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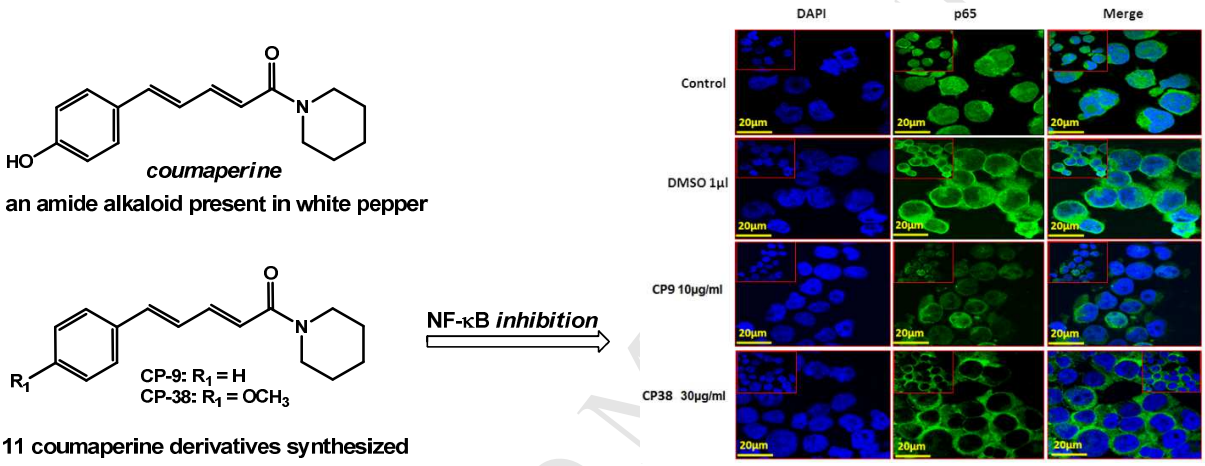
Graphical abstract

Coumaperine, a minor amide alkaloid, present in white piper, its analogs (11 derivatives) were synthesized in less number of synthetic steps and evaluated for NF-κB (a key transcription factor often associated with several inflammatory disorders and different types of cancer) inhibitory activity. They found to effectively inhibit NF-κB proteins in L428 cells in vitro, with a potential to be used as anti-inflammatory compounds.



Synthesis of Coumaperine derivatives: their NF-κB inhibitory effect, inhibition of cell migration and their cytotoxic activity

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Abstract

Coumaperine (an amide alkaloid, present in white piper) and its derivatives were synthesized and investigated for their cytotoxicity against ~~Hodgkin's lymphoma derived~~ L428 ~~cells~~ and A549 ~~lung adenocarcinoma~~ cells and their NF-κB inhibitory activity. It was found that the coumaperine derivatives CP-9 and CP-38 suppress NF-κB subunits p50 and p65 in nuclear fractions by western blot and by NF-κB luciferase reporter gene assay in a dose dependent

manner. Confirmation of these results was obtained by confocal microscopy. CP-9, CP-32 and CP-38 also exhibited dose dependent cell cytotoxicity in a L428 cells expressing constitutively active NF-κB and in A549 cells, with an IC50 value of 43.25 µg/ml, 0.39 µg/ml and 16.85 µg/ml respectively against L428 cells and 57.15 µg/ml, 69.1 µg/ml and 63.2 µg/ml respectively against A549 cells. In addition, the coumaperine derivatives show remarkable inhibitory activity on the cancer cell migration assay against A549 lung adenocarcinoma cells at the concentrations of 5 µg/ml, 10 µg/ml, and 5 µg/ml of CP-9, CP-32 and CP-38 respectively. Aromatic substituents and number of olefinic double bond ~~of~~ in coumaperine derivatives found to influence~~d~~ the inhibitory activity. In luciferase reporter gene assay, di-olefin conjugated coumaperine derivatives, CP-38, CP-32 and PIP exhibited higher inhibitory activity than their corresponding tri-olefin conjugated coumaperine derivatives, CP-102, CP-146 and PIP-155 respectively. CP-32 with a stronger electron donating group (-$N(CH_3)_2$) showed better ~~inhibitionry~~ inhibitory activity in luciferase reporter gene assay and in cell proliferation of L428 cells. Simple coumaperine derivative (CP-9, with no substituent) effectively inhibited A549 cells proliferation and migration than the other coumaperine derivatives. CP-9 and CP-38 diminish significantly the NF-κB subunits (p50 and p65) of L428 cells in nuclear fractions at the dosage of 10 µg/ml and 30 µg/ml respectively. Which clearly shows that CP-9 and CP-38 inactivate the NF-κB pathway in vitro.

