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PRELIMINARY REPORT

Preliminary exploration on anti-inflammatory mechanism of Corilagin (beta-1-*O*-galloyl-3, 6-(*R*)-hexahydroxydiphenoyl-D-glucose) *in vitro*

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Abstract

Corilagin (beta-1-*O*-galloyl-3,6-(*R*)-hexahydroxydiphenoyl-D-glucose) is a novel member of the tannin family which has been discovered from many medicinal plants and has been confirmed in many pharmacological activities. However, the purified Corilagin that was used in experiment is rare, and the anti-inflammatory mechanism of Corilagin has not been investigated clearly. This study is to explore the inner anti-inflammatory mechanism of Corilagin. Inflammatory cellular model was established by lipopolysaccharide (LPS) interfering on RAW264.7 cell line. Levels of TNF-α, IL-1β, IL-6, NO and IL-10 in supernatant, mRNA expression of TNF-α, COX-2, iNOS and HO-1, protein expression of COX-2 and HO-1, translocation of NF-κB were assayed by ELISA or Griess method, real-time quantitative PCR, western blot and immunocytochemistry method, respectively. As a result, Corilagin could significantly reduce production of pro-inflammatory cytokines and mediators TNF-α, IL-1β, IL-6, NO (iNOS) and COX-2 on both protein and gene level by blocking NF-κB nuclear translocation. Meanwhile Corilagin could notably promote release of anti-inflammatory factor HO-1 on both protein and gene level, but suppress the release of IL-10. In conclusion, the anti-inflammatory effects of Corilagin are attributed to the suppression of pro-inflammatory cytokines

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and mediators by blocking NF-κB activation. Corilagin also can promote HO-1 production to induce regression of inflammation but can inhibit IL-10 production like Dexamethasone. Corilagin possesses a potential anti-inflammatory effect by not only abating inflammatory impairment but also promoting regression of inflammation and has a good prospect to be used in many inflammation-related diseases.

1. Introduction

Corilagin (beta-1-*O*-galloyl-3,6-(*R*)-hexahydroxydiphenoyl-D-glucose) is a novel member of the tannin family which has been discovered in many medicinal plants such as *Phyllanthus* species etc. [1]. The molecular formula of corilagin is $C_{27}H_{22}O_{18}$ (Fig. 1) [2]. Corilagin presents acicular crystal powder and can easily be dissolved in MeoH, EtoH, Aceton and DMSO but undissolved in water. It has been reported that Corilagin has strong antioxidative [3], thrombolytic [1], hepatoprotective [3], antiatherogenic [2] and antihypertensive [4] effects and has potential activity on beta-lactams against methicillin-resistant Staphylococcus aureus [5]. A preliminary study has reported Corilagin is a TNF-α inhibitor [6]. However, as in these reports the experimental material was the extract containing Corilagin but not purified substance, the exact pharmacological activities of Corilagin remained limited. Moreover, so far the inner anti-inflammatory mechanism of Corilagin has not been well investigated.

In inflammation discovered at present [7], macrophages play a central role in inflammation. The activation of pro-inflammatory and anti-inflammatory cytokines and mediators from macrophages is regarded as the key procedure of inflammatory reaction and leads consequent inflammatory impairment and restoration. Thereby the inflammation-related cytokines and mediators are frequently chosen as parameters to explore the effect of anti-inflammatory drugs. In experimental practice, RAW 264.7 mouse macrophage cell line stimulated by LPS is widely used as the inflammatory cellular model to study the effect of anti-inflammatory drugs and herbs [8].

In this study, we chose that inflammation cellular model to explore the anti-inflammatory effect of Corilagin and explored the anti-inflammatory mechanisms of that medicinal plant ingredient.

