Anti-inflammatory activities of *Phyllanthus emblica* and *Betula alnoides* through transcription factor NF-κB pathway

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Abstract

Nuclear factor κB (NF-κB) has been recognized as an important transcription factor related to many immune and inflammatory responses. Inactive NF-κB is associated with inhibitor κB (IκB) in the cytoplasm. Upon cell stimulation, such as the treatment with tumor necrosis factor alpha (TNF-α), IκB is phosphorylated followed by a rapid protein degradation and NF-κB is released and translocates into the nucleus. In the present study, 112 medicinal plant extracts were screened for their anti-inflammatory activities using the transcription factor NF-κB pathway as target. Their effects on TNF-α induced IκB-α degradation were tested by using Western blot analysis of IκB-α. Water extracts of Yu Gan Zi (余甘子) *Phyllanthus emblica* L., Meng Zi Hua (蒙自樺) *Betula alnoides* Buch-Ham. ex D. Don, Dang Gui Teng (当帰藤) *Embelia pulchella* Mez, Bian Dan Teng (扁担藤) *Tetrasigma planicumlum* Gag., Du Zi Teng (独子藤) *Monocelastrus monosperma* Wang et Tang and Xiang Xu Shu (香須樹) *Albizzia odoratissima* Benth. (all from Yun Nan Prov., 雲南省; China) showed inhibitory activities on TNF-α induced degradation of IκB-α. By Western blot analysis of NF-κB p65 in the nuclear fraction and immunofluorescence, it was observed that these extracts also inhibited the translocation of NF-κB to the nuclei. The presented results suggest that the method used in our study is suitable to screen the anti-inflammatory extracts and guide the fractionation in order to isolate highly active compounds.

Key words IκB-α, NF-κB, TNF-α, *Phyllanthus emblica, Betula alnoides,* anti-inflammatory activity.

Abbreviations BAEC, bovine carotid artery endothelial cells; IκB-α, inhibitor kappa B alpha; NF-κB, nuclear factor kappa B; PBS, phosphate-buffered saline; PDTC, pyrrolidine dithiocarbamate; SDS/PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor alpha.

Introduction

NF-κB is one of the most important and widely used transcription factor that plays an important role in many cellular responses to environmental changes. Five different subunits of NF-κB have been described, but the most frequent form of NF-κB is a heterodimer complex containing the p50 and p65/RelA subunits.[1] NF-κB complexes exist in the cytoplasm in a latent form. This form is stabilized by an inhibitory subunit, called IκB, which is tightly bound to NF-κB and masks its nuclear localization signal.[2,3] The best characterized IκB is IκB-α, upon cell stimulation IκB-α becomes phosphorylated followed by a rapid protein degradation. As a result, NF-κB is activated and translocates into the nucleus.[4] A large variety of stimulus, such as inflammatory

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cytokines, bacterial and viral infections, oxygen stress, can induce NF-κB activation rapidly. Among the stimulus, TNF-α is a potent activator which can induce degradation of IκB-α and activation of NF-κB in a wide variety of cell types.[5,6] The inducible transcription factor NF-κB regulates the transcription of various inflammatory cytokines,[7,8] and also participates in the regulation of viral promoters including HIV-1 long terminal repeat.[9,10]

Activation of NF-κB is instrumental for the immune response, therefore inhibition of NF-κB may have potentials to modulate inflammation. In this study, we screened 112 medicinal plants for their anti-inflammatory activities using the transcription factor NF-κB as target. Pyrrolidine dithiocarbamate (PDTC) is known as an effective inhibitor on TNF-α induced IκB-α degradation and activation of NF-kappa B[11,12] so we used it as a positive control in this study.

112 medicinal plants were extracted with boiling water according to their traditional usage and their effects on TNF-α mediated IκB-α degradation were tested by using Western blot analysis of IκB-α. Among them, six extracts showed inhibitory activities on TNF-α induced degradation of IκB-α. In order to confirm whether these active extracts inhibit activation of NF-κB induced by TNF-α, Western blot analysis of nuclear NF-κB p65 subunit and immunofluorescence methods were used. The results showed that above six active extracts which inhibited the degradation of IκB-α also inhibited TNF-α induced nuclear translocation of NF-κB. In this paper, we report two of them which inhibited TNF-α induced degradation of IκB-α and nuclear translocation of NF-κB significantly.