Keywords

NF-κB; L428 cells; A549 cells; coumaperine; piperine; amide alkaloid.

1. Introduction

Nuclear factor-κB (NF-κB), a key transcription factor regulates various genes involves in inflammation, cell proliferation and apoptosis [1-5]. ~~Abnormal activation of NF-κB is often associated with several cancer cells [5] especially lymphoma cells [7]. Many synthetic and natural products small molecule inhibitors against NF-κB have been discovered, underscoring it as a validated drug target [7-10]~~. Typically, NF-κB exists in its inactive form as a protein complex with its inhibitor protein IkB in the cytoplasm of resting cells. Activation of NF-κB occurs through a cascade of pro-inflammatory events, leading to the translocation of NF-κB from the cytoplasm to nucleus either through the classical or alternative pathway [3, 9]. Dysregulation of NF-κB is witnessed in several diseased states, hence inhibition of these activation pathway through various synthetic and natural products have been developed, underscoring it as a validated drug target [7-10]. Since natural products have been the source of inspiration for various anticancer agents, it is not surprising that the inhibitors developed against NF-κB are enriched with several natural products including polyphenols (quercetin, curcumin, epigenin and resveratrol) and terpenoids (helenalin, parthenolide, costunolide and thionupharidines/thionuphlutidines) [9, 12]. Nevertheless, lack of any clinical candidate against this target further demand the identification of new chemical entities, especially natural product based inhibitors [7, 12]. Piperine, (**Figure 1**) an amide alkaloid, a major constituent of black *(Piper nigrum* Linn*)* and long (*Piper longum* Linn*)* pepper, was identified as a potent NF-κB inhibitor [13] in lung (adenocarcinoma derived A549 cells [14], and prostate cancer cells (LNCaP, PC-3 and DU-145) [15]. Furthermore, piperine is known to inhibit LPS-mediated activation of NF-κB proteins [16]. It is also know to exhibit a wide variety of biological activities [17-19], including antioxidant [20, 21], anti-inflammatory [22, 23], antimutagenic [24] and antitumor [25] activity. Coumaperine (**Figure 1**), a piperine type amide alkaloid present in white

piper (*Piper nigrum* Linn.) also possess interesting biological activities like hepatocarcinogenesis in rat models [26, 27]. Unlike piperine, coumpaperine is present in lower concentration [28], which makes its isolation not only challenging but also became a bottle-neck for various biological studies. In order to circumvent this situation, we developed an efficient method to access coumparine and its analogs and study their anticancer properties. Herein, we report the synthesis and cytotoxic effect of coumparine and its derivatives against L428 and A549 cancer cell lines and study their NF-κB inhibition pathway in vitro.

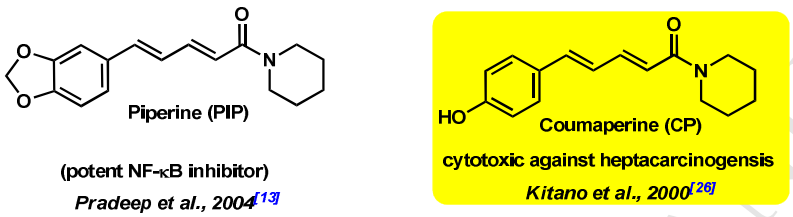


Figure 1. Structure of amide alkaloids, piperine and coumaperine found in *Piper* species

2. Materials and Methods

2.1 Materials

All commercially obtained reagents/solvents for the synthesis of coumaperine and its derivatives were used as received without further purification; chemicals were purchased from Spectrochem®, SRL®, Acros Organics®, RANKEM®, Fisher Scientific®. ~~and used as received without further purification~~. Unless stated otherwise, reactions were conducted in oven-dried glassware and under normal atmospheric conditions.

