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Article

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Effects of Stable Degradation Products of Curcumin on Cancer Cell Proliferation and Inflammation

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Title running head: Curcumin degradation

Abstract

Curcumin is among the most promising dietary compounds for cancer prevention. However, curcumin rapidly degrades in aqueous buffer at physiological pH, making it difficult to understand whether the effects of curcumin are from curcumin itself or its degradation products. Here we studied the anti-proliferative and anti-inflammatory effects of curcumin degradation products, including its total degradation products (a mixture containing all stable degradation products of curcumin) and bicyclopentadione (a dominant stable degradation compound of curcumin). Curcumin potently modulated cell proliferation, progression of cell cycle, and apoptosis in MC38 colon cancer cells, and inhibited lipopolysaccharide (LPS)-induced inflammatory responses and NF-kB signaling in RAW 264.7 macrophage cells. In contrast, neither the total degradation products of curcumin nor bicyclopentadione had such effects. For example, after 24-h treatment in MC38 colon cancer cells, 5 μg/mL curcumin inhibited 39.2±1.8% of cell proliferation, while its degradation products were inactive. Together, these results suggest that the stable chemical degradation products of curcumin are not likely to play a major role in mediating the biological activities of curcumin.

Keywords

Curcumin; degradation; bicyclopentadione; cancer; inflammation

Introduction

Curcumin is among the most promising dietary compounds for cancer prevention and treatment [1]. Animal studies have shown that curcumin inhibits many types of cancers, including but not limited to pancreatic cancer [2], breast cancer [3], cervical cancer [4], lung cancer [5], and colorectal cancer [6]. Human studies also support the anti-cancer effects of curcumin. One Phase IIα human clinical trial has shown that a daily intake of 4 grams of curcumin for a month caused a ~40% reduction of aberrant crypt foci, a biomarker of colorectal cancer [7]. Another Phase II human clinical trial has shown that a daily intake of 8 grams of curcumin displayed anti-cancer efficacy in some patients with advanced pancreatic cancer [8]. Currently the anti-cancer effects of curcumin are being evaluated in many human clinical trials.

Until now, the molecular mechanisms of curcumin are not well understood. Recent studies showed that curcumin has a poor chemical stability at physiological pH: in aqueous solution at physiological pH, it has a half-life of several minutes, thus leading to the formation of various degradation products [9, 10]. These chemical degradation products include two classes of compounds: (1) alkaline hydrolysis products, where the hydroxyl ion (OH^-) attacks the carbonyl group of curcumin, generating hydrolyzed products such as ferulic acid, vanillin, ferulaldehyde, and feruloyl methane [11]; and (2) autoxidation products, where curcumin is first converted to a phenolic radical, which then migrates to the conjugated heptadienedione chain and initiates a chain reaction of curcumin degradation to generate cyclized compounds such as bicyclopentadione (BCP) [9, 10, 12]. Recent studies showed that the alkaline hydrolysis products are minor compounds of curcumin degradation products, and the autoxidation pathway is the dominant mechanism to mediate curcumin degradation [9, 10, 12]. When curcumin was added into mouse macrophage RAW 264.7 cells, the cellular level of BCP was increased by 1.8-fold, suggesting that chemical degradation could happen in biological systems [13]. Because of the short half-life of curcumin at physiological pH, it is difficult to understand whether the observed

biological effects of curcumin in cell culture experiments are from curcumin itself or its degradation products. With the presence of serum protein in cell culture medium, curcumin is more stable, but still could undergo degradation after long-term incubation [14]. Curcumin is much more stable in the presence of serum proteins: in cell culture medium containing 10% fetal calf serum and in human blood, the half-life of curcumin is > 8 hours [14]. Our recent research showed that co-addition of redox active antioxidants dramatically enhanced the chemical stability and biological activity of curcumin, suggesting that curcumin, but not its degradation products, is largely responsible for the observed effects [15]. Until now, the biological activities of the stable autoxidation products, such as BCP, are largely unknown. To this end, here we studied the comparative effects of curcumin and its stable autoxidation products on cancer and inflammation, in order to better understand the mechanism of curcumin.

Materials and Methods

Chemicals

To synthesize curcumin, boric anhydride (0.35 g, 5 mmol) and acetylacetone (1.03 mL, 10 mmol) in 50 mL anhydrous ethyl acetate was stirred at 50℃ for 30 min. Vanillin (3.04 g, 20 mmol) and tributyl borate (10.8 mL, 40 mmol) were added and the mixture was stirred at 50℃ for another 30 min. n-Butylamine (0.4 mL, 5 mmol) dissolved in 15 mL anhydrous ethyl acetate was added dropwise, and the reaction mixture was stirred at 80℃ for 4 hours and at room temperature overnight. To stop the reaction, 30 mL of 1N hydrochloric acid was added and stirred for 30 min. The reaction mixture was extracted with ethyl acetate and dried with anhydrous magnesium sulfate. Curcumin was obtained after recrystallization using methanol. The structure of synthesized curcumin was confirmed using high resolution ESI-MS and 'H NMR, as reported previously [16].

Preparation and analysis of total degradation products (TDP) of curcumin

A 1 L solution of 25 μM curcumin in 0.1 M phosphate buffer (2.59 g/L KH_2PO_4, 11.5 g/L Na_2HPO_4, pH=7.4) was stirred at room temperature for 5 days, then extracted with ethyl acetate. The organic layer, which is a mixture containing various curcumin degradation products, was dried and used as total degradation products (TDP) for the biological experiments.

TDP was dissolved in methanol and analyzed by HPLC on an Agilent 1100 HPLC system, using a 4.6 x 250 mm, 5 μm Supelco Nucleosil 100-5C18 column (Sigma-Aldrich, St. Louis, MO), with flow rate at 1 mL/min and detection wavelength at 254 nm. The mobile phase consisted of methanol with 0.1% acetic acid (mobile phase B) and water with 0.1% acetic acid (mobile phase A). The gradient was 20% solvent B increasing to 65% B in 15 min, increasing to 90% B in 3 min, kept at 90% B for 2 min, decreasing to 20% B in 2 min and kept at 20% B for 3 min.

