

<http://dx.doi.org/10.4314/ajtcam.v11i1.19>

## IN VITRO ANTI-INFLAMMATORY MECHANISM OF FOLIUM HIBISCI MUTABILIS LEAVES ETHANOL EXTRACTS

Jiangning Wang<sup>1#</sup>, Xiaorong Li<sup>2#</sup>, Lei Gao<sup>3</sup>, Xueyan Wang<sup>\*4</sup>,

<sup>1</sup>Beijing Shijitan Hospital, Capital Medical University, <sup>2</sup>Central Laboratory, The Luhe Teaching Hospital of the Capital Medical University, <sup>3</sup>Plastic Surgery, The Luhe Teaching Hospital of the Capital Medical University, <sup>4</sup>Department Of Allergy, Beijing Shijitan Hospital, Capital Medical University

\* E-mail: [xueyanwang135@163.com](mailto:xueyanwang135@163.com)

# Jiangning Wang and Xiaorong Li contributed equally to this research.

### Abstract

**Background:** Folium Hibisci Mutabilis was one of the traditional Chinese medicines with pharmacological activities of wound healing, antibacterial and anti-inflammatory, normally applied to the clinical treatment of local purulent infection, scald, and epidemic parotitis. However, few people reported that its effects of extracts on RAW264.7, cells and TNF- $\alpha$ , IL-6, and NO levels in rats serum of inflammation rats.

**Materials and Methods:** We mainly adopted the RAW264.7, macrophage cell line which was intervened by lipopolysaccharide (LPS), to establish the model of inflammatory cells. The secretion changes of TNF, and IL-6, level in the model cell acted by the FHMAEs, were detected by ELISA, NO level changes were determined by nitrate reductase method; Arthritis model induced by Collagen Type II were chose, and regulatory role of FHMAEs on IL-6 and NO level in serum were observed, FHMAEs mechanism of action were investigated in the treatment of rheumatoid arthritis. The states of the model rats were recorded.

**Results:** Release of TNF- $\alpha$ , IL-6, and NO in LPS-induced RAW264.7, cells were significantly inhibited and has a clear dose-response relationship; The TNF- $\alpha$ , IL-6 and NO levels were reduced by FHMAEs in experimental arthritis rats serum, and the status of rats were improved. At last, we found that IL-6, NO and TNF- $\alpha$  levels in the inflammatory cells could be reduced by FHMAEs greatly.

**Conclusion:** Both synovial inflammation and synovial proliferation could be reduced or inhibited by FHMAEs, all of this may provide experimental effective data for the novel drugs development and the clinical treatment of rheumatoid arthritis.

**Key words:** Folium Hibisci Mutabilis, Arthritic rats, TNF, IL-6, NO

### Introduction

*Folium hibisci mutabilis* was one of the traditional Chinese medicines. Its main chemical ingredients containing glycosides, phenols, amino acids, and sugar, phlegmatic (Li Zhang et al., 2013). Extract of Folium Hibisci Mutabilis leaves have different degrees of inhibition on gram-negative and gram-positive bacteria, like it was stronger on Gram-negative bacteria *Escherichia coli* (Changling Li et al., 2013). At the same time, its extracts has good anti-inflammatory, and analgesic effects (Ya Xu et al., 2013; Shichong Fu Li et al., 2002); and mouse writhing test proved that could significantly reduced writhing times; protection on renal ischemia-reperfusion injury as well as chronic liver injury (Ran Joo Choi et al., 2011). However, few people reported that its effects on RAW264.7, cells and TNF- $\alpha$ , IL-6 and NO levels in rats serum of inflammation rats. This study was to verify the in vitro anti-inflammatory activity action of hibiscus leaves ethanol extracts in both cell model and rats.

