UNIVERSITI PUTRA MALAYSIA

EFFECTS OF XANTHORRHIZOL ON 3T3-L1 ADIPOCYTE HYPERPLASIA AND HYPERTROPHY

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FS 2018 75

EFFECTS OF XANTHORRHIZOL ON 3T3-L1 ADIPOCYTE HYPERPLASIA AND HYPERTROPHY

By

OON SEOK FANG

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

June 2018

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

EFFECTS OF XANTHORRHIZOL ON 3T3-L1 ADIPOCYTE HYPERPLASIA AND HYPERTROPHY

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June 2018

Chair : Meenakshii Nallappan, PhD
Faculty : Science

According to the National Health and Morbidity Survey (NHMS) 2015, 47.7% of the Malaysian population are either obese or overweight. The increased obesity prevalence has caused major health problems such as cardiovascular diseases and diabetes. Although several anti-obesity drugs have been developed, they are limited due to adverse side effects such as stroke, myocardial infarction, and depression. These circumstances have increased the demand for effective and safe anti-obesity agents. Previous studies demonstrated that xanthorrhizol (XNT) reduced the levels of serum free fatty acid and triglyceride *in vivo*, but the detailed anti-obesity activities and its related mechanisms are yet to be reported. In this study, crude oil containing XNT was extracted from *Curcuma xanthorrhiza* Roxb. by supercritical fluid carbon dioxide extraction. It was further purified by column chromatography and centrifugal thin layer chromatography (TLC). The presence of XNT in each eluate was identified by TLC. The purity of XNT was determined by gas chromatography-mass spectrometry (GC-MS) analysis, whilst its structure was confirmed by proton and carbon nuclear magnetic resonance (NMR) spectral analysis. Next, the IC_{50} value of XNT was determined by MTT assay. The mode of cell death was further evaluated by annexin V/7-AAD staining for early apoptosis and TUNEL assay for late apoptosis, respectively. The mechanisms involved were determined by quantitative ELISA analysis of caspase-3 and PARP-1 proteins. On the other hand, the ability of XNT to inhibit adipogenesis was examined by oil red O (ORO) staining and glycerol-3-phosphate dehydrogenase (GPDH) activity. The mechanisms involved were evaluated by quantitative ELISA analysis of PPARγ and FAS proteins. The ability of XNT to induce lipolysis was investigated by quantifying the glycerol amount. The mechanisms were examined by quantitative ELISA analysis of leptin and insulin proteins. Statistical significance was analyzed by one-way ANOVA, where $p < 0.05$ was considered significantly different. Thus, this study aims to evaluate XNT's abilities to induce apoptosis, impede adipogenesis, and

stimulate lipolysis employing 3T3-L1 adipocytes. In this study, XNT purified from centrifugal TLC demonstrated 98.3% purity, and the structure was confirmed by ^{1}H and ^{13}C NMR spectral analysis. The IC_{50} value of XNT in 3T3-L1 adipocytes was 35 ± 0.24 µg/mL. The loss of cell viability was due to 20.01 ± 2.77% of early apoptosis and 24.13 ± 2.03% of late apoptosis ($p < 0.05$). XNT elicited apoptosis via up-regulation of caspase-3 and cleaved PARP-1 protein expression for 4.09-fold and 3.12-fold, respectively. Moreover, XNT decreased adipocyte differentiation and GPDH activity in a dose-dependent manner from 3.13 to 12.5 µg/mL. The highest inhibition of adipogenesis was 36.13 ± 3.64% ($p < 0.05$). The GPDH activity was reduced to 52.26 ± 4.36% by XNT ($p < 0.05$). It was found that XNT reduced adipocyte formation by impairing the expression of PPARγ to 0.36-fold and FAS to 0.38-fold, respectively. On the other hand, XNT increased glycerol release by 45.37 ± 6.08% compared to control ($p < 0.05$). During lipolysis, XNT up-regulated the leptin protein for 2.08-fold but down-regulated the protein level of insulin to 0.36-fold ($p < 0.05$). These results indicated that XNT reduced the volume of adipocytes through modulation of leptin and insulin. To conclude, XNT exerted its anti-obesity mechanisms by suppression of adipocyte hyperplasia through induction of apoptosis (regulation of caspase-3 and PARP-1) and inhibition of adipogenesis (regulation of PPARγ and FAS) whilst reduction of adipocyte hypertrophy through stimulation of lipolysis (regulation of leptin and insulin). Thus, XNT could be developed as a potential anti-obesity agent in the future.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KESAN XANTORIZOL ATAS HYPERPLASIA DAN HYPERTROPHY 3T3-L1 ADIPOSIT