Purification of bicyclopentadione (BCP) from TDP

BCP, the most abundant curcumin degradation compound, was purified from TDP using a Shimadzu 2020 preparative HPLC system equipped with automatic fraction collector (Shimadzu, Marlborough, MA). The HPLC condition was the same as described above. The fractions containing BCP were combined and extracted with ethyl acetate. The organic layer was dried over anhydrous magnesium sulfate and evaporated to dryness using a rotary evaporator. The purity and structure of isolated BCP was confirmed by HPLC and 'H NMR respectively, as described previously [13, 17].

Cell lines

MC38 colon cancer cells (a kind gift from Prof. Ajit Varki at the University of California San Diego [18]) and RAW 264.7 murine macrophage cells were used in cellular assays. Cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza, Allendale, NJ)

supplemented with 10% Fetal Bovine Serum (FBS, Corning Inc., Corning, NY) in a 37 ˚C incubator under an atmosphere with 5% CO_2.

Cell proliferation assay

MC38 cells (6000 cells/well) in DMEM medium were plated into 96-well plates and allowed to attach overnight. Cells were treated with 10-40 μg/mL test compound or DMSO vehicle (0.1%) for 24, 48, and 72 h. Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

Flow cytometry analysis of cell cycle and apoptosis

MC38 cells (200,000 cells/well) in DMEM medium were plated in 6-well plates and allowed to attach overnight. Cells were treated with 5 μg/mL test compounds or DMSO vehicle (0.1%) for 24 h. For cell cycle analysis, cells were collected, washed by ice-cold PBS, and fixed in 70% ethanol at -20°C overnight. Cells were then centrifuged, washed by PBS, and stained with propidium iodide solution (10 μg/mL propidium iodide, 100 μg/mL RNAse) for 30 min at room temperature. For apoptosis analysis, cells were collected, washed by ice-cold DMEM fortified with 2% FBS medium, centrifuged, and stained with propidium iodide and Annexin V solution (10 μg/mL propidium iodide, 0.5 μg/mL Annexin V) for 10 min at room temperature. After staining, cells were analyzed using the BD LSRFortessa™ cell analyzer (BD Biosciences) and data were processed using the FlowJo software.

Griess assay of LPS-induced nitric oxide (NO) production in RAW 264.7 cells

RAW 264.7 cells in 96-well plates were treated with 2.3-9.2 μg/mL test compounds and 0.5 μg/mL LPS for 24 h, then NO concentration in the cell culture medium was analyzed by a Griess assay as previously described [19], and cell proliferation was measured by MTT assay.

157 **Immunoblotting**

158 Immunoblotting was conducted using standard procedures. Briefly, after cellular treatments in
159 RAW 264.7 cells, the medium was decanted and cells were washed with cold PBS buffer then
160 lysed. Nuclear extracts were collected using a nuclear extraction kit (Cayman Chemicals, Ann
161 Arbor, MI) according to the manufacturer's instructions. Cell lysates were resolved using
162 SDS/PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 5%
163 BSA (Sigma Aldrich, St. Louis, MO) for 1 h at room temperature then probed with antibodies
164 against iNOS, COX-2, IκBα, phospho-IKK, NF-κB p65 (Cell Signaling Technology, Danvers,
165 MA), lamin A/C (Santa Cruz), and β-actin (Sigma-Aldrich). Membranes were then probed with
166 LI-COR IRDye® 800CW Goat anti-Rabbit and IRDye® 680RD Goat anti-Mouse secondary
167 antibodies. The Odyssey imaging system (LI-COR, Lincoln, NE) was used to detect the
168 antibodies. ImageJ software was used for western blot quantification.

169

170 **Data Analysis**

171 All cell culture experiments have been performed in at least three independent experiments.
172 The results are expressed as means ± standard deviation (SD), where each of the mean is
173 derived from an independent experiment. All statistical calculations were performed using
174 ANOVA followed by Tukey's honest significance difference test with the assistance of R
175 statistical software. If a significant difference among the interaction between treatment and dose
176 was found, the interaction was separated by either treatment or dose and analyzed again with
177 ANOVA followed by Tukey's. Statistical significance was considered significant at $p<0.05$.

178

179

180 **Results**

181 **Preparation of curcumin, total degradation products of curcumin, and BCP**

We chemically synthesized curcumin, since most commercial samples of curcumin contain other curcuminoids such as demethoxycurcumin and bisdemethoxycurcumin. The purity of the synthesized curcumin was >99% as assessed by HPLC and NMR. To study the biological activities of curcumin degradation products, we prepared the total degradation products of curcumin (TDP), which was a mixture containing all stable degradation products of curcumin after incubating curcumin in phosphate buffer, and BCP, which is was the most abundant degradation compound of curcumin and was purified from TDP by HPLC (**Figure 1**).

Curcumin degradation products have weaker anti-proliferative effects than curcumin

We compared the effects of curcumin, TDP, and BCP on cancer cell proliferation. MC38 colon cancer cells are among the most widely used cells to study colon carcinogenesis [18]. Curcumin potently inhibited MC38 colon cancer cell proliferation in a dose- and time-dependent manner: at a dose of 10 μg/mL, curcumin inhibited ~60% of MC38 proliferation after 24 h treatment, and inhibited >95% of MC38 proliferation after 48-72 h treatment (**Figure 2**). This is consistent with previous studies which showed potent anti-proliferative effects of curcumin in various cancer cells. In contrast to the potent action of curcumin, the degradation products of curcumin, including TDP and BCP, had dramatically reduced anti-proliferative effects. For example, TDP at a dose of 10 μg/mL had no inhibitory effect on MC38 proliferation after 24 h treatment, and only inhibited ~20% of cell proliferation after 48-72 h treatment. BCP was completely inactive to inhibit cancer cell proliferation in the tested dose range (10-40 μg/mL) after 24-72 h treatment (**Figure 2**). Together, these studies showed that curcumin, but not its stable autoxidation products, inhibited cancer cell proliferation.

