# Assessing the role of the transcription factor nuclear factor kappa B (NF-κB) in the anti-inflammatory activity of EQUISETUM ARVENSE (COMMON HORSETAIL) extracts

by

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Assessing the role of the transcription factor nuclear factor kappa B (NF-kB) in the anti-inflammatory activity of EQUISETUM ARVENSE (COMMON HORSETAIL) extracts

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## ABSTRACT

NF-κB is a key regulator of inflammatory signaling in the cell and is a transcription factor for many genes, including interferon-beta. The constitutive activation of NF-κB can cause hyperinflammation, which is implicated in many diseases, including rheumatoid arthritis and inflammatory bowel disease. Many anti-inflammatory drugs and compounds, such as diclofenac, and curcumin, have been found to inhibit NF-κB activation, thus blocking the inflammatory response. Treatment for inflammatory disorders has also included *Equisetum arvense* (common Horsetail) as an herbal remedy for arthritis. Since plant extracts of *E. arvense* have been shown to exhibit medicinal, anti-inflammatory properties, we wanted to investigate the effect of an *E. arvense* extract on NF-κB signaling by looking at the TNFα-mediated activation of the NF-κB promoter and comparing the anti-inflammatory effects of the extract with diclofenac and curcumin. We first show that diclofenac, curcumin, and the *E. arvense* extract all inhibit TNFα-induced NF-κB activation. We then show that treatment of cells with TNFα, a known stimulator of NF-κB mediated inflammation, leads to the activation of NF-κB specific promoters, and that this activation is mitigated by the transfection of cells with IκBΔN. Our results suggest that the anti-inflammatory effects of *Equisetum arvense* may act via the modulation of similar signaling pathways as other commercial anti-inflammatory drugs.

# INTRODUCTION

NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) plays a key role in pro-inflammatory gene expression and is activated in many inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and asthma. The NF-κB family of transcription factors is made up of five members, p50, p52, p65 (RelA), c-Rel, and RelB, which dimerize to positively regulate target genes. NF-κB has a central role in inflammation as a transcription factor for many pro-inflammatory cytokine genes, including interferon-beta interleukin and TNFα, and other genes mediating pain such as cyclooxygenase 2 (COX-2).[1] Interferon-beta, TNFα, and interleukin help initiate the immune response by activating surrounding cells to express inflammatory genes.[2] COX-2 is an enzyme that creates prostaglandins, which cause inflammation, pain, and fever.[3]

Given its role as a target of inflammatory signaling pathways, many pro-inflammatory stimulants activate NF-κB-induced transcription. These stimulants can include cytokines, such as TNFα, DAMPs (damage-associated molecular pattern molecules) like ATP or DNA, and PAMPs (pathogen-associated molecular pattern molecules), which include macromolecules such as lipopolysaccharies (LPS) and nucleic acids derived from the genomes of microbial or viral pathogens. The negative sense single-stranded RNA genome of Sendai virus (SV), a murine parainfluenza virus type 1 of the *Paramyxoviridae* family, is one example of a PAMP and activates a signal transduction pathway that ultimately activates NF-κB and translocates the transcription factor into the nucleus to induce expression of interferon-beta and other anti-viral genes.[4] Therefore, Sendai virus may be used to promote NF-κB signaling in the laboratory. The cytokine TNFα also activates NF-κB, but through a different signaling mechanism. Endogenous

TNFα is produced by many different cell types in the body, but plays a central role in inflammation when released by macrophages.[5] NF-κB can regulate the transcription of TNFα, but is also activated by the cytokine, and therefore, like SV, TNFα can be used in the laboratory to study NF-κB activation and inflammation.

SV and TNFα both activate NF-κB but through different mechanisms (Figure 1). RIG-I, a dsRNA-recognizing RNA helicase, detects the ssRNA of SV intracellularly and interacts with