Materials and Methods

Test extracts and chemicals : Extracts of 112 kinds of medicinal plant were from the division of Natural Products Chemistry of Shenyang Pharmaceutical University of China. Recombinant human tumor necrosis factor alpha (TNF-α) was purchased from Pepro Tech EC Ltd (London, England) and dissolved in distilled water before use. The ammonium salt of pyrrolidine dithiocarbamate (PDTC) was from Sigma Chemical Company (St.Louis, MO, USA).

Cell culture : Bovine carotid artery endothelial cells (BAEC) were cultured at 37°C in the presence of 5 % CO_2 in the minimum essential medium (MEM, GIBCO BRL, Gaithersburg, MD, USA) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum (JRH BIOSCIENCES, Lenexa, KS, USA).

Cytoplasmic and nuclear extraction : After appropriate treatments, the media were removed and the cells were washed with ice-cold phosphate-buffered saline (PBS) for three times. The cells were harvested and lysed in 40 μl of buffer A [10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethane sulfonic acid (HEPES) pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 500 μM (±)-dithiothreitol (DTT), 0.1 % (v/v) (nonylphenoxy)-(polyethoxy)-ethanol (NP-40)]. After incubating at 4°C for 15 min, the cytoplasmic proteins were separated by centrifuging at 1,300 rpm for 5 min at 4°C and used for Western blot analysis of IκB-α. The nuclei pellet were resuspended in 30 μl of buffer B [20 mM HEPES, 1.5 mM $MgCl_2$, 100 mM DTT, 400 mM NaCl, 20 μM phenylmethylsulfonyl fluoride (PMSF)] and incubated for 20 min at 4°C. The supernatant was seperated as nuclear extraction by centrifuging at 14,000 rpm for 10 min at 4°C and used for Western blot analysis of NF-κB p65 subunit. Protein concentrations were determined by the method of Bradford using a commercial kit (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA, USA).

Western blot analysis : Equal amount of cytoplasmic proteins (10 μg) were dissolved in five-fold SDS/PAGE loading buffer [125 mM tris hydroxymethyl aminomethane (Tris) pH 6.8, 25 % (v/v) glycerol, 5 % (m/v) sodium dodecyl sulfate (SDS), 5 % (v/v) 2-mercaptoethanol, 0.25 % (m/v) bromophenol blue] and boiled for 3 min at 100°C. Then the proteins were resolved on 10 % SDS/PAGE, run at 100v in electrophoretic buffer [25 mM Tris, 192 mM glycine, 0.1 % (m/v) SDS]. After running, the proteins were electrotransferred on a Hybond ECL Nitrocellulose membrane for 1.5 hr. The membrane was washed with Tris-buffered saline Tween [TBS-T, 20 mM Tris pH 8.0 ; 150 mM NaCl ; 0.1 % (v/v) Tween-20]. Non-specific sites on the membrane were blocked by incubating the membrane in the blocking solution containing 5 % non-fat dry milk in TBS-T for 60 min

at room temperature on an orbital shaker. The membrane was washed with TBS-T and incubated in diluted (1 : 1000) I κ B-α primary antibody (rabbit, New England Biolabs, Inc., Beverly, MA, USA) for 1hr. The membrane was washed and incubated in diluted (1 : 1000) secondary anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) conjugated to horse radish peroxidase (HRP) for 1 hr. The final washed membrane was reacted with an enhanced chemiluminescence reagent (ECL, Amersham) and exposed to Kodak Scientific Film (EASTMAN KODAK Company, Rochester, NY, USA) to test the immunoblots.

Ten micrograms of nuclear proteins were denatured samely and resolved on 8 % SDS-PAGE. After running, the proteins were electrotransferred on a membrane for 2 hr. The membrane was treated samely until the immunoblots were tested. In this experiment, anti-NF κ B p65/Rel A subunit primary antibody (rabbit, Santa Cruz, CA, USA) was used.