Antibodies against p65, Rel B, p50 and p52 were obtained from Santa Cruz Biotechnology, Anti-mouse and anti-rabbit IgG peroxidase was obtained from Jackson Immuno Research. The anti-β-actin monoclonal antibody from MP Biomedicals, Alexa flour®-488 conjugated goat anti-mouse

IgG and Cy^{TM}3 -conjugated goat anti-rabbit IgG were obtained from Molecular Probes Inc. and Jackson Immuno Research Laboratories Inc. respectively and DAPI was obtained from Sigma.

2.2 NMR, IR, High resolution mass spectrometry, and melting point characterization

^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker 500 MHz spectrometer operating with the ^{13}C resonance frequency of 125 MHz and proton resonance frequency of 500 MHz or Bruker 400 MHz spectrometer operating with the ^{13}C resonance frequency of 100 MHz and proton resonance frequency of 400 MHz. Data from the ^{1}H NMR spectroscopy are reported as chemical shift (δ ppm) with the corresponding integration values. Coupling constants (*J*) are reported in hertz (Hz). Standard abbreviations indicating multiplicity were used as follows: s (singlet), br (broad), d (doublet), t (triplet), q (quartet) and m (multiplet). Data from ^{13}C NMR spectra are reported in terms of chemical shift (δ ppm).

IR spectra were recorded in Thermo Scientific Nicolet Nexus 470 FT-IR spectrometer and band positions are reported in reciprocal centimeters. Samples were made as pellet with KBr and recorded.

High-resolution mass spectral data in Electrospray Ionization mode were recorded on Agilant 6520 (Q-TOF) mass spectrometer in positive (ESI+) ion mode.

Melting points were recorded with REMI DDMS 2545. The instrument is calibrated with benzoic acid before the measurement.

2.3 Cell culture

Hodgkin's lymphoma (HL)—derived L428 cells lack IκBα, therefore NF-κB is constitutively activated and expressed in the nucleus [6, 29]. The cells are maintained in RPMI 1640. The Lung Adenocarcinoma derived A549 cell lines were maintained and DMEM medium. Media was supplemented with 10% heat-inactivated fetal bovine serum, 1% L-glutamine, and 1% Pen- Strep (Beit Haemek, Israel). A549 cells were passaged by trypsinization.

2.4 Cell viability by XTT assay

Cell survival was measured by a tetrazolium-formazan XTT assay kit (Beit Haemek) in 96-well plates. 3 x 10^4 L428 cells/well in 200 µl medium were treated with different concentrations of coumaperine derivatives for 48 h at 37 °C. Similarly, 3 x 10^4 ~~Lung adenocarcinoma~~ A549 cells were seeded in 96-well plates. After the formation of monolayer (24 h) the cells were treated with different doses of coumaperine derivatives. XTT solution (40 µl) was added, and the plates were again incubated for 4 h at 37 °C. Absorbance was read at 450 nm by an ELISA reader.

2.5 Luciferase-NF-κB reporter gene assay

L428 cells stable transfectants with the luciferase NF-κB-Luc reporter gene were generated as described in Ozer *et al.,* 2009 [12] and were maintained in 500 µg/ml of G418. L428 cells (10^6 per well) expressing the luciferase-NF-κB reporter gene were incubated in 1 ml of medium containing the solvent (DMSO) with different concentrations of coumaperine derivatives for 2 h. Cells were then harvested, lysed and monitored by a luciferase reporter assay kit (Promega) according to the manufacturer's instructions. Measurements were carried out using a luminometer at 300 nm. Data were normalized to the protein concentration in each lysate as measured by the Bradford method (BioRad).

2.6 Cell migration assay

The metastatic potential of the cells depends on other properties such as on their migration ability. To investigate how coumaperine derivatives affect the cell migration, A549 human lung carcinoma cells were seeded on 6-well plates without coumaperine derivatives. After the formation of the monolayer (24 h), coumaperine derivatives and the vehicle were added and the monolayers were scratched. After 48 h the results were recorded.