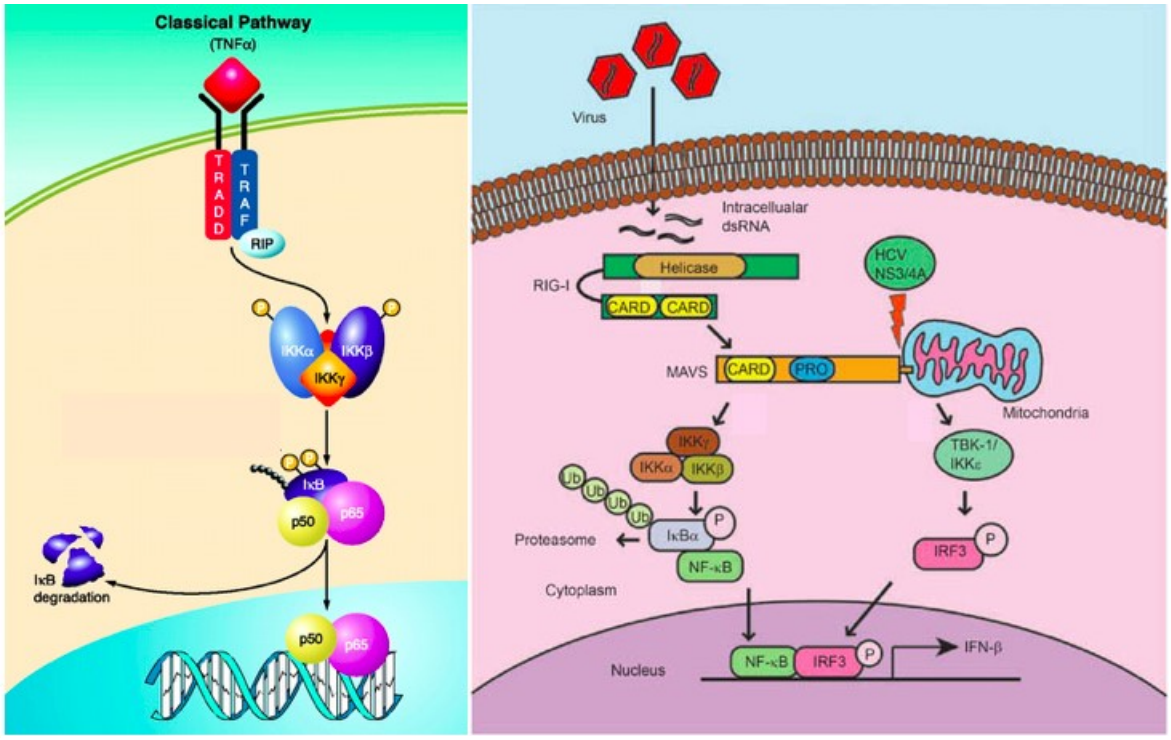


**Figure 1. Mechanism of NF-κB activation through TNFα or virus. (A)** In response to TNFα, TRADD and TRAF are recruited to the receptor and IKK is activated. IKK phosphorylates IκBα, which is degraded in the proteasome, and NF-κB (p50 and p65) is released. **(B)** RIG-I detects viral RNA and interacts with MAVS. IKK is activated and phosphorylates IκBα. IκBα is degraded and NF-κB is released to translocate into the nucleus. MAVS signaling also leads to the activation of IRF-3, which along with NF-κB acts as a transcription factor for interferon-beta.[4]

MAVS (mitochondrial anti-viral signaling) via a CARD domain.[6] MAVS then recruits and activates TRAF6, which further activates IκB Kinase (IKK). TNFα binds to TNFR1, and TRADD is recruited to the receptor through a death domain. TRADD recruits TRAF2 and activates IKK through RIP.[7] SV and TNFα signaling converge at IKK. As a central regulator for

both signaling pathways, IKK phosphorylates IκBα (inhibitor of NF-κB) and controls NF-κB regulation. When phosphorylated, IκBα is quickly ubiquitinated and degraded by the proteasome, releasing NF-κB dimers that translocate to the nucleus.[8] Because IκBα degradation determines the activation of the NF-κB dimers, inhibiting this degradation mitigates the pro-inflammatory response. As one example, IκBΔN, a dominant negative mutant of IκBα, is easily used as a control for inhibiting NF-κB. When the gene for IκBΔN is transfected into the cell, the protein is produced and binds to NF-κB. The N-terminus of IκBΔN is mutated by the removal of the two serines that are normally phosphorylated by IKK, thus, unlike IκBα, IκBΔN can not be phosphorylated, and therefore can not be ubiquitinated and degraded. The nuclear localization signal of NF-κB is kept hidden and NF-κB is unable to translocate to the nucleus and activate gene expression.

NF-κB activation leads to many inflammatory and proliferative effects. As a transcription factor for interferon-beta, NF-κB when activated, can also indirectly lead to the stimulation of the JAK-STAT pathway, thus initiating the transcription of cytokines and a multitude of other immune response-related genes (known collectively as the Interferon Sensitive Genes). The initiation of the immune response leads to the release of cytokines and recruits leukocytes to sites of inflammation creating a spiraling effect of more inflammatory responses and proliferating immune cells. NF-κB also initiates inflammation by inducing the expression of cyclooxygenase-2 (COX-2) which produces prostaglandins and whose gene has a binding site for NF-κB in its promoter region.[9] The release of these cytokines, prostaglandins, and other chemicals from cells causes inflammation.[10] Also acting as a transcription factor, NF-κB can directly affect the ability of a cell to proliferate by inducing the expression of Cyclin D1, which is a cell cycle regulator and has an NF-κB binding site in its promoter.[11] These downstream effects of NF-κB activation

can lead to both short term inflammation and pain through interferon-beta and COX-2 expression. They can also lead to chronic diseases, such as cancer, which can be caused by constitutive activation of NF-κB. The proliferative effects of NF-κB, which includes the activation of cyclin D1 and of inhibitors of apoptosis[11], link NF-κB activation to many cancers, including B-cell lymphomas and colitis-associated cancer. One form of NF-κB (RelA) is a known oncogene that is implicated in several forms of cancer.

