Erythrinae Cortex inhibits Synthesis of Inflammatory Cytokines induced by IL-1β and TNF-α in Cultured Human Synovial Cells

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Our study shows that EC extract has inhibitory effect on pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, iNOS and COX2 in hFLSs. IL-1β, IL-6, iNOS and COX2 mRNA expression is suppressed at a low dasage (1μg/ml) of EC extract. TNF-α was also suppressed at higher dosages (10μg/ml, 100μg/ml). EC extract also inhibited TNF-α, IL-1β and IL-6 production in pro-inflammatory cytokine stimulated-hFLSs. Expecially IL-1β ($p<0.05$) production are suppressed significantly. On the other hand, EC extract did not show any cytotoxicity. Thses data suggest that EC extract has anti-inflammatory effect mostly by inhibiting IL-1β production, and thus could be used to prevent or treat some inflammatory disease such as RA. It remains to be known what are the major components responsible for anti-inflammatory effect and what is the main mechanism.

Key words : Erythrinae Cortex, human synovial cell, pro-inflammatory cytokines

Introdution

Erythrina variegata L. (Leguminosae) is found in many tropical and subtropical regions and is used medicinally as an antibacterial, anti-inflammatory, antipyretic, and antiseptic agent and as a collyrium in China[47]. Phytochemical investigation of the non-alkaloidal secondary metabolites of the genus Erythrina revealed the presence of one cinnamylphenol[49] and several isoflavonoids[11,25,35], some of which exhibit antibacterial[49,48] and anti-inflammatory activities[25] and inhibit the Na^+/H^+ exchange system[35]. Erythrinae Cortex (EC) is the cortex of the Leguminosae plant, Erythrina variegata L. var. orientalis Merr. The thorny bark is gathered in the early summer, then dried in the sun. It taste bitter, pungent and neutral, and is used mainly to dispel wind and dampness and clear the meridians. Main symptoms used for are wind-damp obstruction syndrome manifested as rheumatic joint pain, spasm of the limbs and lower back and knee pain[33]. Rheumatoid arthritis(RA) is a chronic and systemic inflammatory disease characterized by the destructive changes of articular cartilage and bone in a chronic phase[10]. A particularly characteristic feature of RA is the predilection for the synovium to become locally invasive at the synovial interface with cartilage and bone. Although histologic analyses of periarticular trabecular bone have demonstrated that osteoclastic bone resorption is greatly stimulated in RA patients, the mechanism of the joint destruction in RA patients remains to be elucidated[50]. RA that has long been recognised but has only recently risen to prominence, because of an increased understanding of the underlying mechanisms, plays a role of the vasculature in these invasive and destructive processes[41,53]. Although the exact causes of RA remain unknown, immunological dysregulation by inflammatory cytokines has been shown to be involved in its development[17]. The synovial reaction in RA patients is characterized by the abundance of many cytokines, chemokines and growth factors. But some factors are more important, including tumor necrosis factor α(TNF-α), interleukin-1(IL-1)[52], Interleukin-6 (IL-6)[26], inducible nitric oxide synthesis(iNOS)[27] and cyclooxygenase (COX), expecially COX2[40]. The synthesis Erythrina alkaloids has attracted considerable synthetic attention in recent years. This can be attributed to the wide range of biological properties that these compounds exhibit including emetic behaviour, inhibition of protein and DNA synthesis in certain cells; antineoplastic, antiviral, short-term hypotensive and insect antifeedant activity[5]. The in vivo antiplasmodial, analgesic and anti-inflammatory properties of Erythrina senegalensis, an ornamental plant commonly used in Northern Nigeria for the treatment of fevers, was evaluated. Aqueous extracts of the stem bark extract of E. senegalensis exhibited

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only slight antiplasmodial activity while significant ($P<0.05$) analgesic and anti-inflammatory effects were observed[32]. But until now, there are only few reports on anti-inflammatory activity of the E. cortex extract, which is expecially related with RA factors such as TNF-α, IL-1, IL-6, iNOS and COX2 in cultured human synovial cells.

In this study we examined whether E. cortex extract has inhibitory effect on IL-1β and TNF-α induced inflammatory cytokine mRAN expression and production in human fibroblast-like synoviocytes(hFLSs) or not.

Material and Methods

1. Drug

E. cortex(EC) was purchased from Heahwa medicinal material Co.(Daejeon. Korea) and identified as Rhizoma smilacis glabrae by Dr. Young-Bae Seo, Department of Herbology, Oriental college in Daejeon University.

The E. cortex extract was prepared by a common method. Briefly, the EC material was extracted 3 times with 100% MEOH at room temperature for 5 h. After filtering through Kimtex, the extracts were evaporated with Rotary evaporator(Büchi B-480, Switzerland), then lyophilized to obtain powder sample using Freeze dryer(EYELA FDU-540, Japan). This MEOH extract was used for all the present experiment.

2. Reagents

Drugs and reagents used in this study were as follows: Diethyl pyrocarbonate(DEPC), methotrexate(MTX), 3-4,5-dimethylthiazol-2, 5-carboxymethoxyphenyl-2,4-sulfophenyl-2H-tetrazolim(MTS), 2,7,-dichlorodihydrofluorescin diacettate (DCFH-DA), collagen typeⅣ, complete adjuvent, chloroform, RPMI-1640 culture fluid, isopropanol, RBC lysis solution, ethidium bromide(EtBr), Dulbecco's phosphate buffered saline(D-PBS), formaldehyde, lamide, magnesium chloride ($MgCl_2$) were obtained from Sigma(USA), Taq polymerase and Deoxynucleotide triphosphate(dNTP) are from TaKaRa(Japan), Moloey Murine Leukemia Virus Reverse Transcriptase(M-MLV RT) and RNase inhibitor are from Promega(Madison, USA), RNAzolB is from Tel-Test (USA), and fetal bovine serum(FBS) is from Hyclone(Logan, USA), Agarose is from FMC(USA), Propidium Iodide(PI) and RNase are from Pharmingen (Torreyana, USA), rhIL-1β(10U/ml) plus rhTNF-α(100 ng/ml) is from R&D system(Minneapolis, USA), 3H-thymidine is from Amersham(Buckinghamshire, UK).

3. Cytotoxicity measurement

hFLSs cells and mouse lung fibroblast cells(mLFC) are incubated at 37℃, 5% CO_2 incubator. Then cultured cells were washed with Trysin-EDTA to isolate cells. 2.0×10^4 cells were divided into 96 well plate and incubated at 37℃ for 2hours. After incubation, sample cells were treated for 48 hours with EC extract at dose of 200㎍/㎖, 100㎍/㎖, 50㎍/㎖, 10㎍/㎖, 1 ㎍/㎖. After this treatment, medium was removed and cells were washed twice with PBS. On each well, 50㎕ of 50% trichloroacetic acid(TCA) was added and again maintained at 4℃ for 1 hour. Then cells were washed with distilled water 5 times and cells were dried in air. SRB(0.4%/1% acetic acid) fluid 100㎕ per well was added and cells were stained at room temperature for 30 mins. Then cells were washed with 0.1% acetic acid fluid 4-5 times and air dried, then dissolved in 10mM Tris base 100㎕ per well. After dissolving, cells were were resuspended and cell density was measured by spectrophotometer at 540㎚ in ELISA LEADER(molecular devices, USA).