Immunofluorescence : BAEC cells were seeded in the glass bottom microwell dishes (35 mm, poly-d-Lysine coated, Mat Tek Corporation, USA) three days before the experiments. After indicated treatments, cells were washed with PBS for three times and fixed with 2 % formaldehyde in PBS for 15 min. After washes, primary antibody solution [1% bovine serum albumin (BSA), 0.2 % Triton X-100, anti-NF κ B/p65 rabbit 1 : 100] were added for 1 hr. After washing with PBS, secondary antibody solution [fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibody (goat 1 : 100), 1 % BSA, 0.2 % Triton X-100] were added for 45min. After final washes, pictures were taken by using a laser scanning microscope ACAS 570.

Results

TNF-α induced degradation of I κ B-α in BAEC

In order to decide the best condition for screening the medicinal plant extracts, at first we tested the effect of TNF-α on I κ B-α degradation in BAEC. Cells were treated with TNF-α for different periods of time. The cytoplasmic proteins were prepared and tested by using Western blot analysis of I κ B-α. As shown in Fig. 1A, unstimulated cells showed very high protein level of I κ B-α. After 10 min treated with 10 ng/ml TNF-α, I κ B-α protein began to degrade rapidly and became almost undetectable after 20 min. From 45 min after the treatment, I κ B-α was newly synthesized and recovered to the normal level after 60 min. In the next experiment (Fig. 1B), cells were treated with various concentrations of TNF-α for 20 min. The cytoplasmic proteins were prepared and tested for Western blot analysis of I κ B-α. When cells were treated with TNF-α at concentrations higher than 5 ng/ml, significant degradation of I κ B-α was observed. These data suggest that TNF-α induces the

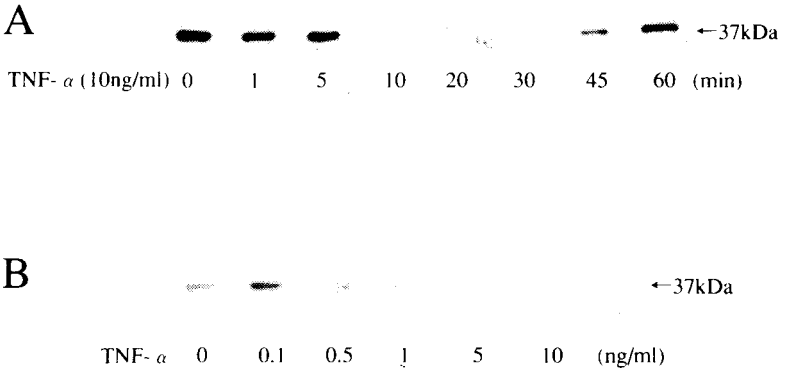

Fig. 1 TNF-α induced degradation of I κ B-α in BAEC. A. Kinetics of TNF-α induced degradation of I κ B-α. BAEC cells were treated for the indicated periods of time with 10 ng/ml TNF-α. Cytoplasmic proteins were prepared from the control and TNF-α treated cells and separated by 10 % SDS/PAGE and transferred on a membrane followed by Western blot analysis using anti-I κ B-α antibody. B. Cells were treated with different concentrations of TNF-α for 20 min. Cytoplasmic proteins were prepared and tested for Western blot analysis of I κ B-α. An arrowhead indicates the position of a 37-kDa I κ B-α-specific signal.

degradation of I κ B-α in BAEC and a concentration of 5 ng/ml is necessary.

The inhibitory effect of PDTC on TNF-α induced degradation of I κ B-α

PDTC is known as a kind of antioxidant which can inhibit TNF-α induced degradation of I κ B-α and activation of NF-κ B in many cell types. We tested its effect on TNF-α induced degradation of I κ B-α in BAEC (Fig. 2). Cells were incubated with 5 ng/ml TNF-α alone for 20 min (lane 2) or pre-incubated with 10 μM (lane 3), 100 μM (lane 4) PDTC for 30 min followed by stimulation with TNF-α for added 20 min. The cytoplasmic proteins were prepared and tested for Western blot analysis of I κ B-α. As shown in Fig. 2, when cells were treated with 100 μM PDTC (lane 4), TNF-α induced degradation of I κ B-α was completely inhibited. The protein level of I κ B-α recovered to 96 % of the normal level (lane 1). In the following screening experiments, PDTC was used as a positive control since it can inhibit the degradation of I κ B-α significantly in BAEC.

