

DOCTORAL THESIS

Phytochemical and pharmacological studies of the root of *Ilex pubescens*

Wang, Jingrong

Date of Award:
2008

[Link to publication](#)

General rights

Copyright and intellectual property rights for the publications made accessible in HKBU Scholars are retained by the authors and/or other copyright owners. In addition to the restrictions prescribed by the Copyright Ordinance of Hong Kong, all users and readers must also observe the following terms of use:

- Users may download and print one copy of any publication from HKBU Scholars for the purpose of private study or research
- Users cannot further distribute the material or use it for any profit-making activity or commercial gain
- To share publications in HKBU Scholars with others, users are welcome to freely distribute the permanent URL assigned to the publication

**Phytochemical and Pharmacological Studies of the
Root of *Ilex pubescens***

WANG Jingrong

A thesis submitted in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Principal Supervisor: Prof. LIU Liang

Hong Kong Baptist University

March 2008

ABSTRACT

Maodongqing (毛冬青, MDQ), the root of *Ilex pubescens* Hook. et Arn. (Aquifoliaceae), is widely used in southern China for treatment of cardiovascular, peripheral vascular and inflammatory diseases. Owing to its efficacy and safety, this herb has been developed into many single herbal preparations and proprietary products. However, the anti-inflammatory and anti-thrombotic activities and their relevant active principles of MDQ have not been well investigated. In the current work, an activity-guided phytochemical study and pharmacological studies were undertaken in order to clarify the underlying scientific foundations of the therapeutic effects of MDQ, and further provide evidence for exploiting and utilizing MDQ. The results were shown as below:

Anti-thrombotic effects of nine fractions isolated from the *n*-BuOH layer of ethanol extract of MDQ by column chromatography were examined by using Kappa-carrageenan-induced tail thrombosis as a testing model. A phenolic fraction, *Fr. 5*, was identified as the bioactive fraction possessing anti-thrombotic activity. Chemical investigation on *Fr. 5* led to the isolation of two novel hemiterpene glucosides whose structures were elucidated on the basis of spectroscopic and chemical evidences as 2-(*trans*-caffeoyloxy)methyl-3-hydroxy-1-butene-4-*O*- β -D-glucopyranoside (pubescenoside A) and 2-hydroxymethyl-3-*trans*-caffeoyloxy-1-butane-4-*O*- β -D-glucopyranoside (pubescenoside B). These two hemiterpene glucosides were found to have anti-platelet aggregation activity by using high shear stress-induced platelet aggregation test.

Anti-inflammatory activities of the nine fractions of MDQ were assayed with carrageenan-induced paw edema in rats. A saponin fraction, *Fr. 8-2*, was identified as the bioactive fraction with the most potent anti-inflammatory effect among those nine fractions. Two novel triterpene saponins named as pubescenosides C and D together with five known triterpene saponins were isolated from *Fr. 8-2*. Based on spectroscopic and chemical evidences, the structures of pubescenosides C and D were elucidated as 3-*O*- β -D-glucopyranosyl(1 \rightarrow 2)- β -D-xylopyranosyl-urs-12,18-dien-(20 β -methyl)-28-oic acid 28-*O*- β -D-glucopyranosyl ester and 3-*O*- α -L-rhamnopyranosyl (1 \rightarrow 2)- β -D-glucopyranosyl (1 \rightarrow 2)- β -D-xylopyranosyl-urs-12,18-dien-(20 β -methyl)-28-oic acid 28-*O*- β -D-glucopyranosyl ester, respectively. The five known triterpene saponins were elucidated as ilexaponin B₁, B₂, B₃, A₁ and chikusetsusaponin IVa. HPLC fingerprinting of *Fr. 8-2* demonstrated that more than 70 % of the components in this fraction could be chemically identified.

Subsequently, *in vivo* anti-inflammatory and analgesic effects of *Fr. 8-2* were

comprehensively evaluated. As a result, it showed a significant suppression on the paw edema of rats induced by subplantar injection of carrageenan or histamine when given intraperitoneally at dosages ranging from 12.5 to 100 mg/kg of *Fr. 8-2* in rats, demonstrating its potent anti-inflammatory activity on acute inflammation model. This fraction was also proven to exert analgesic effect, because it could significantly inhibit acetic acid-induced abdominal writhing response of mice and prolong the time required for mice tail flick after exposure to a source of radiant heat at the oral dosages of 100 and 200 mg/kg. Mechanistic studies showed that the anti-inflammatory effect of *Fr. 8-2* was closely related to the selective inhibition on cyclooxygenase-2 (COX-2) expression, and overall attenuation of the ratio of pro-inflammatory /anti-inflammatory cytokines which was resulted from significant inhibition on the pro-inflammatory cytokines (IL-1, IL-6 and TNF- α) and elevation of the anti-inflammatory cytokines (IL-4 and IL-10) production at the sites of inflammation.