4. Reverse transcription-polymerase chain reation (RT- PCR)

1) RNA extraction : 1×10^6 hFLSs cells were plated to each well of 24 well plate, and treated with EC extract(100㎍/㎖, 10 ㎍/㎖, and 1㎍/㎖) for 1 hour. rhIL-1β(10U/ml) and rhTNF-α(100 ng/ml) were added to each well, then incubated for 6 hours. After centrifugation at 2000 rpm for 5 mins, supernatant was removed. 500㎕ RNAzolB was added and mixed to lyse precipitate, then 50㎕ of chloroform ($CHCl_3$) was added and mixed for 15 secs using voltex. After location at ice box for 15 mins, it was centrifuged at 13,000 rpm and 200㎕ supernatant was obtained. After adding 200㎕ of 2-propanol slowly, it was located at ice box for 15 mins, centrifuged at 3,000 rpm, washed with 80% EtOH, and dried using vaccum pump for 3 mins to extract RNA. Obtained RNA was mixed with 20㎕ of diethyl pyrocarbonate (DEPC).

2) RT-PCR : Reverse transcription reaction was measured as follows. After denaturation of 3㎍ of total RNA at 75℃ for 5 mins, 2.5㎕ of 10mM dNTPs mixture, 1㎕ of random sequence hexanucleotides(25pmole/25㎕), 1㎕ of RNase inhibitor(20U/㎕), 1㎕ of 100 mM DTT, 4.5㎕ of 5×RT buffer(250mM Tris-HCl, pH 8.3, 375mM KCl, 15 mM $MgCl_2$) were added. Then 1㎕ of M-MLV RT(200U/㎕) was added again and DEPC water was added to total volume 20㎕. This 20㎕ was resuspended and used for synthesis of first-strand cDNA. After incubation at 95℃ for 5 mins to inactivate M-MLV RT, synthesized cDNA was used for polymerase chain reaction (PCR).

3) cDNA PCR : PCR is performed using Primus 96 Legal PCR system(with high pressure lid, MWG in germany). 3㎕ of

synthesized cDNA was used as template, β-actin, interleukin-1 β(IL-1β), IL-6, IL-8, tumor necrosis factor-α(TNF-α), cyclo-oxygenase-2 were as primer, and 1$\mu\ell$ of sense primer(20 pmole/$\mu\ell$), antisense primer(20 pmole/$\mu\ell$) was added to amplify NOS-II gene, then 3$\mu\ell$ of 2.5 mM dNTPs, 3$\mu\ell$ of 10× PCR buffer(100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), and 0.18$\mu\ell$ of Taq polymerase (5U/$\mu\ell$) was added, and finally sterilized distilled water was added to final volume 30 $\mu\ell$. Then the cycle of pre-denaturation at 95℃ for 5 mins, denaturation at 95℃, annealing at 55℃ for 1 min, elongation at 72℃ for 1 min was repeated 25 times. For PCR, post-elongation was accomplished at 72℃ for 3 mins. And 20$\mu\ell$ of each products was loaded in 1.2% agarose gel, 120 V, for 20 mins for electrophoresis.

4) Sequence of Oligonucleotide : IL-1β, sence oligonucleotide, 5'-CCTCTTCTTGAGCTTGCAAC-3'; antisence oligonucleo -tide, 5'-AGCCCATGAGTTCCATTCAC-3'. IL-6, sence oligo-nucleotide, 5'-ATGAACTCCTTCTCCACAAGCGC-3'; antisence oligonucleotide 5'-GAAGAGCCCTCAGGCTGGACTG-3'. IL-17, sence oligonucleotide, 5'-TGGAGGCCATA GTGAAGG-3'; antisence oligonucleotide, 5'-GGCCACATGGT GGACAAT-3'. TNF-α, sence oligonucleotide, 5'-AGCGGCTGA CTGAACTCAGATTGT-3'; antisence oligonucleotide, 5'-GT CACAGTTTTCAGCTGTATAGGG -3'. COX-2, sence oligonucl eotide, 5'-TTCAAATgAgATTgTggg AAAAT-3'; antisence oligo-nucleotide, 5'-AgATCATCTCTgCC TgAgTATCTT -3'. MMP-3, sence oligonucleotide, 5'-TGACT CCACTCACATTCTCCAGGC-3'; antisence oligonucleotide, 5'-GGTCTGTGAGTGAGTGATAGAGTGG-3'. MMP-13, sence oligonucleotide, 5'-GTCCGATGTAACTCC TCTGA-3'; antisence oligonucleotide, 5'-TAGAGAGACTGGAT CCCTTG-3'. iNOS, sence oligonucleotide, 5'-CggAggATTgCTCA ACAAC-3'; antisence oligonucleotide, 5'-CggAggATTgCTCA ACAAC-3'. β-actin, sence oligonu-cleotide, 5'-TggAATCCT gTggTCCATgAAAC-3' ; antisence oligonucle-otide, 5'-TAAAACg CAgCTCAgTAACAg TCCg-3'. Height is measured for PCR product using Windows 1D main program (AAB, USA).

5) Measurement of cytokine using ELISA

3 days before, hFLSs cell is subcultured (1x10^5cells/$m\ell$) and 2x10^6 cells were plated on a each well of 12 well plate, then overnight with fetal bovine serum(FBS) deficit RPMI1640 medium fluid. After 1 hour of treatment with EC extract (100 $\mu g/m\ell$, 10$\mu g/m\ell$, 1 $\mu g/m\ell$), rhIL-1β(10U/ml) and rhTNF-α (100ng/ml) was added at each well. Six 6 hours later, each well was washed with RPMI-1640 medium fluid and again was treated with new medium fluid and EC extract, then incubated in CO_2 incubator for 48 hours. After incubation, total medium fluid was centrifuged at 2000 rpm for 5 mins, and supernatant was obtained. To measure cytokine, IL-1β kit (Endogen, USA), IL-6 enzyme-linked immuno-sorbent assay (ELISA) was used and ELISA kit (R&D system) was used for TNF-α.

Result

1. Cytotoxicity effects of EC extract.

MEOH extract of EC did not show any cytotoxic effect on mouse lung fibroblast cells and human fibroblast-like synoviocytes cells at any doses studied(1, 10, 50, 100, 200 μg/ml). We used some of these dosages(1, 10, 100 μg/ml) that did not show any cytotoxicity in all test (Fig. 1).

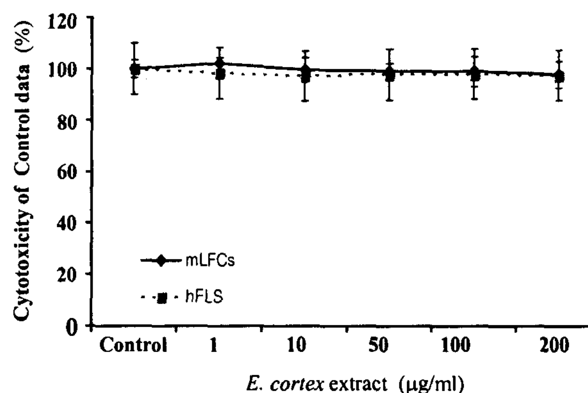

Fig. 1. Cytotoxicity effects of Erythrinae cortex extract on mouse mLFCs and hFLSs. Mouse lung fibroblast cells(mLFCs) and human fibroblast-like synoviocytes(hFLSs) were pretreated with various concentration E. cortex extract. The results are expressed the mear ±S.E(N=6). Statistically significant value compared with control group data by T test(*p<0.05, **p<0.01, ***p<0.001).