### Material and Methods

Murine macrophage cell line RAW264.7, supplied by the CAS Shanghai Institute of Cell Biology. 60 SD, (Sprague Dawley), rats weighing (180~220g), male were purchased from Capital Medical University Experimental Animal Center. Folium Hibisci Mutabilis leave, purchased from Beijing Tong Ren Tang drugstore; identified as *Hibiscus L.* of Malvaceae by capital Medical laboratory. The extracts were dialed by 70% ethanol and prepared to solid dispersion agent. NO kit (nitrate reductase) (Nanjing Jiancheng Institute of Biology), rat TNF- $\alpha$ ELISA kit, rat IL-6 ELISA kit (U.S. R&D Systems), bovine type II collagen, incomplete Freund's adjuvant was purchased from Sigma Chemical Co.; UV spectrophotometer (BIO-RAD company), high-speed desktop centrifuge (Beckman company), MK3 Microplate Reader (USA Thermo Multiskan company).

#### Effects of Hibiscus leaves ethanol extract on RAW264.7 cell viability (Song Wang et al., 2012)

RAW264.7, cell suspension was adjusted to the concentration of  $1 \times 10^5$ /mL, added to 96-well plate, 190 $\mu$ L into each well, 37°C, CO<sub>2</sub> incubated for 12hrs, 10 $\mu$ L different concentrations FHMAEs, added to make a final concentration of 0.25, 0.5, 1g/mL, duplicate three holes and cultured for 24hrs. 20 $\mu$ L 5mg/mL MTT solution added into the experimental wells, incubated 4hrs. Terminated and carefully aspirate hole medium, 150 $\mu$ L DMSO added; shake for 10min., at low speed, the crystals were fully dissolved, measured at 570nm. At last, the inhibition rate was calculated out.

#### Effects of FHMAEs, on the release of TNF-, IL-6, NO in LPS-induced RAW264.7 cells (Hua Qin et al., 2013; Yanyan Wang et al., 2010; Dandan Zhang et al., 2013).

Cell suspension was adjusted to the concentration of  $1 \times 10^5$ /mL, and 1mL was added to 24-well plates, settled at 37°C, 5% CO<sub>2</sub> in incubator until the cells reached 80% confluence. Each well was added with a final concentration of 0.25, 0.5, 1/g/mL of hibiscus leaves ethanol

<http://dx.doi.org/10.4314/ajtcam.v11i1.19>

extracts, cultured for 6h, added with the concentration of 1g/mL of LPS, 4h later, 600 $\mu$ L supernatant was spared. TNF- $\alpha$ , IL-6 levels was determinate by ELISA, and NO content was measured by nitrate reductase.

#### Effects of FHMAEs, on the TNF-, IL-6, NO levels in C $\square$ -induced rat inflammatory model (Xinwei Tian et al., 2011; Lantao Liu et al., 2013)

Under sterile conditions, bovine type II collagen (type II Collagen, C $\square$ ) was fully dissolved with 0.1 mol/L acetic acid to the concentration of 4mg/L, stayed at 4 °C overnight, mixed with an equal volume of Freund's complete adjuvant agent (1mg/mL) to be fully emulsified C $\square$  emulsion. 0.25ml emulsion was injected intra-dermal into each rat neck and back to make inflammatory models, repeated this action 14, days later. After immunization, all rats were randomly divided into four groups, namely the control group and the experimental groups, the experimental group was divided into high, medium and low concentrations groups.

#### Data Analysis

All measurement values were expressed as mean standard deviation, and all experiments were in triplicate. Application of SPSS 18.0 software was used for statistical analysis. The enumeration data was expressed with the  $\chi^2$  test, measurement data was expressed with t test.  $p < 0.05$  was considered statistically significant,  $p < 0.01$  was considered statistically significant difference.

## Results

#### Effects of FHMAEs on the viability RAW264.7 cell

FHMAEs extra [cts], has no obvious effect on the viability RAW264.7 cell at a given concentration when compared with the control group. Thus 0.2, 0.4, 0.8, 1.6g/mL can be used for other subsequent experiments concentrations. (Table 1)

**Table 1:** Effect of FHMAEs on the viability RAW264.7 cell

FHMAEs( $\mu$ g/mL)	A570	IR(%)
0	1.016 $\pm$ 0.042	-
0.2	0.985 $\pm$ 0.039	3.1
0.4	0.980 $\pm$ 0.086	3.5
0.8	0.950 $\pm$ 0.071	6.4
1.6	0.939 $\pm$ 0.683	7.6

#### Effects of FHMAEs on the release of TNF- $\alpha$ , IL-6, NO in LPS-induced RAW264.7 cells

LPS-stimulated RAW 264.7 cell after 4h, TNF- $\alpha$ , IL-6 and NO levels increased significantly ( $P < 0.01$ ). However when it was treated with FHMAEs which ranged from 0.2mg/mL to 1.6mg/mL, TNF- $\alpha$ , IL-6 and NO in cell culture medium were all significantly reduced, and the greater the concentration, the stronger the inhibition (Table 2).