2. Materials and methods

2.1. Chemicals and reagents

Corilagin standard substance (purity>99%) was offered and identified by "China National Institute for the Control of Pharmaceutical and Biological Products". RPMI1640 was from Gibico (Grand Island, NY). Lipopolysaccharide (LPS) (*Escherichia coli* O111:B4) and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma (St. Louis, MO). Affinity-purified goat anti-mouse HO-1 antibody was obtained from R&D Systems (Minneapolis, MN). Affinity-purified goat anti-mouse COX-2 antibody and rabbit anti-mouse NF-κB p65 IgG antibody were gotten from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse TNF-α, IL-1β and IL-6 ELISA kits were purchased from Quantikine, R&D Systems (Minneapolis, MN). Griess reagent nitric oxide assay kit was from Beyotime Biotech (Jiangsu, P. R. China). Mouse IL-10 ELISA kit was obtained from Bender Medsystem (Vienna, Austria). Trizol was purchased from Gibico (Grand Island, NY). M-MLV Reverse Transcriptase was afforded from Promega (Madison, WI). SYBR Green I was taken from Biotium (Hayward, CA). The Oligo(dT18) and primers was synthesized by Shanghai Invitrogen (Shanghai, China). The dNTP was obtained from Promega (Madison, WI). All the material under study is endotoxin free.

2.2. Cellular model establishment and intervention

Murine macrophage cell line RAW264.7 was from China Center for Typical Culture Collection (CCTCC) (Wuhan, China). The cells were cultured in suggested circumstance by American Type Culture Collection (ATCC). 24 h prior to LPS treatment, the cells were inoculated into 6, 24 or 96 micro-well plates. 24 h later, when the cells were observed for adherence at the bottom of the well, cell supernatants were disposed and 10 ng/mL LPS with prepared exact solution were added into the well. The stimulation and intervention lasted for different hours and the supernatants and the cells were harvested for ELISA, real-time PCR, and western blot as well as immunocytochemical test. Based on the literature [3], the Corilagin was diluted by 1640 medium into concentration of 20 ng/mL, 10 ng/mL and 2 ng/mL for interfering in inflammation cellular model. Dexamethasone was chosen as positive control with concentration of 0.5 μg/mL [9]. According to the literature [10], APS with concentration of 100 μg/mL was selected as negative control of pro-inflammatory cytokines for monitoring the procedure. Cells stimulated by LPS without any intervention were observed as blank control, whereas cells incubated by 1640 medium were as normal control.

2.3. MTT assay and cell morphous observation

Cytotoxic effect of the Corilagin was evaluated by MTT assay according to instruction of ATCC. After cell model interfered with Corilagin for 24 h, cell morphology was observed.

2.4. Analysis of TNF-α, IL-1β, IL-6 and nitric oxide in supernatant

After stimulation and intervention on RAW264.7 cells for 24 h, supernatants were harvested and assayed for TNF-α, IL-1β and IL-6 by respective ELISA kits according to the kit instructions. Levels of the nitric oxide (NO) derivative nitrite were determined together

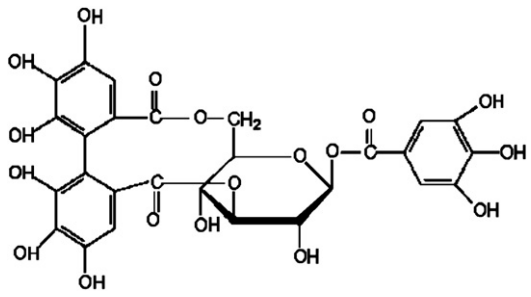

Figure 1 Corilagin (beta-1-*O*-galloyl-3,6-(*R*)-hexahydroxydiphenoyl-D-glucose).

with the Griess reaction according to instructions provided by the manufacturer.