Table 1. Cytotoxicity effects of Erythrinae cortex extract on mouse lung fibroblast cells(mLFCs) and human fibroblast-like synoviocytes (hFLSs)

Drug	Dose (μg/ml)	% of Control Data	
		mLFC	hFLS
Control	0	100 ± 3.6	100 ± 4.6
	1	102.1 ± 2.4	98.4 ± 4.3
	10	99.6 ± 4.9	97.4 ± 3.8
E. cortex	50	99.2 ± 3.1	97.9 ± 4.7
	100	99.0 ± 5.8	98.1 ± 3.5
	200	97.8 ± 5.3	97.5 ± 3.2

Mouse lung fibroblast cells(mLFCs) and human fibroblast-like synoviocytes(hFLSs) were pretreated with various concentration E. cortex extract. The results are expressed the mear ±S.E(N=6). Statistically significant value compared with control group data by T test(*p<0.05, **p<0.01, ***p<0.001).

2. Inhibitory effects of EC extract on pro-inflammatory cytokine TNF-α mRNA expression.

EC extract inhibited TNF-α mRNA expression by 66.8% at 100μg/ml, 42.3% at 10μg/ml, 28.6% at 1mg/ml (Fig. 2, Table 2).

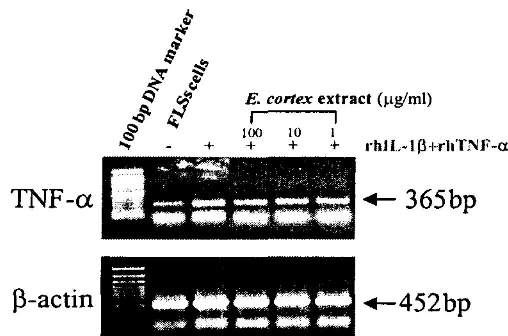

Fig. 2. Inhibitory effects of Eythrinae cortex extract on pro-inflammatory cytokine TNF-α mRNA expression in human fibroblast - like synoviocytes. Human fibro blast like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL -1β (10 U/ml) and TNF-α (100ng/ml) for 6h. Amplified TNF-α PCR products were electrophorsed on 1.2% agarose gel and internal control(β -actin) and the analysis(Ht) was used to 1D-density program and the other methods for assay performed as described in Materials and Methods.

Table 2. Inhibitory effects of Eythrinae cortex on pro-inflammatory cytokines mRNA expression in human fibroblast-like synoviocytes.

Drug	E. Cortex extract (μg/ml)	Cytokines m-RNA expression (Ht)	
		TNF-α	β-actin
Media control	0	43	241
hrIL-1β (10U/ml) plus hrTNF-α (100ng/ml)	0	196	248
	100	65	246
	10	113	245
	1	140	248

Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and TNF-a (100 ng/ml) for 6h. Amplified PCR products were electrophoresed on 1.2% agarose gel, and the analysis (Ht) was used to 1D-density program. The other methods for assay were performed as described in Materials and Methods. Pro-inflammatory cytokines were observed TNF-a mRNA expression in hFLSs.

3. Inhibitory effects of EC extract on pro-inflammatory cytokine IL-1β mRNA expression.

EC extract inhibited IL-1β mRNA expression by 90.1% at 100μg/ml, 82.5% at 10μg/ml, 50.5% at 1mg/ml (Fig. 3).

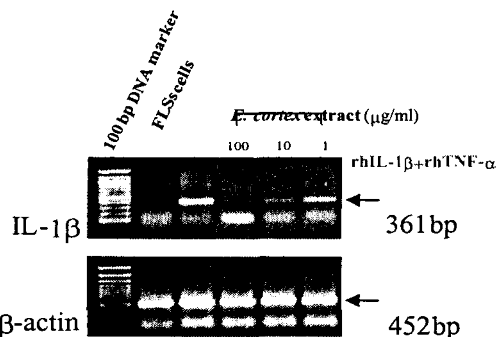

Fig. 3. Inhibitory effects of Eythrinae cortex extract on pro-inflammatory cytokine IL-1b mRNA expression in human fibroblast-like synoviocytes. Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and TNF-a (100 ng/ml) for 6h .Amplified IL-1b PCR products were electrophoresed on 1.2% agarose gel and internal control (b-actin) and the analysis (Ht) was used to 1D-density program and the other methods for assay were performed as described in Materials and Methods.

Table 3. Inhibitory effects of Eythrinae cortex on pro-inflammatory cytokines mRNA expression in human fibroblast-like synoviocytes.

Drug	E. Cortex extract (μg/ml)	Cytokines m-RNA expression (Ht)	
		IL-1β	β-actin
Media control	0	22	241
hrIL-1β (10U/ml) plus hrTNF-α (100 ng/ml)	0	212	248
	100	21	246
	10	37	245
	1	105	248

Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and TNF-a (100 ng/ml) for 6h. Amplified PCR products were electrophoresed on 1.2% agarose gel, and the analysis (Ht) was used to 1D-density program. The other methods for assay were performed as described in Materials and Methods. Pro-inflammatory cytokines were observed IL-1b mRNA expression in hFLSs.

4. Inhibitory effects of EC extract on pro-inflammatory cytokine IL-6 mRNA expression.

EC extract inhibited IL-6 mRNA expression by 81.6% at 100mg/ml, 80.4% at 10mg/ml, 67.6% at 1mg/ml (Fig 4 & Table 4).

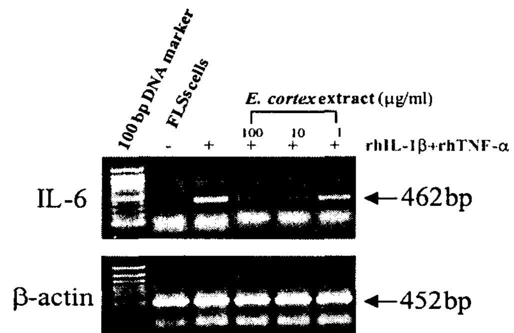

Fig. 4. Inhibitory effects of Eythrinae cortex extract on pro-inflammatory cytokine IL-6 mRNA expression in human fibroblast-like synoviocytes. Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and TNF-a (100 ng/ml) for 6h . Amplified IL-6 PCR products were electrophoresed on 1.2% agarose gel and internal control (b-actin) and the analysis (Ht) was used to 1D-density program and the other methods for assay were performed as described in Materials and Methods.

Table 4. Inhibitory effects of Eythrinae cortex on pro-inflammatory cytokines mRNA expression in human fibroblast-like synoviocytes.

