 39439 M^{-1} and binding constant of 45709 M^{-1}. Antiglycation assay showed that RRE exhibited almost equivalent glycation inhibitory effect to that of RC, with IC_{50} values of 39.7, and 37.3 µg/mL, respectively, but higher than that of RD (IC_{50} of 50.4 µg/mL), RN (IC_{50} of 89.5 µg/mL) as well as the positive control, rutin (IC_{50} of 41.5 µg/mL). **Conclusion:** The potent superoxide scavenging and albumin glycation inhibitory effect of RRE rationalized its therapeutic application in various chronic diseases especially in the complications of diabetes.

KEYWORDS: Antiglycation, antioxidant, rhinacanthins-rich extract**,** *Rhinacanthus nasutus*

ABBREVIATIONS USED: RRE: rhinacanthins-rich extract; RC: rhinacanthin-C; RD: rhinacanthin-D; RN: rhinacanthin-N; IC_{50}: 50% inhibitory concentration; Kao: antioxidant activity coefficient; Kb: binding constant; E_rC_i: reversible electron transfer followed by an irreversible chemical reaction; DM: diabetes mellitus; AGEPs: advanced glycation end products; NMR: nuclear magnetic resonance; HPLC: high performance liquid chromatography; CV: cyclic voltammetry; DMSO: dimethyl sulfoxide; Ipa: anodic peak current; Ipc: cathodic peak current; HSA: human serum albumin; MOE: molecular operating environment; PASSonline: online prediction of activity spectra for substances

SUMMARY

- Rhinacanthins-rich extract exhibited potent superoxide scavenging activity.
- RRE and rhinacanthin-C showed remarkable and comparable antiglycating effect.
- Rhinacanthins exhibited antiglycation activity by masking specific residues of albumin.

INTRODUCTION

Type-2 diabetes mellitus (DM) is a serious global health concern. The international prevalence of DM increased from 4.7-8.5% during the last three decades, and the number of diabetic patients was estimated as 422 million in 2014.[1] Oxidative stress caused by an imbalance of free radicals, is implicated in various chronic disease conditions including DM. Chronic DM leads to serious complications including nephropathy, neuropathy, retinopathy and cardiovascular problems and increase the risk of mortality. The overproduction of free radicals can be attenuated by intake of antioxidants.[2] Diabetic complications are instigated by 'macromolecule aging' phenomena involving non-enzymatic glycation reactions such as nucleophilic addition reactions between the amino groups of proteins and the carbonyl groups of reducing sugars in chronic hyperglycemic conditions.[3-5] Hyperglycemia and oxidative stress are the major factors in accelerating the formation of early glycation products that

subsequently rearrange and dehydrate into more stable compounds known as advanced glycation end products (AGEPs).[3,6] Formation of AGEPs acts as positive feedback for oxidative stress that further damages cells and intensifies diabetic complications.[7]

A number of compounds have been used to inhibit AGEPs formation such as aminoguanidine but toxicity and adverse effects limit use of these agents.[8] Plant extracts and isolated phytochemicals are recognized as highly valuable sources of novel therapeutic molecules which offer a potential alternative to currently used drugs which may be associated with side effects. Various plant extracts and phytochemicals have been reported to offer potential as antidiabetic drugs, which function via antioxidant and anti-AGEPs mechanisms.[9,10] In particular, *Rhinacanthus nasutus* (L.) Kurz (Family Acanthaceae), a medicinal herb native to Thailand and Southeast Asia, has traditionally been used in the treatment of various disorders including DM.[11] In China and Taiwan, *R. nasutus* has been consumed as an herbal drink.[12,13] Methanol extracts of *R. nasutus* leaf have been investigated extensively for antidiabetic activity.[14-18] Ethanol and aqueous extracts of *R. nasutus* leaves have also been reported to exhibit antioxidant and antiglycation activities.[19,20] Rhinacanthin-C (RC), a major phytochemical of *R. nasutus* leaf has recently been shown to elicit antidiabetic, hyperlipidemic and pancreatic protection effects in diabetic rats.[21] However, the multi-stage and high cost purification process of RC hinders drug development. Rhinacanthins-rich extract (RRE) is a semi-purified extract obtained from *R. nasutus* leaf that contains almost 70% w/w rhinacanthins in total, with 60% w/w of RC as the major constituent.[22] In the present study, RRE was obtained using a simple, environment-friendly, 'green' extraction method to investigate its superoxide scavenging and AGEPs inhibitory activity. RRE offers significant advantages as an alternative to RC in term of lower production cost and potentially equivalent or higher bioactivity due to synergism between RRE components.[23,24] *In silico* studies were conducted to identify the relationships between rhinacanthins structure and antiglycating activity and to predict their antioxidant potential.

MATERIALS AND METHODS

Chemicals

Tetrabutylammonium perchlorate was obtained from TCI, Japan and DMSO of HPLC grade was obtained from Merck, Germany. Human serum albumin (HSA), fraction V was purchased from Advent Bio, USA. Rutin was obtained from Alfa Aesar, Germany. All other chemicals used were of analytical grade.

Plant material source, extraction and isolation

The fresh leaves of *R. nasutus* were collected from the Botanical Garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai Campus, Thailand and the voucher specimen (No. 0011814) was field in the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand. Leaves were washed with tap water and dried at 60°C for 24 h in a hot air oven and reduced to powders using a grinder, which were passed through a No. 45 sieve.

RRE was prepared using ethanol by previously described method[22] with some modifications using green extraction process. RC, RD and RN were purified form the RRE using a silica gel column eluted by hexane and ethyl acetate (99:1, v/v). The structures of all three compounds [Figure 1] were confirmed by comparing the ^{1}H and ^{13}C-NMR spectral data with those from the literature.[25,26]

HPLC analysis of RRE

HPLC analysis of RRE was performed as previously described[22] using a UFLC Shimadzu system incorporating a Discovery® C18 (5 µm, 4.6 × 150 mm) column (Supelco, PA, USA) equipped with a photodiode-array detector and autosampler (Shimadzu Corp. Kyoto, Japan).

Assay for antioxidant activity using cyclic voltammetry

Cyclic voltammograms were obtained using an Autolab PGSTAT 302 Potentiostat in combination with software GPES 4.9 (Eco Chemie, Utrecht, Netherland). The electrochemical experiments were carried out in a conventional three electrodes

comprising glassy carbon as the working electrode, saturated calomel as the reference electrode, and platinum wire as the counter electrode. The working electrode was polished with alumina prior to making cyclic voltammetry (CV) measurements. All CV runs were carried out at 25±1 ºC at 25 mV/s sweep rate.[27]

To assess the antioxidant capacity of the target compounds, CV was performed in 0.1M tetrabutylammonium perchlorate in DMSO.[27] CV was first carried out at 25 mV/s scan rate without any sample or blank solution (0.1 M Tetrabutylammonium perchlorate in DMSO) to produce the superoxide. Aliquots (μL) of samples (4 mg/mL) were then added to DMSO and CV measurements were recorded together with the response of anodic and cathodic peaks. An addition of further aliquots of sample was terminated when the anodic peak was diminished. The percent superoxide scavenging effect was calculated as follows:

% Inhibition = $Ip^o - Ip \times 100 / Ip^o$

Where, Ip and Ip^o are the anodic peak currents of superoxide with and without sample.

Kinetic parameters were also measured, namely the antioxidant activity coefficient (Kao), binding constant (Kb) and spontaneity of the interaction (−ΔG) of RRE and its marker compounds.[28,29]

Antiglycation assay

Fructose mediated HSA glycation inhibitory activity of REE, RC, RD and RN was performed as previously described.[30] Rutin was used as a positive control. The percent protein glycation inhibition of the test compounds and positive control was calculated as follows:

% Inhibition = (1- fluorescence of test sample/ fluorescence of the control) x 100

Molecular docking simulations

Molecular docking simulations of RC, RD and RN were carried out to help rationalize the observed antiglycation activity of the REE and its marker compounds. RC, RD

and RN were docked against HSA, which contains multiple binding sites having preference for specific chemotypes. Molecular docking simulations focused on two sites having significant correlation with drug activity. Site I was identified using the coordinates of warfarin (PDB 2BXD) while site II was characterized by ibuprofen binding (PDB 2BXG). Both the structures were subjected to protein correction module in MOE2015.10 prior to docking. Protonate 3D was used to add missing hydrogen atoms. Docking was carried out using the default rigid receptor protocol. The interaction pattern was observed by PLIP; a web server for automatic detection of protein-ligand interactions.[31] All the visuals were rendered using Chimera[32] and MOE.[33]

In silico **biopredicition**

In silico bioactivity screening of RC, RD and RN was performed using the Prediction of Activity Spectra for Substances (PASSonline) webserver based on their chemical structure.[34]

RESULTS AND DISCUSSION

Determination of rhinacanthins contents in RRE

On the basis of HPLC analysis, RRE used in this study contained RC as a major constituent (62.2% w/w), while rhinacanthin-D (7.9 % w/w) and rhinacanthin-N (3.6 % w/w) were present as minor components.

Antioxidant activity of RRE and its marker compounds

A reversible cyclic voltammogram was obtained with cathodic peak current (Ipc) 4.69 µA and anodic peak current (Ipa) 4.59 µA [Figure 2A]. The forward to reverse peak current ratio of almost unity confirmed the generation of superoxide free radical which is decreased in the presence of scavenging agents resulting in a reduction of anodic peak current.[27] The scavenging ability of RRE and its marker compounds was assessed by adding increasing volumes of RRE (10 to 25 µL), RC (12 to 40 µL), RD (50 to 350 µL) and RN (20 to 200 µL) solutions to react with the electrochemically

generated superoxide [Figure 2B-2E]. RRE and RC exhibited the highest and almost equivalent superoxide scavenging activity with IC_{50} values of 8.0 µg/mL and 9.6 µg/mL, respectively. These results are significantly higher than the minor naphthoquinone esters, RD (IC_{50} value 91.4 µg/mL) and RN (IC_{50} value 45.1 µg/mL) [Table 1]. In the present study, the added volume of the different compounds reflected their scavenging impact. RRE was found to be the most powerful scavenger and interestingly, the data revealed that the scavenging potency of RRE is identical to the combined activity of its specific chemical composition. These *in vitro* findings support a previous *in vivo* study on enhanced antioxidative enzymes in liver and pancreas of diabetic rats by *R. nasutus* leaf methanol extract and RC.[16,21]

The relative superoxide scavenging capacity of the test compounds was expressed as the antioxidant activity coefficient (*Kao*)[29], which is the ratio of current density, in the presence and absence of substrate to the electrochemically generated superoxide free radicals. The relative antioxidant activity of each compound was quantified using the following equation:

$$Kao = \Delta J / ((J_o - J_{res}) \Delta c)$$

Where, ΔJ is the change in oxygen current density in the presence of analyte, J_o is the limiting current density of oxygen in the absence of analyte, J_{res} is the residual current density of dissolved oxygen and ΔC is the change in the concentration of the analyte in mol/L. The equation is valid only for the region in which there is a linear change in the value i.e. at low sample concentration. The antioxidant activity of the compounds was determined using a modified expression where ΔC is replaced by ΔV_{ext}. The tabulated data showed that the antioxidant activity of the samples is; RRE>RC>RN>RD [Table 1].

The degree of interaction between the superoxide anionic radical and each test compound was expressed in terms of the binding constant '*Kb*'[28,29] derived from the reduction in peak current and determined using the following equation:

$$\log [1/(AO)] = \log Kb + \log [Ip/Ip^o - Ip]$$

Where *Ip* and Ip^o are the peak currents of electrochemically generated superoxide anion radical in the presence or absence of the test compound respectively, *[AO]* is the compound concentration which was replaced by the volume of the compound (ΔV_{ext}). Since the volume of the solution containing ($O_2^{\bullet -}$) is fixed, volumetric addition of the samples is proportional to their number of moles i.e. concentration. Compounds resulting in higher *Kb* values show enhanced interaction with the free radical. From [Table 2] it is evident that the *Kb* values follow the order: RRE>RC>RN>RD. Moreover, the *Kb* values reveal strong interaction even at very low concentration. The ΔG values calculated using the following equation and displayed in Table 1 indicate the degree of spontaneity of the interaction between the superoxide free radicals and the test compounds and support the scavenging capacity as measured by *Kao*. Together with the *Kb* value, a threshold 'ΔG' value may provide a useful factor to classify sample scavenging ability.

$$\Delta G = -RT \ln Kb$$

Based on the irreversible scavenging of superoxide (Fig. 2B) and the values of kinetic parameters [Table 1], RRE may be classed as a potent superoxide scavenger, operating *via* E_rC_i mechanism and probably involving a synergistic effect due to the combination of rhinacanthins.

Antiglycation activity of RRE and its marker compounds

The fructose mediated HSA glycation inhibitory activity of RRE and its marker compounds was evaluated to explore their potential role in treating diabetic complications. Previous reports rationalized the anti-AGEPs activity of RRE, RC, RD and RN on the basis of their 1, 4-naphthoquinone skeletal structure.[20,35] In the present study, both RRE and RC were found to exhibit significant glycation inhibitory activity with IC_{50} values of 39.7 and 37.3 µg/mL, respectively that were slightly higher than that of the positive control, rutin (41.5 µg/mL) [Table 2]. RRE and RC showed almost equivalent antiglycation activity, similar to previously reported anti-inflammatory and antimicrobial activities.[23,24] Furthermore, the antiglycation activity of RC supports the findings of a study by Adam et al[21] in which RC caused a reduction of glycated hemoglobin levels in diabetic rats. RD and RN, the minor

naphthoquinone compounds of RRE also showed impressive antiglycation activity with IC_{50} values of 50.4 and 89.5 µg/mL, respectively [Table 2]. Diabetes and age-related diseases including neurotoxic disorders are mainly caused by the unusual protein aggregation.[7] Thus, the potent anti-AGEPs activity of RRE, measured in this study recommends further evaluation of these compounds as therapeutics for treatment of a range of conditions of major clinical and global significance.