2.7 Western blot analysis

Nuclear and cytoplasmic protein lysates (10 x 10^6 cells per sample) were prepared. Briefly, 10 x 10^6 cells per samples were incubated for 2 hours with different concentrations of coumaperine derivatives, after incubation, cells were collected by centrifuged at 1000 g for 5 min at 4 ˚C. Then the cell pellet was washed with 1x PBS for 3 times. Then protease inhibitor 3.2 mg/ml (PI cocktail tablets, Roche) was added to the cells in Buffer A, containing Tris 1M, NaCl 5M, EDTA 0.5 M and allowed in ice for 15 min. Then the lysates were sheared several times through a 21 gauge needle, followed by centrifugation at 13,000 g for 10 min at 4 ˚C. The supernatant (cytosolic fraction) was collected and stored at -80 ^{o}C, then remaining cell pellet was washed again with 1x PBS for 3 times at 2000 g for 5 min at 4 ˚C. Then 40 µl buffer B (Tris 1M, NaCl 5M, $MgCl_2$) was added and kept in ice for 3 min, then 10 µl of 5M NaCl was added, allowed in ice for 25 min, and centrifuged at 13,000 g for 30 min 4 ˚C. The supernatant was collected (nuclear fraction). Protein quantification of lysates was determined by the Bradford method (BioRad). RIPA lysis buffer containing (10 mM Tris pH 8.0, 100 mM NaCl, 5 mM EGTA, 0.1% SDS, 1% NP-40, 45 mM β-mercaptoethanol, 50 mM NaF) was added to lysates after the quantification of protein and Protein lysates (40µg) were separated in 10% SDS-polyacrylamide

gel and blotted onto nitrocellulose membranes. The membranes were incubated with several primary antibodies and subsequently with peroxidase-linked anti-mouse or anti-rabbit IgG. Protein bands were detected by chemiluminescence with ECL (Amersham).

2.8 Immunocytochemistry

Cellular localization of NF-κB subunits in treated L428 cells was done in experiments where the cells were incubated for 2 h at 37 °C with coumaperine derivatives CP-9 and CP-38. After incubation, the cells were cyto-centrifuged (Shandon Cytospin 4) at 900 rpm for 5 min and fixed in 8% formalin for 20 min. Samples of these cells were incubated separately with antibodies against p65 and p50 and then with their respective fluorescent anti-mouse or anti-rabbit IgG-peroxidase-linked secondary antibody, and the nucleus was stained with DAPI and the signals were detected using Olympus FV1000-IX81 confocal microscope.

2.9 Statistical analysis

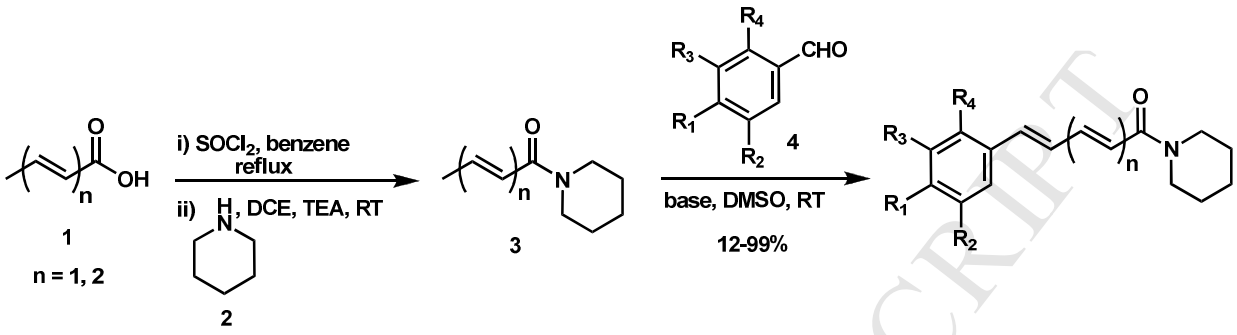
The results are representatives of three independent experiments. For reporter gene assay, each sample was tested in triplicate.