2.5. Real-time PCR for detecting mRNA of TNF-α, COX-2, iNOS and HO-1

The RNA on TNF-α, inducible nitric oxide synthase (iNOS) and cycloxygenase-2 (COX-2) was extracted 4 h after stimulation and intervention and the RNA on HO-1 was obtained 18 h after stimulation and intervention. The primer was as below: Mus-COX-2: Forward: 5′-GAAGTCTTTGGTCTGGTGCCTG-3′, Reverse: 5′-GTCTGCTGGTTTGGAATAGTTGC-3′; Mus-iNos: Forward: 5′-GGAGCGAGTTGTGGATTGTC-3′, Reverse:5′-GTGAGGGCTTGGCTGAGTGAG-3′; Mus-TNF-α: Forward: 5′-GTGGAACTGGCAGAAGAGGC-3′, Reverse: 5′-AGACAGAAGAGCGTGGTGGC-3′; Mus-HO-1: Forward: 5′-CACAGATGGCGTCACTTCGTC-3′, Reverse: 5′-GTGAGGACCCACTGGAGGAG-3′. Quantitative PCR was performed in ABI-7700 Sequence Detector (Applied Biosystems, Foster City, CA). Each 50 μL real-time PCR system contained 1/50 of the original cDNA synthesis reaction, 7 μL (25 mM) $MgCl_2$, 0.8 μL (20 pmol/μL) of each primer, 1 μL (10 mM) dNTP, 1 μL SYBR Green I, 0.5 μL (5 U/μL) Taq and 5 μL 10× Buffer. Fifty cycles of amplification were performed: after 94 °C, 3 min, reaction cycle with 94 °C, 30 s, to 57 °C, 30 s, then to 72 °C, 30 s was carried out for 50 times. The fluorescence signal was detected at the end of each cycle. Melting curve analysis was used to confirm the specificity of the products. The $2^{-\Delta\Delta CT}$ method was performed to analyze the results [11].

2.6. Western blot analysis of COX-2 and HO-1

The expressive level of protein of COX-2 and HO-1 was tested by western blot analysis after intervention for 24 h. Briefly describing, the lysis buffer included 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 100 mg/mL phenyl methylsulfonyl fluoride, 1 mg/mL aprotinin, and 1% Triton X-100. The 2× sodium dodecyl sulfate (SDS) loading buffer contained 100 mM Tris–HCl, pH 6.8, 4% SDS, 20% glycerine, 10% b-mercaptoethanol, and 0.2% bromophenol blue. The goat polyclonal anti-COX-2, anti-HO-1 and horseradish peroxidase-conjugated secondary immunoglobulin G (IgG, Kangcheng, Shanghai, P.R. China) were used. At last the signals were detected by exposure of the membranes to X-ray films (Kodak, Rochester, NY, USA).

2.7. Immunocytochemistry assay on NF-κB

SP immunocytochemical assay was employed to detect expression of the nuclear translocation of NF-κB after intervention for 2 h. Five fields of vision were selected randomly and activation rate was calculated.

2.8. Statistical analysis

Each test was performed and then repeated two times. Data were presented as mean ± SD. Comparisons of the measurement data between multiple groups were performed with one-way ANOVA test. Rates were compared with Chi-square test. Statistical significance was considered significant when $p<0.05$. The statistical process was performed with SPSS 12.0 software.

3. Results

3.1. *In vitro* cytotoxicity of Corilagin

Based on MTT assay, it showed that pretreatment on unstimulated and stimulated RAW264.7 cell lines with prepared solution of Corilagin (concentration mentioned above) for 24 h did not significantly affect cell viability (data not shown). The max uncytotoxic concentration of Corilagin on unstimulated and stimulated RAW264.7 cell lines was 5 μg/mL. Cell morphous observation presented the same result.