Molecular interaction studies

Molecular docking studies are commonly performed in the process of drug discovery to predict the occurrence of protein-ligand-binding and its possible lead to therapeutic effect. We applied molecular docking protocols to explore binding between HSA, the major transport protein in the circulatory and lymphatic system[36] and RRE and its marker compounds (RC, RN and RD) and thereby help explain their observed anti-glycation activity. The non-enzymatic glycation of lysine and arginine residues in HSA; in the case of diabetes, impairs the transport of several moieties leading to detrimental physiological effects.[37] Masking of the lysine and arginine residues has therefore been proposed as an effective strategy to inhibit non-enzymatic glycation of HSA. There are two main sites in the HSA structure which offer opportunities for drug action; Sudlow's site I and site II. Docking simulations were performed using both sites to investigate ligand binding. Sudlow's site I was identified using the coordinates of warfarin from the PDB: 2BXD while, ibuprofen from PDB: 2BXG identified Sudlow's site II.[38] The docking scores of each compound calculated for the Sudlow's site I and II of HSA are presented in [Table 3].

The binding mode of RC, RD and RN in each site is presented in [Figure 3]. The compounds establish polar contacts with surrounding arginine and lysine residues. In Sudlow's site I, RC forms hydrogen bonds with Lys195, and Arg222, RD binds to Lys199 and Arg257 and RN interacts with Lys195, Lys199, Arg218 and Arg222. The rhinacanthins are also involved in the formation of salt bridges. In the case of Sudlow's site II, RC, RD and RN were found to interact with Arg410 and Lys 414. The complexes are also stabilized by Van der Waal's forces between the ligands and other amino acid residues at Sudlow's site I, namely Tyr150, Leu238, and Leu260.

Residues Ile388, Asn391 and Phe403 lining Sudlow's site II provided anchorage for the ligands via formation of hydrophobic and aromatic contacts. Interestingly, the binding pattern of RC, RD an RN in this analysis is consistent with docking studies of cinnamic acid reported earlier.[39]

In silico **predictions of bioactivity**

PASSonline as used to predict the potential targets and pharmacological effect of the marker compounds of RRE based on structural information. The analysis presents the ratio of probability of being active (Pa) or inactive (Pi) with regards to a particular biological effect.[40] The antioxidant potential and selection of predicted biological activities for the marker compounds of RRE presented in [Table 4], provides additional, strong support for the superoxide scavenging activity of RRE.

CONCLUSIONS

This is the first report on antioxidant and antiglycation potential of RRE and its marker compounds, rhinacanthin-C, -D and -N. The docking studies determined the binding mode of rhinacanthins with respect to human serum albumin. Rhinacanthins exhibited antiglycation activity by masking different residues of albumin. The potent superoxide scavenging and remarkable protein glycation inhibitory effects of RRE further rationalized its therapeutic application in various chronic diseases especially in the complications of diabetes.

AUTHOR CONTRIBUTION

MAS and PP conceived and designed the research study. MAS, HM and YM conducted the experiments. RK and ZU designed and carried out *in silico* calculations. MAS, HM, YM, RK, ZU and PP analyzed the data, discussed the findings and prepared the manuscript**.**

ACKNOWLEDMENTS

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Table 1 Super oxide scavenging effect and kinetic parameters of rhinacanthins-rich extract, rhinacanthin-C, rhinacanthin-D and rhinacanthin-N in cyclic voltammetry of 0.1 M tetrabutylammonium perchlorate in DMSO at glassy carbon electrode, at 25 mV/s scan rate.

Compounds	IC_{50} (µg/mL)	Kao (M^{-1})	Kb (M^{-1})	−ΔG (KJ/mole)
Rhinacanthins-rich extract	8.0	39439	45709	2468
Rhinacanthin-C	9.6	38281	43199	2448
Rhinacanthin-D	91.4	30463	24769	2312
Rhinacanthin-N	45.1	35720	36529	2408

Table 2 Antiglycation activity of rhinacanthins-rich extract and its marker compounds**.**

Compounds	Antiglycation activity IC_{50} (µg/mL)
Rhinacanthins-rich extract	39.7 ± 2.90[a]
Rhinacanthin-C	37.3 ± 2.59[a]
Rhinacanthin-D	50.4 ± 2.67[b]
Rhinacanthin-N	89.5 ± 3.73[c]
Rutin	41.5 ± 2.37[d]

Inhibition concentrations are expressed as the mean ± SEM (n=3). Mean values followed by different letters are significantly different (*P ≤ 0.05*).

Table 3 The docking scores of the top ranked pose of rhinacanthin-C, rhinacanthin-D and rhinacanthin-N with the druggable sites in human serum albumin.

Ligands	Docking site	
	Sudlow's site I	Sudlow's site II
Rhinacanthin-C	−7.78	−7.93
Rhinacanthin-D	−7.62	−8.00
Rhinacanthin-N	−8.12	−8.12

Table 4 The pharmacological activities prediction of rhinacanthin-C (RC), rhinacanthin-D (RD) and rhinacanthin-N (RN) by PASSonline.

Compounds	Predicted biological activity									
	Lipid peroxidase inhibition		Membrane integration agonist		Free radical scavenger		Reductant		Antioxidant	
	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi	Pa	Pi
RC	0.713	0.005	0.306	0.191	0.537	0.008	0.681	0.007	0.451	0.009
RN	0.508	0.016	0.698	0.055	0.460	0.017	0.543	0.017	0.336	0.018
RD	0.609	0.009	0.899	0.011	0.349	0.022	0.605	0.011	0.298	0.023

Figure 1 Chemical structures of rhinacanthin-C (**1**), rhinacanthin-D (**2**) and rhinacanthin-N (**3**).

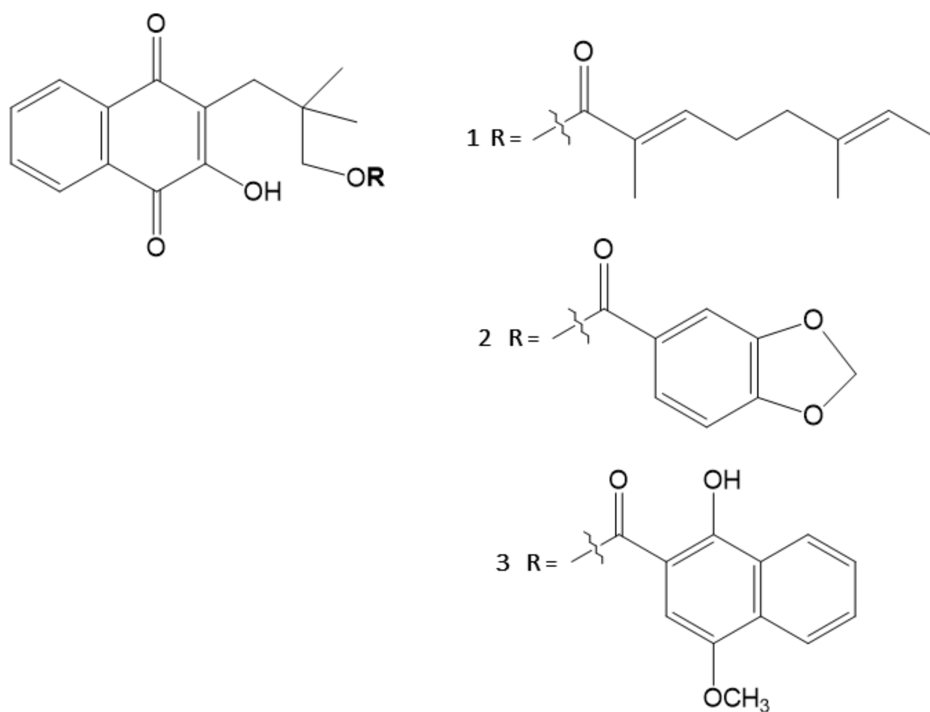


Figure 2 Cyclic voltammogram of 0.1M tetrabutylammonium perchlorate in DMSO (A) with different concentration of RRE (B), RC (C), RD (D) and RN (E) at the glassy carbon electrode (25 mV/s scan rate).

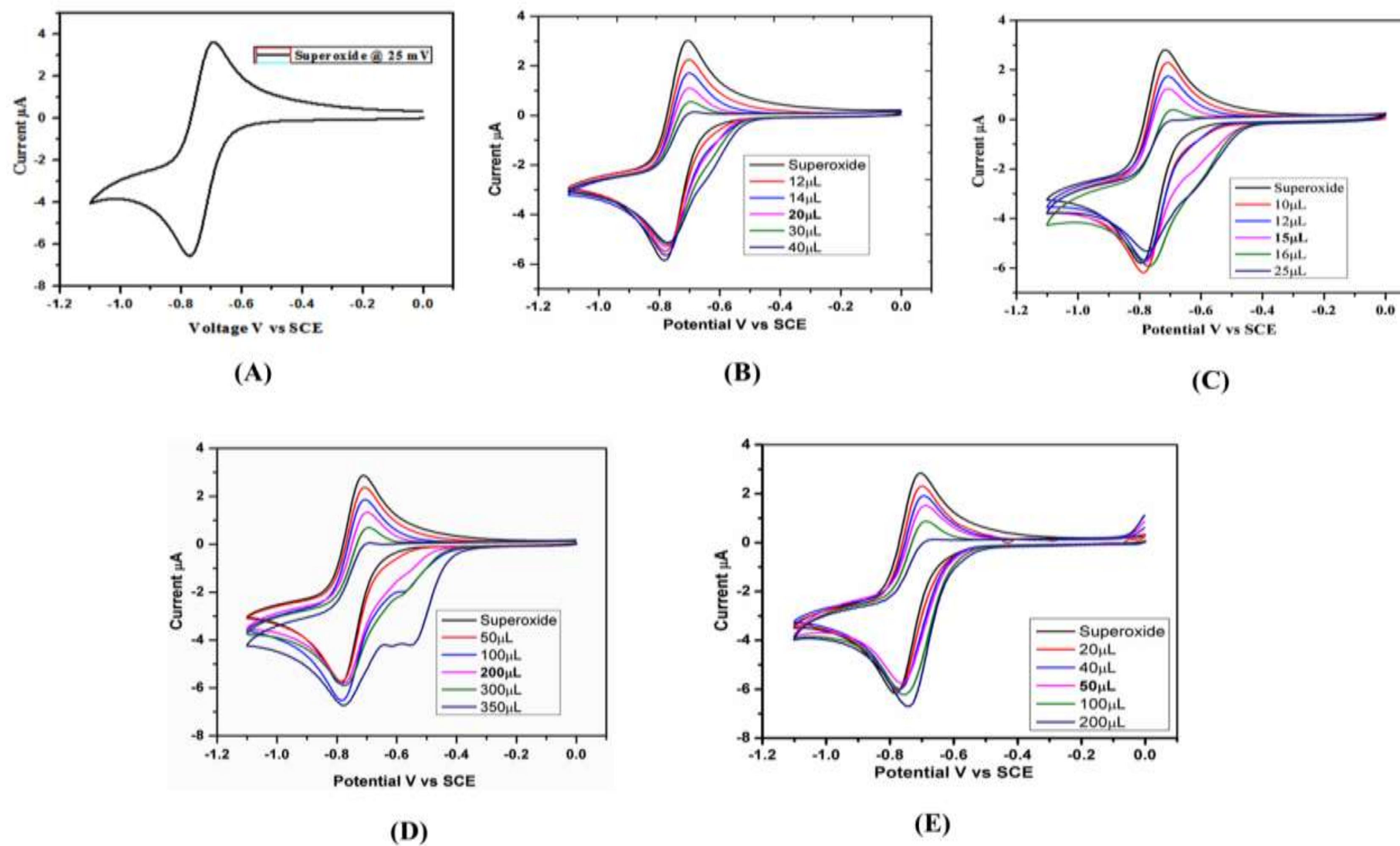
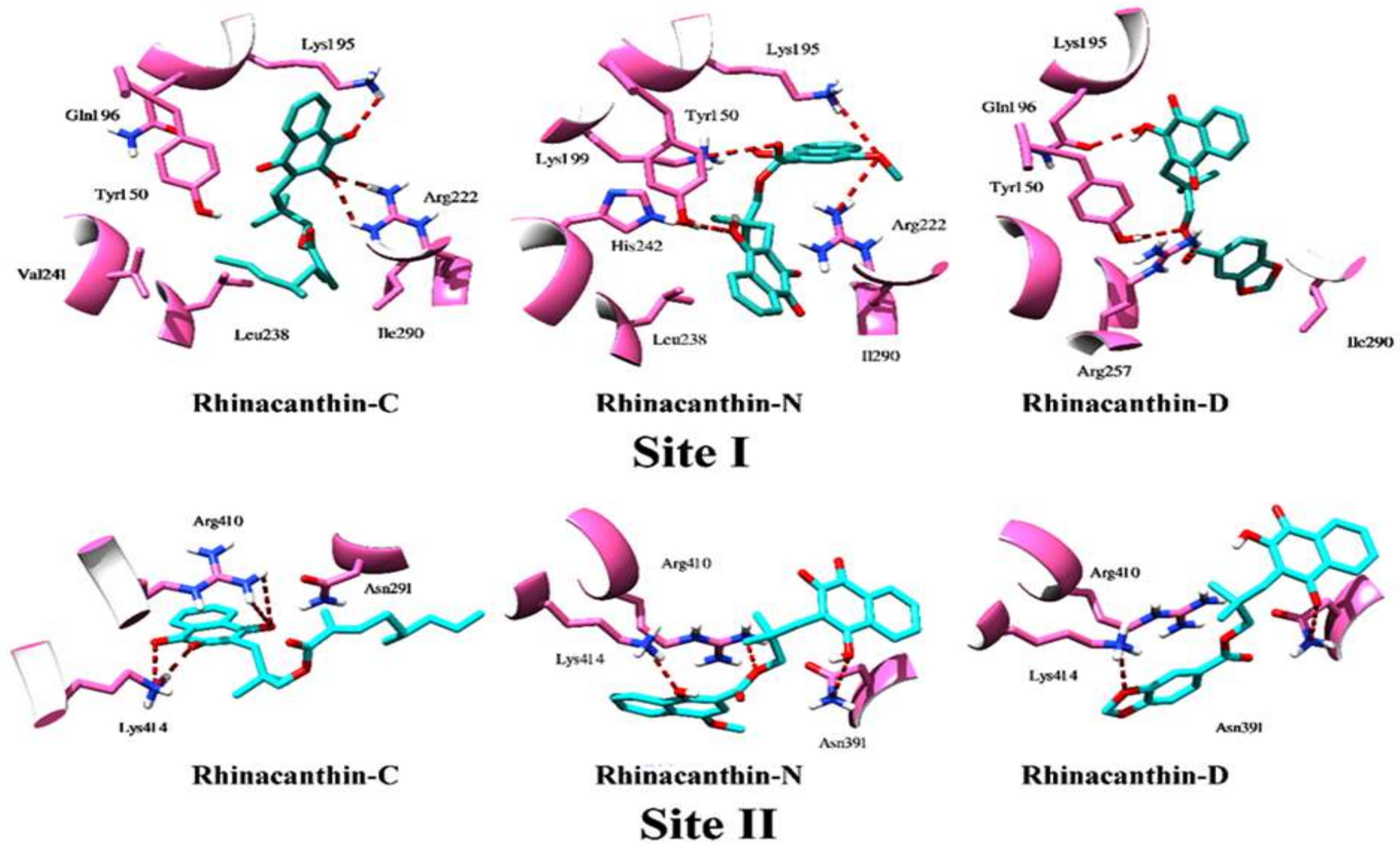


Figure 3 Visualization of the binding mode of rhinacanthin-C, rhinacanthin-D and rhinacanthin-N in the Sudlow's sites of human serum albumin.