Curcumin degradation products have weaker effects on cell cycle progression and apoptosis than curcumin

We further compared the effects of curcumin, TDP, and BCP on two critical processes involved in regulation of cell proliferation: the progression of cell cycle and apoptosis. After 24 h treatment, curcumin significantly induced G2 cell cycle arrest and apoptosis in MC38 colon cancer cells; in contrast, neither TDP nor BCP had such effects (**Figure 3**). These results further support that curcumin, but not its degradation products, played a major role in modulating cancer cell proliferation and associated cellular responses.

Curcumin degradation products have weaker anti-inflammatory effects than curcumin

We compared the effects of curcumin, TDP, and BCP on LPS-induced inflammatory responses in RAW 264.7 macrophage cells, which are widely used to study inflammatory responses. Consistent with previous studies, curcumin inhibited LPS-induced NO production in a dose-dependent manner. In contrast, TDP had a much weaker inhibitory effect and BCP had no effect (**Figure 4A**). Consistent with the NO result, curcumin dose-dependently inhibited LPS-induced expression of iNOS, which is the major enzyme involved in production of NO; while TDP or BCP had no effect (**Figure 4B**). We also found that curcumin, but not its degradation products, inhibited LPS-induced expression of inflammatory protein COX-2 (**Figure 4C**). Together, these results showed that curcumin, but not its degradation products, have anti-inflammatory effects.

Curcumin degradation products have weaker inhibitory effects on NF-kB signaling than curcumin

We compared the effects of curcumin, TDP, and BCP on LPS-induced NF-kB signaling in RAW 264.7 macrophage cells. Consistent with previous studies, curcumin inhibited LPS-induced IKK phosphorylation, IκBα degradation, and nuclear translocation of p65, demonstrating that curcumin inhibited LPS-induced activation of NF-kB signaling. In contrast, neither TDP nor BCP had such inhibitory effect (**Figure 5**). Together, these results further suggest that curcumin, but not its degradation products, have anti-inflammatory effects.

Discussion

Due to the rapid degradation of curcumin at physiological pH, recent research suggests that the resulted degradation products, including the reactive and unstable degradation products, could contribute to the observed biological activities of curcumin [20, 21]. Here in this study, we compared the anti-proliferative and anti-inflammatory effects of curcumin with its stable degradation products. Our central finding is that compared with curcumin, the stable degradation products of curcumin such as TDP and BCP have dramatically reduced biological effects in vitro, suggesting that these stable degradation products are not likely to play a major role in mediating the anti-proliferative and anti-inflammatory effects of curcumin.

Chemical degradation of curcumin in aqueous buffer occurs through multiple mechanisms. The first identified mechanism is the hydrolysis pathway: a hydroxyl ion in the aqueous buffer attacks the carbonyl group of curcumin and generates break-down products such as vanillin, ferulic acid and feruloyl methane [14]. Substantial studies have investigated the biological activities of hydrolysis-derived degradation products of curcumin, and found that these compounds such as vanillin and ferulic acid have dramatically reduced biological activities compared with curcumin. For example, in terms of cell proliferation in peripheral-blood mononuclear cells, the IC_{50} value of vanillin was ~260 times higher than that curcumin [22]. In addition, recent studies showed that the hydrolysis-derived compounds are minor degradation products of curcumin [13, 17, 21]. Therefore, it is unlikely that these hydrolysis-derived degradation products contribute to the biological activities of curcumin.

Besides the hydrolysis pathway, recent research showed that curcumin degradation is mainly mediated by a radical-dependent autoxidation pathway, leading to formation of cyclized compounds such as BCP [13, 17, 21]. The roles of the autoxidation pathway in biological activities of curcumin remain largely unknown, and here we studied the biological effects of stable

autoxidation-derived degradation products of curcumin. In our experiment, we treated cells with curcumin in cell culture medium containing 10% FBS, at this condition curcumin is relatively stable (half-life > 8 hours, based on previous study [14]). Therefore, the biological effects of curcumin observed in our study, in particular the NF-κB assays in which the cells were treated curcumin for 1-2 hours, were at least in part mediated by intact curcumin. Consistent with previous studies for the anti-cancer and anti-inflammatory effects of curcumin [23], our results showed that curcumin inhibited cell proliferation, and modulated cell cycle progression and apoptosis in MC38 colon cancer cells, and inhibited LPS-induced inflammatory responses (NO, iNOS, COX-2, and NF-kB signaling) in RAW 264.7 cells. In contrast, its stable autoxidation products such as TDP (mixed degradation products of curcumin, in which BCP is the most abundant compound) and purified BCP have dramatically reduced effects in these cellular assays. In particular, BCP, the most abundant compound in TDP, was completely inactive in these assays. TDP still has some effects to suppress cell proliferation and production of NO, this effect is at least in part mediated by the remaining curcumin. Previous studies have characterized the chemical components of curcumin degradation products in detail [13, 17, 21], though we need to address that due to the difference in terms of preparation method, the degradation products prepared by us could have some unidentified compounds. The different effects of curcumin and BCP are likely due to the presence and number of α,β-unsaturated carbonyl groups in their chemical structures. Curcumin has two α,β-unsaturated carbonyl groups, which are thiol-reactive and have been suggested to be the pharmacophore of curcumin; in contrast, BCP has no reactive α,β-unsaturated carbonyl group, which could in part explain why this compound has dramatically reduced anti-proliferative and anti-inflammatory effects compared with curcumin. Due to the lack of potent effects of TDP and BCP, it is not likely that these compounds play a major role in mediating the activities of curcumin. We need to point out that here we studied the anti-proliferative and anti-inflammatory effects of stable degradation products of curcumin. It is likely that during the radical-dependent autoxidation process of

curcumin, some highly reactive intermediates could be formed and could play a role in the biological activities of curcumin [21]. In fact, a study showed that curcumin alone or BCP could not increase topoisomerase II-mediated DNA cleavage. When combining curcumin and potassium ferricyanide, an oxidizing agent, cleavage was increased, suggesting that curcumin's biological effects could be due to reactive and unstable intermediates [24]. Further studies are needed to better characterize the molecular mechanisms of curcumin.