3. Results

3.1 Synthesis of coumaperine and its derivatives

In order to generate coumaperine derivatives for structure activity relationship studies, it is necessary to have a robust methodology for the preparation. Methodologies reported in the literature for the preparation of coumaperine, and its derivatives were associated with several limitations: A number of synthetic steps to the target, expensive reagents, or tedious synthetic

procedure [30-33]. In order to have easy access to coumaperine and its derivatives, we modified the literature procedure [34] to overcome the aforementioned difficulties.



When n = 1
CP = R_1 = OH, R_2 = H, R_3 = H, R_4 = H
CP-9 = R_1 = H, R_2 = H, R_3 = H, R_4 = H
CP-10 = R_1 = OCH_3, R_2 = OCH_3, R_3 = H, R_4 = H
CP-27 = R_1 = OCH_3, R_2 = OCH_3, R_3 = OCH_3, R_4 = H
CP-32 = R_1 = $N(CH_3)_2$, R_2 = H, R_3 = H, R_4 = H
CP-38 = R_1 = OCH_3, R_2 = H, R_3 = H, R_4 = H
CP-50 = R_1 = OCH_3, R_2 = H, R_3 = OCH_3, R_4 = OCH_3
CP-158 = R_1 = OAc, R_2 = H, R_3 = H, R_4 = H
CP-184 = R_1 = Ocyclopentyl, R_2 = H, R_3 = H, R_4 = H
PIP = R_1 = R_3 = -OCH_2O-, R_2 = H, R_4 = H

When n = 2
CP-102 = R_1 = OCH_3, R_2 = H, R_3 = H, R_4 = H
CP-146 = R_1 = $N(CH_3)_2$, R_2 = H, R_3 = H, R_4 = H
PIP-155 = R_1 = R_3 = -OCH_2O-, R_2 = H, R_4 = H

Scheme 1. General reaction scheme for the synthesis of coumaperine and its derivatives.

Coumaperine and its derivatives were prepared from crotonic acid (or sorbic acid), piperidine and benzaldehyde (or substituted benzaldehyde). At first, crotonic acid (or sorbic acid) is converted to the corresponding acid chloride by refluxing over thionyl chloride and reacted with piperidine to form crotonyl piperidine amide (**3**). It is then reacted with benzaldehyde (or substituted benzaldehyde) in the presence of a base (KOH or *t*-BuOK), to achieve the target molecule (detail synthetic procedure is given in ESI). Following the above described procedure, coumaperine and its derivatives, shown in **figure 2** were synthesized. Attempt to synthesize the nitro derivative was not successful. Changing the reaction conditions *viz* performing the reaction at elevated temperature (90-110 ˚C), with different bases (K_2CO_3 or NaH) or solvents (DMF,

toluene or NMP) were not fruitful. Piperine (PIP) was isolated from black pepper by following the modified literature procedure [35]. ~~A modified literature procedure [35] was adopted for the extraction~~. All the compounds were characterized by spectroscopic techniques (^{1}H NMR, ^{13}C NMR, IR and GC-MS). The unreported compounds, CP-27, CP-50, CP-184 and CP-146 were additionally characterized by HRMS.