PAPER IV

Rhinacanthins-rich Extract Enhance Glucose Uptake and Inhibit Adipogenesis in 3T3-L1 Adipocytes and L6 Myotubes

Muhammad Ajmal Shah[1], Chanawee Jakkawanpitak[2], Decha Sermwittayawong[2], Pharkphoom Panichayupakaranant[1], [3*]

[1]Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

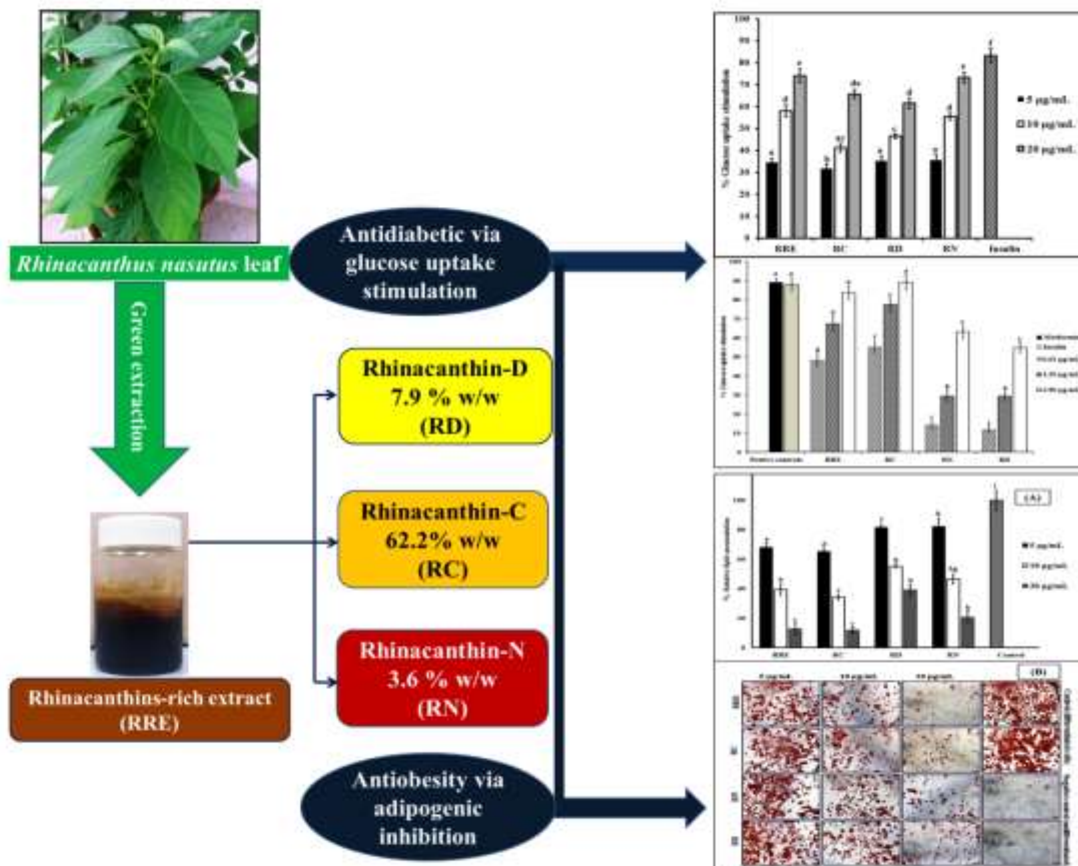
[2]Department of Biochemistry, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

[3]Phytomedicine and Pharmaceutical Biotechnology Excellence Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

Abbreviated running title: Antiadipogenic and glucose uptake activity of rhinacanthins-rich extract

*Corresponding author: Pharkphoom Panichayupakaranant. Tel. /fax: +66 74 428220.
E-mail address: pharkphoom.p@psu.ac.th (P. Panichayupakaranant).

GRAPHICAL ABSTRACT



ABSTRACT

Background: Obesity is one of the imperative dynamic in the incidence and intensification of type 2 diabetes mellitus (T2DM). *Rhinacanthus nasutus* leaf extracts are previously reported for their antidiabetic and antiobesity potential. **Objective:** The present study was performed to evaluate glucose uptake stimulatory and antiadipogenic activities of a standardized rhinacanthins-rich extract (RRE) and its marker compounds namely rhinacanthin-C (RC), rhinacanthin-D (RD) and rhinacanthin-N (RN) in 3T3-L1 and L6 cells. **Materials and methods:** RRE was prepared by a green extraction process and the marker compounds (RC, RD and RN) were isolated from the RRE using a silica gel column chromatography. Glucose uptake stimulation in both 3T3-L1 and L6 cells was performed by quantification of residual glucose in the media using glucose oxidase kit. Antiadipogenic activity in 3T3-L1 adipocytes was performed by intracellular lipids quantification using oil red O dye. **Results:** At the highest effective dose, RRE (20 µg/mL) exhibited satisfactory glucose uptake stimulatory effect in 3T3-L1 adipocytes that equivalent to RN (20 µg/mL) and the positive control insulin (0.58 µg/mL), but higher than RC (20 µg/mL) and RD (20 µg/mL). In addition, treatments of L6 myotubes showed that RRE (2.5 µg/mL) exhibited potent and equivalent glucose uptake stimulation (>80%) to RC (2.5 µg/mL) and the standard drugs, insulin (2.90 µg/mL) and metformin (219.5 µg/mL), but higher than RD (2.5 µg/mL) and RN (2.5 µg/mL). Furthermore, RRE (20 µg/mL) exhibited potent antiadipogenic effect in 3T3-L1 adipocytes, which equivalent to RC (20 µg/mL) but higher than RD (20 µg/mL) and RN (20 µg/mL). **Conclusion:** The undertaken study suggests that RRE could be used as an effective remedy in the treatment of obesity associated T2DM.

KEYWORDS: antidiabetic, antiobesity, rhinacanthin-C**,** rhinacanthin-D, rhinacanthin-N

ABBREVIATION USED: T2DM: type-2 diabetes mellitus; RRE: rhinacanthins-rich extract; RC: rhinacanthin-C; RD: rhinacanthin-D; RN: rhinacanthin-N; WHO: world health organization; α-MEM: α-minimum essential medium; DMEM: Dulbecco's modified Eagle's medium; HS: horse serum; FBS: fetal bovine serum; BSA: bovine serum albumin; IBMX: 3-isobutyl-1-methyl-xanthine; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; GO: glucose oxidase; NMR: nuclear magnetic resonance; HPLC: high performance liquid chromatography;

SUMMARY

- Rhinacanthins-rich extract and its marker compounds showed potent glucose uptake stimulatory activity in 3T3-L1 adipocytes and L6 myotubes.
- Rhinacanthins-rich extract and rhinacanthin-C showed comparable antiadipogenic effect in 3T3-L1 adipocytes.
- RRE could be used as an effective remedy in the treatment of obesity associated T2DM.

INTRODUCTION

According to WHO, type 2 diabetes mellitus (T2DM) is a major type of diabetes comprising 90% of total diabetic cases.[1] Hyperglycemia and hyperlipidemia are prime characteristics in the progression of T2DM and chronic cardiovascular disorders.[2,3] Insulin resistance, the main cause of T2DM is linked with the release of free fatty acids and proinflammatory cytokines from adipose tissues in obesity or excessive adiposity, which stimulate beta cells for over secretion of insulin and its receptors reduction.[4,5] The global prevalence of obesity and overweight raised to almost double with the reported 600 million obese adults and 41 million obese children having high mortality than underweight.[6] Along with other adverse effects, both insulin and non-insulin therapy in T2DM promote weight gain probably via adipogenesis, the foremost cause of T2DM.[7,8] The therapeutic molecule that can effectively control hyperglycemia with antiadipogenic potential would be an ideal antidiabetic agent. Therefore, adipogenic inhibition and glucose uptake stimulation in adipose and muscle tissue present the prominent strategies to control obesity associated T2DM.[9]

Plant extracts and purified phytochemicals are known as highly valuable sources of novel therapeutic molecules that offer a potential alternative to currently used drugs, which may be associated with side effects. Various plant extracts and phytochemicals have been reported to offer potential as antidiabetic and antiobesity drugs.[10,11] *Rhinacanthus nasutus* (L.) Kurz (Family Acanthaceae), a medicinal herb native to Thailand and Southeast Asia, has traditionally been used in the treatment of various disorders including DM.[12] In China and Taiwan, *R. nasutus* has been consumed as an herbal drink.[13,14] Methanol extracts of *R. nasutus* leaf have been investigated extensively for antidiabetic and hypolipidemic activity.[15-19] *R. nasutus* leaf extracts have also been reported for antiobesity effect.[20,21] Rhinacanthin-C (RC), a major phytochemical of *R. nasutus* leaf has been recently reported for antidiabetic, hyperlipidemic and pancreatic protection effects in diabetic rats.[22] However, the multi-stage and high cost purification process of RC hinders drug development. Standardized rhinacanthins-rich extract (RRE) is a semi-purified extract obtained from *R. nasutus* leaf that contains almost 70% w/w rhinacanthins in total, with 60% w/w of RC as the major constituent.[23] RRE offers significant advantages as an alternative to RC in term of lower production cost and potentially equivalent or higher bioactivity due to synergism among RRE components.[23-25] In the present study, RRE was obtained using a simple, environment friendly, green extraction process to investigate its glucose uptake stimulatory and antiadipogenic effects in 3T3-L1 adipocytes and L6 myotubes.

MATERIALS AND METHODS

Chemicals

α-Minimum essential medium (α-MEM), Dulbecco's modified Eagle's medium (DMEM), horse serum (HS), fetal bovine serum (FBS) and bovine serum albumin (BSA) were obtained from Gibco, Canada. Dexamethasone, 3-isobutyl-1-methyl-xanthine (IBMX), oil red O dye, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), penicillin, streptomycin, metformin, insulin and glucose oxidase (GO) kit were purchased from Sigma-Aldrich, USA. All others chemicals used were of analytical grade.

Cell lines

The 3T3-L1 pre-adipocytes and L6 myocytes were obtained from the American Type Culture Collection (ATCC, USA).

Plant material, extraction and isolation

The fresh leaves of *R. nasutus* were collected from the Botanical Garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai Campus, Thailand, and a voucher specimen (No. 0011814) was kept at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Thailand. The leaves were washed with tap water and dried at 60ºC for 24 h in a hot air oven and reduced to powders using a grinder, and the powders were passed through a sieve No. 45. RRE was prepared using ethanol by previously described method[23] with some modifications using green extraction process. RC, RD and RN were purified form the RRE using a silica gel column eluted by hexane and ethyl acetate (99:1, v/v). The structures of all three rhinacanthins [Figure 1] were confirmed by comparing the ^{1}H and ^{13}C-NMR spectral data with those from the literature.[26,27]

HPLC analysis of RRE

HPLC analysis of RRE was performed as previously described method[23] using a UFLC Shimadzu system incorporating a Discovery® C18 (5 µm, 4.6 × 150 mm) column (Supelco, PA, USA) equipped with a photodiode-array detector and autosampler (Shimadzu Corp. Kyoto, Japan). HPLC analysis showed that RRE contained RC (62.2% w/w) as a major compound, and RD (7.9 % w/w) and RN (3.6 % w/w) were the minor compounds.

Determination of cell viability

Cell viability of both 3T3-L1 and L6 cells was determined using MTT reduction assay. After treatment with various concentrations of RRE and its maker compounds, the supernatant was removed, and the cells were incubated with 200 µL MTT solution (0.5 mg/mL) for 4 h at 37ºC under 5% CO_2. The supernatant was carefully removed and 200 µL of DMSO was added to dissolve the formazan. Absorbance was measured with a microplate reader at 570 nm. Cell viability was expressed as a percentage of control.

Glucose uptake stimulation assay in 3T3-L1 adipocytes

Glucose uptake stimulatory effect of RRE and its marker compounds was evaluated in 3T3-L1 adipocytes by previously described methods.[28,29] Briefly the cells were grown in 48 well plates with serum-free DMEM containing 0.2% BSA for 12 h. The cells were washed and incubated with different concentrations of samples in low glucose medium supplemented with 10% FBS for 24 h. Insulin was used as a standard drug. The medium was collected in 96-well plate and glucose uptake assay was performed by the glucose oxidase method using commercial GO kit.

