

## DOCTORAL THESIS

### Anti-arthritic properties of a herbal formula comprising *Rosae Multiflorae Fructus* and *Lonicerae Japonicae Flos*

Cheng, Chi Yan

*Date of Award:*  
2016

[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

## ABSTRACT

Rheumatoid arthritis (RA), the most common autoimmune disease, affects billions of people worldwide. Conventional therapeutics do not provide satisfactory efficacy and even cause severe adverse reactions. Researchers are seeking new approaches for RA management. Toll-like receptor 4 (TLR4) signalling plays a pivotal role in the pathogenesis of RA, and has been proposed as a potential therapeutic target for RA. Chinese medicines are believed to be alternative options for conventional RA therapeutics. A herbal formula RL, consisting of *Rosae Multiflorae Fructus* and *Lonicerae Japonicae Flos*, has traditionally been used in treating various inflammatory disorders including RA. In this study we assessed the anti-arthritic efficacy of RL in animals, and investigated the involvement of TLR4 signalling in RL's effects *in vivo* and *in vitro*.

*In vivo* anti-arthritic efficacy of RL was evaluated using CIA (collagen-induced arthritis) rats, a model that is well established for studying human RA. Articular disease manifestations were investigated grossly, radiographically, and histologically. Isolated splenocytes were used to determine the effects of RL on immune responses. Molecular events in the TLR4 pathways upon RL treatment were examined in sera and joint tissues of CIA rats as well as in cultured lipopolysaccharide (LPS)-stimulated murine RAW264.7 and human THP-1 cells.

In CIA rats, RL significantly increased food intake and weight gain of CIA rats without any observable adverse effect; ameliorated joint erythema and swelling; and inhibited immune cell infiltration, bone erosion and osteophyte formation in joints. RL also reduced the upregulated protein expression levels of TLR4, phospho-transforming growth factor  $\beta$ -activated kinase 1 (p-TAK1), phospho-nuclear factor- $\kappa$ B (p-NF- $\kappa$ B), phospho-c-Jun, and phospho-interferon regulatory factor 3 (p-IRF3) in

joint tissues; modulated the levels of inflammatory factors [lowered tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin (IL)-6, IL-1 $\beta$ , IL-17A and monocyte chemoattractant protein-1 (MCP-1) in sera, and TNF $\alpha$ , IL-6, IL-1 $\beta$  and IL-17A in joints; and elevated IL-10 in sera and joints]; reinvigorated the declined activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in liver tissues and sera; reduced T helper 17 (Th17) cell proportions in splenocytes; inhibited splenocyte proliferation and activation; and lowered serum immunoglobulin G (IgG) levels.

In LPS-stimulated RAW264.7 and THP-1 cells, RL inhibited the production of pro-inflammatory mediators (e.g. TNF $\alpha$ , IL-6, IL-1 $\beta$ , and MCP-1), the phosphorylation and nuclear localization of transcription factors downstream of TLR4 [NF- $\kappa$ B, activator protein 1 (AP-1) and IRF3], and the activation/phosphorylation of inhibitor NF- $\kappa$ B  $\alpha$  (I $\kappa$ B $\alpha$ ), I $\kappa$ B kinase  $\alpha/\beta$  (IKK $\alpha/\beta$ ), TAK1, TANK-binding kinase 1 (TBK1) and interleukin-1 receptor-associated kinase 1 (IRAK1). RL's inhibitory effects on IRF3 phosphorylation reduced gradually along with the increase in LPS concentrations.

In conclusion, RL possesses anti-arthritic effects in CIA rats and had no observable adverse effect. The therapeutic effect of RL is, at least in part, attributed to its inhibition on the IRAK1/TAK1/NF- $\kappa$ B, IRAK1/TAK1/AP-1, and TBK1/IRF3 pathways. Findings of this study provide a pharmacological justification for the use of RL in the control of RA, and suggest that RL is a safe and effective alternative anti-RA agent.