**Table 2:** Effects of FHMAEs on the factors in RAW264.7 cells

Group	IL-6(pg·mL <sup>-1</sup> )	NO( $\mu$ M)	TNF- $\alpha$ (pg·mL <sup>-1</sup> )
Normal group(NG)	32.15 $\pm$ 11.25	35.41 $\pm$ 22.13	191.2 $\pm$ 17.81
Model group(MG)	92.31 $\pm$ 8.56**	105.35 $\pm$ 2.41**	872.32 $\pm$ 124.26**
FHMAEs group (mg·mL <sup>-1</sup> )	0.2	68.73 $\pm$ 7.43 <sup>#</sup>	760.41 $\pm$ 90.23 <sup>#</sup>
	0.4	52.28 $\pm$ 6.22 <sup>#</sup>	72.46 $\pm$ 2.64 <sup>#</sup>
	0.8	32.67 $\pm$ 2.57 <sup>##</sup>	61.18 $\pm$ 3.02 <sup>##</sup>
	1.6	15.43 $\pm$ 3.55 <sup>##</sup>	47.86 $\pm$ 2.31 <sup>##</sup>

\* indicated  $p < 0.05$ , \*\* indicated  $p < 0.01$ , vs NG; <sup>#</sup> indicated  $p < 0.05$ , <sup>##</sup>:  $p < 0.01$ , vs MG

#### FHMAEs on experimental arthritis serum TNF- $\alpha$ , IL-6 and NO(Ok-Kyoung Kwon et al., 2010; Eun Myoung Shin et al., 2008)

Normal groups were given 2ml/100g, saline once daily by intra-gastric administration; model group were also taken 2ml/100g, saline once daily by intra-gastric administration; In FHMAEs group, four different groups were taken different concentration of FHMAEs, respectively

<http://dx.doi.org/10.4314/ajtcam.v11i1.19>

which containing crude drug 160mg/kg, 80mg/kg, 40mg/kg, 20mg/kg, 2, times a day by intra-gastric administration. 14, days later, detection of TNF- $\alpha$ , IL-6 and NO in arthritis rats serum were significant different from model group, which were much lower than that in the normal group.

**Table 3:** Effects of FHMAEs on the factors in experimental arthritis rats serum

Group	IL-6(pg·mL <sup>-1</sup> )	NO( $\mu$ M)	TNF- $\alpha$ (pg·mL <sup>-1</sup> )
Normal group(NG)	33.18 $\pm$ 10.24	32.08 $\pm$ 9.81	199.2 $\pm$ 8.93
Model group(MG)	846.21 $\pm$ 97.73**	425.35 $\pm$ 80.23**	923.46 $\pm$ 34.57**
FHMAEs (mg·mL <sup>-1</sup> )	20	725.62 $\pm$ 92.45	710.15 $\pm$ 36.82 <sup>#</sup>
	40	594.58 $\pm$ 96.28 <sup>#</sup>	592.57 $\pm$ 33.21 <sup>##</sup>
	80	536.37 $\pm$ 76.39 <sup>##</sup>	420.33 $\pm$ 28.60 <sup>##</sup>
	160	490.77 $\pm$ 72.83 <sup>##</sup>	371.36 $\pm$ 30.81 <sup>##</sup>

\* indicated  $p < 0.05$ , \*\* indicated  $p < 0.01$ , vs NG; <sup>#</sup> indicated  $p < 0.05$ , <sup>##</sup>:  $p < 0.01$ , vs MG

## Discussion

This study was divided into three parts, the first part was the effect of FHMAEs, on RAW264.7, cell viability, and the second part was the action on the release of TNF- $\alpha$ , IL-6, and NO in the LPS-induced RAW264.7 cells; The third part was the changes of three factors affected by the extracts in the serum of experimental arthritis rats. Firstly we recognize the extract has few apparent inhibitions on RAW264.7 cells; Secondly results show that the capacity of TNF- $\alpha$ , IL-6, and NO release from LPS-induced RAW264.7, cells could be well suppressed by FHMAEs. Similarly, experiment proved that the three factors could also be inhibited in experimental arthritis rats' serum obviously. Therefore further separation and purification on FHMAEs may get more effective monomer compounds for anti-inflammatory drug development.