Oleh

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Merujuk kepada Kajian Kesihatan dan Morbiditi Kebangsaan (NHMS) 2015, terdapat 47.7% daripada penduduk Malaysia adalah obes atau berlebihan berat badan. Kadar obesiti yang semakin meningkat telah menyebabkan masalah kesihatan utama seperti penyakit kardiovaskular dan diabetes. Penggunaan kebanyakan ubat rawatan obesiti adalah tersekat oleh kesan-kesan sampingan yang serius seperti strok, infarksi miokardium dan kemurungan. Kesan-kesan tersebut menunjukkan bahawa ubat rawatan obesiti yang berkesan dan selamat perlu dipertingkatkan untuk memerangi obesiti. Kajian yang sebelumnya menunjukkan bahawa xantorizol (XNT) menurunkan tahap asid lemak dan trigliserida pada peringkat *in vivo*, tetapi setakat ini *belum ada kajian* khusus yang dijalankan terhadap aktiviti anti-obesiti dan mekanisme XNT. Dalam kajian ini, minyak mentah yang mengandungi XNT diekstrak daripada *Curcuma xanthorrhiza* Roxb. dengan pengekstrakan cecair karbon dioksida superkritikal. Kemudiannya, XNT ditulenkan dengan kromatografi lajur dan sentrifugal dan kromatografi lapisan nipis (TLC). Kehadiran XNT pada setiap fraksi dikenalpasti dengan TLC. Ketulenan XNT ditentukan oleh analisis kromatografi gas-spektrometri jisim (GC-MS) manakala struktur XNT disahkan oleh analisis spectrum resonans magnet nuklear (NMR) proton dan karbon. Seterusnya, nilai IC_{50} XNT ditentukan menggunakan ujian MTT. Mod sel mati dianalisis seterusnya dengan pewarnaan annexin V/7-AAD untuk apoptosis awal manakala ujian TUNEL untuk apoptosis akhir. Mekanisme yang terlibat dikenalpasti oleh analisis kuantitatif ELISA bagi protein caspase-3 dan PARP-1. Sebaliknya, kebolehan XNT untuk menghalang adipogenesis dianalisis oleh pewarnaan minyak merah O (ORO) dan aktiviti gliserol-3-fosfat dehydrogenase (GPDH). Mekanisme yang terlibat dikenalpasti oleh kuantitatif analisis ELISA

bagi protein PPARγ dan FAS. Keupayaan XNT untuk merangsang lipolisis dianalisis dengan pengiraan jumlah gliserol. Mekanisme yang terlibat dikenalpasti oleh analisis kuantitatif ELISA bagi protein leptin dan insulin. Signifikansi statistik dianalisis dengan ujian "One-way ANOVA" dan data adalah signifikan apabila $p < 0.05$. Oleh itu, kajian ini bertujuan untuk menilai potensi xantorizol dalam induksi apoptosis, perencatan terhadap generasi adiposit dan rangsangan lipolisis dengan menggunakan 3T3-L1 adiposit. Kajian ini mendapati ketulenan XNT adalah 98.3% dengan menggunakan kaedah kromatografi sentrifugal. Struktur XNT dianalisis dengan spektrum daripada ^{1}H and ^{13}C resonans magnet nucleus. Nilai IC_{50} XNT pada 3T3-L1 adiposit adalah 35 ± 0.24 µg/mL. Kemusnahan sel adalah disebabkan 20.01 ± 2.77% apoptosis awal dan 24.13 ± 2.03% apoptosis akhir ($p < 0.05$). XNT didapati merangsangkan apoptosis melalui peningkatan pengekspresan protein caspase-3 sebanyak 4.09 kali ganda dan pecahan PARP-1 sebanyak 3.12 kali ganda. Selain itu, perencatan XNT dalam pembezaan adiposit dan aktiviti gliserol-fosfat dehidrogenase adalah bergantung pada kepekatan dari 3.13 hingga 12.5 µg/mL. Penghalangan yang tertinggi dalam pembezaan adipositi adalah 36.13 ± 3.64% ($p < 0.05$). Aktiviti GPDH pula diturunkan oleh XNT kepada 52.26 ± 4.36% ($p < 0.05$). Kajian ini mendapati bahawa XNT menghalang generasi adiposit dengan mengurangkan pengekspresan protein PPARγ kepada 0.36 kali ganda dan FAS protein kepada 0.38 kali ganda. Sebaliknya, XNT meningkatkan pembebasan gliserol sebanyak 45.37 ± 6.08% berbanding dengan kawalan ($p < 0.05$). Semasa lipolisis, XNT meningkatkan 2.08 kali ganda pengekpresan protein tetapi menurunkan pengekpresan protein insulin kepada 0.36 kali ganda ($p < 0.05$). Hasil kajian ini menunjukkan bahawa XNT mengurangkan saiz adiposit melalui pengawalan leptin dan insulin. Kesimpulannya, mekanisme anti-obesiti XNT melibatkan penurunan hiperplasia adipositi melalui aruhan apoptosis (pengawalan caspase-3 and PARP-1) dan merencat pembezaan adipositi (pengawalan PPARγ and FAS), manakala pengurangan hipertropik melalui aruhan lipolisis (pengawalan leptin. dan insulin). Oleh yang demikian, XNT boleh digunakan sebagai agen anti-obesiti yang berpotensi pada masa akan datang.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God Almighty for giving me the opportunity, knowledge and strength to complete this research work. I would not have achieved all that I have without his blessings.