Inhibitory effects of Phyllanthus emblica and Betula alnoides on TNF-α induced degradation of I κ B-α

In order to investigate whether the extracts of medicinal plant interfere with the activation of tran-

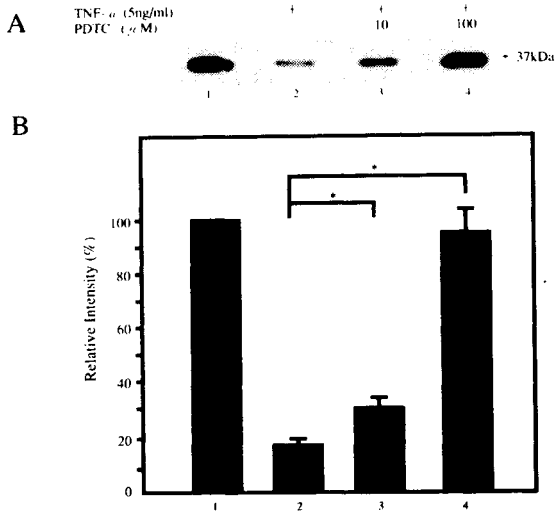

Fig. 2 The inhibitory effect of PDTC on TNF-α induced degradation of I κ B-α. A. Cells were treated with 5 ng/ml TNF-α alone for 20 min (lane 2) or pretreated with 10 μM (lane 3) and 100 μM PDTC (lane 4) for 30 min followed by simulation with TNF-α for added 20 min. Cytoplasmic proteins were prepared from the control (lane 1) and treated cells (lanes 2-4) and separated by 10 % SDS/PAGE and transferred on a membrane followed by Western blot analysis of I κ B-α. An arrowhead indicates the position of a 37-kDa I κ B-α -specific signal. B. Densitometric analysis of Western blot from two separate experiments. The data are expressed as mean±S.D. (*$p <$ 0.01).

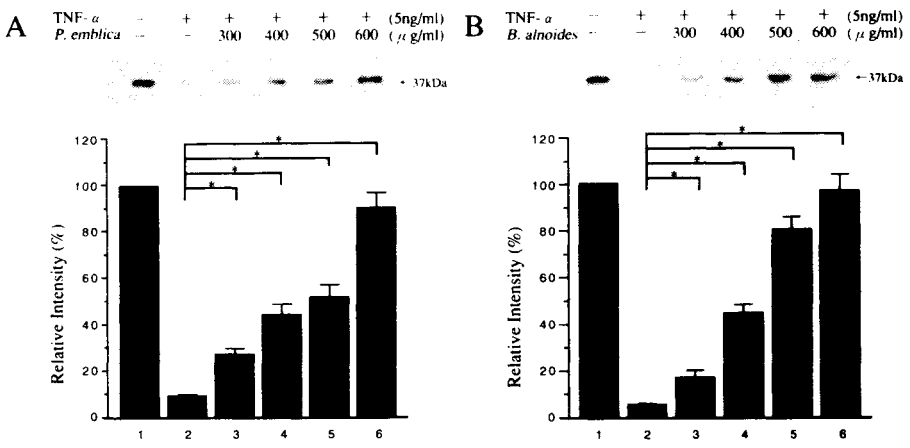

Fig. 3 Effects of *Phllanthus emblica* and *Betula alnoides* on TNF-α induced degradation of I κ B-α. Cells were treated with 5 ng/ml TNF-α alone for 20 min (lane 2) or pretreated with different concentrations of indicated extract solution (lanes 3-6) for 30 min followed by stimulation with TNF-α for added 20 min. Cytoplasmic proteins were prepared from the control (lane 1) and treated cells (lanes 2-6) and separated by 10 % SDS/PAGE and transferred on a membrane followed by Western blot analysis of I κ B-α. An arrowhead indicates the position of a 37-kDa I κ B-α -specific signal. Densitometric analysis of Western blot from two separate experiments. The data are expressed as mean±S.D. (*$p<0.01$). A. The effect of *Phyllanthus emblica* on TNF-α induced degradation of I κ B-α. B. The effect of *Betula alnoides* on TNF-α induced degradation of I κ B-α.