In conclusion, this research revealed that MDQ has multiple functions, such as anti-thrombotic, anti-inflammatory and analgesic actions, which may attribute to the unusual hemiterpene glucosides and triterpene saponins in this herb. These results supported the clinical usage of MDQ in treating cardiovascular, peripheral vascular diseases and various inflammatory conditions. Meanwhile, *Fr. 8-2*, a triterpene saponin fraction whose chemical components were largely identified, and pharmacological activities were well verified, is expected to be developed as a botanical drug for anti-inflammation.

TABLE OF CONTENTS

DECLARATION	i
ABSTRACT.....	ii
ACKNOWLEDGEMENTS.....	iv
TABLE OF CONTENTS	vi
LIST OF FIGURES	xii
LIST OF TABLES	xv
LIST OF SCHEMES	xvi
LIST OF ABBREVIATIONS	xvii
CHAPTER 1 INTRODUCTION AND DESIGN OF THE RESEARCH	1
1.1 General Introduction on <i>Ilex pubescens</i> Hook. et Arn.....	2
1.2 Clinical Applications of MDQ and Ilexonin A	12
1.2.1 Treatment for Heart Diseases.....	12
1.2.2 Treatment for Peripheral Vascular Diseases	15
1.2.3 Treatment for Cerebrovascular Diseases	18
1.2.4 Treatment for Respiratory Infectious Diseases.....	19
1.2.5 Treatment for Acute and Chronic Inflammatory Diseases	20
1.2.6 Other Applications	22
1.2.7 Adverse Effects.....	22
1.3 Pharmacological Activities of MDQ and Ilexonin A.....	23
1.3.1 Actions on the Cardiovascular System	23
1.3.2 Anti-thrombotic Effect.....	31
1.3.3 Anti-inflammatory Effect.....	34

1.3.4 Other Activities	36
1.3.5 Toxicity	36
1.3.6 Summary	37
1.4 Chemical Investigations on <i>Ilex pubescens</i>	40
1.4.1 Triterpenes	40
1.4.2 Triterpene Saponins	41
1.4.3 Coumarins	43
1.4.4 Lignans.....	43
1.4.5 Phenylpropanoid	44
1.4.6 Phenolics and Other Compounds.....	45
1.5 Introduction to Triterpene and Triterpene Saponins	47
1.5.1 Definition.....	48
1.5.2 Structural Types	49
1.5.3 Occurrence	49
1.5.4 Biological Activities	51
1.6 Structural Elucidation of Organic Compounds with Spectroscopic Techniques	53
1.6.1 Mass Spectrometry	54
1.6.2 NMR Spectroscopy.....	59
1.6.3 X-ray Crystallography	64
1.7 Aims, Objectives and Design of the Study	64
1.7.1 Aims and Objectives.....	64
1.7.2 Design of the Study.....	66
1.8 Structure of the Thesis	71
CHAPTER 2 SCREENING FOR THE BIOACTIVE FRACTIONS WITH ANTI-THROMBOTIC ACTIVITY AND IDENTIFICATION OF THE RELEVANT CONSTITUENTS	73
2.1 Introduction.....	74
2.2 Experimental.....	81

2.2.1	Materials and Methods.....	81
2.2.2	Preparation of Fractions of MDQ.....	83
2.2.3	Carrageenan-induced Tail Thrombosis in Mice.....	86
2.2.4	Isolation of Pubescenosides A (1) and B (2).....	86
2.2.5	Shear-induced Platelets Aggregation.....	89
2.2.6	Statistical Analysis.....	90
2.3	Results.....	90
2.3.1	Effects of MDQ Fractions on Carrageenan-induced Tail Thrombosis in Mice	90
2.3.2	Structure Elucidation of Pubescenosides A (1) and B (2).....	93
2.3.3	Activities of Pubescenosides A and B on Platelet Aggregation Induced by High Shear Stress.....	101
2.4	Discussions.....	103
2.4.1	Identification of the Bioactive Fractions.....	103
2.4.2	Chemical Constituents of the Bioactive Fraction.....	104
2.4.3	<i>In vitro</i> Activities of Pubescenosides A and B on Platelets Aggregation....	108
2.5	Conclusions.....	110
CHAPTER 3 SCREENING FOR THE BIOACTIVE FRACTIONS WITH ANTI-INFLAMMATORY ACTIVITY AND IDENTIFICATION OF THE RELEVANT CONSTITUENTS		111
3.1	Introduction.....	112
3.1.1	<i>In Vivo</i> Models for Anti-inflammatory Studies.....	114
3.1.2	<i>In Vitro</i> Models of Inflammation.....	117
3.1.3	Models in the Current Study.....	121
3.2	Experimental.....	122
3.2.1	Materials and Methods.....	122
3.2.2	Induction of Acute Inflammation in Rat Hind Paws by Carrageenan.....	124
3.2.3	Isolation of Triterpene Saponins from <i>Fr. 8-2</i>	124