During my PhD journey, I would like to express my deepest gratitude and appreciation to my supervisor, Dr. Meenakshii Nallappan for her endless patience in giving me invaluable supervision, guidance, suggestions and encouragement during my research. Without her kind supervision and constructive advices, I would not have been able to accomplish all the objectives.

My sincerest thanks go to my supervisory committees, Dr. Nur Kartinee Kassim, Dr. Shamarina Shohaimi and Dr. Mohd Shazrul Fazry Sa'ariwijaya for sharing their expertise and providing me helpful ideas, comments and support that contribute to my research work. Their supervision and invaluable assistance have helped my research to run smoothly.

My special thanks go to Dr. Tee Thiam Tsui and Dr. Cheah Yew Hoong from ZACH Biotech Depot Sdn. Bhd. for their generous investment of time, energy and grant in my research work. Without their constant support, this achievement would not have been possible. Their insightful comments, suggestions and inspiration have sustained me along the way.

Also, I would like to extend my great appreciation to Raymond from Faculty of Health Sciences, Universiti Kebangsaan Malaysia for assisting me on the use of flow cytometry in order to complete this research work successfully.

Not forget, I am very thankful to my labmates, Nithiyaa Perumal, Muhammad Akram b Mohd Noordin, Ibrahim Mahmood and Malina Kumaran for their friendship, assistance and encouragement throughout this research.

Last but not least, I want to deeply thank my beloved family and friends for giving me their love, understanding and support throughout the period of my study. I am also grateful to Chik Kiew Choon for his patience and care at all times. Special thanks to Ministry of Education Malaysia for the scholarship under myBrain 15.

I certify that a Thesis Examination Committee has met on 25th June 2018 to conduct the final examination of Oon Seok Fang on her thesis entitled "Effects of Xanthorrhizol on 3T3-L1 Adipocyte Hyperplasia and Hypertrophy" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
Akt	Protein kinase B
ANOVA	One-way analysis of variance
AP-1	Activator protein 1
ap2	Adipocyte fatty acid binding protein
Apaf-1	Apoptotic protease activating factor 1
Asp	Aspartate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BMI	Body mass index
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
CAB	7-chloroarctinone-b
CAD	Caspase-activated DNase
cAMP	Cyclic adenosine monophosphate
$CDCl_3$	Deuterated chloroform
C/EBPβ	CCAAT/enhancer-binding protein beta
$CHCl_3$	Chloroform
CNS	Central nervous system
COX-2	Cyclooxygensae-2
CRP	C-reactive protein
CVD	Cardiovascular diseases
DHAP	Dihydroxyacetone phosphate
DMBA	7,12-dimethylbenz[a]anthracene
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EDTA	Ethylenediaminetetraacetic acid
EGCG	(-)-epigallocatechin-3-gallate
EI	Electron ionization
EIMS	Electron ionization mass spectrometry
ELISA	Enzyme linked immunosorbent assay
ERE	Estrogen responsive element
ERK1	Extracellular signal-regulated kinase 1
ESCP	Endocrine Society Clinical Practice
Et_2O	Diethyl ether
FA	Fatty acid
FAS	Fatty acid synthase
FBS	Fetal bovine serum