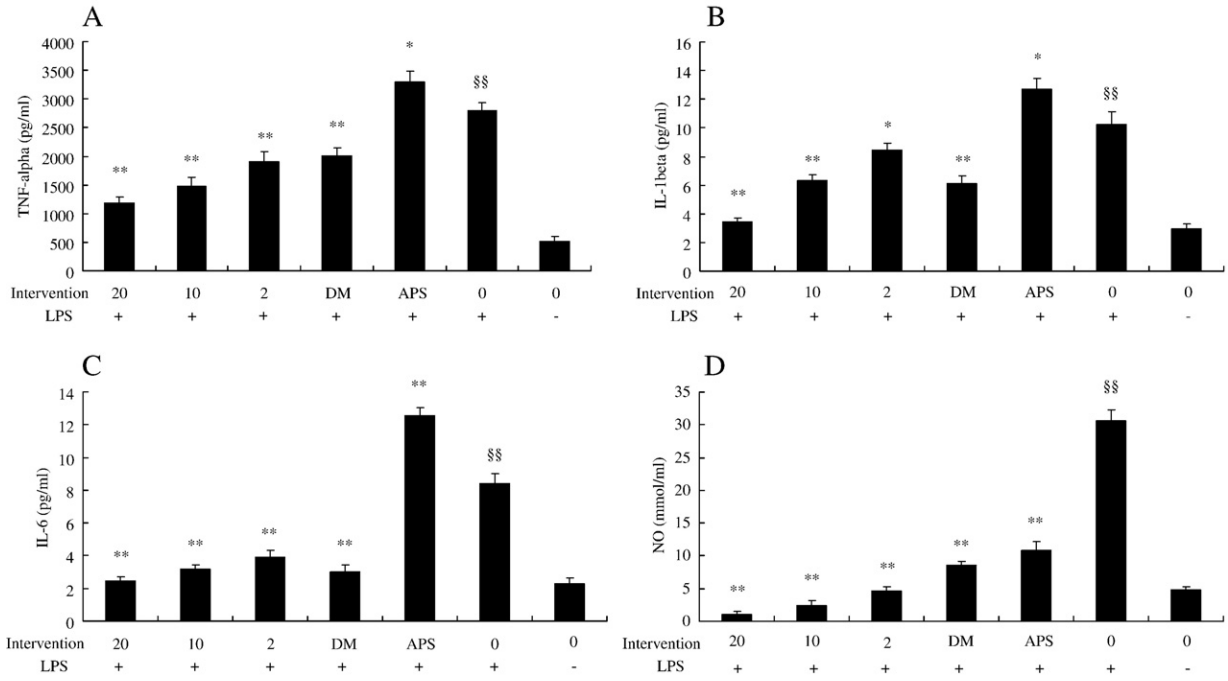

Figure 2 Effects of Corilagin on pro-inflammatory cytokine and mediator production. Data were shown as mean ± SD ($n=3$). *$p<0.05$ compared to LPS alone; **$p<0.01$ compared to LPS alone; §§$p<0.01$ compared to normal cell. A: effect on TNF-α production; B: effect on IL-1β production; C: effect on IL-6 production; D: effect on NO production.

3.2. Inflammatory model establishment and procedure monitoring

As shown from figures on pro-inflammatory cytokines and mediators, the levels of cells stimulated by LPS were significantly higher than those of normal cells ($p<0.01$), which implied the successful establishment on model of inflammation. Meanwhile, the levels of those factors from cells by Dexamethasone intervention were significantly lower than those from single LPS stimulation ($p<0.01$). The levels of those factors from cells by APS intervention, an immunomodelator for enhancing immune response on TNF-α, IL-1β and IL-6, were significantly higher than those from single LPS stimulation ($p<0.05$ or 0.01). The effects of Dexamethasone and APS intervention demonstrated experimental procedure was proper.

3.3. Effect of Corilagin on pro-inflammatory cytokines stimulated by LPS

As shown in Fig. 2A–C, after LPS stimulation with Corilagin intervention for 24 h, secretion of TNF-α, IL-1β and IL-6 were significantly decreased than that in single LPS stimulation ($p<0.01$). Furthermore, when the dosage of Corilagin increased, the effects of antagonizing pro-inflammatory cytokines were significantly elevated ($p<0.05$ or 0.01), which showed dose-dependent relation between anti-inflammatory effect and Corilagin.

3.4. Effect of Corilagin on protein of COX-2 stimulated by LPS

Corilagin displayed strikingly decreased level of COX-2 protein as shown in Fig. 3 by the method of western blot analysis ($P<0.01$). It is suggested that Corilagin could control pro-inflammatory mediator production at protein levels.