scription factor NF-κ B, we screened the extracts for their inhibitory effects on TNF-α induced degradation of I κ B-α. Cells were pre-incubated with every extract solution for 30 min before stimulation of TNF-α. The cytoplasmic proteins were prepared and tested for Western blot analysis of I κ B-α. Six active extracts were found from 112 tested extracts. Water extracts of Yu Gan Zi (余甘子) *Phyllanthus emblica* L. and Meng Zi Hua (蒙自樺) *Betula alnoides* Buch-Ham. ex D. Don (Yun Nan Prov., 雲南省 ; China) showed significant inhibitory activities on TNF-α induced degradation of I κ B-α (Fig. 3A and 3B). Both of them completely inhibited the degradation of I κ B-α at a concentration of 600 μg/ml and the inhibitory effects exhibited very good dose-dependency. At the same time, extracts of Dang Gui Teng (当帰藤) *Embelia pulchella* Mez, Bian Dan Teng (扁担藤) *Tetrastigma planicumlum* Gag., Du Zi Teng (独子藤) *Monocelastrus monosperma* Wang et Tang and Xiang Xu Shu (香須樹) *Albizzia odoratissima* Benth. (all from Yun Nan Prov., 雲南省 ; China) also strongly inhibited TNF-α induced degradation of I κ B-α (data not shown).

In unstimulated cells, NF-κ B heterodimers are kept as inactive complexes in the cytoplasm by inhibitory protein I κ B-α. After cell stimulation, I κ B-α is phosphorylated and degraded, and the free NF-κ B dimers translocate into the nucleus. The data above have shown that six active extracts inhibited degradation of I κ B-α induced by TNF-α. In order to investigate whether these extracts inhibit activation of NF-κ B, Western blot analysis and immunofluorecence were carried out to confirm the nuclear translocation of NF-κ B. Cells were treated with TNF-α for indicated times, the nuclear proteins were prepared as described in the materials and methods and tested for Western blot analysis of NF-κ B p65 subunit. As shown in Fig. 4A, NF-κ B p65 subunit was not detected in the nucleus in unstimulated cells (lane 1). Upon the stimulation of TNF-α, NF-κ B was activated and translocated into the nucleus and reached maximum after 60 min (lane 5). In the experiments expressed by Fig. 4B, cells were incubated with 600 μg/ml *Phyllanthus emblica* (lane 3), *Betula alnoides* (lane 4) and 100 μM PDTC (lane 5) for 30 min and subsequently incubated with 5 ng/ml TNF-α for added 1 hr. The