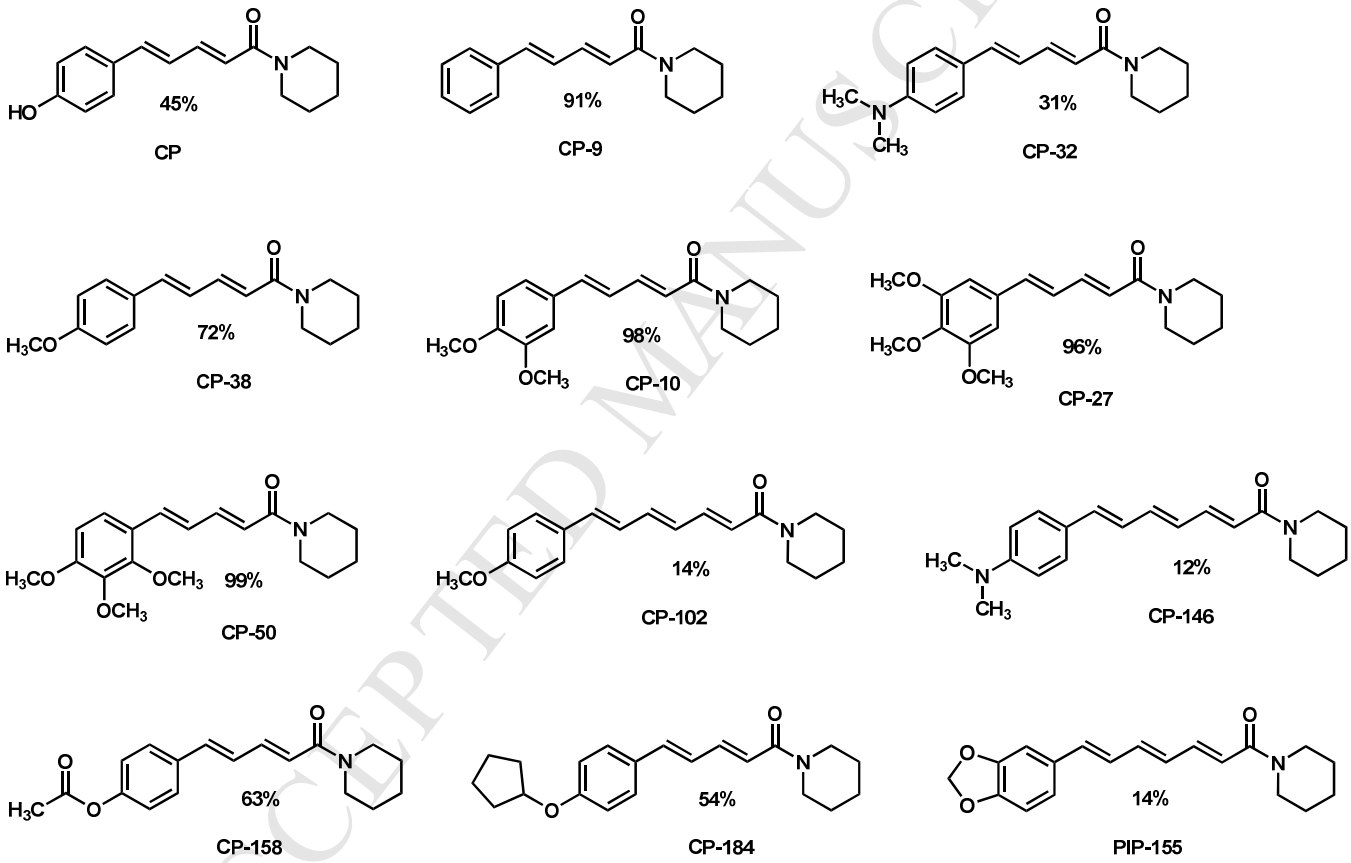


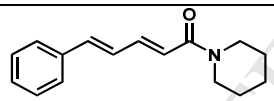
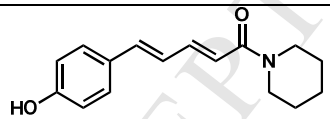
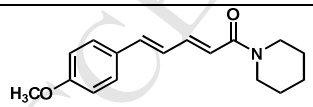
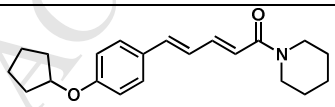
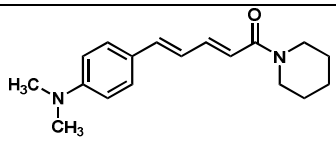
Figure 2. Coumaperine derivatives synthesized.