3.5. Effect of Corilagin on mRNA of pro-inflammatory mediators stimulated by LPS

Corilagin displayed strikingly decreased level of TNF-α and COX-2 mRNA as shown in Fig. 4A–B ($p<0.01$). It is suggested that Corilagin could control pro-inflammatory cytokine production at gene levels. Furthermore, when the dosage of Corilagin increased, the effects of antagonizing pro-inflammatory gene expression were significantly elevated ($p<0.05$ or 0.01), which showed dose-dependent relation between controlling effect on inflammatory gene and Corilagin.

3.6. Effect of Corilagin on NO and iNOS stimulated by LPS

As shown in Fig. 2D, after LPS stimulation with Corilagin intervention, secretion of nitric oxide (NO) was significantly decreased than that in single LPS stimulation ($p<0.01$). Furthermore, when the dosage of Corilagin increased, the effects

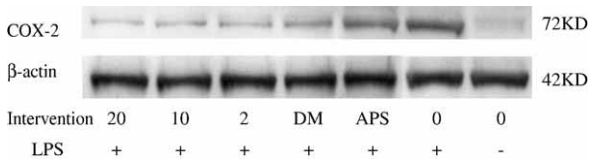

Figure 3 Effects of Corilagin on COX-2 protein expression assayed by western blot analysis.

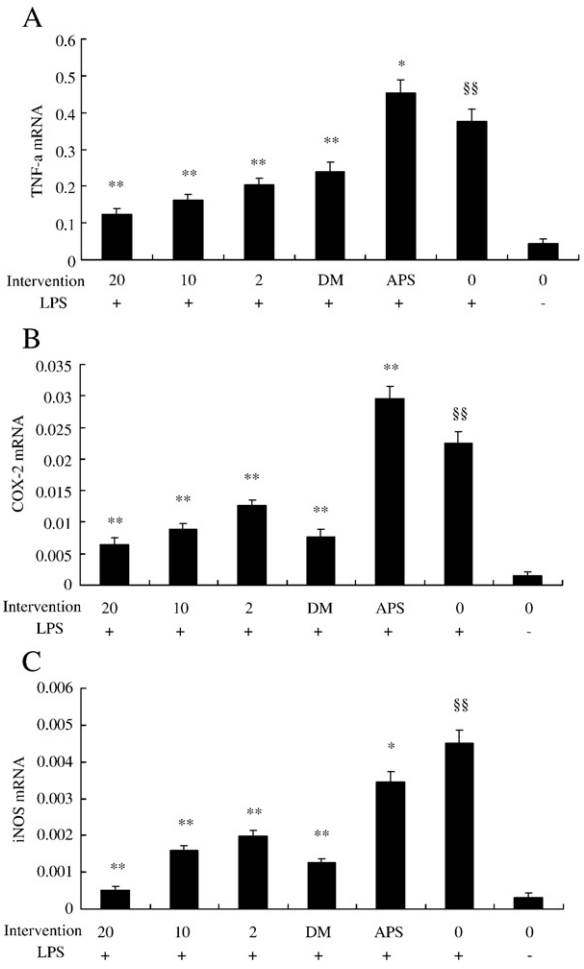

Figure 4 Effects of Corilagin on pro-inflammatory cytokine and mediator mRNA expression. Data were shown as mean ± SD ($n=3$). *$p<0.05$ compared to LPS alone; **$p<0.01$ compared to LPS alone; $^{§§}p<0.01$ compared to normal cell. A: effect on TNF-α mRNA expression; B: effect on COX-2 mRNA expression; C: effect on iNOS mRNA expression.

of antagonizing NO releasing significantly elevated ($p<0.05$), which showed dose-dependent relation on Corilagin. As shown from Fig. 4C, effect of Corilagin on mRNA expression of iNOS was coincidence with that on NO ($p<0.01$ or 0.05).

3.7. Effect of Corilagin on IL-10 stimulated by LPS

As shown in Fig. 5A, the level of IL-10 decreased the most under APS condition, which illustrated the effect of APS on reinforcing cellular immunity and inhibiting humoral immunity. Increase of IL-10 on single LPS stimulation displayed the regulatory action on cells after inflammatory reaction. The levels of IL-10 on Corilagin intervention were similar to that on Dexamethasone intervention.