Glucose uptake stimulation assay in L6 myotubes

Glucose uptake stimulatory effect of RRE and its marker compounds was determined in L6 myotubes by previously reported method.[30] Briefly, L6 myocytes were grown in α-MEM with 10% FBS at 37ºC under 5% CO_2. Differentiation to L6 myotubes was performed by 2% HS containing medium in 48-well culture plates. The various amounts of samples were incubated with the cells for 24 h. Insulin and metformin were used as positive controls. After incubation, the media was collected in 96 well plate and the glucose contents were measured by glucose oxidase method using commercial GO kit.

Antiadipogenic assay in 3T3-L1 adipocytes

Antiadipogenic effect of RRE and its marker compounds was determined by previously described method.[31] Briefly, the 3T3-L1 pre-adipocytes were cultured in high glucose DMEM supplemented with 10% FBS at 37°C under an atmosphere of 95% air and 5% CO_2. 2 days post-confluent the cells were incubated in differentiation medium (1 µM dexamethasone, 10 µg/mL of insulin and 0.5 mM IBMX in DMEM) along with various concentrations of samples. The level of differentiation or adipogenesis was determined using oil red O staining.[31]

RESULTS AND DISCUSSION

Glucose uptake stimulatory effect of RRE in 3T3-L1 and L6 cells

On the basis of MTT assay, RRE, RC, RD and RN at various concentrations (0.63-20 µg/mL) showed low cytotoxicity on both 3T3-L1 and L6 cells with cell viability of 80-100% [Figure 2]. Insulin resistance is the major cause of T2DM, the search of small molecules with insulin like glucose uptake stimulation potential is an effective

approach in diabetic treatment. Based on the previous glucose uptake report of RC[22], RRE and its naphthoquinone constituents, RC, RD and RN were evaluated for their glucose uptake stimulation effect in differentiated 3T3-L1 adipocytes by glucose oxidase method. The results showed that RRE and RN exhibited higher glucose uptake stimulation effect than RC and RD, and in a dose dependent manner (5, 10 and 20 µg/mL). The activity at concentration of 20 µg/mL was almost equivalent to the positive control, insulin (0.58 µg/mL) [Figure 3]. The mechanism of glucose uptake enhancement by 1,4-naphthoquinones of RRE may be *via* an insulin independent tyrosine kinase pathway, which is previously reported for shikonin, a 1,4-naphthoquinone of *Lithospermum erythrorhizon*.[32] This is a preliminary study; however it provides an interesting research insight to elucidate in depth and exact glucose enhancement mechanism of rhinacanthins in 3T3-L1 adipocytes. Furthermore, the glucose uptake stimulation along with adipogenic inhibitory potential of REE provides an interesting strategy to control obesity associated T2DM and other related complications.

Regarding the body mass, skeletal muscles are the major body part which utilizes 80 % of blood glucose, presenting a prominent therapeutic target for diabetic treatment.[29] Based on the previous reports on muscular glucose uptake stimulatory potential of 1,4 naphthoquinone[33,34], RRE and its naphthoquinone compounds (RC, RD and RN) were determined for their glucose uptake enhancement potential in L6 myotubes. RRE possessed higher glucose uptake enhancing activity than RC, RD and RN in a dose dependent manner (0.63, 1.25 and 2.5 µg/mL) [Figure 4]. RRE at a dose of 2.5 µg/mL showed potent glucose uptake stimulating activity (>80%) that equivalent to insulin (2.90 µg/mL) and almost 87-folds higher than that of metformin (219.5 µg/mL). The strong glucose uptake stimulatory potential of RRE might be due to the possible synergism among the component rhinacanthins as previously reported in antimicrobial and anti-inflammatory activities.[24,25] These results provide a strong base for further detail mechanistic study of glucose uptake stimulation by rhinacanthins in L6 myotubes that could be insulin dependent via GLUT4 or insulin independent calcium dependent pathway, as previously reported for other natural 1,4 naphthoquinones.[33,34]

Adipogenic inhibitory effect of RRE in 3T3-L1 adipocytes

Adipogenesis or excess intracellular lipid accumulation is the main factor behind obesity and insulin resistance that leads to T2DM. Adipogenic inhibitory property is therefore an effective strategy to control these pathological disorders.[35] RRE and its naphthoquinone compounds (RC, RD and RN) showed potent and comparable dose dependent adipogenic inhibitory activity in 3T3-L1 adipocytes [Figure 5A]. At the highest effective dose (20 μg/mL), the antiadipogenic activity of RRE (<20% intracellular lipids) was significantly equivalent to RC but higher than RD (20.5 % intracellular lipids) and RN (39 % intracellular lipids). The microscopic images of stained lipid droplets in various treated cells further confirmed the consistent dose dependent adipogenic inhibition by RRE and its marker compounds [Figure 5B]. The antiadipogenic potential of RRE correlated with the previous report of shikonin that inhibited adipogenesis *via* inhibiting FABP4 and LPL genes expression.[36] 1,4-Naphthoquinones exert their antiadipogenic activity by both upstream (SREBP1C) and downstream (PPARγ and C/EBPα) regulations. [36] Rhinacanthins should be therefore subjected to further studies on antiadipogenic molecular mechanism. Apart from diabetes, obesity has been reported to be linked with atheromas, cardiovascular disorders and malignant tumors.[37] The epidemiological reports interlinked obesity with metabolic disorders which is further associated with the increased circulation of inflammatory adipocytokines such as leptin, interleukin-6 and tumor necrotic factor, which resulting in malignant growth enhancement.[38] Adipocytes are supposed to be responsible for the release of tumor enhancing adipocytokines.[39] The antiadipogenic effect of rhinacanthins could protect against malignancy *via* reduction in tumor enhancing and inflammatory adipocytokines, which can be correlated with the previous anti-inflammatory and anticancer activity of rhinacanthins.[24]

CONCLUSIONS

This is the first report on the glucose uptake enhancer and antiadipogenic constituents from *R. nasutus* leaf extracts. RRE obtained by green extraction method with 62.2% w/w of RC showed potent glucose uptake stimulatory and antiadipogenic effects in 3T3-L1 adipocytes and L6 myotubes. RRE may be used as a potential candidate for

antidiabetic and antiobesity drug development. Further mechanistic *in vivo* studies of RRE and safety assessment are recommended.

AUTHOR CONTRIBUTION

MAS, DS and PP conceived and designed the research study. MAS and CJ conducted the experiments. MAS, CJ, DS and PP analyzed the data, discussed the findings and prepared the manuscript.

ACKNOWLEDGMENTS

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Conflict of interest

The authors declared that they have no conflicts of interest.

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Figure 1 Chemical structures of rhinacanthin-C (**1**), rhinacanthin-D (**2**) and rhinacanthin-N (**3**).

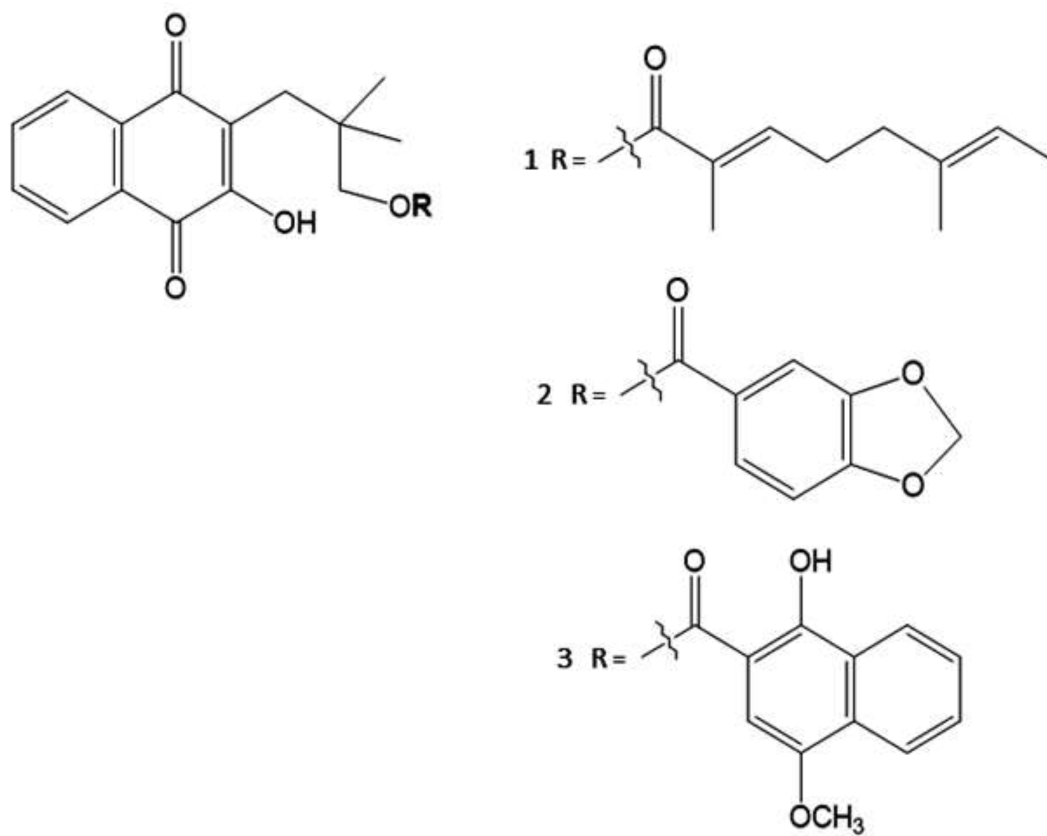


Figure 2 Percentage cell viability of 3T3-L1 (**A**) and L6 (**B**) cells after treatment with various concentrations of RRE (rhinacanthins-rich extract), RC (rhinacanthin-C), RD (rhinacanthin-D) and RN (rhinacanthin-N). Results are expressed as mean ± SD (n=3).

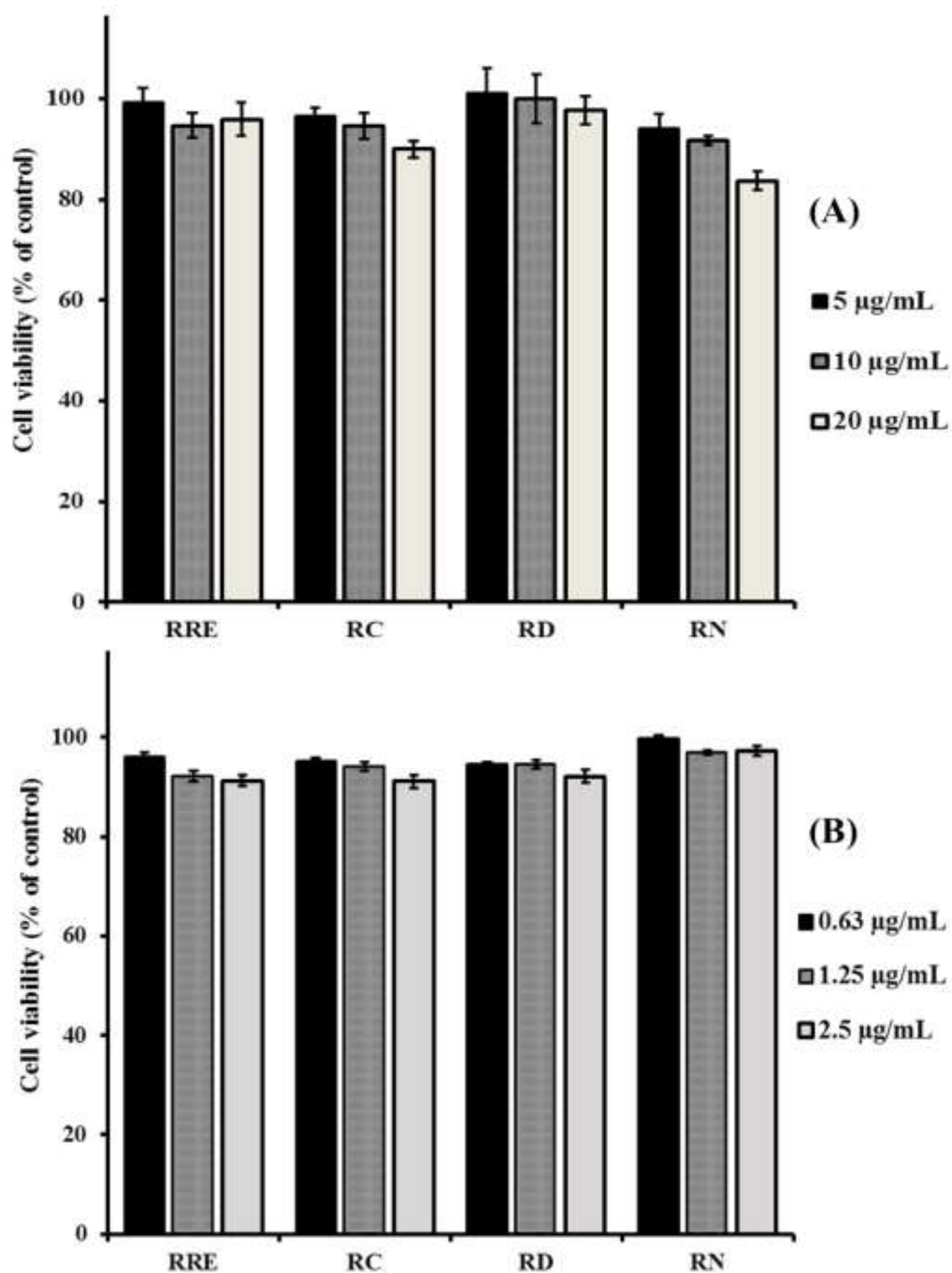


Figure 3 Dose dependent glucose uptake stimulation in 3T3-L1 adipocytes by RRE (rhinacanthins-rich extract), RC (rhinacanthin-C), RD (rhinacanthin-D) and RN (rhinacanthin-N) in comparison with positive control (Insulin= 0.58 µg/mL). Results are expressed as mean ± SEM (n=3). Mean values followed by different letters are significantly different (*P ≤ 0.05*).