Our current finding is consistent with our recent study which showed that when curcumin degradation is suppressed, the anti-proliferative effects of curcumin are significantly enhanced [15]. Indeed, our recent study showed that the co-addition of a various redox active antioxidants, dramatically enhanced curcumin stability in aqueous buffer. We further showed that when MC38 cells are treated with curcumin in basal cell culture medium (without FBS), curcumin rapidly degraded and had a weak anti-proliferative effect while co-addition of these redox active antioxidants, which enhanced chemical stability of curcumin, significantly increased the anti-proliferative effect of curcumin [15]. Together, these results suggest that curcumin, but not its degradation products, has potent anti-proliferative effects. Co-addition of antioxidants increases levels of curcumin and reduces levels of its degradation products in cellular systems, leading to enhanced activities. It remains to test whether co-addition of antioxidants could be used as a practical strategy to enhance the effects of curcumin in cell culture and animal models.

In conclusion, here our results showed that the stable degradation products of curcumin, including TDP (mixed degradation products of curcumin, in which BCP is the most abundant compound) and purified BCP have dramatically reduced anti-proliferative and anti-inflammatory effects compared with curcumin, suggesting that these compounds are not likely to contribute to the effects of curcumin. A limitation of the study is that we only performed our study in cultured cells with short treatment time. It is important to validate the results in animal models, in

311 particular with long-term treatment of curcumin to mimic human consumption of curcumin.
312 Previous study has shown that long-term treatment of curcumin has potent biological effect on
313 inflammation, cancer, and immune responses [25-27]. Further studies are needed to understand
314 how curcumin exerts various beneficial effects despite its poor chemical and metabolic stability.
315

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Figure Legends

Figure 1. Preparation and analysis of curcumin, total degradation products of curcumin (TDP), and bicyclopentadione (BCP). (***A***) Scheme to prepare curcumin, TDP and BCP, and (***B***) HPLC analysis of curcumin, TDP, and BCP.

Figure 2. Curcumin degradation products have weaker anti-proliferative effects than curcumin. MC38 cells were treated with curcumin, TDP, BCP, or DMSO vehicle (0.1%) in complete DMEM medium for 24-72 h and cell proliferation was assessed by MTT assay. The results are expressed as % of cell viability of compound-treated cells to vehicle-treated cells, expressed as mean ± SD from at least three independent experiments. * $P < 0.05$.

Figure 3. Curcumin degradation products have weaker effects on cell cycle progression and apoptosis than curcumin. MC38 cells were treated with 5 μg/mL of curcumin, TDP, BCP, or DMSO vehicle (0.1%) in complete DMEM medium for 24 h, then cell cycle progression (***A***) and apoptosis (***B***) were analyzed. *Left*: representative images of flow cytometry analysis. *Right*: quantification of cell population. The results are mean ± SD from at least three independent experiments, * $P < 0.05$.

Figure 4. Curcumin degradation products have weaker anti-inflammatory effects than curcumin. RAW 264.7 cells were treated with curcumin, TDP, BCP, or DMSO vehicle (0.1%) and 0.5 μg/mL LPS in complete DMEM medium for 24 h. (***A***) NO in cell culture medium, the results are expressed as % of NO production of compound-treated cells to vehicle-treated cells. (***B-C***) expressions of iNOS and COX-2, *Top panel*: representative western blot, *Bottom panel*: quantitative analysis of protein expression, the results are mean ± SD from at least three independent experiments, * $P < 0.05$.

Figure 5. Curcumin degradation products have weaker inhibitory effects on NF-kB signaling than curcumin. RAW 264.7 cells were pre-treated with curcumin, TDP, BCP, or DMSO vehicle (0.1%) for 1.5 h, then stimulated with 0.5-2 μg/mL LPS in complete DMEM medium for 20-30 min. (***A***) representative western blots of phospho-IKK, IKBα, and nuclear p65, (***B***) quantitative analysis of protein expression, the results are mean ± SD from at least three independent experiments, * $P < 0.05$.