3.8. Effect of Corilagin on HO-1 mRNA and protein expression stimulated by LPS

As shown in Figs. 5B and 6, the levels of HO-1 mRNA and protein on single LPS stimulated cells and Dexamethasone interfered cells were not different to the level on normal incubated cell.

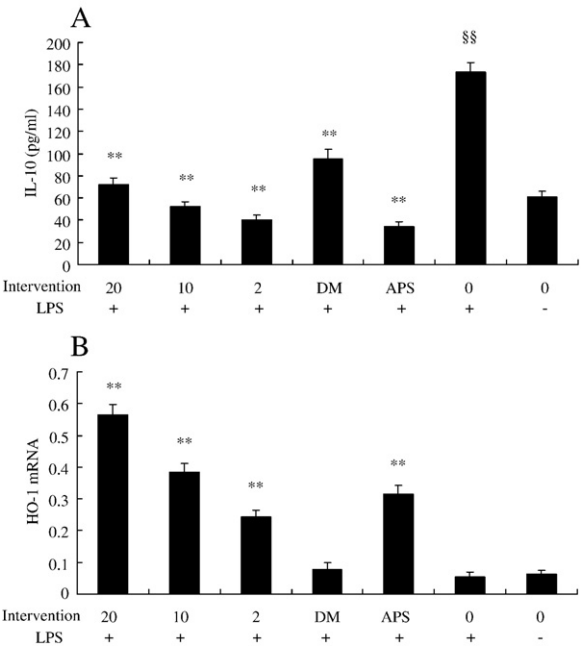

Figure 5 Effects of Corilagin on anti-inflammatory cytokine and mediator expression. Data were shown as mean ± SD ($n = 3$). **$p < 0.01$ compared to LPS alone; §§$p < 0.01$ compared to normal cell. A: effect on IL-10 production; B: effect on HO-1 mRNA expression.

The levels of HO-1 mRNA and protein on Corilagin and APS intervention were significantly higher than those on single LPS stimulation and normal incubation ($p < 0.01$), which suggested that Corilagin could promote regression of inflammatory. Furthermore, the higher the concentration of Corilagin was, the stronger the expression of HO-1 mRNA and protein ($p < 0.01$) was, which showed dose-dependent relation between promoting anti-inflammatory mediators effect and Corilagin.

3.9. Inhibition effect of Corilagin on NF-κB nuclear translocation

The results of immunocytochemistry in Fig. 7 showed NF-κB nuclear translocation was significantly blocked by Corilagin ($p < 0.01$). Those results suggested that suppression of IL-1β, TNF-α, iNOS, and COX-2 expression by Corilagin might be due to the attenuation on NF-κB activation.

4. Discussion

In inflammation progress, a series of cytokines and mediators contribute to evoking and regression of inflammation. Tumor

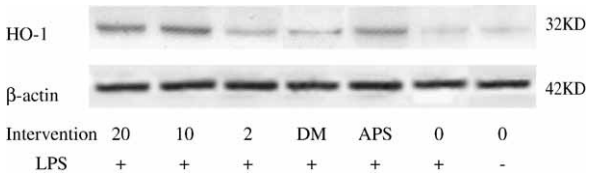

Figure 6 Effects of Corilagin on HO-1 protein expression assayed by western blot analysis.

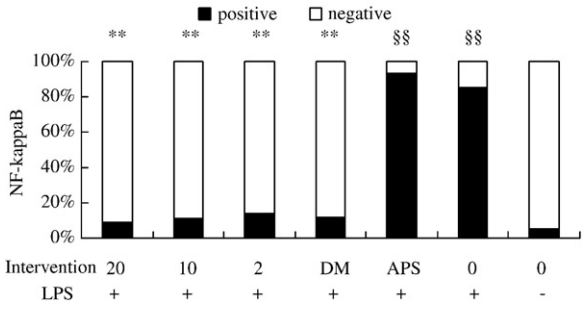

Figure 7 Effects of Corilagin on NF-κB translocation. Data were shown as percentage. **$p < 0.01$ compared to LPS alone; §§$p < 0.01$ compared to normal cell.