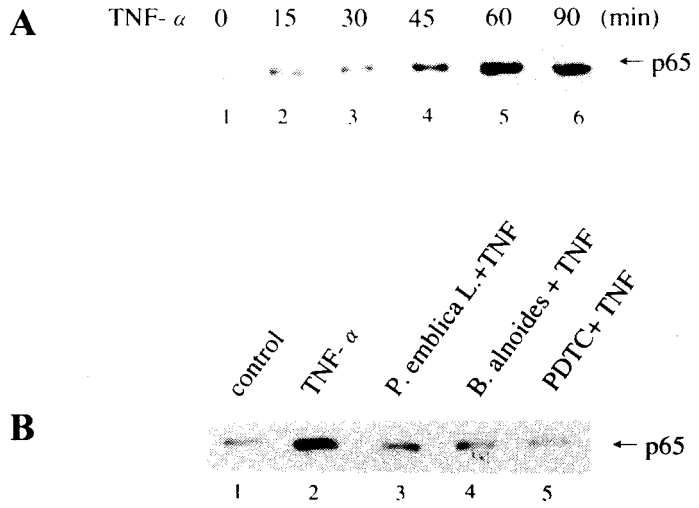

Fig. 4 Effects of PDTC, *Phyllanthus emblica* and *Betula alnoides* on the nuclear translocation of NF-κ B p65 subunit. A. Kinetics of TNF-α induced nuclear translocation of NF-κ B p65 subunit. Cells were treated for the indicated periods of time with 5 ng/ml TNF-α. Nuclear proteins were prepared from the control (lane 1) and TNF-α treated cells (lanes 2-6) and separated by 8 % SDS/PAGE and transferred on a membrane followed by Western blot analysis using anti-NF κ B p65 antibody. B. Cells were treated with 5 ng/ml TNF-α alone for 1hr (lane 2) or pretreated with 100 μM PDTC (lane 5) and 600 μg/ml each extract solution (lanes 3-4) for 30 min followed by stimulation with TNF-α for added 1 hr. Nuclear proteins were prepared and tested by Western blot analysis of NF-κ B p65 subunit. An arrowhead indicates the position of NF-κ B p65 specific signal.

results showed that PDTC completely inhibited the nuclear translocation of NF-κ B p65 subunit at the same concentration which inhibited the degradation of I κ B-α. At the same time, extracts of *Phyllanthus emblica* and *Betula alnoides* also inhibited the nuclear translocation of p65 completely at the concentration of 600 μg/ml. The results also showed that other four extracts which inhibited the degradation of I κB-α also strongly inhibited the nuclear translocation of p65 subunit (data not shown).

In the next experiments, cells were grown in glass bottom dishes, treated as indicated, fixed, and the location of p65 was determined by immunofluorescence using an antibody specific against p65. As shown in Fig. 5, in the unstimulated state p65 localized exclusively to the cytoplasm (Fig. 5A). After stimulation with TNF-α, p65 was mainy found in the nucleus (Fig. 5B). However, treatment with TNF-α plus PDTC or the extracts of medicinal plant resulted in prevention to nuclear translocation of p65 (Fig. 5C-E).

Discussion

In the field of inflammation research the inducible transcription factor NF-κ B became an important pharmacological target, since activation of NF-κ B is instrumental for inflammatory response.[13] The aim of our study was to find out anti-inflammatory extracts by using the transcription factor NF-κ B as target. The present results have shown that six extracts among tested 112 medicinal plants inhibited TNF-α induced degradation of I κ B-α and activation of NF-κ B in BAEC. Among them the extracts of *Phyllanthus emblica* and *Betula alnoides* showed significant inhibitory activities.

Phyllanthus emblica has been used for the anti-inflammatory and antipyretic treatment in its growing areas in subtropical and tropical parts of China, India, Indonesia and the Malay Peninsula. The chemical compounds so far isolated from the leaves of *Ph. emblica* are polyphenolic constituents, a gallotannin, amlaic acid, and alkaloids, phyllantidine and