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Fig. 1

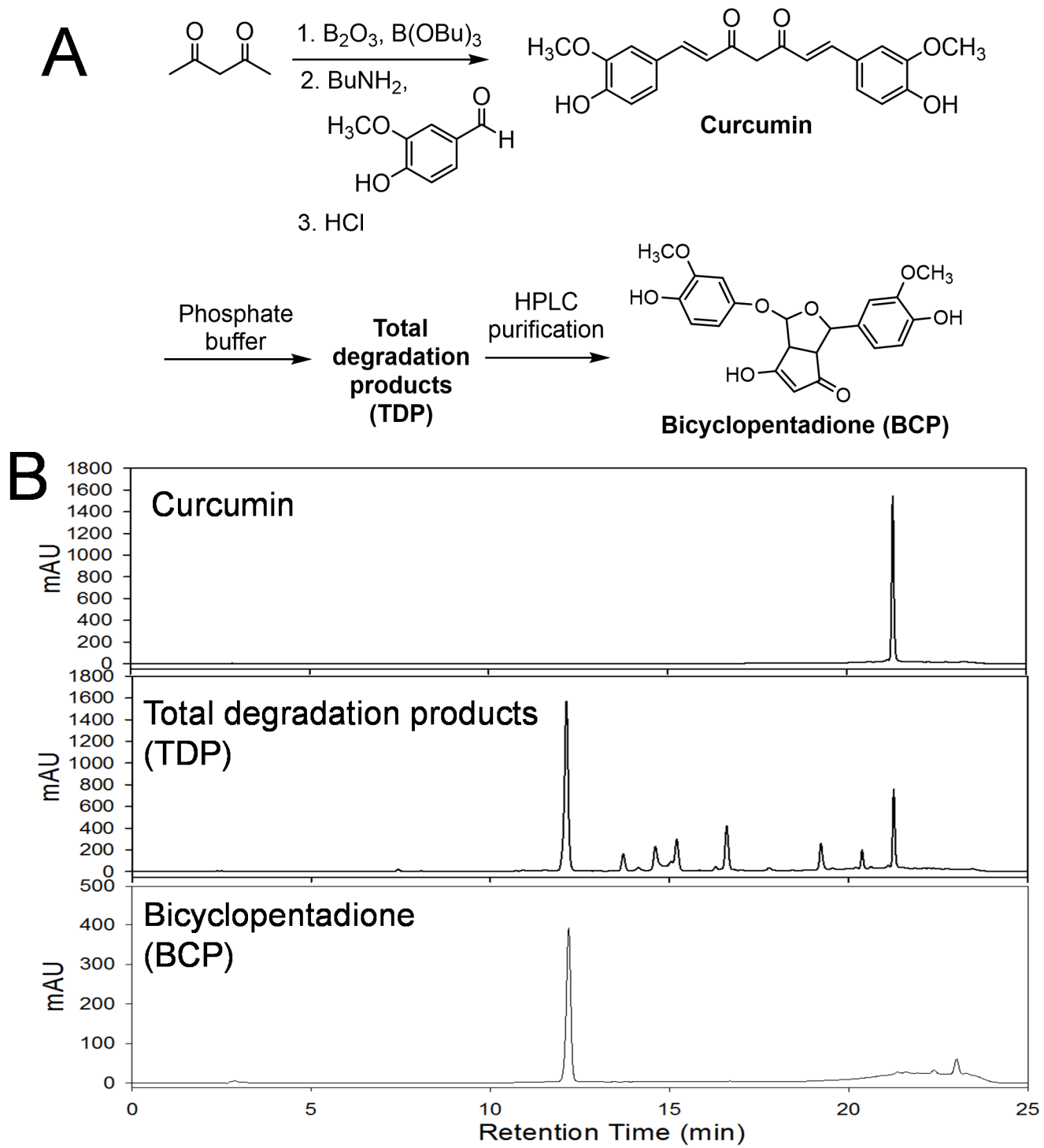

A
1. B_2O_3, $B(OBu)_3$
2. $BuNH_2$,
3. HCl
H_3CO
HO
OH
OCH_3
Curcumin
Phosphate buffer
Total degradation products (TDP)
HPLC purification
Bicyclopentadione (BCP)
B
Curcumin
Total degradation products (TDP)
Bicyclopentadione (BCP)
mAU
Retention Time (min)