Drug	E. Cortex extract (μg/ml)	Cytokines m-RNA expression (Ht)	
		IL-6	β-actin
Media control	0	31	241
hrIL-1β (10U/ml) plus hrTNF-α (100 ng/ml)	0	179	248
	100	33	246
	10	35	245
	1	58	248

Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and TNF-a (100 ng/ml) for 6h. Amplified PCR products were electrophoresed on 1.2% agarose gel, and the analysis (Ht) was used to 1D-density program. The other methods for assay were performed as described in Materials and Methods. Pro-inflammatory cytokines were observed IL-6 mRNA expression in hFLSs.

5. Inhibitory effects of EC extract on pro-inflammatory cytokine iNOS mRNA expression.

EC extract has inhibitory effect on iNOS mRNA expression by 91.7% at 100mg/ml, 72.8% at 10mg/ml and 23.8% at 1mg/ml (Fig 5.).

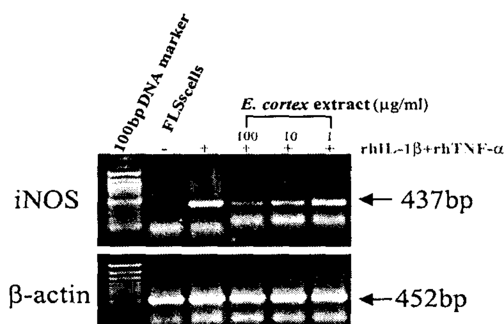

Fig. 5. **Inhibitory effects of Eythrinae cortex extract on pro-inflammatory cytokine iNOS mRNA expression in human fibroblast-like synoviocytes.** Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and TNF-a (100 ng/ml) for 6h . Amplified iNOS PCR products were electrophoresed on 1.2% agarose gel and internal control (b-actin) and the analysis (Ht) was used to 1D-density program and the other methods for assay were performed as described in Materials and Methods.

Table 5. Inhibitory effects of Eythrinae cortex on iNOS mRNA expression in human fibroblast-like synoviocytes

Drug	E. cortex extract (μg/ml)	Cytokines m-RNA expression (Ht)		
		iNOS	COX-2	β-actin
Media control	0	29	35	241
hrIL-1β (10U/ml) plus hrTNF-α (100 ng/ml)	0	202	223	248
	100	37	34	246
	10	55	61	245
	1	154	166	248

Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and TNF-a (100 ng/ml) for 6h. Amplified PCR products were electrophoresed on 1.2% agarose gel, and the analysis (Ht) was used to 1D-density program. The other methods for assay were performed as described in Materials and Methods. Inflammatory cytokines were observed iNOS mRNA expression in hFLSs.

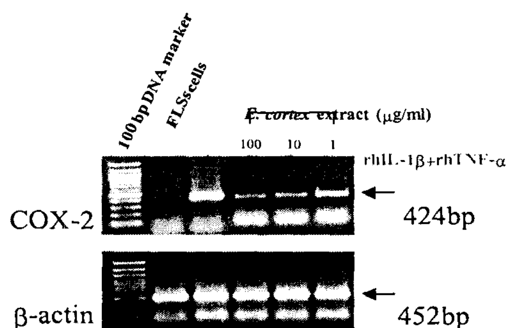

Fig. 6. **Inhibitory effects of Eythrinae cortex extract on pro-inflammatory cytokine cyclooxygenase-2(COX-2) mRNA expression in human fibroblast-like synoviocytes.** Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and TNF-a (100 ng/ml) for 6h. Amplified COX-2 PCR products were electrophoresed on 1.2% agarose gel and internal control (b-actin) and the analysis (Ht) was used to 1D-density program and the other methods for assay were performed as described in Materials and Methods.

6. Inhibitory effects of EC extract on pro-inflammatory cytokine COX-2 mRNA expression.

EC extract has an inhibitory effect on COX-2 mRNA expression by 84.8% at 100mg/ml, 72.6% at 10mg/ml, 25.6% at 1mg/ml (Fig 6.).

Table 6. Inhibitory effects of Eythrinae cortex on COX-2 mRNA expression in human fibroblast-like synoviocytes

Drug	E. cortex extract (μg/ml)	Cytokines m-RNA expression (Ht)		
		iNOS	COX-2	β-actin
Media control	0	29	35	241
hrIL-1β (10U/ml) plus hrTNF-α (100 ng/ml)	0	202	223	248
	100	37	34	246
	10	55	61	245
	1	154	166	248

Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and TNF-a (100 ng/ml) for 6h. Amplified PCR products were electrophoresed on 1.2% agarose gel, and the analysis (Ht) was used to 1D-density program. The other methods for assay were performed as described in Materials and Methods. Inflammatory cytokines were observed COX-2 mRNA expression in hFLSs.

7. Inhibitory effect of EC extract on the TNF-α production in pro-inflammatory cytokine stimulated-hFLSs.

EC extract inhibits TNF-α production at dose of 100μg/ml in rhIL-1β and rhTNF-α stimulated-hFLSs but does not inhibit significantly (Fig. 7).

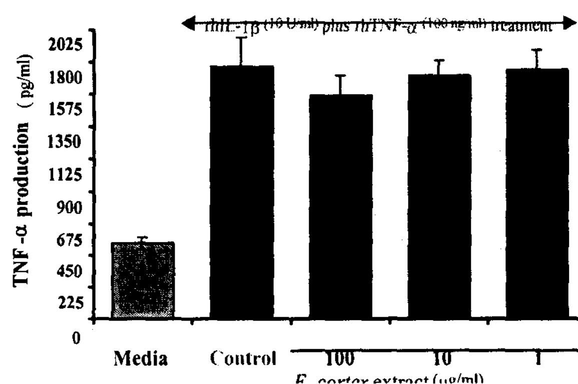

Fig. 7. **Inhibitory effect of E. cortex extract on the TNF-a production in pro-inflammatory cytokine stimulated-hFLSs.** Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and rhTNF-a (100 ng/ml) for 6h. After 6h, RPMI1640-free washed two repeat and fresh RPMI 1640 media supplemented with 1%FBS. The supernatants were collected after 72 h and TNF-a concentration in the culture supernatants was assay by ELISA kit. and the other methods for assay were performed as described in Materials and Methods. RPMI-1640 media control was not treated. rIL-1b + rhTNF-a was control. The results are expressed the mean ± S.E (N=6). Statistically significant value compared with control group data by T test (*p<0.05, **p<0.01, ***p<0.001).

8. Inhibitory effect of EC extract on the IL-1β production in pro-inflammatory cytokine stimulated-hFLSs.

EC extract inhibits IL-1β production significantly($p<0.05$) at dose of 100mg/ml in rhIL-1β and rhTNF-α stimulated-hFLSs (Fig 8).

Table 7. Inhibitory effect of Eythrinae cortex on the TNF-a production in pro-inflammatory cytokine stimulated-hFLSs.