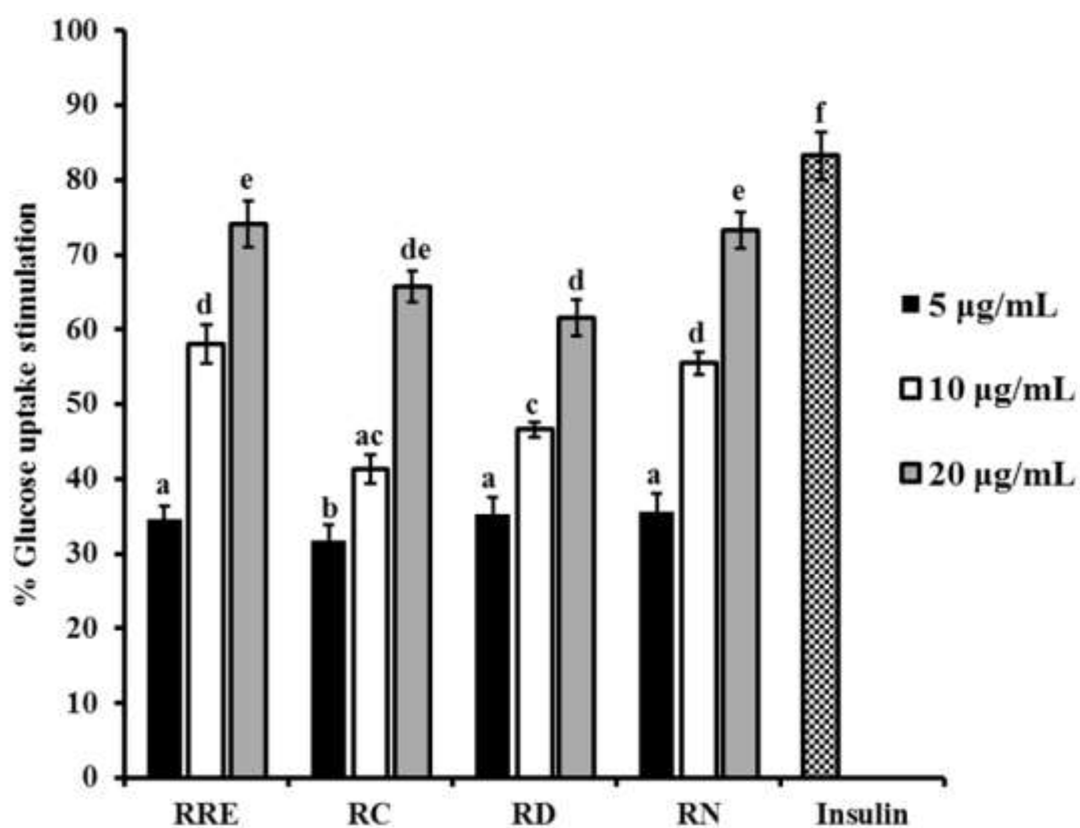


Figure 4 Dose dependent glucose uptake stimulation in L6 muscle cells by RRE (rhinacanthins-rich extract), RC (rhinacanthin-C), RD (rhinacanthin-D) and RN (rhinacanthin-N), in comparison with positive controls (Metformin= 219.5 µg/mL; Insulin= 2.90 µg/mL). Results are expressed as mean ± SEM (n=3). Mean values followed by different letters are significantly different ($P \leq 0.05$).

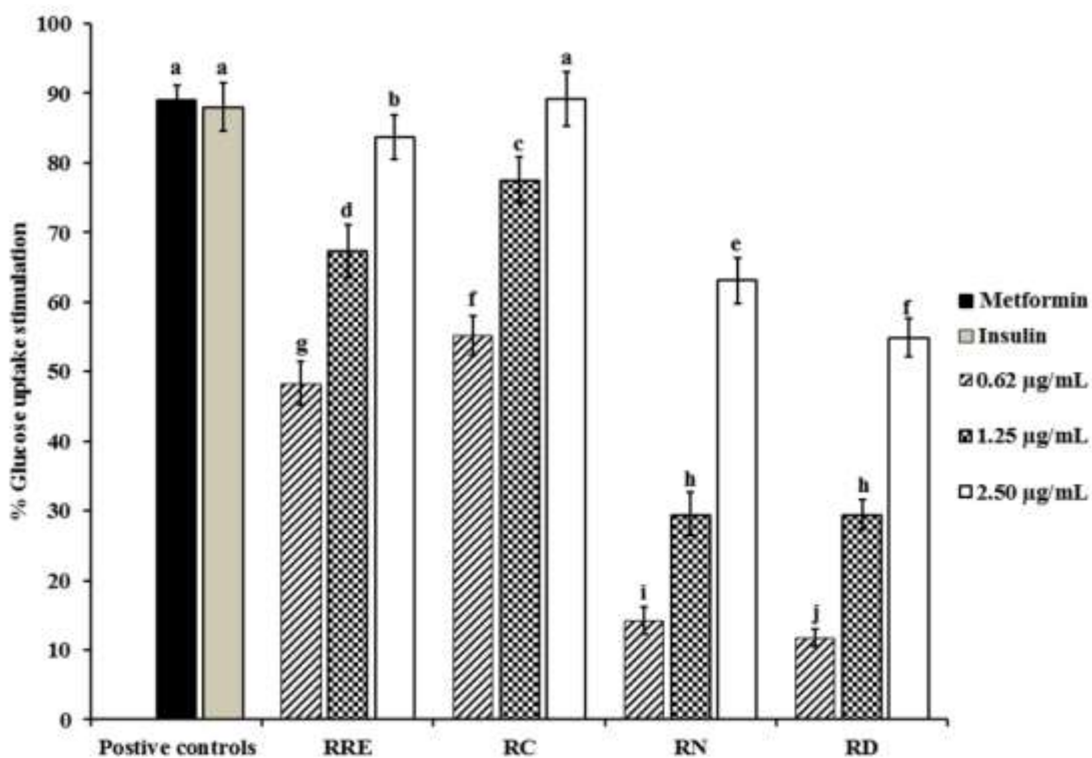
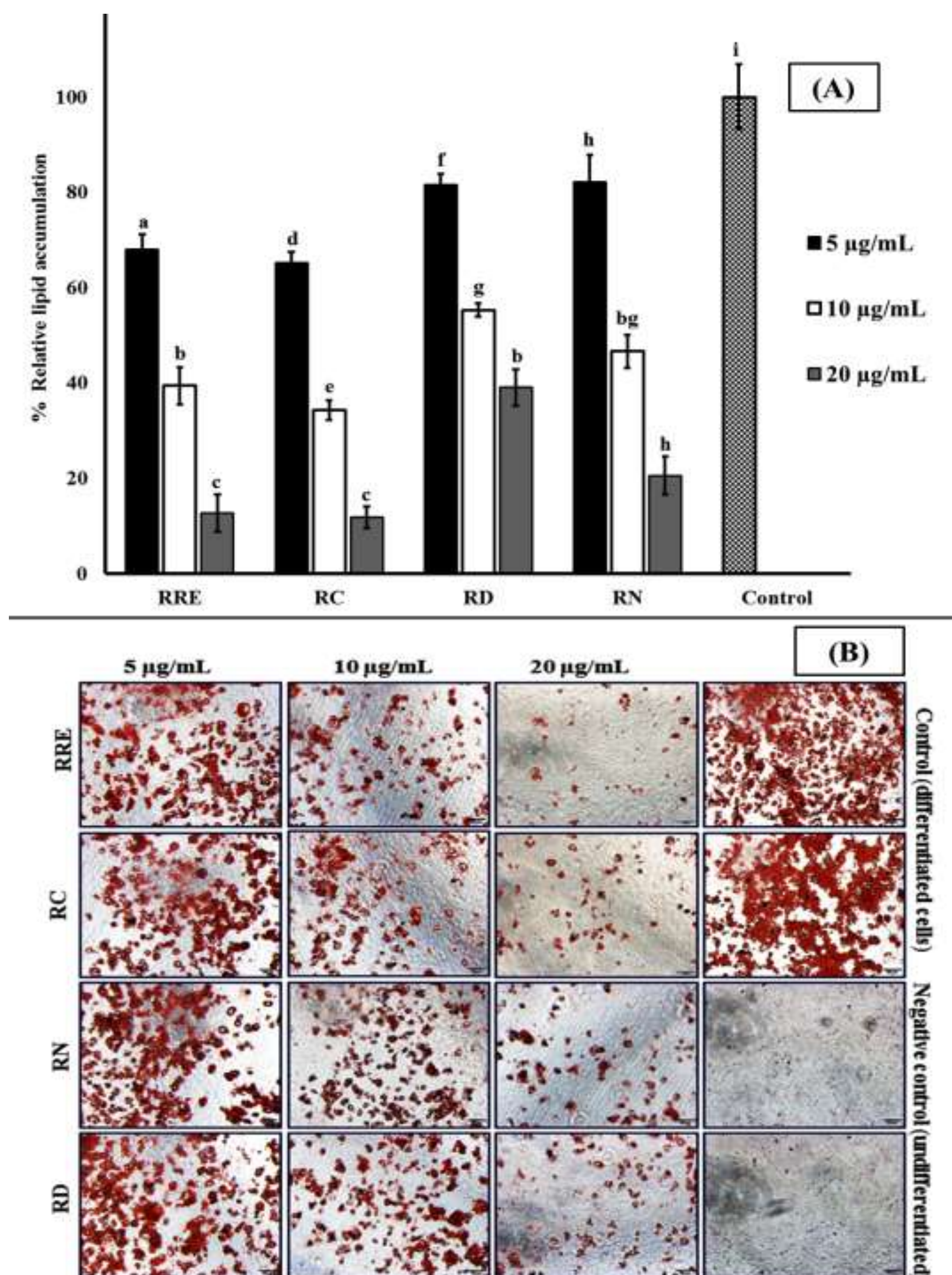


Figure 5 Dose dependent adipogenic inhibition (**A**) by RRE (rhinacanthins-rich extract), RC (rhinacanthin-C), RD (rhinacanthin-D) and RN (rhinacanthin-N) in 3T3-L1 adipocytes and microscopic images (**B**) of treated and untreated cells. Results are expressed as mean ± SEM (n=3). Mean values followed by different letters are significantly different (*P ≤ 0.05*).



PAPER V

Hypoglycemic and hypolipidemic effects of rhinacanthins-rich extract from *Rhinacanthus nasutus* leaves in nicotinamide-streptozotocin induced diabetic rats

Muhammad Ajmal Shah[1], Wantana Reanmongkol[2], Nisaudah Radenahmad[4], Ruqaiya Khalil[5], Zaheer Ul-Haq[5], Pharkphoom Panichayupakaranant[1, 3*]

[1]Department of Pharmacognosy and Pharmaceutical Botany, [2]Department of Clinical Pharmacy, [3]Phytomedicine and Pharmaceutical Biotechnology Excellence Center, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

[4]Department of Anatomy, Faculty of Science, Prince of Songkla University, Hat-Yai, Songkhla 90112, Thailand

[5]Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

Abbreviated running title: Antidiabetic and hypolipidemic effects of rhinacanthins-rich extract

* Corresponding author. Pharkphoom Panichayupakaranant Tel. /fax: +66 74 428220. E-mail address: pharkphoom.p@psu.ac.th (P. Panichayupakaranant).

ABSTRACT

Rhinacanthins-rich extract (RRE) is a semipurified *Rhinacanthus nasutus* leaf extract that contains 60 % w/w of rhinacanthin-C (RC) with not less than 70 % w/w of total rhinacanthins content obtained by green extract process using ethanol. In the present study, oral administration of RRE (24.11 mg/kg equivalent to 15 mg/kg RC content), RC (15 mg/kg) and the standard drug glibenclamide (600 µg/kg) were comparatively assessed for their hypoglycemic and hypolipidemic activity in nicotinamide-streptozotocin induced diabetic rats for 28 days. Various parameters including body weight, daily food and water intake, fasting blood glucose (FBG), insulin level, HbA1c level, lipid profile (TC, TG, HDL and LDL), liver (AST and ALT) and kidney function markers (creatinine and BUN), and histopathology of pancreas were studied in both diabetic and normal rats. *In silico* study was also performed to predict the pharmacokinetic and toxicity profile of RC. RRE and RC significantly reduced the FBG, HbA1c and food/water intake, while increased the insulin level and body weight in diabetic rats without affecting the normal rats. The serum lipid, liver and kidney biomarkers were markedly normalized by both RRE and RC in diabetic rats without affecting the normal rats. Moreover, the histopathology of pancreas revealed that RRE and RC evidently restored the islets of Langerhans in diabetic rats. The overall results indicated that RRE has significantly equivalent antidiabetic potential to that of RC. Furthermore, the *in silico* pharmacokinetic and toxicity analysis predicts that RC is orally non-toxic, non-carcinogenic and non-mutagenic with a decent bioavailability. The undertaken study suggests that RRE could be used as an effective natural remedy in the treatment of diabetes.

Keywords: Diabetes, dyslipidemia, *Rhinacanthus nasutus***,** rhinacanthins-rich extract

1. Introduction

Diabetes mellitus (DM) is a multifactorial disorder that needs effective multidimensional therapeutic approaches for glycemic control. The number of diabetic patients was dramatically increased in the last three decades from 108 to 422 million globally, with rising prevalence of 3.8 % [1]. According to WHO, 90% of diabetic patients are suffering from type-2 DM (T2DM) [2]. T2DM is mainly associated with insulin resistance, which is related with the release of free fatty acids and proinflammatory cytokines from adipose tissues in obesity, which stimulates beta cells for over secretion of insulin and its receptors reduction [3-5]. Clinically available oral antidiabetic drugs which exert their hypoglycemic effect by different mechanisms are still associated with certain health hazards such as diarrhea, lactic acidosis, weight gain and cardiovascular problems [6,7]. The rising diabetic prevalence and adverse effects of currently used antidiabetic drugs necessitate the investigation of potent, effective and safe, novel antidiabetic remedies preferably from natural resources.