Fig. 2

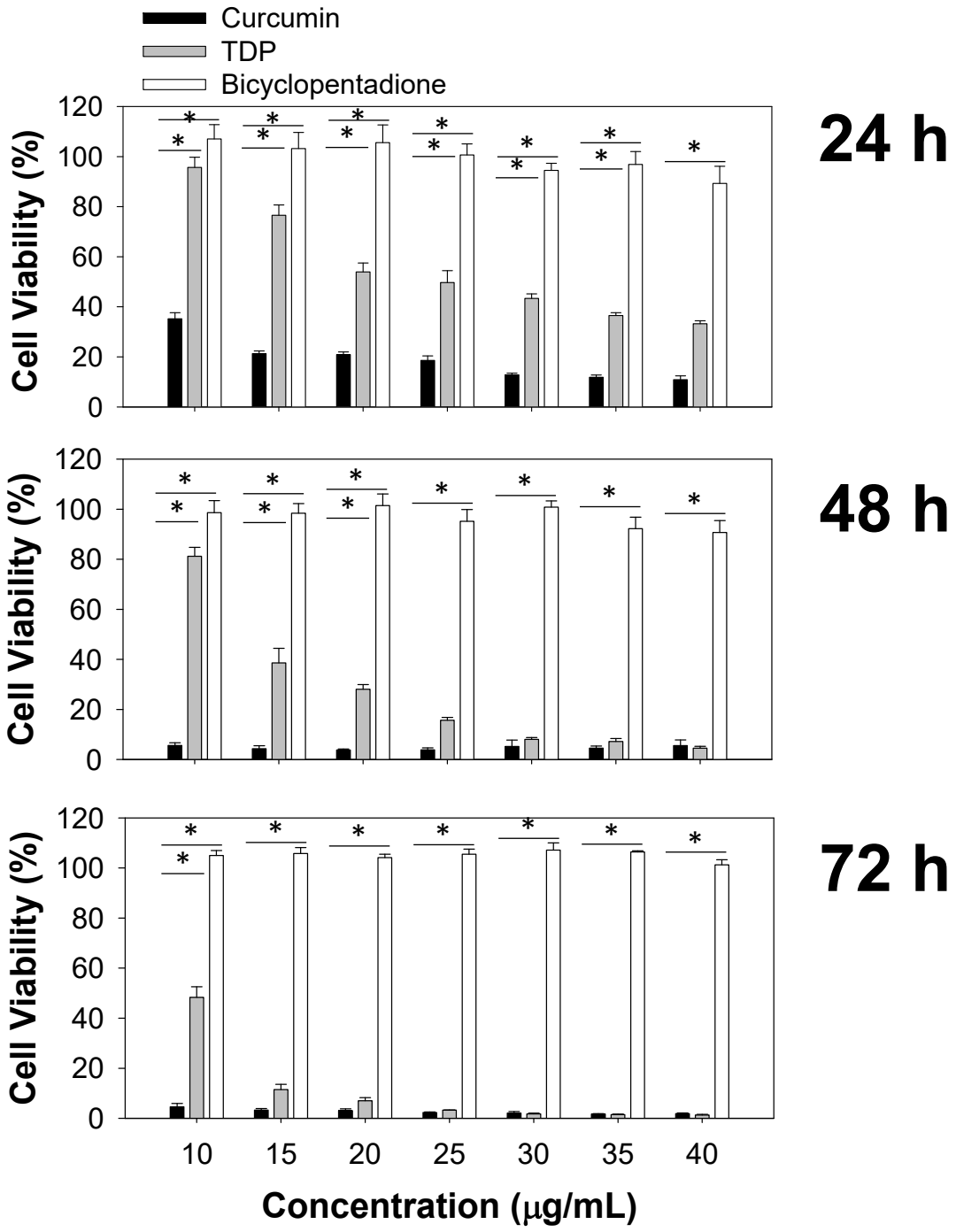

Curcumin
TDP
Bicyclopentadione
Cell Viability (%)
24 h
48 h
72 h
Concentration (μg/mL)
10
15
20
25
30
35
40

Fig. 3

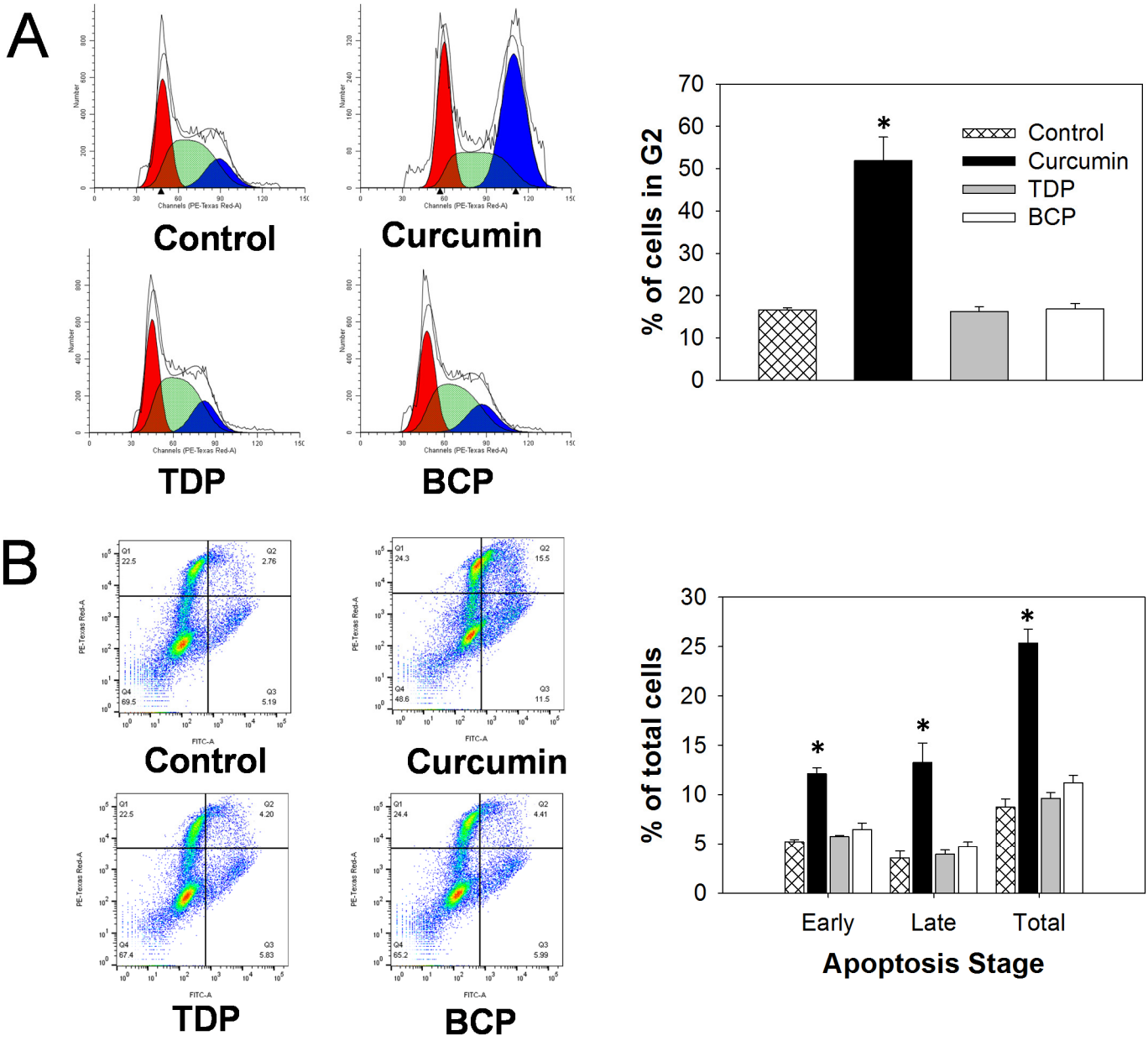

A
Control
Curcumin
TDP
BCP
% of cells in G2
Control
Curcumin
TDP
BCP
B
Control
Curcumin
TDP
BCP
% of total cells
Early
Late
Total
Apoptosis Stage