FDA	Food and Drug Administration
FFA	Free fatty acid
GC	Gas chromatography
GC-MS	Gas chromatography- mass spectrometry
GLUT4	Glucose transporter type 4
Gly	Glycine
GLP	Glucagon-like peptide
GPDH	Glycerol-3-phosphate dehydrogenase
GPT	Glutamate pyruvate transaminase
GOT	Glutamate–oxaloacetate transaminase
G3P	Glycerol-3-phosphate
HCl	Hydrochloric acid
HDL	High-density lipoprotein
hERα	Human estrogen receptor-α
HFD	High-fat diet
HIF	Hypoxia-inducible fact□r
HRP	Horseradish peroxidase
H_2O_2	Hydrogen peroxide
ICAD	Inhibitor caspase-activated DNase
IC_{50}	Half-maximal inhibitory concentration
IGF-1	Insulin-like growth factor 1
IkBα	IkappaBalpha
IL-1β	interleukin-1ß
IL-6	Interleukin-6
Inos	Inducible nitrc oxide synthase
IRS	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
LDL	Low-density lipoprotein
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein
mRNA	Messenger ribon□cleic acid
MSD	Mass Selective Detector
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetraolium bromide
NaCl	Sodium chloride
NAD^+	Nicotinamide adenine dinucleotide$^+$
NADH	Nicotinamide adenine dinucleotide dehydrogenase
NADPH	Nicotinamide adenine dinucleotide phosphate dehydrogenase
NAFLD	Nonalcoholic fatty liver disease
NF-Kb	Nuclear factor kappaB
NHMS	National of Health and Morbidity Survey
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance

NP-40	Nonyl phenoxypolyethoxylethanol.
NPY	Neuropeptide
OD	Optical density
ODC	Ornithine decarboxylase
ORO	Oil red O
PAI-1	Plasminogen activator inhibitor-1
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PE	Petroleum-ether
Ph	Phenyl
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
PS	phosphatidylserines
pS2	trefoil factor 1
R_f	Retention factor
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
RT	Room temperature
RXR	Retinoid X receptor
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
SPSS	Statistical Package for the Social Sciences
ssDNA	Single-stranded DNA
Tc	Control growth
TdT	Terminal deoxynucleotidyl transferase
Ti	Test growth
TIC	Total ion chromatogram
TG	Triglyceride
TLC	Thin layer chromatography
TMB	3,3',5,5'-tetramethylbenzidine
TMS	Trimethylsilane
TNFα	Tumor necrosis factor α
TPA	12-O-tetradecanoylphorbol-13-acetate
TRITC	tTtramethylrhodamine
TUNEL	Terminal dUTP nick end labelling
UPM	Universiti Putra Malaysia
UTP	Uridine-5'-triphosphate
UV	Ultraviolet
VLDL	Very -low-density lipoprotein
XNT	Xanthorrhizol
WAT	White adipose tissue
WHO	World Health Organization
7-AAD	7-amino-actinomycin

CHAPTER 1

INTRODUCTION

1.1 Introduction to Study

The word obesity is derived from Latin obesitas and defined as stout, fat or plump (Mohamed, Ibrahim, Elkhayat, & Dine, 2014). At the cellular level, obesity is known as increased number (hyperplasia) and increased size (hypertrophy) of adipocytes (Swick, 2011; Kim et al, 2016). Obesity represents a major risk factor of metabolic disorders throughout the world (Hsu & Yen, 2007; Tu & Tawata, 2014). It is often associated with cardiovascular diseases (CVDs), hyperlipidemia, coronary heart disease, hypertension, type 2 diabetes mellitus, respiratory complications, osteoarthritis, and cancer (Ko et al., 2013; Tu & Tawata, 2014). In obesity treatment, many synthetic anti-obesity drugs exhibit severe side effects, whilst bariatric surgery poses potential health risks. From the development of anti-obesity drugs to surgical treatment of obesity, a new platform is required to combat obesity. Numerous studies elucidated that dietary phytochemicals could be served as alternative treatments for obesity.