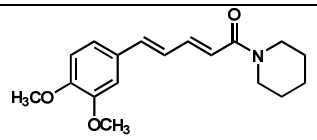
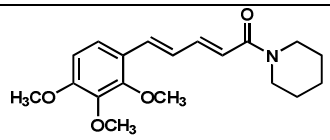
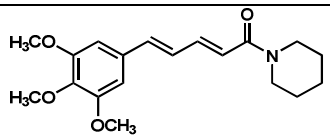
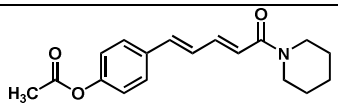
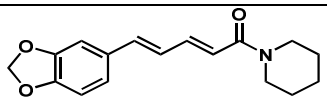
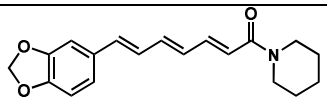
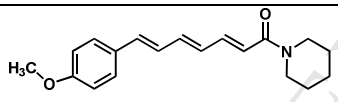
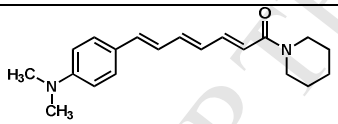
3.2 Screening ~~of~~ coumaperine derivatives for inhibition of NF-κB in a reporter gene assay.

In order to understand whether coumaperine and its derivatives inhibit the NF-κB in reporter gene assay, different concentration of coumaperine and its derivatives (10-50 µg/ml) were

incubated with L428 cells constitutively expressing NF-κB and the luciferase- NF-κB reporter gene for 2 h. A dose dependent inhibition of NF-κB was observed (**Table 1**).

Table 1. Effect of coumaperine derivatives on NF-κB luciferase reporter gene assay. Triplicate wells of L428 cells expressing the luciferase-NF-κB reporter gene were incubated with Coumaperine derivatives or with solvent and a positive control, Nuphar extract [12] for 2 h. The cells were then harvested and lysed; cell extracts were monitored by a luciferase reporter assay kit. NF-κB-luciferase activity in control cells was taken as 100%. The average and standard deviation of three independent experiments are shown. CP-9, CP-38 and CP-32 showed the better inhibition at lower doses.

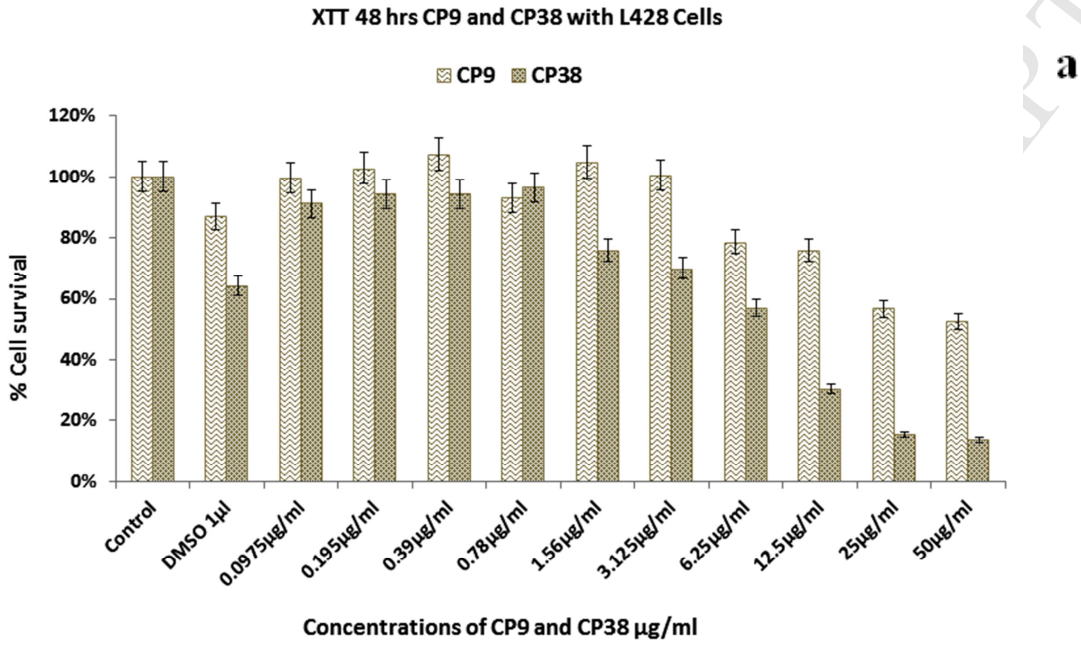
Compound code	**Structure**	**% NF-κB inhibition–Luciferase activity Concentration of coumaperine derivatives in µg/mL**				
		10µg/mL	**20µg/mL**	**30µg/mL**	**40µg/mL**	**50µg/mL**
CP-9		71.9%	73.9%	82.2%	85.9%	88.2%
CP		68.4%	79.6%	89.8%	91.5%	92.2%
CP-38		69.7%	76.3%	77.2%	86.9%	89.9%
CP-184		57.6%	61.7%	63.2%	69.7%	72.2%
CP-32		95.5%	96.6%	97.3%	97.7%	97.9%