Fig. 4

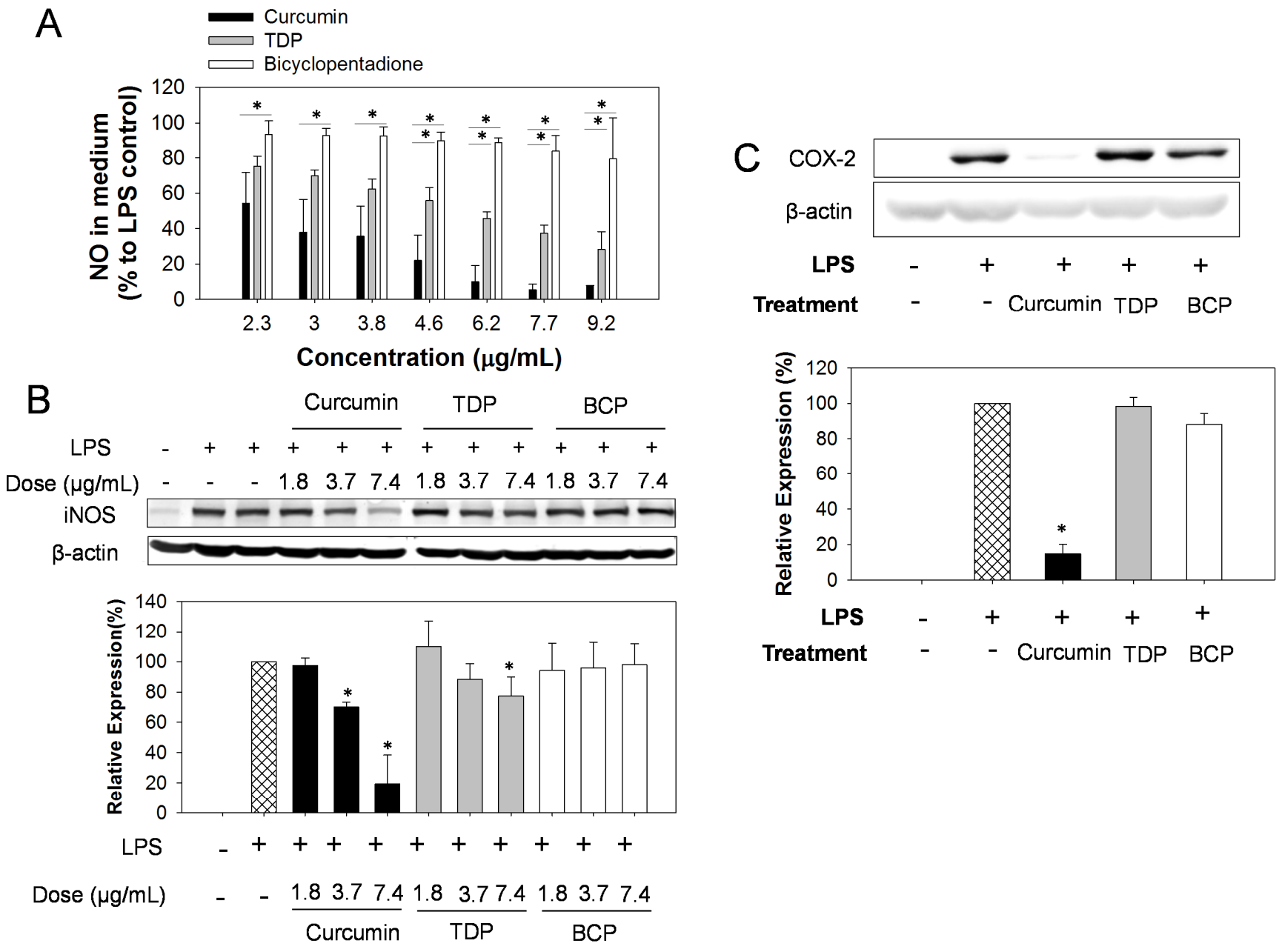
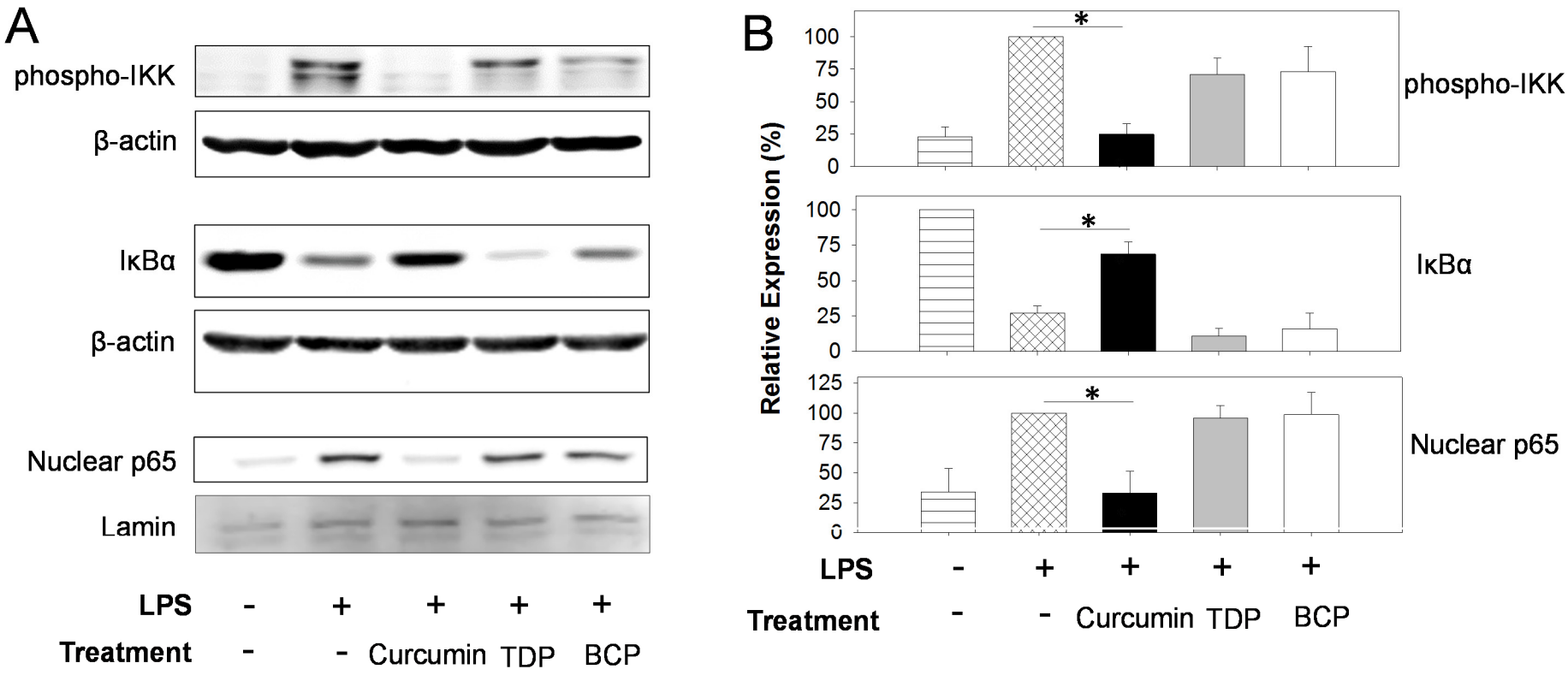
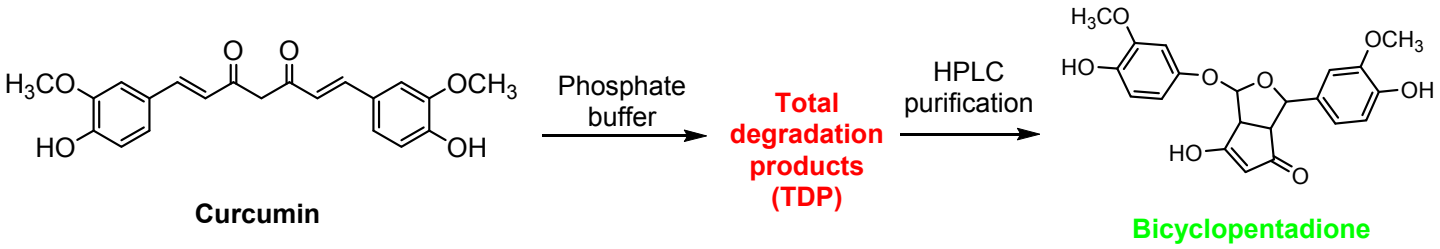
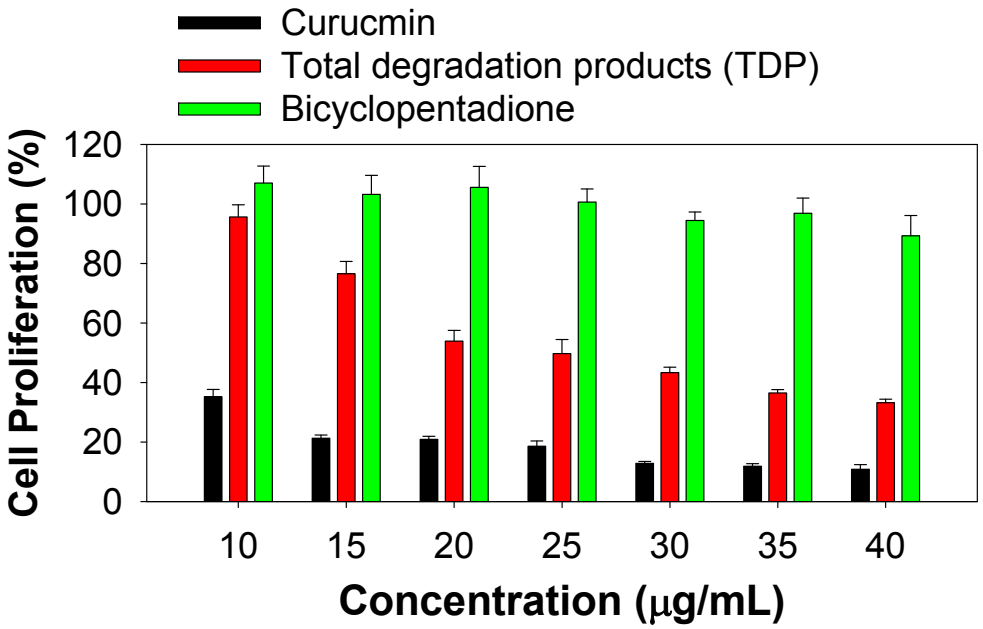