Treatment group		Dose	TNF-α Production (pg/ml)
RPMI1640-Media		0	530 ± 43.1
rIL-1β plus rhTNF-α	Control	0	1768 ± 203.5
	E. cortex extract (mg/ml)	100	1572 ± 137.3
		10	1701 ± 114.2
		1	1745 ± 139.0

Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and rhTNF-a (100 ng/ml) for 6h. After 6h, RPMI1640-free washed two repeat and fresh RPMI 1640 media supplemented with 1%FBS. The supernatants were collected after 72 h and TNF-a concentration in the culture supernatants was assay by ELISA kit. and the other methods for assay were performed as described in Materials and Methods. RPMI-1640 media control was not treated, rIL-1b + rhTNF-a was control. The results are expressed the mean ± S.E (N=6). Statistically significant value compared with control group data by T test (*p<0.05, **p<0.01, ***p<0.001).

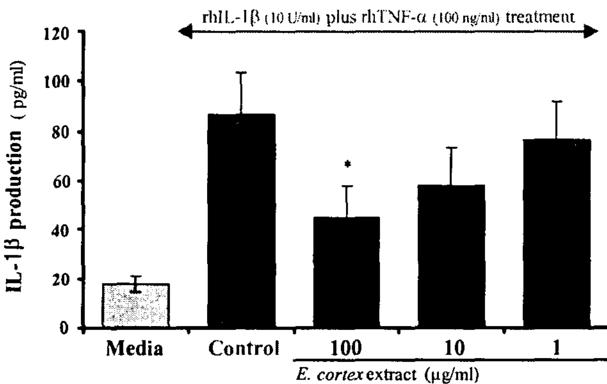

Fig. 8. Inhibitory effect of E. cortex on the IL-1b production in pro-inflammatory cytokine stimulated-hFLSs. Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and rhTNF-a (100 ng/ml) for 6h. After 6h, RPMI1640-free washed two repeat and fresh RPMI 1640 media supplemented with 1%FBS. The supernatants were collected after 72 h and IL-1b concentration in the culture supernatants was assay by ELISA kit. and the other methods for assay were performed as described in Materials and Methods. RPMI-1640 media control was not treated, rIL-1b + rhTNF-a was control. The results are expressed the mean ± S.E (N=6). Statistically significant value compared with control group data by T test (*p<0.05, **p<0.01, ***p<0.001).

9. Inhibitory effect of EC extract on the IL-6 production in pro-inflammatory cytokine stimulated-hFLSs.

EC extract inhibited IL-6 production at dose of 100μg/ml in rhIL-1β and rhTNF-α stimulated-hFLSs but does not inhibit significantly (Fig 9).

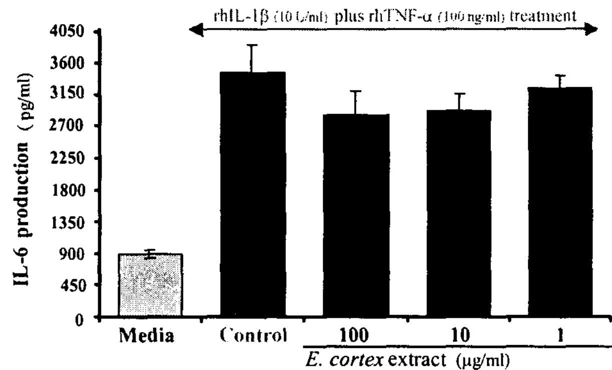

Fig. 9. Inhibitory effect of E. cortex extract on the IL-6 production in pro-inflammatory cytokine stimulated-hFLSs. Human fibroblast-like synoviocytes were pretreated with various concentration E. cortex extract in the presence or absence rIL-1b (10 U/ml) and rhTNF-a (100 ng/ml) for 6h. After 6h, RPMI1640-free washed two repeat and fresh RPMI 1640 media supplemented with 1%FBS. The supernatants were collected after 72 h and IL-6 concentration in the culture supernatants was assay by ELISA kit. and the other methods for assay were performed as described in Materials and Methods. RPMI-1640 media control was not treated, rIL-1b + rhTNF-a was control. The results are expressed the mean ± S.E (N=6). Statistically significant value compared with control group data by T test (*p<0.05, **p<0.01, ***p<0.001).

Discussion

RA is a chronic inflammatory disease eventually leading to joint destruction. Also RA is a chronic and destructive disease, which typically affects the peripheral joints but may affect any synovial joint in the body. The synovium in RA becomes inflamed and increases greatly in mass, because of hyperplasia of the lining cells. Inflammatory synovitis in RA involves the migration of leukocytes into the synovial tissue. These leukocytes and other cells in the synovial tissue produce various inflammatory mediators including many kind of cytokines[12,13].

The mechanism of the joint destruction in RA patients remains to be elucidated. Although the cause of RA is unknown, advances in molecular technology have facilitated the identification of novel therapeutic targets, including cell-subsets and cytokines, which contribute to the inflammatory and destructive components of the disease[42]. The synovial reaction in RA patients is characterized by the abundance of many cytokines, chemokines and growth factors. But some factors such as TNF-α, IL-1, IL-6, iNOS and COX2 are more important[60].

It has been reported that isolated component from EC has anti-inflammatory activity[25], which suggests that the extract from EC may be effective on preventing or treating RA. In this study we tested whether EC MEOH extract has any inhibitory effect on the expression and production of inflammatory cytokine (TNF-α, IL-1, IL-6, iNOS and COX2) in both IL-1β and TNF-α treated hFLS cells.

First, MEOH extract of EC did not show any cytotoxic effect on mouse lung fibroblast cells and human fibroblast-like synoviocytes cells at any doses studied ; 1, 10, 50, 100 and 200 μg/ml. We used these dosages that did not show any cytotoxicity in all test. These results suggest that inhibitory effect on inflammatory cytokine mRAN expression and production is not due to cytotoxicity. (Fig. 1. & Table 1.)

It is now well accepted that the spontaneous production of proinflammatory cytokines, in particular, TNF and IL-1 produced locally in the inflamed synovial joint contribute directly/indirectly to the pathogenesis of RA[16)]. TNF is a multifunctional cytokine that is secreted by monocytes (TNF-α) or lymphocytes (TNF-β)[7)]. TNF has potent proinflammatory effects, and is implicated in many inflammatory and autoimmune diseases, such as rheumatoid arthritis[14,38)]. IL-1 is also important pro-inflammatory cytokine abundantly expressed in RA synovium. It stimulates resorption of cartilage and bone through activation of osteoclasts and inhibits synthesis of proteoglycan and articular collagen[3,15,19)].

It is now generally accepted that TNF and IL-1 are the master cytokines in the process of chronic joint inflammation and the concomitant erosive changes in cartilage and bone[28)]. We tested whether EC extract has Inhibitory effects on TNF-α and IL-1β mRNA expression and TNF-α and IL-1β production in rhIL-1β and rhTNF-α stimulated-hFLSs. Results show that EC extract inhibited TNF-α mRNA expression by 66.8% at 100μg/ml, 42.3% at 10μg/ml, 28.6% at 1mg/ml (Fig. 2 & Table 2), and IL-1β by 90.1% at 100μg/ml, 82.5% at 10μg/ml, 50.5% at 1mg/ml (Fig. 3 & Table 3).

Also, TNF-α and IL-1β production are similarly inhibited in rhIL-1β and rhTNF-α stimulated-hFLSs ; EC extract inhibits IL-1β production significantly($p<0.05$) at 100mg/ml (Fig. 8 & Table 8) but does not inhibit TNF-α production significantly (Fig. 7 & Table 7). These results indicate that EC extract is more effective in inhibiting IL-1β than TNF-α.