Natural resources in general and plants in particular provide high value source of novel therapeutic moieties which offer a potential alternative to currently used drugs which may be associated with side effects. Numerous phytochemicals and active constituents enriched plant extracts have been reported for their antidiabetic and hypolipidemic potential [8-12]. *Rhinacanthus nasutus* (L.) Kurz (Family Acanthaceae), a medicinal herb native to Thailand and Southeast Asia, has traditionally been used in the treatment of various disorders including DM [13]. In China and Taiwan, *R. nasutus* has been consumed as an herbal drink [14,15]. Methanol extracts of *R. nasutus* leaf have been explored extensively for antidiabetic and hypolipidemic activity [16-20]. Rhinacanthin-C (RC), a major constituent of *R. nasutus* leaf has been recently reported for antidiabetic, antihyperlipidemic and pancreatic protective effects in diabetic rats [21]. However, the commercial unavailability, multi-stage and high cost purification process with the consumption of large amount of toxic organic solvents in the purification of RC obstructs its application in drug development process. Rhinacanthins-rich extract (RRE) is a semi-purified extract obtained from *R. nasutus* leaf that contains not less than 70% w/w rhinacanthins in total, with 60% w/w of RC as the major constituent [22]. RRE offers

remarkable benefits as a substitute to RC in term of lower production cost, green extraction process and potentially equivalent or higher bioactivity due to synergism among RRE components [23, 24]. In the present study, RRE was prepared by simple, environment-friendly, green extraction process to investigate its hypoglycemic and hypolipidemic effects in nicotinamide-streptozotocin induced type-2 diabetic rats. *In silico* studies were also conducted to predict pharmacokinetic and toxicity profile of RC.

2. Materials and methods

2.1. Drugs and chemicals

Streptozotocin (STZ), nicotinamide and glibenclamide (Glb) were purchased from Sigma Aldrich (St Louis, MO, USA). All other chemicals were of analytical grade.

2.2. Plant material, extraction and isolation

The fresh leaves of *R. nasutus* were collected from the Botanical Garden of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai Campus, Thailand, a voucher specimen (No. 0011814) was kept at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Thailand. Leaves were washed with tap water and dried at 60ºC for 24 h in a hot air oven and reduced to powders using a grinder, and the powders were passed through sieve No. 45.

RRE was prepared using ethanol as previously described methods [22] with some modifications. The present method used a microwave assisted extraction followed by a simple step of fractionation with Amberlite® column. Moreover, only the green solvents, ethanol and water, were used in the extraction and fractionation processes. RC, rhinacanthin-D (RD) and rhinacanthin-N (RN) were purified from the RRE using a silica gel column eluted by hexane and ethyl acetate (99:1, v/v). The structures of all three compounds were confirmed by comparing the ^{1}H and ^{13}C-NMR spectral data with those from the literature [25, 26].

2.3. HPLC analysis of RRE

HPLC analysis of RRE was conducted as previously reported [22] using a UFLC Shimadzu system incorporating a Discovery® C18 (5 μm, 4.6 × 150 mm) column (Supelco, PA, USA) equipped with a SPD-M20A photodiode-array detector, SIL-20A HT auto-sampler, and CTO-20AC oven (Shimadzu Corp. Kyoto, Japan).

2.4. Experimental animals

Adult male Wistar rats weighing approximate 200-250 g were obtained from the National Laboratory Animal Center, Mahidol University, Nakorn Pathom, Thailand and supplied with a standard feed protocol from the Southern Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. The animals were housed for at least 1 week in the laboratory prior to testing. Animals were allowed free access to food and water under standard environmental conditions of room temperature 24 ± 2°C, 55 ± 10 % humidity and 12 h light: 12 h dark cycle. All experimental protocols were approved by the Institutional Animal Care and Use Committee, Prince of Songkla University (MOE 0521.11/326, Ref. 01/2016).

2.6. Induction of diabetes

Rats were divided into two groups; diabetic and non-diabetic. Diabetes was induced according to previously described method [21] by single intraperitoneal injection of STZ (55-60 mg/kg) dissolved in 0.1 M cold citrate buffer (pH 4.5) with pre-nicotinamide (100 mg/kg) injection to reduce pancreatic destruction and to create T2DM [28]. Diabetes was confirmed from the higher level of fasting blood glucose (FBG) using a glucometer (One Touch, LifeScan, Zug, Switzerland) after 72 h of STZ injection. The tail vein of animals was pricked to collect the blood for FBG determination. Animals with FBG above 300 mg/dL having symptoms such as hyperphagia, polydipsia and polyuria were marked as diabetic.

Treatment with 24.11 mg/kg/day of RRE (equivalent to 15 mg/kg RC) or 15 mg/kg/day of RC was started after 72 h of STZ injection and this was recorded as day first. RRE and RC were dissolved in cosolvent system consisted of propylene glycol, tween 80 and water (4:1:4). 1 ml of the solution containing 24.11 mg/kg of

RRE (equivalent to 15 mg/kg of) or 15 mg/kg of RC was administered once daily to the rats via feeding tube for 28 days.

2.6. Experimental design

The rats were assigned into seven groups with six (6) rats per group:

1. Normal control rats receiving cosolvent
2. Normal control rats receiving 24.11 mg/kg of RRE
3. Normal control rats receiving 15 mg/kg of RC
4. Diabetic control rats
5. Diabetic rats receiving 24.11 mg/kg of RRE
6. Diabetic rats receiving 15 mg/kg of RC
7. Diabetic rats receiving standard drug Glb (600 µg/kg)

2.7. Body weight, food and water intake, and FBG determination

The initial and final body weights, daily food and water consumptions of all animals were recorded. The FBS at day 0, 7, 14 and 28 was measured using the glucometer (One Touch, LifeScan, Zug, Switzerland) from the tail blood. At the end of the experiment (28th day), the overnight fasted rats were euthanized using phenobarbital (100-150 mg/kg i.p). Blood was collected from each rat through cardiac puncture and centrifuged at 4°C for 15 min at 800 g and the serum was used for biochemical analysis.

2.8. Measurement of HbA1c and insulin analysis

HbA1c level in whole blood was analyzed by Clover A1c self-analyzer (Infopia, Gyeonggi-do, Korea). Serum insulin level was measured by Electro-chemiluminescence method using Cobas 6000, Roche kit (Roche Diagnostics, Rotkreuz, Switzerland).

2.9. Biochemical analysis

Biochemical tests of the serum total cholesterol (TC), triglyceride (TG), high density lipoprotein (HDL), low density lipoprotein (LDL), aspartate

aminotransferase (AST), alanine aminotransferase (ALT) and blood urea nitrogen (BUN) were performed by Stanbio Diagnostic kits (Stanbio Laboratory, Texas, USA). Serum creatinine level was determined using BioSystem Diagnostic Kit (BioSystems, Barcelona, Spain). All the results were calculated using an A25 automated analyzer (BioSystems, Barcelona, Spain).

2.10. Histopathology of pancreas

The pancreas from euthanized rats was preserved in 10% buffered formalin. The tissue was embedded in molten paraffin and cut into 5 μm thick sections. The sections were stained with hematoxylin and eosin and visualized under light microscope (Olympus DP73) to study the histopathological changes.

2. 11. *In silico* pharmacokinetic and toxicity predictions of RC

Candidates with poor pharmacokinetics should be identified early in drug design process to reduce the number of experiments required for compound selection and development [29]. In this context, drug-likeliness, blood brain barrier permeability, and toxicity of the RC were evaluated using MOE2016.0801 [30]. Additionally, ADMET profile of RC was established using admetSAR [31] a free tool for evaluation of ADMET properties. Further to validate our finding PreADMET v.02 was used to evaluate various toxicity and ADMET associated descriptors using the default parameters [32]. The web-based server, Xenosite was used to calculate the pattern of Cytochrome P-450 based metabolism of RC [33].

3. Results and discussion

3.1. Determination of rhinacanthins content in RRE

HPLC analysis showed that the total rhinacanthins content were 73.7% w/w. RRE used in the current study contained RC (62.2% w/w) as a major compound, and RD (7.9 % w/w) and RN (3.6 % w/w) were the minor compounds. The quantitative results of RRE analysis are consistent with the previous report [22].

3.2. Effect of RRE on body weight, food and water consumptions

Polydipsia (thirst), hyperphagia (appetite) and weight loss are the characteristic symptoms of diabetes [34]. As shown in Table 1, the initial water and food intake and body weight of nondiabetic and diabetic rats were comparable. After four weeks a significant rise in water and food intake with reduction in body weight was observed in diabetic rats in comparison with the normal rats. In T2DM, insulin deficiency causes abnormal glucose metabolism and protein catabolism resulting in muscular degeneration and weight loss [35]. Oral administration of RRE (24.11 mg/kg) and RC (15 mg/kg) significantly normalized the water and food intake, and body weight of diabetic rats without any significant effect on the normal rats (Table 1).

3.3. Effect of RRE on FBG, HbA1c and insulin

Insulin is an important hormone which acts as a metabolic regulator of carbohydrate, lipid and protein by promoting their synthesis, and prevents them from degrading and releasing into the circulatory system. Insulin also functions as glucose, amino acid and fatty acid uptake stimulator and certain metabolic enzymes expression enhancer [36]. In T2DM, due to partial destruction of pancreas the insulin level becomes lower that affect the overall metabolic process. In the current study, STZ along with nicotinamide has been used for partial destruction of pancreas to develop T2DM model which was confirmed by higher FBG and lower serum insulin levels in the diabetic rats (Fig. 1 and Fig. 2A). In nondiabetic control and nondiabetic rats receiving 24.11 mg/kg of RRE and 15 mg/kg of RC, the FBG and insulin levels were consistently normal throughout the 28 days (Fig. 1 and Fig. 2A). Diabetic rats treated with RRE (24.11 mg/kg), RC (15 mg/kg) or the standard drug Glb (600 μg/kg) gradually decreased the hyperglycemia and markedly increased the serum insulin throughout the experimental period of 28 days (Fig. 1 and Fig. 2A). Furthermore, it is evident from the histopathological images of pancreas (Fig. 3) that RRE markedly decreased beta cells destruction in diabetic rats. Pancreatic protective effect of REE can be correlated to the previous study on its marker compound RC [21]. It has been

reported that RC is a potent antioxidant [37], anti-inflammatory [24] and antiapoptotic agent [21] which can be correlated to the pancreatic protective effect.

During hyperglycemia the excess sugar reacts with the protien resulting in glycated hemoglobin (HbA1c) which is a laboratory marker of diabetes and the associated risk of diabetic complications due to the formation of advanced glycation products [37]. In the present study the increase HbA1c level in diabetic rats was significantly reduced by RRE (24.11 mg/kg) and RC (15 mg/kg) comparatively with the standard drug Glb at a dose of 600 µg/kg (Fig. 2B). However, there was no significant effect on the HbA1c level of normal treated rats. The results can be correlated with our previous *in vitro* study on the antiglycation potential of RRE and RC [38].

3.4. Effect of RRE on lipid profile

In DM, hyperglycemia is closely associated with hyperlipidemia. The linkage of hyperglycemia with hyperlipidemia often leads to fatal cardiovascular problems in diabetic patients [21]. Insulin deficiency instigates the hormone sensitive lipase which stimulates fatty acid release from adipocytes [39]. The extra fatty acid enhances the production of phospholipids and cholesterol in hepatocytes. The elevated levels of phospholipids and cholesterol along with triglycerides in serum are the biomarkers of hyperlipidemia [40]. In the present study, the serum level of TC, TG, HDL and LDL was significantly normalized by REE (24.11 mg/kg), RC (15 mg/kg) and Glb (600 µg/kg) (Table 2). However, there was no significant lipid profile alteration of normal rats treated with RRE and RC at same dose. The hypolipidemic results of RRE are consistent with previous report about hypolipidemic activity of crude methanol extract [17] and RC [21].

3.5. Effects of RRE on liver and kidney functions

Liver is the insulin dependent organ for production of carbohydrate metabolizing enzymes essential for accumulation and consumption of glycogen. Low insulin level particularly in DM markedly reduced the expression of these enzymes and other important proteins [41]. Further, the insulin deficiency severely disturbs the

carbohydrate and fat metabolism [20]. AST and ALT are the key enzymes which are known to be the liver function marker [42]. Table 3 indicates that diabetic rats have elevated level of AST and ALT which is significantly reduced by REE (24.11 mg/kg), RC (15 mg/kg) and Glb (600 μg/kg). However, there is no effect of RRE and RC administration on liver markers of normal control rats (Table 3). These results show the hepatic safety of RRE (24.11 mg/kg) and (15 mg/kg).

DM is a metabolic disorder which affects all the vital organs including kidney, diabetic nephropathy is a one of the major diabetic complication. Oxidative stress, protien glycation and hyperlipidemia are the prominent diabetic factors which are responsible in the occurrence of diabetic nephropathy [43]. The serum BUN and creatinine levels are the important markers to evaluate the kidney function in nephropathic condition [44]. In the current study, the elevated level of BUN and creatinine were markedly reduced by RRE (24.11 mg/kg), RC (15 mg/kg) and the standard drug Glb (600 μg/kg) in diabetic rats (Table 4). However, there was no significant effect of RRE and RC administration on BUN and creatinine level of normal rats which indicate the renal safety of the selected dose of RRE and RC. The results can be correlated with previous *in vitro* reports on the antioxidant and antiglycation potential of crude extract, RRE and RC [18,21,38].

3.6. ADMET profile of RC

In order to identify the pharmacokinetic and toxicity properties of RC, various *in silico* calculations were performed. The RC complies well with the Lipinski's rule of drug-likeliness with a violation count value of 0 (Table 4). RC was found to be BBB+ (0.44) that suggests it can penetrate in the blood brain barrier which can be correlated with a recent report on the modulatory effect of RC through high-mobility group box 1 related pathway to attenuate brain apoptosis in the pathogenesis of subarachnoid hemorrhage [45]. In the Table 4, ADMET profile of RC is presented as established by using admetSAR, a free web based tool for evaluation of ADMET properties.