Inflammatory diseases are widespread and there are many pharmacological approaches that are used for their treatment. One current treatment to reduce inflammation includes NSAIDS (nonsteroidal anti-inflammatory drugs), which is a large class of drugs prescribed for their analgesic and anti-inflammatory effects.[11] Common NSAIDS are aspirin, ibuprofen, and celecoxib. Corticosteroids are another treatment for inflammation, especially for arthritis and asthma, and inhibit the formation of prostaglandins.[10] While not necessarily prescribed as medications, some natural products, like curcumin, which is the active ingredient in turmeric, are known for their anti-inflammatory properties. Another natural product, *Equisetum arvense* (common horsetail) is a plant of the Equisetopsida family that has exhibited anti-inflammatory effects and has been used as an herbal remedy to treat anti-inflammatory diseases, such as wounds and arthritis.[12] Previous studies have shown that extracts of *E. arvense* inhibit T cell proliferation and lymphocyte activation.[12]

Since *E. arvense* has a long history of use as an anti-inflammatory product, we investigated its effect on the activation of NF-κB. We tested the ability of the extract to inhibit TNFα-mediated NF-κB activation. We predicted that the *E. arvense* extract would inhibit NF-κB activation in the presence of TNFα. To compare the anti-inflammatory properties of *E. arvense* with other products, we also studied the ability of diclofenac and curcumin to inhibit TNFα-

induced activation of NF-κB. We then looked at the NF-κB signaling pathway in response to TNFα and IκBΔN.

## MATERIALS AND METHODS

### *Cell lines and Culture*

The 293HEK (human embryonic kidney fibrobast) cell line was used for all the experiments. The cells were maintained in Dulbecco's modified eagle's medium (DMEM, SIGMA) supplemented with 10% heat inactivated fetal bovine serine (FBS), penicillin-streptomycin (88 U/mL penicillin and 88 μg/mL streptomycin), L-glutamine (0.88 mM), and MEM non-essential amino acids (SIGMA #M7145).

### *Anti-inflammatory compounds*

Leaves of the *E. arvense* plant were dried, grounded, washed, and then distilled in 50% ethanol to make the crude extract. The plant was harvested in Malvern, Worcestershire, United Kingdom, and supplied by Professor Leigh T. Canham (Nanoscale Physics, Chemistry, and Engineering Research Laboratory of the University of Birmingham, Birmingham, UK). Differing concentrations of the extract and curcumin were dissolved in 2% DMSO and 1% DMSO, respectively, while different concentrations of diclofenac sodium salt was dissolved in $H_2O$.

### *Luciferase Assay*

The effect of anti-inflammatory compounds on the TNFα-induced activation of NF-κB was analyzed by a luciferase assay. 293HEK cells were plated in 12-well plates and were transfected 24 hours later with CMV-LUC (5 ng/mL; Renilla sp.) and PRDII-LUC (10 ng/mL firefly) using Lyovec (Invivogen) following the manufacturer's protocol. The PRDII domain contains the binding site for NF-κB on the enhancer region for the interferon-beta gene. As a

control, cells were also transfected with a Renilla luciferase gene under control of the Cytomegalovirus Immediate Early promoter (CMV IEp-Luc) and with 0.05 μg/mL of the IκBΔN gene for some experiments. After 48 hours, cells were treated with the indicated anti-inflammatory compound. One hour later, the cells were stimulated with 10 ng/mL of TNFα and after five hours, the cells were harvested and lysed with passive lysis buffer according to protocol. The luciferase assays were performed according to the Promega Dual-Luciferase Reporter Assay protocol, and firefly and Renilla luciferase activity was measured using a SIRIUS luminometer.

*Western Blot*

The effect of anti-inflammatory compounds on the degradation of IκBα was analyzed using a western blot. The cells were treated with an anti-inflammatory compound and TNFα by the same method as described above. Cells were harvested with 1X PBS (phosphate-buffered saline), lysed with SDS lysis buffer, and samples were prepared with SDS sample buffer according to protocol. The proteins were separated by PAGE (9% acrylamide) and transferred to a PVDF transfer membrane [Millipore, which was blotted with 5% BLOTTO (5% milk in 1X TBST, tris-buffered saline with 0.1% Tween)]. The membrane was immunoblotted with anti-β-actin and anti-IκBα primary antibodies, followed by alkaline phosphatase-conjugated secondary anti-mouse and anti-rabbit antibodies, respectively.