As shown above, in our study EC extract inhibited IL-1β expression by 90.1% at 100μg/ml and by 50.5% even at 1mg/ml. This suggest that EC extract could prevent or treat RA condition mainly by inhibiting IL-1β expression or production. Importantly, synergy between IL-1 and TNF has been demonstrated[9)]. IL-1 is much more potent than TNF in inducing cartilage destruction in vivo. Tiny amounts of IL-1 are sufficient to cause proteoglycan synthesis inhibition in chrondrocytes[1)].

Considering our results, EC extract could mainly affect on RA condition, by inhibiting IL-1 production and expression via blockade of the synergistic interaction between TNF-α and IL-1β.

IL-6 is a multifunctional cytokine, which is produced during the inflammatory response. IL-6 has major regulating effects upon the inflammatory response[21,54)]. IL-6 is a pleiotropic cytokine with a wide range of biological activities in immune regulation, hematopoiesis, inflammation, and oncogenesis.

It is considered that IL-6 is one of cytokines which is fundamentally related with RA. Whatever the mechanism is, IL-6 is one of the cytokines which concerned with RA. IL-6 regulates the production of acute phase proteins by hepatocytes, and activates bone absorption by osteoclasts[42)]. And uncontrolled IL-6 overproduction appears to be responsible for the clinical symptoms and abnormal laboratory findings in RA[52)]. In fact, a large amount of IL-6 has been observed in both sera and synovial fluids from the affected joints of patients with RA[26)].

In our study, we found that EC extract inhibited IL-6 mRNA expression by 81.6% at 100mg/ml, 80.4% at 10mg/ml, 67.6% at 1mg/ml (Fig 4. & Table 4) but did not inhibit production in rhIL-1β and rhTNF-α stimulated-hFLSs significantly (Fig 9. & Table 9). In production test, EC extract shows more effective on IL-1 production($p<0.05$) than TNF-α and IL-6 (no significant). And in inhibitory effect on mRNA expression, EC extract most effective on IL-1 expression by 82.5% and IL-6 by 80.4% but TNF-α by 42.3% at dose of 10mg/ml.

It was reported that IL-1β stimulates muscle IL-6 production at least in part by activating the MAP kinase pathway and NF-kappaB[36)]. Even though there are no reports that same mechanism was discovered at human RA synovium, the possibility that IL-1β also stimulate synovial IL-6 production could be existed. So, such hypothesis that EC extract affect RA condition by inhibiting IL-1 production and eventually showing double effects on IL-6 reduction and weakening the synergistic role between IL and TNF-α.

NO plays important roles in the normal physiology and pathophysiology of many organ systems[44)]. NO first identified as an endothelium-derived relaxation factor, is now recognized to regulate the functions of many mammalian cells and tissues[39)]. NO is a gaseous molecule enzymically generated from L-arginine by a family of NO synthases[20)]. The overexpression of iNOS in a variety of inflammatory tissues had led many to conclude that the modulation of NO synthesis and action could represent a new approach to treatment of inflammatory and autoimmune diseases[30)], including RA[24)].

NO is known to play critical roles in cell signaling by way of soluble guanylate cyclase and cyclic guanosine

monophosphate formation, cell-mediated immune responses, cytotoxicity, and cytostasis[51]. Inflammatory cytokines such as IL-1, interferon-gamma, TNF-α induces iNOS expression in various cells including macrophages[37].

Evidence exists that NO is an initiator of apoptotic signals, owing to its reaction with superoxide to form peroxynitrite, and thereby inducing oxidative stress-induced apoptosis[8,31]. And studies suggest that it may play a role in apoptosis of chondrocytes and synovial cells[2,43]. Endothelial cells, synovial lining cells and interstitial cells expressed both eNOS and iNOS with high frequency in RA synovium compared with OA synovium. It seemed to be correlated with NO production. These results suggest that expression of iNOS may be involved in the induction of arthritis and eNOS may be participated in augmentation of inflammation in RA[27].

Our result shows that EC extract has inhibitory effect on iNOS mRNA expression by 81.7% at 100mg/ml, 72.8% at 10mg/ml and 23.8% at 1mg/ml (Fig.5 & Table. 5). IL-1 and TNF-α induce iNOS expression, and as showed above, EC extract inhibits IL-1β production and inhibits mRAN expression. These results could be connected with the inhibition effect of EC extract on iNOS mRNA expression.

It is now accepted that NOS inhibitors could be of therapeutic value for the prevention and treatment of RA[6]. Recent studies also showed that the selective inhibition of iNOS attenuates the direct endothelial damage induced by IL-1β in vitro[46]. This suggests the possibility that EC extract could attenuate damage induced by IL-1β through inhibiting of IL-1β production directly and of NO production indirectly.

COX are a group of proinflammatory enzymes that release prostaglandins including prostaglandin E2, and act in prostaglandin G/H synthase. COX is known to exist as two isoforms: COX-1 and COX-2. COX-1 expression is ubiquitous and its activity predominates during normal physiologic conditions. COX-2 expression is restricted to a few specialized loci during basal conditions.

In response to factors such as inflammation and synaptic activity, COX-2 expression is markedly enhanced by mediators, including growth factors, cytokines, and mitogens[18]. Generally, COX-1 is expressed constitutively in most tissues and is involved primarily in cellular homeostasis, whereas COX-2 is highly inducible and plays a major role in inflammation[56,23]. Recent evidence suggests that COX-2 is a mediator of angiogenesis, and COX-2 activity is known to be upregulated in the RA synovium[29]. Studies in animal models provide optimism about the use of angiogenesis inhibitors for RA[34,56]. In short, angiogenesis appears to be a key event in the initiation and persistence of rheumatoid disease and can be strategically inhibited to reduce inflammation in animal models of RA. Joints from RA patients exhibited abundant COX expression, to higher levels than found in synovial tissue from osteoarthritis patients or nonarthritic synovial tissue[45]. Later studies with human and animal tissues used specific COX antibodies and demonstrated COX-2 immunostaining of synovial blood vessels, synovial lining cells, chondrocytes, lymphoid aggregates, and subsynovial fibroblast-like cells[29].

The increased expression of iNOS and COX-2 in OA chondrocytes is largely due to the increased expression of pro-inflammatory cytokines, particularly IL-1, which act in an autocrine/paracrine fashion to perpetuate a catabolic state that leads to progressive destruction of articular cartilage[4]. Both NOS and COX are involved in the inflammatory cascade of arthritis.

In this study we found that EC extract has an inhibitory effect on COX-2 mRNA expression by 84.8% at 100mg/ml, 72.6% at 10mg/ml, 25.6% at 1mg/ml (Fig.6 & Table.6). As mentioned above, EC extract mostly inhibits mRNA expression and production of IL-1β. In cultured bovine chondrocytes and explants of human osteoarthritic cartilage, both NOS and COX activities were induced by the inflammatory mediators, lipopolysaccharide, and IL-1β or TNF-α[22]. The present data that EC extract inhibits mRAN expression of COX2 are connected with the fact that NOS and COX activities are induced by IL-1β or TNF-α and NO may modulate COX activity.