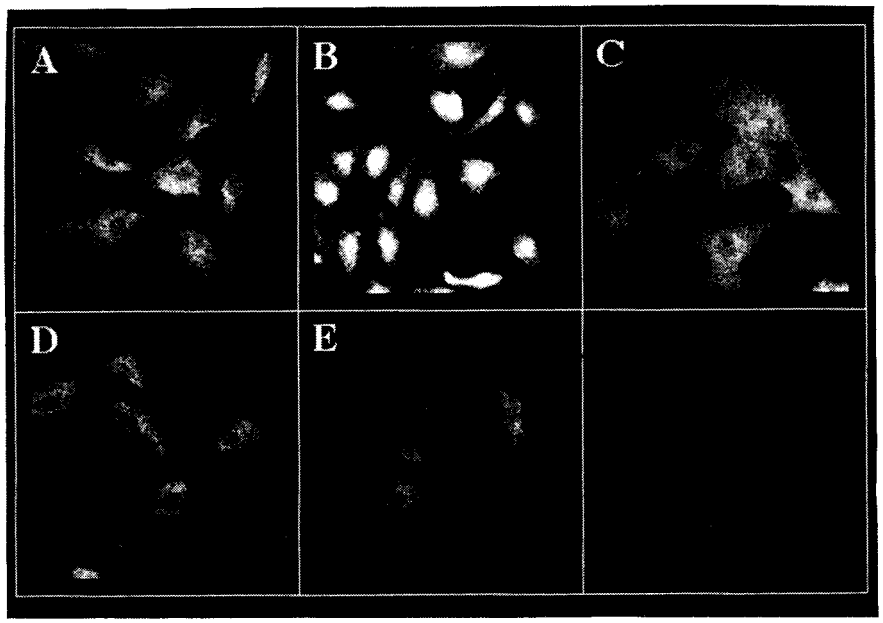

Fig. 5 Immunofluorescence of nuclear translocation of NF-κ B p65 subunit. Cells were incubated with 5 ng/ml TNF-α alone for 1 hr (B) or pretreated with 600 μg/ml *Phyllanthus emblica* (C), *Betula alnoides* (D) and 100 μM PDTC (E) for 30min followed by stimulation with TNF-α for added 1 hr. The control (A) and treated cells were washed with PBS and fixed with 2 % formaldehyde in PBS for 15 min. After washes, primary antibody solution were added for 1 hr. After washing with PBS, secondary antibody solution were added for 45 min. After final washes, pictures were taken by using a laser scanning microscope ACAS 570.

phyllantine. Some compounds isolated from fruits of *Ph. emblica* were reported to inhibit human immunodeficiency virus-1 reverse transcriptase.[14] But reports of the possible anti-inflammatory components of *Ph. emblica* are rare. Recently, methanol, tetrahydrofuran and 1,4-dioxane extracts from fruits of *Ph. emblica* were reported to have inhibitory activities against human polymorphonuclear leukocyte (PMN) and platelet functions which confirmed its anti-inflammatory and antipyretic properties.[15] However, according to the TLC experiments, the methanol extract of *Ph. emblica* does not contain significant amounts of ellagic acid or gallic acid methyl ester. Though some rutin or gallic acid monohydrate may be present, these compounds do not provide a comprehensive understanding of its anti-inflammatory properties. This means that the active anti-inflammatory agents in this plant may be as yet unidentified compounds.

Betula alnoides is a large tree growing wild in the forest of the provinces Cao Bang, Lang Son, Tuyen Quang in North Vietnam. It is used in the dyestuff industry and for the treatment of wounds. There are only three publications on the phytochemistry of this plant. Two of them described constituents from the bark oil of this plant [16,17] and another one reported four triterpenoides lupeol, 3-O-acetoxyoleanolic acid, betulinic acid and betulin isolated from chloroform extract of its barks.[18] Until now there is no report on its anti-inflammatory activity of this plant.

In summary, the results of our present study have shown that water extracts of *Phyllanthus emblica* and *Betula alnoides* have significant inhibitory activity on TNF-α induced degradation of I κ B-α and activation of NF-κ B, which suggest that the products isolated from these extracts may be used as lead compounds for development of novel anti-inflammatory drugs. We consider the method used in the present study as a suitable model for a bio-guided fractionation to isolate anti-inflammatory agents from the active extracts. This method will be helpful for further research.

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和文抄録

核内因子 NF-κ B は様々な炎症反応，免疫反応などに関連する遺伝子の発現を制御する重要な転写因子として発見され，通常 I κ B と複合体を形成しており，細胞質内に不活性の状態で存在している。細胞外刺激（腫瘍壊死因子 TNF-α など）により I κ B のリン酸化およびそれに続く分解が急速に起こり，フリーになった NF-κ B は核内に移行し，目的遺伝子の発現誘導を行うことになる。そこで本稿では，NF-κ B をターゲットにし，112種類の生薬抽出物の抗炎症作用を検討した。ウェスタンブロットの方法を用いて，生薬抽出物の TNF で誘導された I κB-α の分解に対する作用を調べた。その中で，6種類の水抽出物（余甘子，豪自樺，当帰藤，扁担藤，独子藤，香須樹）が TNF で誘導された I κ B-α の分解に対して抑制作用を示した。さらに，この6種類の生薬抽出物の NF-κ B の核内移行に対する影響を NF-κ B のサブユニットである p65 のウェスタンブロットと抗体染色方法を用いて調べたところ，これらの生薬抽出物は核内移行も有意に抑制した。このように NF-κ B, I κ B の挙動を調べることは，生薬抽出物のスクリーニングに適しているとともに，これら抽出物からの抗炎症活性成分の単離，同定にも利用できると考えている。

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