## TABLE OF CONTENTS

DECLARATION .....	I
ABSTRACT.....	II
ACKNOWLEDGEMENTS .....	IV
TABLE OF CONTENTS.....	V
LIST OF TABLES .....	X
LIST OF FIGURES .....	XI
LIST OF ABBREVIATIONS.....	XIII
CHAPTER 1 Introduction.....	1
1.1 Rheumatoid arthritis (RA).....	1
1.1.1 Epidemiology of RA .....	2
1.1.2 Factors affecting RA susceptibility.....	3
1.1.2.1 Genetic factors of RA .....	3
1.1.2.2 Environmental factors.....	7
1.1.2.3 Constitutional factors.....	9
1.1.3 The current management options of RA and their limitations .....	10
1.1.3.1 Non-pharmacologic therapies .....	10
1.1.3.2 Pharmacologic therapies.....	11
1.1.4 Emerging molecular targets of RA.....	18
1.1.5 TLRs in RA pathophysiology .....	19
1.1.5.1 Induction phase.....	19
1.1.5.2 Inflammation phase .....	21
1.1.5.3 Self perpetuation and destruction phase .....	22
1.2 TLR4 activation and intracellular signalling.....	24
1.2.1 MyD88-dependent signalling .....	26
1.2.2 TRIF-dependent signalling.....	27
1.2.3 Targeting TLR4 signalling: an emerging approach for development of novel therapeutics.....	28
1.2.4 Targeting TLR4 signalling in RA treatment.....	29
1.3 Traditional Chinese Medicine (TCM) and RA .....	31
1.4 A TCM formula comprising Rosae Multiflorae Fructus and Lonicerae Japonicae Flos.....	32
1.4.1 Rosae Multiflorae Fructus ( <i>Yingshi</i> ).....	32
1.4.2 Lonicerae Japonicae Flos ( <i>Jinyinhua</i> ).....	35
1.5 Hypothesis.....	36
1.6 Objectives.....	37
CHAPTER 2 Extraction of herbal materials.....	38

2.1 Chapter summary .....	38
2.2 Introduction .....	40
2.3 Materials and methods .....	42
2.3.1 Herbal materials and extractions .....	42
2.3.2 Cell culture .....	43
2.3.3 Cell viability test.....	43
2.3.4 Anti-NO bioassay .....	44
2.3.5 Quality control of herbal materials by HPLC fingerprinting .....	44
2.3.6 Pro-inflammatory mediator assays .....	45
2.3.7 RNA extraction and RT-PCR.....	45
2.3.8 Immunoblotting .....	46
2.3.9 DPPH assay .....	47
2.3.10 ABTS assay .....	48
2.3.11 Statistical analysis.....	48
2.4 Results .....	49
2.4.1 RL3 ( <i>Yingshi: Jinyinhua</i> = 5:3) exhibited the most potent suppression of NO production in LPS-stimulated RAW264.7 cells .....	49
2.4.2 RL3 prepared using 95% EtOH exhibited the most potent suppression of NO production among extracts prepared with different concentrations of ethanol .....	51
2.4.3 RL's inhibitory activities on pro-inflammatory mediators.....	53
2.4.3.1 RL suppressed the upregulated mRNA and protein expression levels of iNOS in LPS- stimulated RAW264.7 macrophages.....	53
2.4.3.2 RL suppressed the production and mRNA expression of various pro-inflammatory mediators.....	55
2.4.3.3 RL exhibited anti-oxidant properties.....	59
2.4.4 HPLC analysis of RL.....	61
2.5 Discussion and conclusion .....	63
CHAPTER 3 RL exerted anti-arthritic effects in CIA rats .....	65
3.1 Chapter summary .....	65
3.2 Introduction .....	66
3.3 Methods.....	67
3.3.1 Induction of CIA.....	67
3.3.2 Animal treatment .....	68
3.3.3 Macroscopic scoring of CIA.....	69
3.3.4 Change in body weight and food intake .....	69
3.3.5 Radiographic analysis.....	69
3.3.6 Histological analysis.....	70