## RESULTS

*Diclofenac inhibits TNFα-induced activation of NF-κB*

To determine whether the *E. arvense* extract contained anti-inflammatory activity, we first needed to establish some anti-inflammatory controls. To do this we first tested the effects of

a commercial medication, diclofenac, on the TNFα-induced activation of NF-κB. Increasing concentrations of diclofenac reduced NF-κB sensitive promoter activation as seen by the reduction in luciferase activity (Figure 2). The lowest concentration of 89 μg/mL exhibited similar levels of luciferase activity as the cells not exposed to drug, but the higher concentrations of diclofenac (370 μg/mL and 740 μg/mL) decreased luciferase activity. These results suggest that diclofenac inhibits TNFα-induced NF-κB activation in a dose-dependent manner. As a positive control for inhibition, cells were transfected with IκBΔN, a dominant negative inhibitor of NF-κB.

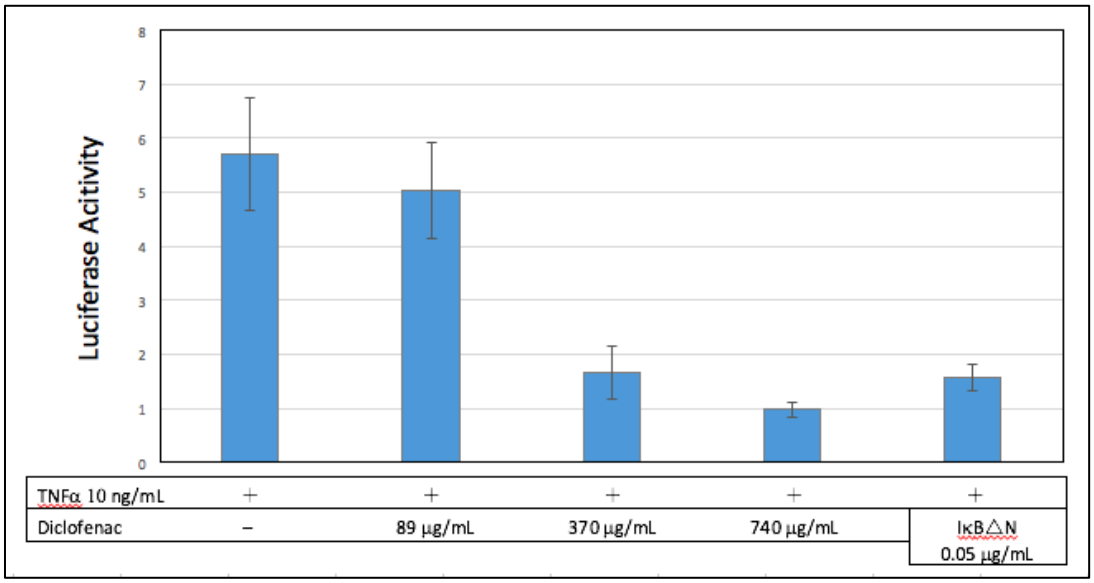


**Figure 2. Diclofenac inhibits the TNFα-induced activation of NF-kB.** 293HEK cells were transfected with PRDII-LUC and CMV-LUC as described in Methods After 48 hours, the cells were treated with differing concentrations of Diclofenac sodium salt dissolved in water. After one hour, cells were then treated with TNFα. Five hours later, the cells were harvested and a luciferase assay was performed on the lysates. As a control, some cells were also transfected with IκBΔN and did not receive Diclofenac.

*Curcumin inhibits TNFα-induced activation of NF-κB*

We next looked at the effect of a natural product on NF-κB activation. Curcumin is a natural product that has been shown to have anti-inflammatory properties by inhibiting NF-kB

activation and IκBα degradation.[11,14] As seen in Figure 3, increasing concentrations of curcumin led to a decrease in NF-κB sensitive promoter activation as seen by the reduction in luciferase activity. Only the two higher concentrations, 0.92 μg/mL and 9.2 μg/mL, decreased luciferase activity compared to the control that did not receive curcumin. From these results we can conclude that curcumin inhibits the TNFα-induced activation of NF-κB in a dose-dependent manner.