In Malaysia, one of the local herbs namely *Curcuma xanthorrhizza* Roxb., is believed to have anti-obesity bioactive component known as xanthorrhizol (XNT). XNT is the most active and abundant compound isolated from the essential oil of *C. xanthorrhiza* (Handayani, Sakinah, Nallappan, & Pihie, 2007; Jantan, Saputri, Qaisar, & Buang, 2012; Kim, Kim, Song, & Hwang, 2014). As extensive research has evaluated on XNT's antiproliferative and antitumor properties (Ismail, Pihie, & Nallappan, 2005; Cheah, Azimahtol, & Abdullah, 2006; Chung et al., 2007; Handayani et al., 2007; Cheah et al., 2008; Park et al., 2008; Cheah et al., 2009; Kang, Park, Chung, Hwang, & Lee, 2009; Joo et al., 2010; Sai, 2011; Kim et al., 2013; Musfiroh et al., 2013; Udin, 2013), there are little scientific studies being conducted on metabolic effect of XNT. Therefore, the potential of XNT to combat obesity could be studied through its ability to inibit adipocyte hyperplasia and hypertrophy.

1.2 Problem Statement and Justification of the Study

Anti-obesity drugs seem to be a solution to obesity but their application has been limited by possible side effects and severe drug reactions (Sun, Wu, & Chao, 2016). Although numerous pharmacological approaches for obesity therapy have been studied, only several drugs have been used clinically (Jang & Choung, 2013). Most of the anti-obesity drugs are limited to apply long-term use due to their severe side effects (Jang & Choung, 2013; Ko et al., 2013). Initially, fenfluramine and dexfenfluramine were withdrawn from the market in 1997 because they may adversely affect heart valves (Sun et al., 2016). In 2010, sibutramine was stopped by Food and Drug Administration (FDA) due to

increased risk of myocardial infarction and stroke. Also, orlistat has been reported to cause severe liver damage once in a while. Hence, a revised drug label that stated its potential side effect was approved by FDA. On the other hand, rimonabant was withdrawn due to the side effect of depression (Adan, 2013).

The accelerating risk of obesity to global health and side effects of anti-obesity drugs have prompted scientists to discover alternative anti-obesity agents (Sun et al., 2016). Efficacy of plants should be evaluated due to insufficient safe modern drugs (WHO, 1980; Daisy, Santosh, & Rajathi, 2009). Sun et al. (2016) revealed that natural products are effective in weight management and other non-communicable diseases. Traditional herbal treatment was found to suppress appetite and reduce body weight (Chandrasekaran et al., 2012). Also, bioactive compounds including apigenin, genistein, and catechins are potentially useful to combat obesity (Jang & Choung, 2013). Since anti-obesity effects of natural products have been elucidated in many studies, they may give promising effects in the treatment of obesity (Ko et al., 2013).

Previous studies demonstrated that XNT reduced the serum levels of free fatty acid (FFA) and triglyceride (TG) in high-fat diet-(HFD)-induced obese mice (Kim et al., 2014). It also reduced the epididymal adipocyte size and its fat pad mass. However, the detailed anti-obesity activities and related mechanisms are yet to be reported. Hence, this research study was initiated by extracting, isolating and purifying XNT (≥ 95% GC-MS) from *C. xanthorrhiza*. Next, IC_{50} value of XNT on 3T3-L1 adipocytes was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. Apoptosis studies of XNT in 3T3-L1 adipocytes were conducted by annexin V/7-amino-actinomycin **(**7-AAD) staining and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. The apoptotic mechanisms were evaluated by protein analysis of caspase-3 and PARP. Based on the dose range finding results, XNT concentrations that did not significantly affect cell viability were used for *in vitro* efficacy studies in 3T3-L1 adipocytes. The potential of XNT to inhibit adipogenesis was examined through oil red O staining and glycerol-3-phosphate dehydrogenase **(**GPDH) activity. Its related mechanisms were evaluated by protein analysis of PPARγ and FAS. Then, the ability of XNT to induce lipolysis was evaluated by glycerol assay and its related lipolytic mechanisms were determined by protein analysis of insulin and leptin.

1.3 Objectives

The objectives of this research are:

(I) to extract, isolate, and purify XNT from *C. xanthorrhiza*.

(II) to determine the highest tolerated dose of XNT on 3T3-L1 adipocytes employing MTT assay.

(III) to evaluate the ability of XNT to induce apoptosis (annexin V/7-AAD staining and TUNEL assay) in 3T3-L1 adipocytes and its apoptotic mechanisms.

(IV) to evaluate the ability of XNT to inhibit adipogenesis (oil red O staining and GPDH activity) in 3T3-L1 adipocytes and its related mechanisms.

(V) to evaluate the ability of XNT to induce lipolysis (glycerol assay) in 3T3-L1 adipocytes and its lipolytic mechanisms.

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