3.3.7 Statistical analysis.....	70
3.4 Results.....	71
3.4.1 RL improved clinical arthritic conditions in CIA rats.....	71
3.4.2 RL improved food intake and reduced weight loss in CIA rats .....	73
3.4.3 RL attenuated the radiographic damage of CIA rats .....	75
3.4.4 RL ameliorated the histological parameters of CIA rats .....	77
3.5 Discussion and conclusion .....	80
CHAPTER 4 Involvement of the TLR4 signalling pathway in the anti-arthritic effects of RL in CIA rats .....	81
4.1 Chapter summary .....	81
4.2 Introduction .....	83
4.3 Methods.....	84
4.3.1 Immunoblotting .....	84
4.3.2 Splenocytes culture.....	84
4.3.3 Splenocytes proliferation assay .....	84
4.3.4 Splenocytes activation assay .....	85
4.3.5 Flow cytometric analysis of T <sub>h</sub> 17 cell proportion .....	85
4.3.6 Cytokine, chemokine, antioxidant and anti-collagen antibody assays .....	85
4.3.7 Statistical analysis.....	86
4.4 Results .....	87
4.4.1 RL inhibited TLR4 signalling in CIA rats.....	87
4.4.2 RL modulated the production of cytokines regulated by NF- $\kappa$ B, AP-1 and IRF3 in CIA rats .....	90
4.4.3 RL rejuvenated the declined activities of endogenous antioxidant enzymes in CIA rats. ....	94
4.4.4 RL suppressed pathogenic immune responses in CIA rats.....	96
4.5 Discussion and conclusion .....	100
CHAPTER 5 Involvement of the TLR4 signalling pathways on the effects of RL <i>in vitro</i> .....	101
5.1 Chapter summary .....	102
5.2 Introduction .....	103
5.3 Methods.....	104
5.3.1 Reagents.....	104
5.3.2 Cell culture .....	104
5.3.3 Cell Stimulation.....	105
5.3.4 Cell viability assay.....	105
5.3.5 Determination of NO production.....	106
5.3.6 NF- $\kappa$ B reporter gene assay .....	106

5.3.7 Preparation of Cytosolic and Nuclear Fractions.....	107
5.3.8 Immunoblotting and cytokine/chemokine assay .....	107
5.3.9 Statistical analysis.....	108
5.4 Results .....	108
5.4.1 RL inhibited NO production in LPS-stimulated RAW264.7 cells .....	108
5.4.2 RL reduced IL-1 $\beta$ , IL-6 and TNF $\alpha$ production in LPS-stimulated RAW264.7 cells.....	112
5.4.3 RL lowered NF- $\kappa$ B transcriptional activity in LPS-stimulated RAW264.7 cells.....	114
5.4.4 RL inhibited NF- $\kappa$ B p65 phosphorylation and, NF- $\kappa$ B (p50/p65) nuclear localization in LPS-stimulated RAW264.7 and THP-1 cells .....	116
5.4.5 RL reduced I $\kappa$ B and IKK $\alpha$ / $\beta$ phosphorylation in LPS-stimulated RAW264.7 and THP-1 cells.....	120
5.4.6 RL reduced MCP-1 and MIP-1 $\alpha$ production in LPS-stimulated RAW264.7 cells.....	123
5.4.7 RL reduced c-Jun phosphorylation in LPS-stimulated RAW264.7 and THP-1 cells.....	124
5.4.8 RL reduced MAPK phosphorylation in LPS-stimulated RAW264.7 and THP-1 cells .....	127
5.4.9 RL reduced TAK1 phosphorylation in LPS-stimulated RAW264.7 and THP-1 cells.....	131
5.4.10 RL inhibited the degradation of IRAK1 but not IRAK4 in LPS-stimulated RAW264.7 and THP-1 cells.....	133
5.4.11 RL reduced CCL5 production and IRF3 phosphorylation in LPS-stimulated RAW264.7 and THP-1 cells .....	135
5.4.12 RL reduced TBK1 phosphorylation in LPS-stimulated RAW264.7 and THP-1 cells.....	138
5.4.13 Contribution of TLR4 signalling in the effects of RL.....	140
5.5 Discussion and conclusion .....	142
CHAPTER 6 General discussion, Conclusion and Future plan.....	146
6.1 General discussion.....	146
6.2 Conclusion.....	148
6.3 Future plans .....	149
6.3.1 Does RL inhibit endogenous-ligand-triggered TLR4 signalling? .....	149
6.3.2 Does RL affect TLR4 homodimerization on the cell membrane?.....	149
6.3.3 Does RL affect LPS-TLR4 binding?.....	150
6.3.4 Does RL affect the activation of T <sub>h</sub> 17 effector cells <i>in vitro</i> ?.....	150
6.3.5 Does RL affect the differentiation of T <sub>h</sub> 17 <i>in vitro</i> ? .....	151
6.3.6 Does RL affect T <sub>h</sub> 17 differentiation and activation <i>in vivo</i> ?.....	152
6.3.7 Multi-omics approach.....	153

6.3.8 Randomized controlled clinical trial.....	154
References.....	155
CURRICULUM VITAE.....	186