As shown in Table 5, RC can be absorbed by both human intestine and brain. It was also predicted to display Caco-2 monolayer permeability. The Caco-2

serves as *in vitro* model of human intestinal mucosa for prediction of the oral bioavailability of a drug [46]. The observation is consistent with the fact that the compound was found to comply with Lipinksii rules which define properties of orally available drugs [47]. Though, the drug was found to be both substrate and inhibitor of P-glycoprotein (Table 5), the probability of being an inhibitor is greater. The P-glycoprotein plays a significant role in drug absorption and deposition by actively transporting a drug from cell cytoplasm to the intestine, thus limiting the oral bioavailability of the drug [46,48]. As an inhibitor of P-glycoprotein, RC may present good bioavailability and the drug concentration shall remain stable.

Human Ether-A-Go-Go-Related gene has emerged as an important anti-target in the drug development. Therefore, the hERG inhibitory potential of a drug is assessed at the preclinical stages during pharmaceutical testing [49]. The hERG inhibitory potential of the RC was evaluated using Pre-ADMET and admetSAR web servers. RC presented medium to low risk of being hERG inhibitor. However, as suggested by experimental results, the drug is non-toxic and non-lethal. The compounds having LD_{50} between 500-5000 mg/kg are included in category III of acute oral toxins. However, as suggested by the experimental results, RC was found to be effective at a dose of 15 mg/kg which suggest the therapeutic window of the drug dose is many folds lower than the lower LD_{50}.

Cytochrome P450 is a family of isozymes responsible for the bio-transformation of several drugs [50]. Table 4 shows the tendency of RC as CYP450 3A4 Substrate. The presence of this isoform in liver and intestine pronounce these organs as sites of clearance of RC. Server Xenosite was used to identify the potential sites of metabolic-oxidation carried out by CYP450 3A4 (Fig. 4). The terminal methyl group seems to be most vulnerable to oxidation based degradation followed by the benzene ring.

4. Conclusion

RRE containing 62.2% w/w RC was obtained from *R. nasutus* leaves using a green extraction method. RRE showed significant hypoglycemic and hypolipidemic effects comparable to RC. In term of green processing and low production cost, RRE is more suitable candidate for antidiabetic drug development.

Author contribution

MAS and PP conceived and designed the research study. MAS conducted the experiments. RK and ZU designed and carried out *in silico* calculations. MAS, RK, ZU, RW and PP analyzed the data, discussed the findings and prepared the manuscript.

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Compliance with ethical standards

Conflict of interest

The authors declared that they have no conflicts of interest.

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Table 1 Effect of rhinacanthins-rich extract (RRE) and rhinacanthin-C (RC) on body weight, food and water intake.

Parameters	Normal	Normal treated		Diabetic	Diabetic treated		
		RRE (24.11 mg/kg)	RC (15 mg/kg)		RRE (24.11 mg/kg)	RC (15 mg/kg)	Glb (0.6 mg/kg)
Initial body weight (g)	225.83 ± 4.16[a]	229.17 ± 6.64[a]	228.33 ± 7.20[a]	232.83 ± 8.75[a]	231.67 ± 5.84[a]	230.83 ± 8.24[a]	232.50 ± 6.37[a]
Final body weight (g)	293.35 ± 9.93[a]	286.34 ±10.37[a]	290.53 ± 10.23[a]	215.74 ± 11.31[b]	255.23 ± 7.93[c]	250.37 ± 11.49[c]	272.67 ± 10.94[d]
Water intake (mL/rat/day)	34.53 ± 6.72[a]	32.75 ± 7.12[a]	33.81 ± 5.37[a]	53.93 ± 7.81[b]	40.32 ± 6.95[c]	43.5 ± 8.75[c]	41.2 ± 7.04[c]
Food intake (g/rat/day)	22.13 ± 4.35[a]	21.59 ± 5.21[a]	22.84 ± 3.98[a]	39.73 ± 6.45[b]	29.3 ± 7.14[c]	31.6 ± 6.24[c]	29.32 ± 3.98[c]

Values are ± S.E.M for each group (n=6). Values that share different letters are significantly different *($P<0.05$).*

Table 2 Effect of rhinacanthins-rich extract (RRE) and rhinacanthin-C (RC) on lipid profile.

Parameters	Normal	Normal treated		Diabetic	Diabetic treated		
		RRE (24.11 mg/kg)	RC (15 mg/kg)		RRE (24.11 mg/kg)	RC (15 mg/kg)	Glb (0.6 mg/kg)
TC (mg/dL)	101.34 ± 5.59[a]	103.56 ± 7.36[a]	105.28 ± 4.79[a]	152.79 ± 15.16[b]	120.25 ± 9.71[c]	123.37 ± 8.97[c]	111.53 ± 11.15[d]
TG (mg/dL)	82.73 ± 4.78[a]	85.13 ± 8.19[a]	83.39 ± 7.39[a]	133.83 ± 12.89[b]	103.79 ± 8.99[c]	107.85 ± 5.98[c]	94.57 ± 5.69[d]
HDL (mg/dL)	49.71 ± 2.99[a]	51.75 ± 3.18[a]	47.98 ± 5.31[a]	23.98 ± 7.12[b]	33.31 ± 4.38[c]	32.98 ± 5.12[c]	38.98 ± 3.97[d]
LDL (mg/dL)	11.31 ± 1.09[a]	9.31 ± 0.97[a]	12.72 ± 0.89[a]	57.98 ± 5.73[b]	29.45 ± 2.78[c]	31.81 ± 4.31[c]	20.89 ± 2.91[d]

Values are ± S.E.M for each group (n=6). Values that share different letters are significantly different *($P<0.05$)*.

Table 3 Effect of rhinacanthins-rich extract (RRE) and rhinacanthin-C (RC) on liver and kidneys function.

Parameters	Normal	Normal treated		Diabetic	Diabetic treated		
		RRE (24.11 mg/kg)	RC (15 mg/kg)		RRE (24.11 mg/kg)	RC (15 mg/kg)	Glb (0.6 mg/kg)
AST (IU/dL)	105.47 ± 7.79[a]	107.12 ± 5.71[a]	103.98 ± 8.94[a]	195.54 ± 12.1[b]	135.93 ± 8.19[c]	151.79 ± 5.32[d]	123.83 ± 7.73[e]
ALT (IU/dL)	51.85 ± 4.17[a]	49.98 ± 3.95[a]	52.73 ± 7.14[a]	89.38 ± 9.39[b]	72.89 ± 5.73[c]	69.19 ± 4.34[c]	61.39 ± 6.38[d]
Creatinine (mg/dL)	0.47 ± 0.05[a]	0.43 ± 0.07[a]	0.49 ± 0.03[a]	0.95 ± 0.08[b]	0.68 ± 0.09[c]	0.71 ± 0.04[c]	0.59 ± 0.03[d]
BUN (mg/dL)	37.39 ± 3.59[a]	36.79 ± 5.03[a]	35.39 ± 4.78[a]	64.31 ± 7.89[b]	47.31 ± 5.31[c]	54.93 ± 4.19[d]	42.98 ± 5.73[e]

Values are ± S.E.M for each group (n=6). Values that share different letters are significantly different *($P<0.05$)*.

Table 4 Pharmacokinetic and toxicity profile predictions of rhinacanthin-C obtained from admetSAR server.

Model	Result	Probability
Absorption		
Blood-brain barrier	bbb+	0.8552
Human intestinal absorption	Hia+	0.9957
Caco-2 permeability	Caco2+	0.7258
P-glycoprotein substrate	Substrate	0.7276
P-glycoprotein inhibitor	Inhibitor	0.9335
Renal organic cation transporter	Non-inhibitor	0.8205
Metabolism		
CYP450 2c9 substrate	Non-substrate	0.8317
CYP450 2d6 substrate	Non-substrate	0.8926
CYP450 3a4 substrate	Substrate	0.7221
CYP450 1a2 inhibitor	Inhibitor	0.8228
CYP450 2c9 inhibitor	Inhibitor	0.7242
CYP450 2d6 inhibitor	Non-inhibitor	0.7797
CYP450 2c19 inhibitor	Inhibitor	0.7216
CYP450 3a4 inhibitor	Non-inhibitor	0.6793
CYP inhibitory promiscuity	High cyp inhibitory promiscuity	0.6948
Toxicity		
Human Ether-A-Go-Go-Related	Weak inhibitor	0.9891
Gene inhibition	Non-inhibitor	0.8434
Ames toxicity	Non ames toxic	0.7815
Carcinogens	Non-carcinogens	0.8585
Acute oral toxicity	III	0.5385

Table 5 The values of different descriptors of rhinacanthin-C and their comparison with the standard Lipinski's drug like values.

Descriptor	Calculated value	Standard Value
Hydrogen bond donor	2	≤5
Hydrogen bond acceptor	4	≤10
Molecular mass	410	≤500
Octanol-water partition coefficient (log P)	4.138	≤5
Number of atoms	60	20-70
Polar Surface Area	84 Å2	140 Å2

Figure 1 Effect of rhinacanthins-rich extrct (RRE) and rhinacanthin-C (RC) on fasting blood glucose level at weekly interval. Values are ± S.E.M for each group (n=6). Bars that share different letters are significantly different (*P<0.05*). C: Non-diabetic control, D: Non-treated diabetic, C+RRE: non-diabetic receiving 24.11 mg/kg of RRE, C+RC: non-diabetic receiving 15 mg/kg of RC, D: non-treated diabetics, D+RRE: diabetic receiving 24.11 mg/kg of RRE, D+RC: diabetic receiving 15 mg/kg of RC and D+Glb: diabetic receiving 600 µg/kg of glibenclamide.

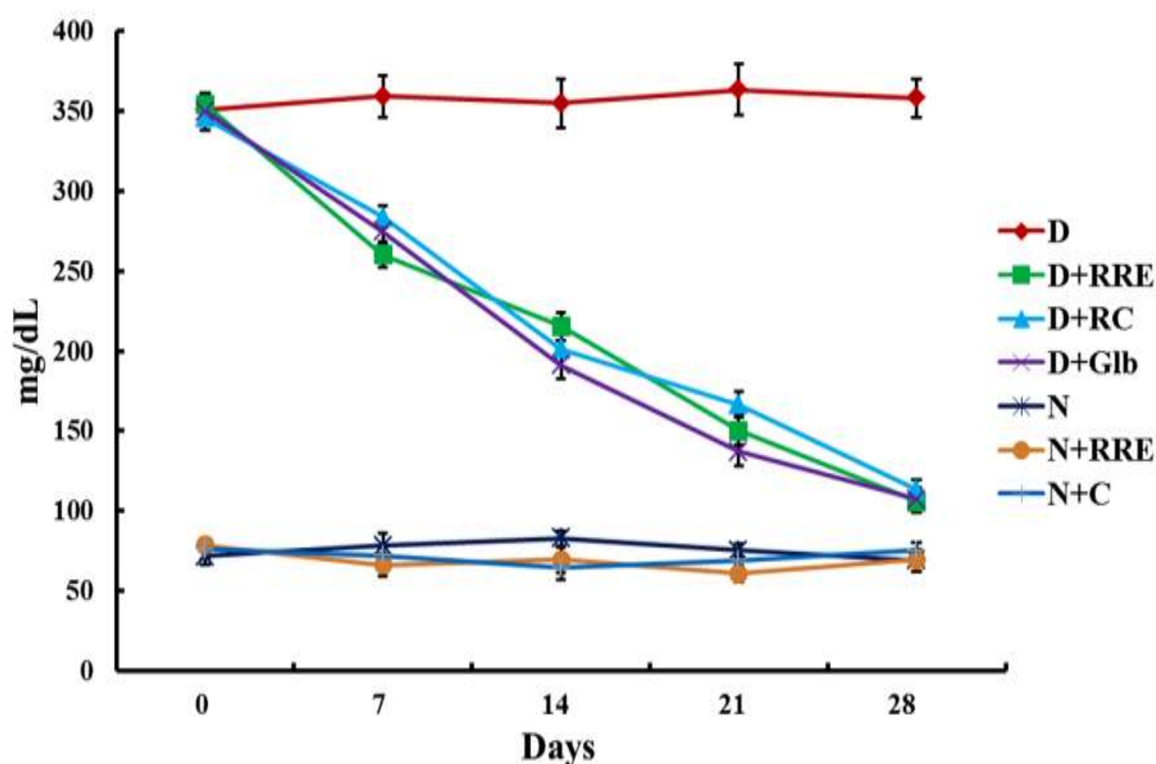


Figure 2 Effect of rhinacanthins-rich extrct (RRE) and rhinacanthin-C (RC) on insulin **(A)** and HbA1c level **(B)**. Values are ± S.E.M for each group (n=6). Bars that share different letters are significantly different (*P<0.05*). C: Non-diabetic control, D: Non-treated diabetic, C+RRE: non-diabetic receiving 24.11 mg/kg of RRE, C+RC: non-diabetic receiving 15 mg/kg of RC, D: non-treated diabetics, D+RRE: diabetic receiving 24.11 mg/kg of RRE, D+RC: diabetic receiving 15 mg/kg of RC and D+Glb: diabetic receiving 600 μg/kg of glibenclamide.