Considering the result that EC extract most effective on inhibiting IL-1 expression, EC extract might be effective on RA treatment by attenuating the connection between IL-1 and iNOS, IL-1 and COX-2. Also, EC extract could act as an inhibitor of mediation of angiogenesis by preventing upregulation of COX-2 in the RA synovium.

However, it remains to be known what are the major chemical components having such an anti-inflammatory effect among those isolates mentioned above, and waht is the main mechanism. Presumably EC extract contains various chemical component, thus the inhibition effect on inflammatory cytokines (TNF-α, IL-1, IL-6, iNOS and COX2) might be caused not by one component but by synergistic interactions among various components containing chemical component discovered already. Further studies would be needed to reveal major components and main mechanism.

Reference

1. AAJ Van de Loo., WB van den Berg : Effects of murine recombinant IL-1 on synovial joints in mice : measurement of patellar cartilage metabolism and joint inflammation,

Ann Rheum Dis ; 49 : 238-245, 1990.
2. A Amin., S Abramson. : The role of nitric oxide in articular cartilage breakdown in osteoarthritis, Curr Opin Rheumatol ; 10 : 263-268, 1998.
3. A.A. van de Loo, O.J. Arntz, A.C. Bakker, P.L. van Lent, M.J. Jacobs and W.B. van den Berg : Role of interleukin-1 in antigen-induced exacerbations of murine arthritis, Am. J. Pathol. ; 146 : 239-249, 1995.
4. Abramson SB., Attur M., Amin AR, Clancy R. : Nitric oxide and inflammatory mediators in the perpetuation of osteoarthritis, Curr Rheumatol Rep ; 3 : 535-41, 2001.
5. Andrew F. Parsons and David A. J. Williams : Radical Cyclisation Reactions Leading to Polycyclics Related to the Amaryllidaceae and Erythrina Alkaloids, Tetrahedron ; 56(37) : 7217-7228, 2000.
6. Armour KE., Van'T Hof RJ., Grabowski PS., Reid DM., Ralston SH. : Evidence for a pathogenic role of nitric oxide in inflammation-induced osteoporosis, J Bone Miner Res ; 14 : 2137-42, 1999.
7. B. Beutler and A. Cerami : The biology of cachectin/TNF-A primary mediator of the host response, Annu Rev Immunol ; 7 : 625, 1989.
8. B. Brune, A.V. Knethen and K.B. Sandau : Nitric oxide and its role in apoptosis, Eur J Pharmacol ; 351 : 261-272, 1998.
9. B Henderson., ER Pettipher : Arthritogenic actions of recombinant IL-1 and TNF in the rabbit : evidence for synergistic interactions between cytokines in vivo, Clin Exp Immunol ; 75 : 306-310, 1989.
10. Bresnihan B. : Rheumatoid arthritis ; principles of early treatment, J. Rheumatol Suppl. ; 66 : 9-12, 2002.
11. Deshpande, V.H., Pendse, A.D. and Pendse, R. : Erythrinins A, B & C, three new isoflavones from the bark of Erythrina variegata, Indian Journal of Chemistry ; 15B : 205-207, 1977.
12. E.D. Harris : Rheumatoid arthritis: pathophysiology and implications for therapy, N. Engl. J. Med. ; 332 : 1277-1287, 1990.
13. Emery P. : Magnetic resonance imaging: opportunities for rheumatoid arthritis disease assessment and monitoring long-term treatment outcomes, Arthritis Res ; 4 : S6-S10, 2002.
14. Eun Bong Lee, Jeong Yeon Kim, Yun Jong Lee, Myoung Hee Park and Yeong Wook Song : TNF and TNF receptor polymorphisms in Korean Behcet&rsquos disease patients, Human Immunology ; 64(6) : 614-620, 2003.
15. F.A.J. van de Loo, L.A.B. Joosten, P.L. van Lent, O.J. Arntz and W.B. van den Berg : Role of interleukin-1 tumor necrosis factor , and interleukin-6 in cartilage proteoglycan metabolism and destruction: effect of in situ blocking in murine antigen- and zymosan-induced arthritis, Arthritis Rheum ; 38 : 164-172, 1995.
16. Feldmann M, Brennan FM, Maini RN : Role of cytokines in rheumatoid arthritis, Annu Rev Immunol ; 14 : 397-440, 1996.
17. Feldmann M., Elliott MJ., Woody JN., Maini RN. : Anti-tumor necrosis factor-α therapy of rheumatoid arthritis, Adv Immunol ; 64 : 283-350, 1997.
18. Franz F. Immer, Alexsandra S. Immer-Bansi, Nathalie Trachsel, Pascal A. Berdat, Verena Eigenmann, Michele Curatolo and Thierry P. Carrel : Pain treatment with a COX-2 inhibitor after coronary artery bypass operation: a randomized trial, The Annals of Thoracic Surgery ; 75(2) : 490-495, 2003.
19. G. Fantuzzi and C.A. Dinarello : The inflammatory response in IL-1 deficient mice. Comparison with other cytokine-related knock-out mice, J. Leukoc. Biol. ; 59 : 489-493, 1996.
20. G. Wolf : Nitric oxide and nitric oxide synthase: Biology, pathology, localization, Histol Histopathol ; 12 : 251-261, 1997.
21. Gadient, R.A. and Otten, U.H. : Interleukin-6 (IL-6) - a molecule with both beneficial and destructive potentials, Prog. Neurobiol. ; 52 : 379-390, 1997.
22. Grand A., Fermor B., Fink C., Pisetsky DS., Weinberg JB., Vail TP., Guilak F. : Interleukin-1, tumor necrosis factor alpha, and interleukin-17 synergistically up-regulate nitric oxide and prostaglandin E2 production in explants of human osteoarthritic knee menisci, Arthritis Rheum ; 44 : 2078-83, 2001.
23. H.R. Herschman : Prostaglandin synthase 2, Biochim. Biophys. Acta ; 1299 : 125-140, 1996.
24. H. Sakurai, H. Kohsaka, M.F. Liu, H. Higashiyama, Y. Hirata, K. Kanno, I. Saito and N. Miyasaka : J. Clin. Invest ; 96 : 2357-2363, 1995.
25. Hegde, V.R., Dai, P., Patel, M.G., Puar, M.S., Das, P., Pai, J., Bryant, R. and Cox, P.A. : Phospholipase A2 inhibitors from an Erythrina species from Samoa, Journal of Natural Products ; 60 : 537-539, 1977.
26. Hirano T., Matsuda T., Turner M., Miyasaka N., Buchan G., Tang B., Sato K., Shimizu M., Maini R., Feldman M., Kishimoto T. : Excessive production of interleukin 6/B cell stimulatory factor-2 in rheumatoid arthritis, Eur J Immunol ; 18 : 1797-1801, 1998.
27. Ishiuchi N., Yoshino S., Yokoyama M., Asano G. : Expression of endothelial nitric oxide synthase and inducible nitric oxide synthase in synovium of rheumatoid

arthritis, Ryumachi ; 39 : 749-56, 1999.