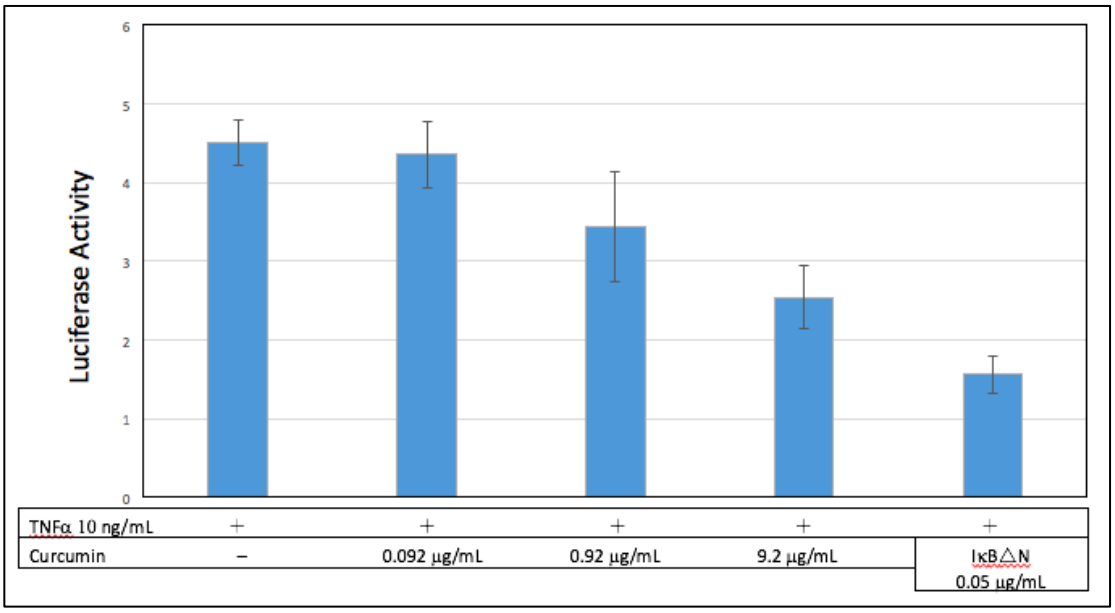


**Figure 3. Curcumin inhibits TNFα-induced activation of NF-κB.** 293HEK cells were transfected with PRDII-LUC and CMV-LUC as described in Methods After 48 hours, the cells were treated with differing concentrations of curcumin dissolved in 1% DMSO. After one hour, cells were then treated with TNFα. Five hours later, the cells were harvested and a luciferase assay was performed on the lysates. As a control, some cells were also transfected with IκBΔN and did not receive curcumin.

*E. arvense extract inhibits TNFα-induced activation of NF-κB*

After testing the effects of a commercial medication, Diclofenac, and a natural product, curcumin, on the TNFα-induced activation of NF-kB, we compared the effect of the *E. arvense* extract, which was prepared as described in Materials and Methods. Increasing concentrations of the extract reduced NF-κB sensitive promoter activation as seen by the reduction in luciferase

activity (Figure 4). At lower concentrations of extract, 2 μg/mL and 20 μg/mL, there was no change in luciferase production. At these concentrations, the extract did not inhibit NF-κB activation. At higher concentrations of extract, 200 μg/mL and 340 μg/mL, luciferase production decreased. We can therefore infer that at these higher concentrations, the extract inhibited the TNFα-induced activation of NF-κB. These results suggest that the *E. arvense* extract inhibits TNFα-induced activation of NF-κB in a dose-dependent manner.