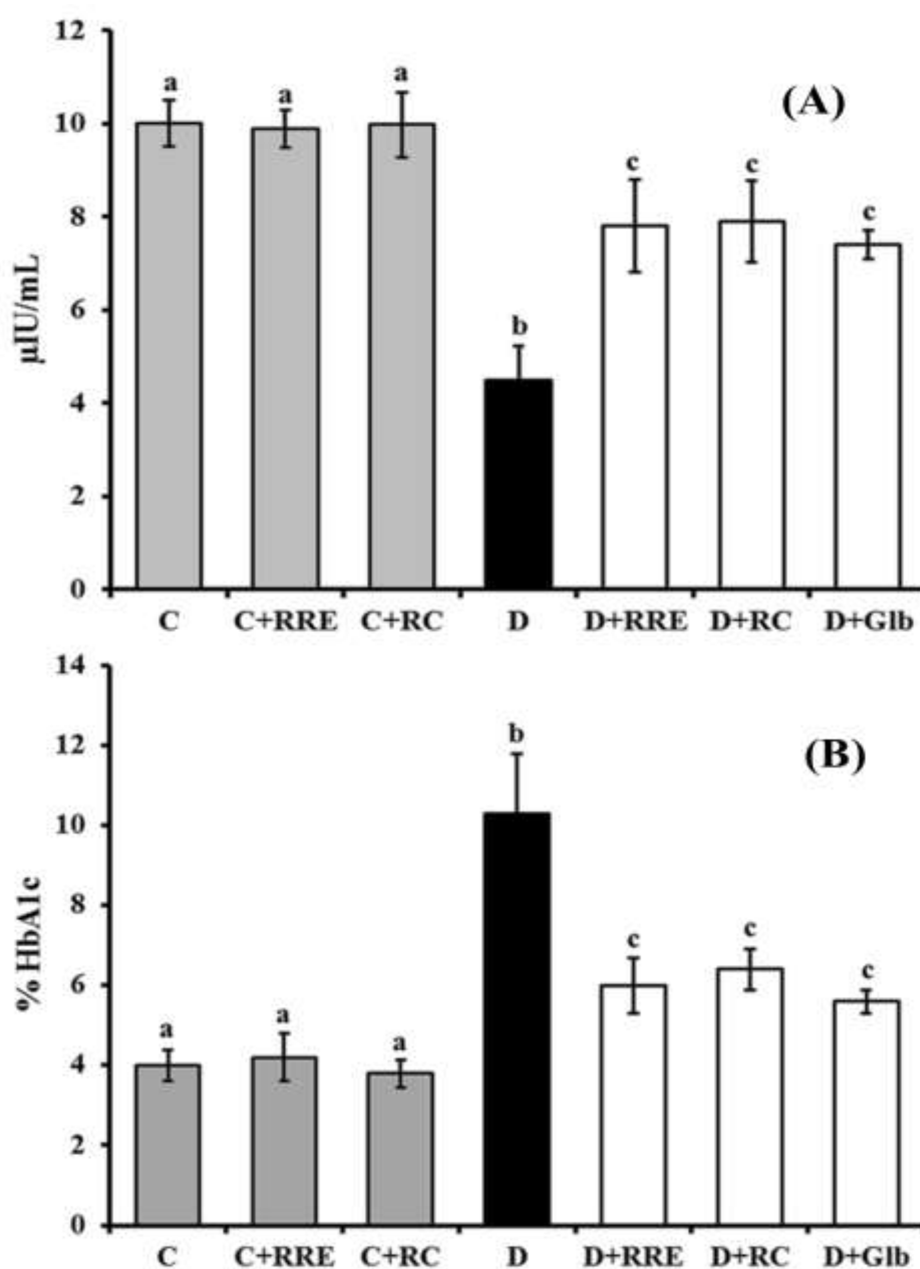


Figure 3 Effect of rhinacanthins-rich extrct (RRE) and rhinacanthin-C (RC) on histopathological changes in the pancreas. White lining indicates islet of Langerhans. C: Non-diabetic control, D: Non-treated diabetic, C+RRE: non-diabetic receiving 24.11 mg/kg of RRE, C+RC: non-diabetic receiving 15 mg/kg of RC, D: non-treated diabetics, D+RRE: diabetic receiving 24.11 mg/kg of RRE, D+RC: diabetic receiving 15 mg/kg of RC and D+Glb: diabetic receiving 600 μg/kg of glibenclamide.

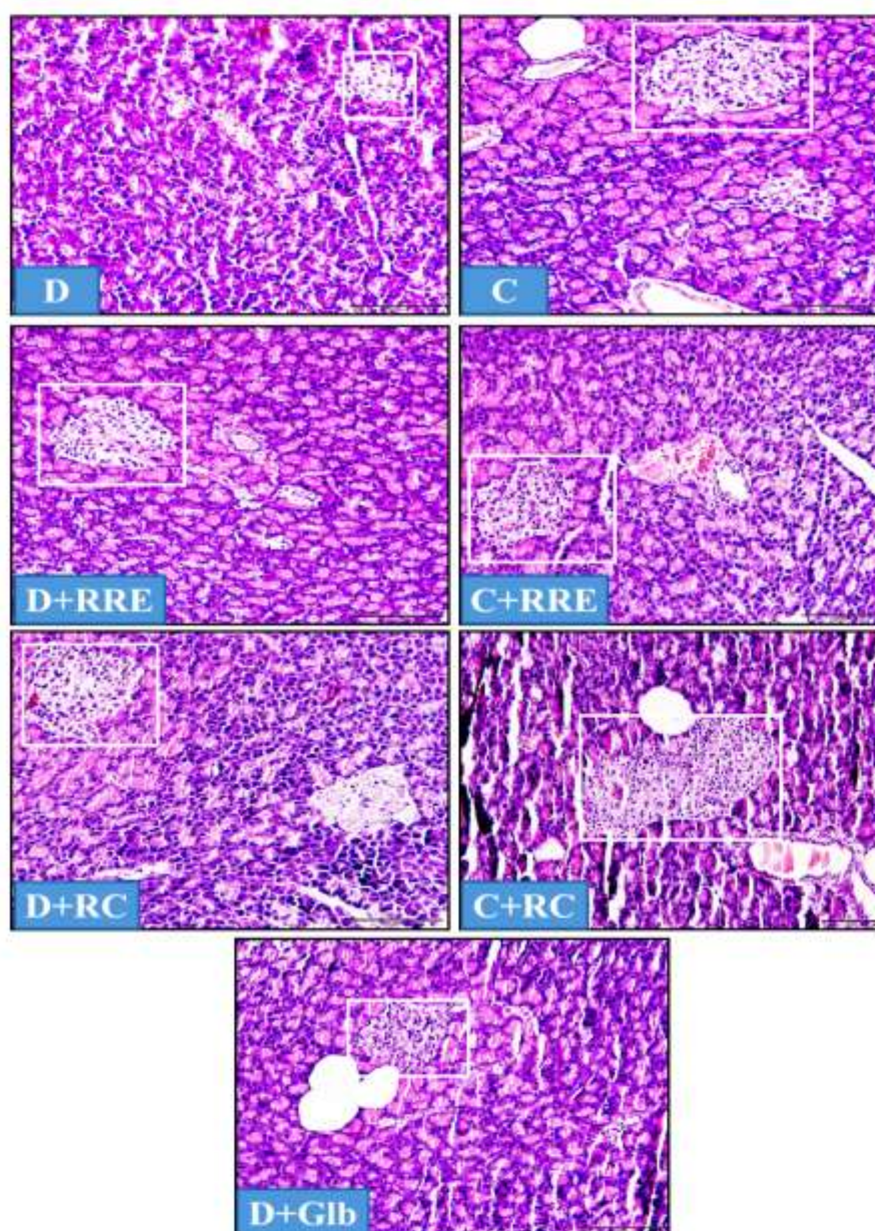
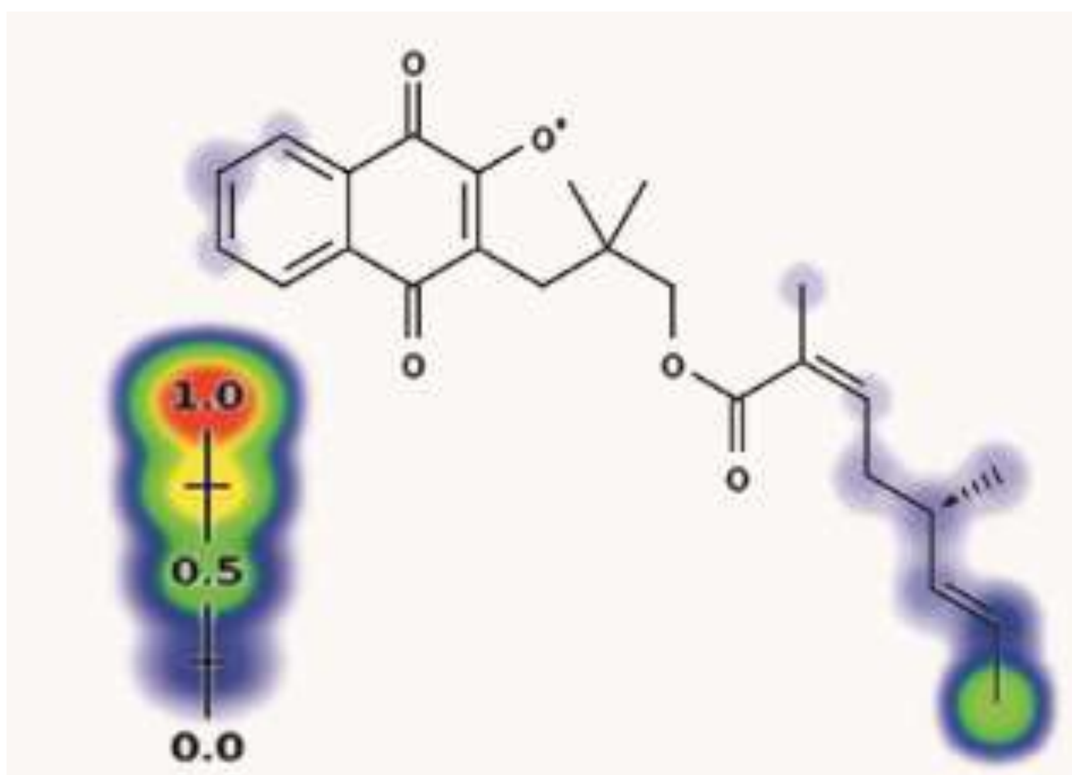


Figure 4 The potential sites of CYP450 mediated oxidation in rhinacanthin-C obtained from the Xenosite server



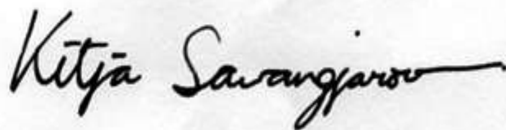
ANIMAL ETHIC CERTIFICATE

PRINCE OF SONGKLA UNIVERSITY
15 Karnjanawanij Road, Hat Yai, Songkhla 90110, Thailand
Tel (66-74) 286940 Fax (66-74) 286961
Website : www.psu.ac.th

MOE 0521.11/ 326 Ref.01/2016

March 23 , 2016

This is to certify that the research project entitled "Antidiabetic activity of rhinacanthins-rich extract and rhinacanthin-C" which was conducted by Assoc. Prof. Dr. Wantana Reanmongkol, Pharmaceutical Sciences, Prince of Songkla University, has been approved by The Animal Ethic Committee, Prince of Songkla University.



Kitja Sawangjaroen, Ph.D.
Chairman,
The Animal Ethic Committee, Prince of Songkla University

VITEA

Name Mr. Muhammad Ajmal Shah

Student ID 5710730002

Educational Attainment

Degree	Name of Institution	Year of Graduation
Doctor of Pharmacy (PharmD, RPh)	Federal Urdu University of Arts, Science & Technology Karachi, Pakistan	2010
Master of Philosophy (Pharmacognosy)	Federal Urdu University of Arts, Science & Technology Karachi, Pakistan	2013

Scholarship Awards

1. Thailand Education Hub for ASEAN Countries (TEH-AC) PhD Award 2014, granted by Graduate School, Prince of Songkla University.
2. Thesis Support Grant 2016, granted by Graduate School, Prince of Songkla University.
3. Conference Support Grants 2016-17, awarded by Faculty of Pharmaceutical Sciences, Prince of Songkla University.
4. GA Travel Grant-2017, awarded by GA Society for Medicinal Plant and Natural Product Research Germany to present poster in their 65th GA Annual Meeting at Basel Switzerland, 6-10 September 2017.

List of Publication and Proceeding

Publications

1. **Shah MA**, Keach J, Panichyupakaranant P. Antidiabetic naphthoquinones and their plant resources in Thailand**:** Review. Chemical and Pharmaceutical Bulletin, (accepted).

2. **Shah MA**, Khalil R, Haq ZU, Panichyupakaranant P. α-Glucosidase inhibitory effect of rhinacanthins-rich extract from *Rhinacanthus nasutus* leaf and synergistic effect in combination with acarbose. Journal of Functional Foods, 36 (2017) 325–331.
3. **Shah MA**, Muhammad H, Mehmood Y, Khalil R, Haq ZU, Panichyupakaranant P. Superoxide scavenging and antiglycation activities of rhinacanthins-rich extract from *Rhinacanthus nasutus* leaves. Pharmacognosy Magazine, (accepted).
4. **Shah MA**, Jakkawanpitak C, Decha S, Panichyupakaranant P. Rhinacanthins-rich extract enhance glucose uptake and inhibit adipogenesis in 3T3-L1 adipocytes and L6 myotubes. Pharmacognosy Magazine, (submitted).
5. **Shah MA**, Reanmongkol W, Radenahmad N, Khalil R, Haq ZU, Panichyupakaranant P. Hypoglycemic and hypolipidemic effect of rhinacanthins-rich extract in nicotinamide-streptozotocin induced diabetic rats. Biomedicine & Pharmacotherapy, (submitted).

Conference Presentation

1. **Shah MA,** Haji M, Khalil R, Haq ZU, Panichyupakaranant P. Rhinacanthins-rich extract: A potent superoxide scavenger and advanced glycation end-product formation inhibitor. 65th GA Annual Meeting, 3-7 September 2017, Basel Switzerland (poster-accepted).
2. **Shah MA,** Decha S, Khalil R, Haq ZU, Panichayupakaranant P. Rhinacanthins-rich extract: A potent synergistic α-glucosidase inhibitor and glucose uptake enhancer in muscles cells. 2nd International Symposium on Phytochemicals in Food and Medicine, 7-10 April 2017, Fuzhou China (poster).
3. **Shah MA,** Panichyupakaranant P. α-Glucosidase inhibitory effect of rhinacanthins-rich extract and rhinacanthin-C. 4th Current Drug Development Conference, 1-3 June 2016, Phuket Thailand (oral).