28. J Keffer, L Probert, H Cazlaris, S Georgopoulos, E Kaslaris, D Kioussis, G Kollias : Transgenic mice expressing human tumor necrosis factor : a predictive genetic model of arthritis, EMBO J ; 13 : 4025-4031, 1991.
29. James M. Woods, Andres Mogollon, Mohammad A. Amin, Rita J. Martinez and Alisa E. Koch : The role of COX-2 in angiogenesis and rheumatoid arthritis, Experimental and Molecular Pathology ; 74(3) : 282-290, 2003.
30. J.R. Vane, J.A. Mitchell, I. Appleton, A. Tomlinson, D. Bishop-Bailey, J. Croxtall and D.A. Willoughby : Proc. Natl. Acad. Sci. ; 91 : 2046-2050, 1994.
31. J.S. Beckman, T.W. Beckman, J. Chen : Apparent hydroxyl radical production by peroxynitrite: Implications for endothelial injury from nitric oxide and superoxide, Proc Natl Acad Sci ; 87 : 1620-1624, 1990.
32. K. Saidu, J. Onah, A. Orisadipe, A. Olusola, C. Wambebe and K. Gamaniel : Antiplasmodial, analgesic, and anti-inflammatory activities of the aqueous extract of the stem bark of Erythrina senegalensis, Journal of Ethnopharmacology ; 71(1-2) : 275-280, 2000.
33. Kang BS., etc. : Herbology. younglim co. pp.266-267. Seoul, 1991.
34. Kim, J.M., Ho, S.H., Park, E.J., Hahn, W., Cho, H., Jeong, J.G., Lee, Y.W. and Kim, S. : Angiostatin gene transfer as an effective treatment strategy in murine collagen-induced arthritis, Arthritis Rheum. ; 46 : 793-801, 2002.
35. Kobayashi, M., Mahmud, T., Yoshioka, N., Shibuya, H. and Kitagawa, I. : Indonesian medicinal plants. XXI. Inhibitors of Na+/H+ exchanger from the bark of Erythrina variegata and the roots of Maclura cochinchinensis, Chemical and Pharmaceutical Bulletin ; 45 : 1615-1619, 1997.
36. Luo, Guangjo, Hershko Dan D., Robb Bruce W., Wray Curtis J., Hasselgren Per-Olof : IL-1beta stimulates IL-6 production in cultured skeletal muscle cells through activation of MAP kinase signaling pathway and NF-kappa B, American Journal Of Physiology. Regulatory, Integrative And Comparative Physiology ; 284(5) : 1249-1254, 2003.
37. Miyasaka N, Hirata Y. : Nitric oxide and inflammatory arthritides, Life Sci ; 61: 2073-81, 1997.
38. M.J. Elliott, R.N. Maini, M. Feldmann, A. Long-Fox, P. Charles, P. Katsikis, F.M. Brennan, J. Walker, H. BijL, J. Ghrayeb and J.N. Woody : Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha, Arthritis Rheum. ; 36 : 1681, 1993.
39. Nathan, C; Xie, Q W : Nitric oxide synthases: roles, tolls, and controls, Cell ; 78(6) : 915-918, 1994.
40. O'Banion, M. K., Winn, V. D., Young, D. A. : cDNA Cloning and Functional Activity of a Glucocorticoid-Regulated Inflammatory Cyclooxygenase. Proc. Natl. Acad. Sci. USA ; 89 : 4888-4892, 1992.
41. Paleolog EM. : Angiogenesis in rheumatoid arthritis. Arthritis Res, in press, 2002.
42. Peter C Taylor : Antibody therapy for rheumatoid arthritis, Current Opinion in Pharmacology ; 3(3) : 323-328, 2003.
43. S Hashimoto., RL Ochs., F Rosen., J Quach., G McCabe., J Solan., JE Seegmiller., R Terkeltaub., M Lotz : Chondrocyte-derived apoptotic bodies and calcification of articular cartilage, Proc Natl Acad Sci ; 95 : 3094-3099, 1998.
44. S Moncada, RM Palmer and EA Higgs : Nitric oxide: Physiology, pathophysiology, and pharmacology, Pharmacol Rev ; 43 : 109-142, 1991.
45. Sano, H., Hla, T., Maier, J.A., Crofford, L.J., Case, J.P., Maciag, T. and Wilder, R.L. : In vivo cyclooxygenase expression in synovial tissues of patients with rheumatoid arthritis and osteoarthritis and rats with adjuvant and streptococcal cell wall arthritis, J. Clin. Invest. ; 89 : 97-108. 1992.
46. Shimizu Toru, Sakamoto Atsuhiro, Ogawa Ryo : Inhibition of inducible nitric oxide synthase attenuates interleukin-1 beta induced vascular hyporeactivity in the rabbit, Journal Of Nippon Medical School ; 69(2) : 149-153, 2002.
47. Tanaka H, Hirata M, Etoh H, Shimizu H, Sako M, Murata J, Murata H, Darnaedi D, Fukai T. : Eryvarins F and G, two 3-phenoxychromones from the roots of Erythrina variegata, Phytochemistry ; 62(8) : 1243-6, 2003.
48. Tanaka H, Sato M, Fujiwara S, Hirata M, Etoh H, Takeuchi H. : Antibacterial activity of isoflavonoids isolated from Erythrina variegata against methicillin-resistant Staphylococcus aureus, Lett Appl Microbiol. ; 35(6) : 494-8, 2002.
49. Telikepalli, H., Gollapudi, S.R., K-Shokri, A., Velazquez, L., Sandmann, R.A., Veliz, E.A., Rao, K.V.J., Madhavi, A.S. and Mitscher, L.A. : Isoflavonoids and a cinnamylphenol from root extracts of Erythrina variegata, Phytochemistry ; 29 : 2005-2007, 1990.
50. Udagawa N., Kotake S., Kamatani N., Takahashi N., Suda T. : The molecular mechanism of osteoclastogenesis in rheumatoid arthritis, Arthritis Res. in press, 2002.
51. U.K. Messmer and B. Brune : Nitric oxide induced apoptosis: p53-dependent and p53-independent signaling pathways, Biochem J ; 319 : 299-305, 1995.
52. Van den Berg WB. : Anti-cytokine therapy in chronic destructive arthritis, Arthritis Res ; 3 : 18-26, 2001.
53. Van der Heijde D. : Structural damage in rheumatoid

arthritis as visualized through radiographs, Arthritis Res ; 4 : pp.29-33, 2002.
54. Van Wagoner, N.J. and Benveniste, E.N. : Interleukin-6 expression and regulation in astrocytes, J. Neuroimmunol. ; 100 : 124-139, 1999.
55. W.L. Smith, R.M. Garavito and D.L. DeWitt : Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2, J. Biol. Chem. ; 271 : 33157-33160, 1996.
56. Yin, G., Liu, W., An, P., Li, P., Ding, I., Planelles, V., Schwarz, E.M. and Min, W. : Endostatin gene transfer inhibits joint angiogenesis and pannus formation in inflammatory arthritis, Mol. Ther. ; 5 : 547-554. 2002.
57. Yoshizaki K., Nishimoto N., Mihara M., Kishimoto T. : Therapy of RA by blocking IL-6 signal transduction with humanized anti-IL-6 receptor antibody, Springer Semin Immunopathol ; 20 : 247-259, 1998.