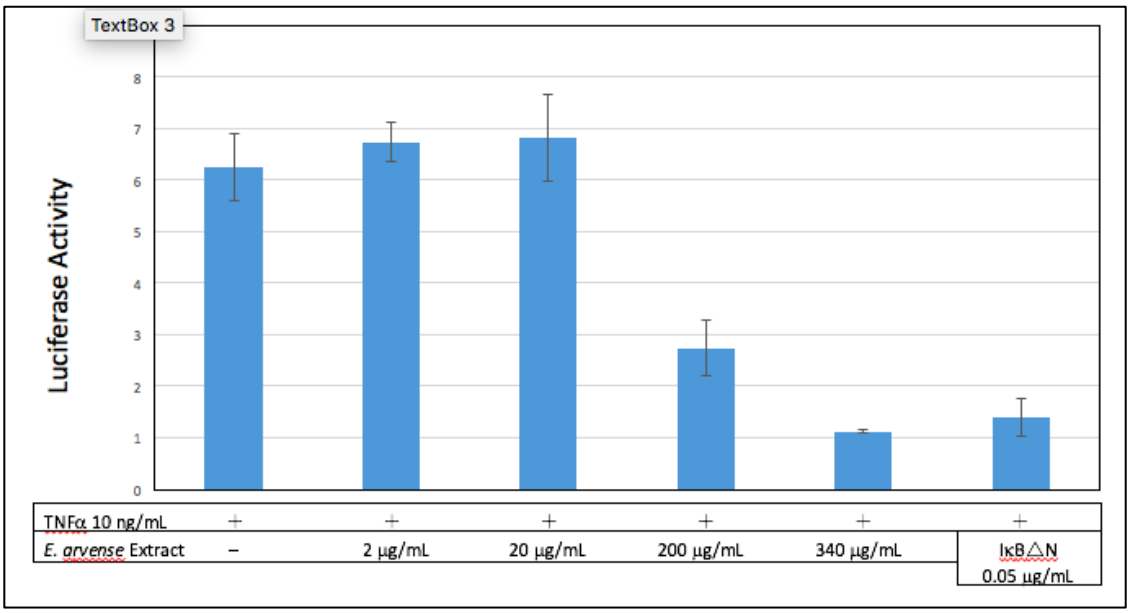


**Figure 4. *E. arvense* extract inhibits TNFα-induced activation of NF-κB.** 293HEK cells were transfected with PRDII-LUC and CMV-LUC as described in Methods After 48 hours, the cells were treated with differing concentrations of the *E. arvense* extract dissolved in 2% DMSO. After one hour, cells were then treated with TNFα. Five hours later, the cells were harvested and a luciferase assay was performed on the lysates. As a control, some cells were also transfected with IκBΔN and did not receive the extract.

*TNFα leads to the degradation of IκBα*

Many inflammatory signals, such as cytokines, can activate the NF-κB signaling pathway. Cytokines like TNFα activate NF-κB by inducing the degradation of its inhibitor, IκBα.[8] Therefore, we decided to look at the degradation of IκBα after stimulation of the NF-κB

activation pathway with TNFα. The amount of IκBα in the cell can be assessed using a Western blot. When treated with 10 ng/mL of TNFα at different time points (Figure 5), IκBα levels in the cell initially decreased. After 60 minutes IκBα levels increased due to a negative feedback loop because IκBα is one of the genes expressed by NF-κB-induced gene expression.[8] These results show that when treated with TNFα, IκBα is degraded as expected. This establishes a baseline to compare the effect of inhibitors of NF-κB.

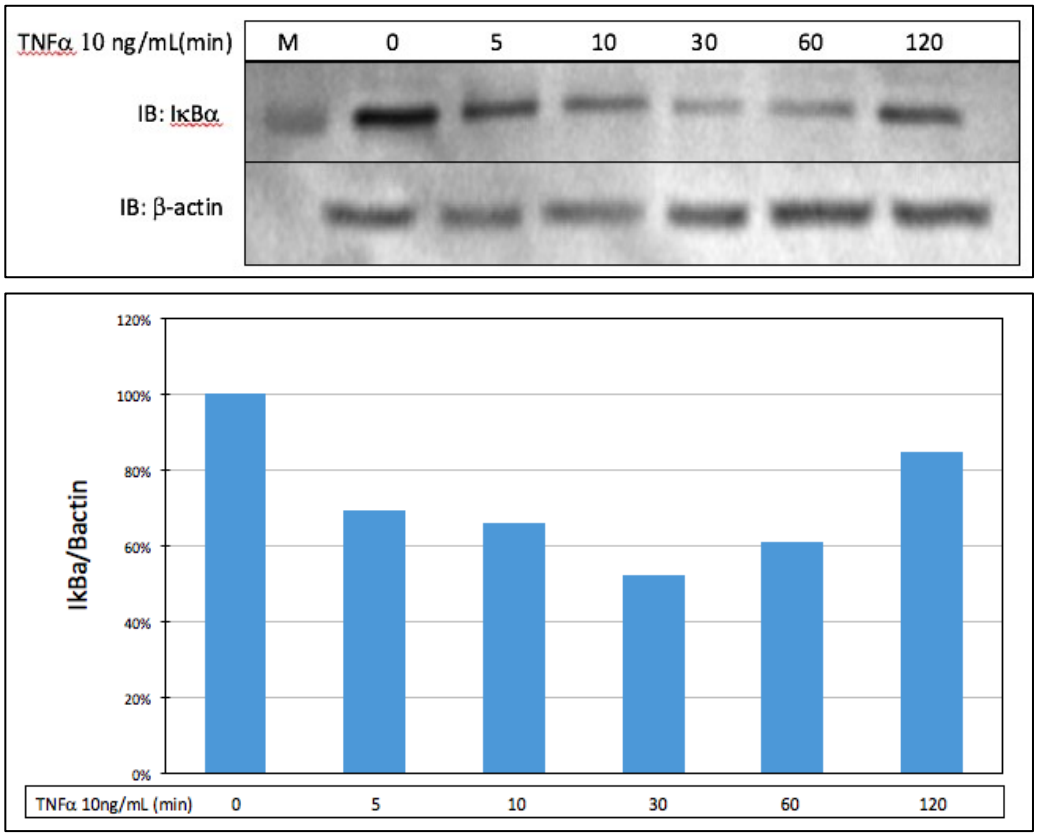


**Figure 2. Degradation of IκBα in the presence of TNFα. (A)** 293HEK cells were treated with TNFα at different time intervals. Cells were harvested at 120 minutes after the first treatment and then immunoblotted with the indicated antibodies. **(B)** The normalized density of the bands from **(A)**.