3.2.4	Chemical Profiling Analysis of <i>Fr. 8-2</i> Using HPLC Technique.....	131
3.2.5	Statistical Analysis	132
3.3	Results and Discussions.....	132
3.3.1	Effects of MDQ Fractions on Carrageenan-induced Paw Edema in Rats ...	132
3.3.2	Structure Elucidation of Two New Triterpene Saponins Pubescenosides C (3) and D (4)	134
3.3.3	Structure Elucidation of the Known Triterpene Saponins	144
3.3.4	Chemical Profiles of <i>Fr. 8-2</i> Characterized by HPLC Fingerprinting	154
3.4	Discussions and Conclusions.....	155

CHAPTER 4 *IN VIVO* ANTI-INFLAMMATORY AND ANALGESIC

ACTIVITIES OF <i>FR. 8-2</i>.....	158	
4.1	Introduction.....	159
4.2	Experimental.....	162
4.2.1	Drugs and Reagents	162
4.2.2	Animals.....	162
4.2.3	Induction of Acute Inflammation in Rat Hind Paws by Injection of Carrageenan	163
4.2.4	Induction of Acute Inflammation in Rat Hind Paws by Injection of Histamine	164
4.2.5	Visceral Nociceptive Model Induced by Acetic Acid Stimulation in Mice .	164
4.2.6	Central Nociceptive Model Induced by Radiant Heat Stimulation in Mice	165
4.2.7	Western Blot Analysis for Determination of COX-1 and COX-2 Protein Expressions	166
4.2.8	Bio-plex Assay for Determination of IL-1 β , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN- γ , TNF- α in Rat Paw Tissues.....	167
4.2.9	Statistical Analysis	168
4.3	Results.....	169
4.3.1	Inhibitory Effect of <i>Fr. 8-2</i> on Carrageenan-induced Paw Edema of Rats..	169

4.3.2	Inhibitory Effect of <i>Fr: 8-2</i> on Histamine-induced Paw Edema of Rats.....	171
4.3.3	Analgesic Effect of <i>Fr: 8-2</i> in Nociceptive Models of Mice.....	173
4.3.4	Inhibition of <i>Fr: 8-2</i> on COX-2 Protein Expression in Rat Paw Tissues	176
4.3.5	Effects of <i>Fr: 8-2</i> on Levels of IL-1 β , IL-2, IL-4, IL-6, IL-10, GM-CSF, IFN- γ and TNF- α in Paw Tissues of Rats	178
4.4	Discussions	183
4.5	Conclusions.....	188
CHAPTER 5 SUMMARIES AND PROSPECTS OF THE RESEARCH.....		190
5.1	Summary and Conclusion of the Research	191
5.1.1	Identification of the Bioactive Fraction with Anti-thrombotic Activity and the Relevant Chemical Constituents	192
5.1.2	Identification of the Anti-inflammatory Fraction and the Relevant Chemical Constituents	194
5.1.3	<i>In vivo</i> Anti-inflammatory and Analgesic Activities of <i>Fr: 8-2</i> and the Mechanism.....	196
5.1.4	Conclusions.....	198
5.2	Prospects of the Future Studies.....	199
5.2.1	Bioassay of Pharmacological Activities of the Single Compounds Isolated from the Bioactive Fractions of MDQ.....	199
5.2.2	Further Investigation on the Chemical Constituents of the Bioactive Fractions.....	199
5.2.3	Interaction of the Chemical Constituents Existed in the Active Fractions ..	200
5.2.4	Combinative Pharmacological Effects of Two Active Fractions	200
5.2.5	Studies on <i>Fr: 8-2</i> as a Potential Botanical Drug.....	201
REFERENCES		202
PUBLICATIONS		217
PRESENTATIONS AND ABSTRACTS		218

PATENTS.....	219
CURRICULUM VITAE.....	220