A
Curcumin
TDP
Bicyclopentadione
NO in medium (% to LPS control)
120
100
80
60
40
20
0
2.3
3
3.8
4.6
6.2
7.7
9.2
Concentration (μg/mL)
B
Curcumin
TDP
BCP
LPS
Dose (μg/mL)
1.8 3.7 7.4 1.8 3.7 7.4 1.8 3.7 7.4
iNOS
β-actin
Relative Expression(%)
140
120
100
80
60
40
20
0
LPS
Dose (μg/mL)
1.8 3.7 7.4 1.8 3.7 7.4 1.8 3.7 7.4
Curcumin
TDP
BCP
C
COX-2
β-actin
LPS
Treatment
Curcumin
TDP
BCP
Relative Expression (%)
120
100
80
60
40
20
0
LPS
Treatment
Curcumin
TDP
BCP

Fig. 5


A
phospho-IKK
β-actin
IκBα
β-actin
Nuclear p65
Lamin
LPS - + + + +
Treatment - - Curcumin TDP BCP
B
Relative Expression (%)
100
75
50
25
0
*
phospho-IKK
IκBα
125
Nuclear p65
LPS - + + + +
Treatment - - Curcumin TDP BCP

TOC


H3CO
HO
O
O
OCH3
OH
Curcumin
Phosphate buffer
Total degradation products (TDP)
HPLC purification
H3CO
HO
OCH3
OH
O
O
HO
O
Bicyclopentadione


Curucmin
Total degradation products (TDP)
Bicyclopentadione
Cell Proliferation (%)
120
100
80
60
40
20
0
10
15
20
25
30
35
40
Concentration (μg/mL)