*Treatment with IκBΔN inhibits IκBα degradation*

IκBΔN is a dominant-negative mutant of IκBα. When the gene for IκBΔN is transfected into the cell, it binds irreversibly and dominantly to NF-κB, which subsequently cannot be

activated. As a control for inhibition of NF-κB, we tested the TNFα-induced degradation of IκBα in cells expressing with IκBΔN. As shown in Figure 6, IκBα levels did not decrease, but rather stayed constant, in cells transfected with IκBΔN and treated with TNFα. This is in part because of the overexpression of IκBΔN, which is picked up by the same antibody.

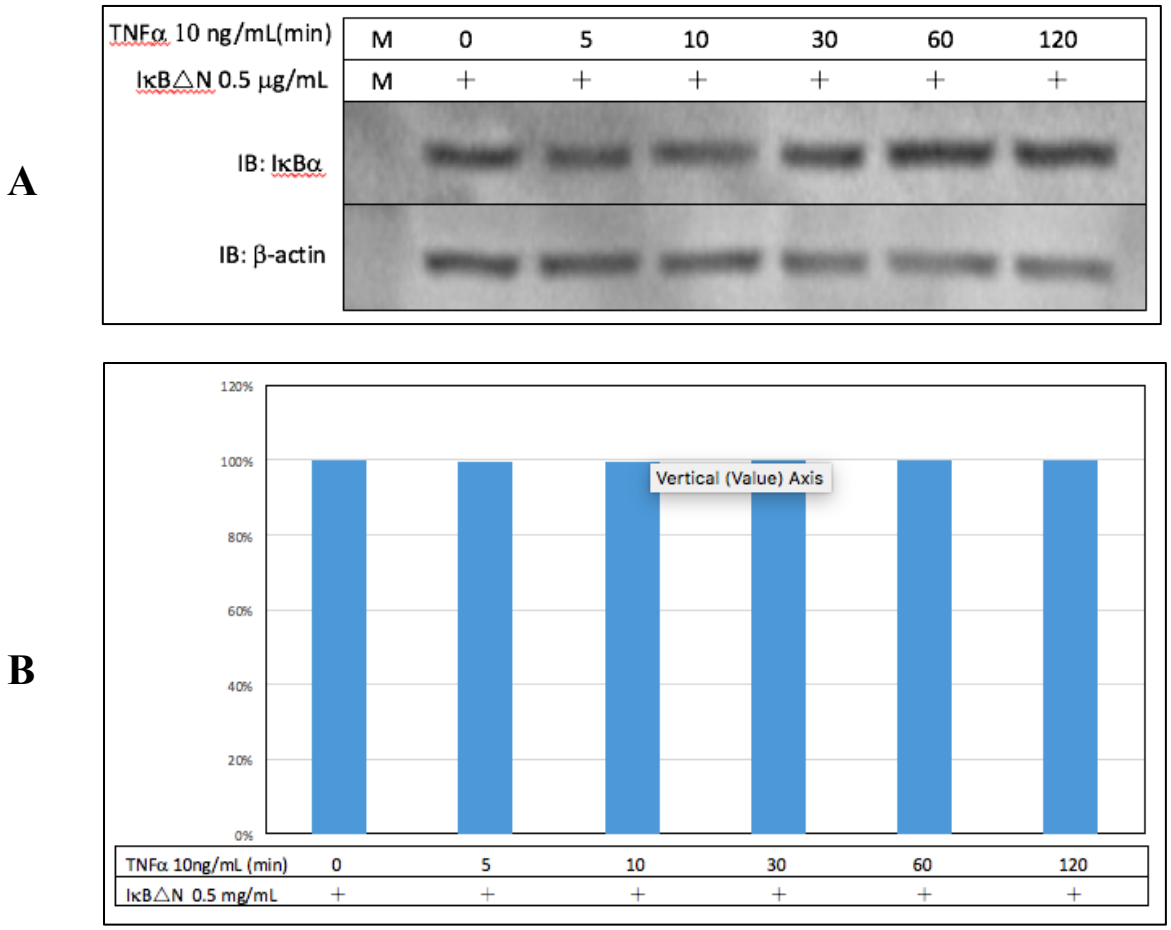


**Figure 3. IκBΔN blocked the degradation of IκBα. (A).** 293HEK cells were transfected with IκBΔN and then treated with TNFα after 48 hours at different time intervals. Cells were harvested at 120 minutes after the first treatment and then immunoblotted with the indicated antibodies. **(B)** The normalized density of the bands from **(A)**.