Hibiscus leaves was extracted with different polar solvents, its extracts has strong antibacterial activity on G<sup>-</sup> and G<sup>+</sup> bacteria which were tested by disk-diffusion method and double dilution method. Results show that its inhibitory effect on E. coli was more obvious, and found that 70% ethanol extract had the strongest inhibitory effect (Changling Li et al., 2009). Water extracts of Hibiscus had a certain effect on secretion of HBsAg, HBeAg have, and the role of HBsAg, in cloned hepatitis B virus DNA transfected HepG2 cell obviously. IL-6 is an important pro-inflammatory cytokine that enhances a variety of inflammatory mediators and is a sensitive indicator of the early inflammatory response in the body. The root of *Scutellaria baicalensis* Georgi, *Andrographis paniculata* (Burm.F.) Nees, the aerial part of *Glossogyne tenuifolia* Cass., all showed anti-inflammatory effects by decreasing the expression of IL-6. TNF- $\alpha$  is one of the major pro-inflammatory cytokines with diverse biological activities. TNF- $\alpha$  activates monocyte-macrophagen cells in an autocrine, and paracrine manner to cause monocyte-macrophage cells to release large amounts of IL-1, IL-6, IL-8, PGE2 and other inflammatory mediators. (Wittmann et al., 1996). Several CMM that have inhibited TNF- $\alpha$  expression in anti-inflammatory experiments, like the fruits of *Zanthoxylum schinifolium* Sieb. Et Zucc., the roots of *Angelica sinensis* (Oliv.) Diels, the roots of *Sophora flavescens* Ait., the roots of *Clematis chinensis* Osbeck, and others.(Wang Q et al., 2013).

Anti-inflammatory effects of hibiscus cream could be tested by xylene-induced mouse ear swelling test, rat carrageenan-induced paw edema degree experiment, mice granuloma hyperplasia test, found its significant inhibition on acute inflammation and chronic inflammation reaction (Qi Zhang et al., 2011). In the observation of extracts effected rats renal ischemia-reperfusion injury test, TNF- $\alpha$  mRNA expression in the treatment group was also significantly lower than the control group, indicated that has a protective effect on the inhibition of TNF- $\alpha$  and other inflammatory cytokine production (Shihua Luo et al., 2005).