necrosis factor-alpha (TNF-α), interleukin-1 (IL-1) and IL-6 are three critical cytokines involved in inflammation and inhibition of them are regarded as a treatment strategy on inflammation-related diseases [12,13,14]. So we chose TNF-α (including its mRNA), IL-1β and IL-6 as a parameter to investigate the anti-inflammatory effect of Corilagin. In our research, Corilagin showed the significantly anti-inflammatory efficacy, which presents the potential of Corilagin to treat typical inflammation-related disorders.

Cyclooxygenease-2 (COX-2) is the central mediator of inflammation. Each step of cyclooxygenease-2 regulation can be used as potential therapeutic target [15]. Thereby, the results showed that Corilagin could suppress the COX-2 expression at both gene and protein levels, which showed evidence that Corilagin have activity to inhibit inflammation.

Nitric oxide (NO) is recognized as a mediator and regulator of inflammatory responses and is produced in high amounts by inducible nitric oxide synthase (iNOS) in activated inflammatory cells [16]. Cyclooxygenease-2 also can be affected directly at its enzymatic activity by nitric oxide and inducible nitric oxide synthase (iNOS) [15]. From the results Corilagin has a significant effect on the mediator. Furthermore, as nitric oxide can contribute to reperfusion injury when excessive amount produced during reperfusion (following a period of ischemia) reacts with superoxide to produce the damaging free radical peroxynitrite [17], it deserved an investigation whether Corilagin can relieve reperfusion injury after ischemia.

NF-kappaB is one of the most important mediators which play a key role in the regulated expression of a large number of pro-inflammatory mediators which may lead to organ destruction in some inflammatory and autoimmune diseases [18,19]. During the detection of anti-inflammation on Corilagin, we found NF-kappaB activity was potently inhibited by that medicinal plant ingredient, which implied the effect on intronuclear mechanism of inflammatory pathway.

IL-10 has attracted much attention because it is capable of inhibiting synthesis of pro-inflammatory cytokines [20,21]. In literature, Dexamethasone can inhibit the release of IL-10 at high dosage and promote the release at low dosage [22]. As the dosage of Dexamethasone in common cellular experiment is high dosage, in our research we chose the dosage of Dexamethasone as control and found Corilagin had a similar effect to the control. As Dexamethasone at high dosage can induce some immune cells into apoptosis, it

deserved further investigation whether Corilagin can perform anti-inflammation on some conditions by inducing apoptosis on some immune cells.

Heme oxygenase-1 (HO-1) is the inducible isoform of heme oxygenase [23] which is discovered as an important anti-inflammatory enzyme featured by its antioxidant activity [24]. Experimental models of acute inflammation have demonstrated that the induction of HO-1 can prevent or mitigate the symptoms associated with related ailments [25]. Inferred from result of our research, Corilagin has anti-inflammatory activity via anti-oxidation by inducing HO-1. Moreover, it can be presumed that the effect on HO-1 rendered Corilagin more activities on peroxidative and vasoconstrictive pathological status such as antiatherogenic and antihypertensive.

Although a few pharmacological and biochemical actions of Corilagin were identified in the past, the effect of Corilagin on pro-inflammatory cytokines and mediators, on blocking translocation of NF-kappaB, on anti-inflammatory mediators and on comparison to Dexamethasone is first explored. Additionally, from the anti-inflammatory mechanisms it could be deduced that there would be of more pharmacological efficacies of Corilagin that will be discovered in the future. When acute inflammatory and supercoagulative response occurs, when hepatitis and jaundice takes place, when viral encephalitis happens, a strong anti-inflammatory agent is just necessary. Based on our previous experience in plant medicine [26,27] and inflammation-related disorders [28,29], our next-step exploration is to disclose the effects of Corilagin on those above disorders and the molecular biological mechanisms.

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