We are currently testing the effects of curcumin and the *E. arvense* extract on the TNFα-induced degradation of IκBα with a Western blot.

## DISCUSSION

Our goal was to test if an *E. arvense* extract can inhibit the TNFα-induced activation of NF-κB. To establish a control, we first tested the effects of diclofenac and curcumin on NF-κB activation. With differing concentrations, diclofenac, curcumin, and the *E. arvense* extract all inhibited TNFα-induced NF-κB activation as seen by a dose-dependent decrease in NF-κB sensitive promoter activation. As both diclofenac and curcumin are known to reduce inflammation, our results suggest that *E. arvense* might also exhibit anti-inflammatory properties by inhibiting NF-κB. We then showed that TNFα leads to the degradation of IκBα. Our results indicated that when provided an inflammatory signal, IκBα levels decreased in the cell, but were then restored.

Many studies have looked at the effects of inflammatory signals on IκBα degradation.[8] Much research has found that in response to stimuli like TNFα, phosphorylation of IκBα targets the protein for proteasomal degradation, which is required for the nuclear import of NF-κB. Generating a negative feedback loop, NF-κB drives the gene expression for IκBα, which restores IκBα levels in the cell.[8] Our results support these findings and illustrate the decrease and recovery of IκBα levels.

We then showed that diclofenac, a commercial NSAID used to treat inflammatory diseases such as pain and rheumatoid arthritis, inhibited the TNFα-induced activation of NF-κB. Most NSAIDS reduce inflammation by inhibiting cyclooxygenase enzymes, but studies have also found that Diclofenac inhibits NF-κB as well. Fredriksson et al. found that diclofenac inhibits IκBα phosphorylation, thus blocking NF-κB activation.[13] Aggarwal et al. also showed that diclofenac inhibits both TNFα-dependent IκBα degradation and NF-kB activation.[11] As our

results also showed, these findings suggest diclofenac acts as anti-inflammatory medication by blocking both COX-2 and NF-κB.

Since the *E. arvense* extract of interest in this study is a natural product, we tested the effects of another natural product, curcumin, on NF-κB activation. Similar to other studies,[11,14] we found that curcumin blocks the TNF$\alpha$-induced activation of NF-κB. Inhibition of NF-κB signaling could be the predominant method in which curcumin reduces inflammation. While diclofenac and other NSAIDS inhibit both COX and NF-κB, there is little research on the effects of curcumin on cyclooxygenase activity. Jurenka reports that COX-2 inhibition by curcumin is most likely accomplished due to the suppression of NF-κB activation.[14] That conclusion, along with our results from this study, support the idea that NF-κB signaling is a major pathway involved in inflammation, and inhibition of the pathway could be effective in reducing inflammation.

Our results show that a natural product, curcumin, can inhibit the TNFα-induced activation of NF-κB. While *E. arvense*, another natural product, has been used to treat inflammatory diseases, little is known about its mechanism of action. We found that an *E. arvense* extract inhibits the TNFα-mediated activation of NF-κB, and thus may exhibit its anti-inflammatory effects through this inhibition. There has been little research on the effects of *E. arvense* extracts specifically regarding NF-κB, but other studies suggest that the plant exerts a multitude of anti-inflammatory effects in the cell. Grundemann et al. found that *E. arvense* blocks T cell proliferation through inhibition of lymphocyte activation,[12] and studies by Jiang showed that a horsetail mixture reduces TNFα levels in the cell.[15] In conjunction with these studies, our results provide a mechanism in which *E. arvense* reduces inflammation. While the extract used in this study was a crude extract, the major components of an ethanol-water extract

of *E. arvense* have been identified. The three main compounds found in the extract include monocaffeoyl-tartaric acid, kaempferol-3-*O*-sophoroside-7-*O*-glucoside, and quercetin-3-*O*-glucoside.[12] Quercetin is believed to be the active compound in *E. arvense* and has been found to inhibit NF-κB activation. [16] It is also likely that quercetin inhibited TNFα-induced activation of NF-kB in our study. In the future, we would like to test how *E. arvense* inhibits NF-kB activation and its effects on virus-mediated activation of NF-κB. Advertised as an anti-inflammatory, *E. arvense* is sold on the market despite little information about its regulatory activity. It is important to know how these drugs, and other common medications, such as NSAIDS, behave in our body. This study shows that NSAIDS, natural products, and *E. arvense* may work as an inflammatory by inhibiting NF-κB signaling, and that inhibition of the NF-κB pathway, especially in regards to IκBα degradation, could further be exploited as an anti-inflammatory treatment option.

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