## References

1. Changling Li, Fanlong Zheng, Bo Tian, Xing Hu, Shenggui Liu, Di Wu.(2013). The in vitro antibacterial effects of Hibiscus mutabilis leaves extracts on E.coli and Staphylococcus aureus. Science and Technology of Food Industry, 34(01): 57-59.
2. Changling Li, Shenggui Liu, Di Wu, Xing Hu, Zongjin Feng.(2009). Antibacterial effects of *Folium Hibisci Mutabilis* leaves extracts. Science and Technology of Food Industry, 30(11):97-101.
3. Dandan Zhang, Ning Tang, Xiaodong Hua, Ka Bian.(2013). Effects of mulberry extracts and their combinations on macrophage inflammatory mediators. Chinese Traditional and Herbal Drugs, 44(02):186-192.
4. Eun Myoung Shin, Hong Yu Zhou, Lian Yu Guo, Jeong Ah Kim, Seung Ho Lee, Irmgard Merfort, Sam Sik Kang, Hak Sung Kim, Sanghee Kim, Yeong Shik Kim.(2008). Anti-inflammatory effects of glycyrol isolated from Glycyrrhiza uralensis in LPS-stimulated RAW264.7 macrophages, International Immunopharmacology, 8(11):1524-1532.
5. Hua Qin, Yan Zhang, Xiaoyan Du, Yan Han, Ru Wang.(2013). The mechanisms and protective effect of Astragaloside  $\square$  on RAW264.7 cell injury induced by endotoxin. Progress in Modern Biomedicine, 13(11):2040-2043.
6. Lantao Liu, Shangfu Guo, Hongwei Wang, Huilin Guan, Jixi Wang, Yudong Xu.(2013). Effects of tripterygium glycosides on IL-10 in collagen-induced arthritis rats. Anatomy Research, 35(01):43-46.
7. Li Zhang, Changzheng Zhou.(2013). Research progress of the *Olium Hibisci Mutabilis* leaves. Guide of China Medicine, 11(1): 453-455.
8. Ok-Kyoung Kwon, Mee-Young Lee, Ji-Eun Yuk, Sei-Ryang Oh, Young-Won Chin, Hyeong-Kyu Lee, Kyung-Seop Ahn.(2010). Anti-inflammatory effects of methanol extracts of the root of *Lilium lancifolium* on LPS-stimulated Raw264.7 cells. Journal of Ethnopharmacology, 130(1): 28-34.
9. Qi Zhang, Qingsheng Yu, Jinfang Pan, Jianfeng Shuai, Shengbing Wu, Juda Liu.(2011). In vitro anti-inflammatory effects of hibiscus paste from old TCM experience side. Anhui Medical Journal, 32(11):1904-1906.
10. Ran Joo Choi, Eun Myoung Shin, Hyun Ah Jung, Jae Sue Choi, Yeong Shik Kim.(2011). Inhibitory effects of kaurenoic acid from *Aralia continentalis* on LPS-induced inflammatory response in RAW264.7 macrophages, Phytomedicine, 18(8): 677-682.

<http://dx.doi.org/10.4314/ajtcam.v11i1.19>

11. Shichong Fu, Zhengxing Rong, Fenghua Zhang, Liyun Yao, Weibin Shi, Ning Du.(2002). of the Study on anti-inflammatory and analgesic effects of Hibiscus leaves effective parts. Chinese Journal of Integrated Traditional and Western Medicine, **S1**: 222-224.
12. Shihua Luo, Shichong Fu, Fenghua Zhang, Xiaopeng Li, Lingzhu Zhou, Ning Du.(2005). Effective components of Hibiscus leaf on TNF- $\alpha$ in renal ischemia-reperfusion injury rats. Chinese Journal of Integrated Traditional and Western Medicine, **S1**:78-81.
13. Song Wang, Wei Wang, Meng Luo, Xiuhua Zhao, Yuangang Zu, Yanli Zhao.(2012). Regulation Effects of glycyrrhizin preparation on the secretion of inflammatory cytokines in RAW264.7 cells induced by LPS. Progress in Pharmaceutical Sciences, 36(**10**):465-470.
14. Wang Q, Kuang H, Su Y, Sun Y, Feng J, Guo R, Chan K.(2013). Naturally derived anti-inflammatory compounds from Chinese medicinal plants. J Ethnopharmacol, 146(**1**):9-39.
15. Wittmann, D.H., Schein, M., Condon, R..(1996). Management of secondaryperitioitis. Annals Surgery, 224, 10–18.
16. Xinwei Tian, Chunjing Liu, Xuehan Wang, Di Xu, Boqiong Jing.(2011). Study on the effect of Chubi Tang Peng grass on the IL-1 $\beta$ , TNF- $\alpha$  and PGE2 in serum of arthritis rats induced by collagen type  $\square$ . Henan Traditional Chinese Medicine, 31(**02**):138-140.
17. Yanyan Wang, Xin Liu, Hongwei Cao, Ning Wang, Yongling Lu, Jiang Zheng.(2010). Inhibition of chloroquine on TLR4-MyD88 independent signal transduction pathways in LPS/endotoxin-induced RAW264.7 cells. Acta Academiae Medicinae Militaris Tertiae, 32(**9**):869-873.
18. Ya Xu, Youlan Zheng, Jianfeng Lin, Lihua Zhang.(1989). Anti-inflammatory effects and toxicity of Hibiscus mutabilis leaves. Fujian Medical Journal, 11(**03**): 24-26.