CP-10		43.4%	67.5%	73.92%	78.9%	80.1%
CP-50		39.2%	52.9%	59.2%	59.8%	62.9%
CP-27		34.1%	44.6%	53.1%	56.9%	59.8%
CP-158		70.3%	71.4%	75.3%	78.1%	80.1%
PIP		67.3%	67.5%	85.1%	86.6%	90.2%
PIP-155		34.0%	56.6%	65.8%	71.0%	71.2%
CP-102		26.4%	34.0%	44.8%	50.7%	51.8%
CP-146		37.2%	56.2%	56.8%	57.2%	59.9%

3.3 Cytotoxic effect of coumaperine derivatives CP-9, CP-32, CP-38 on cell proliferation

To investigate the potential inhibition of coumaperine derivatives on cancer cell proliferation, they were incubated with L428 and A549 cells in triplicate for 48 h and the treatment was evaluated using the XTT assay. Control cells represent 100% survival. The average and standard deviation of three independent experiments are shown (in **Figures 3a-b**). Coumaperine derivatives CP-9, CP-32 and CP-38 markedly inhibited cell proliferation in a dose dependent

manner against L428 cells with the IC50 value of 43.25 µg/ml, 0.39 µg/ml and 16.85 µg/ml respectively. The IC50 values against A549 cells were found to be at the concentrations of 57.15 µg/ml, 69.1 µg/ml and 63.2 µg/ml respectively (**Figures 4a-b**).



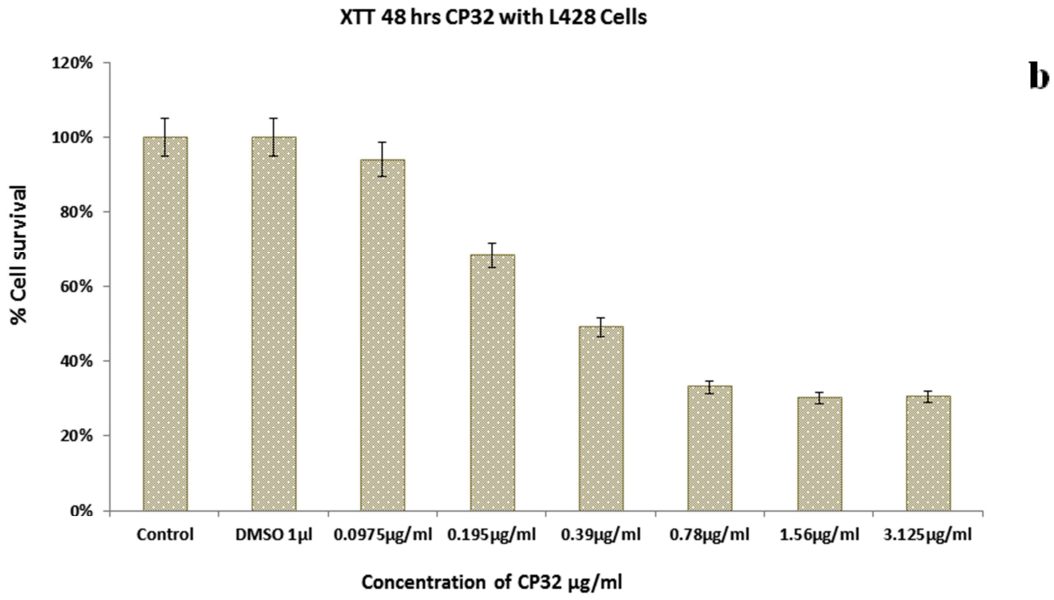
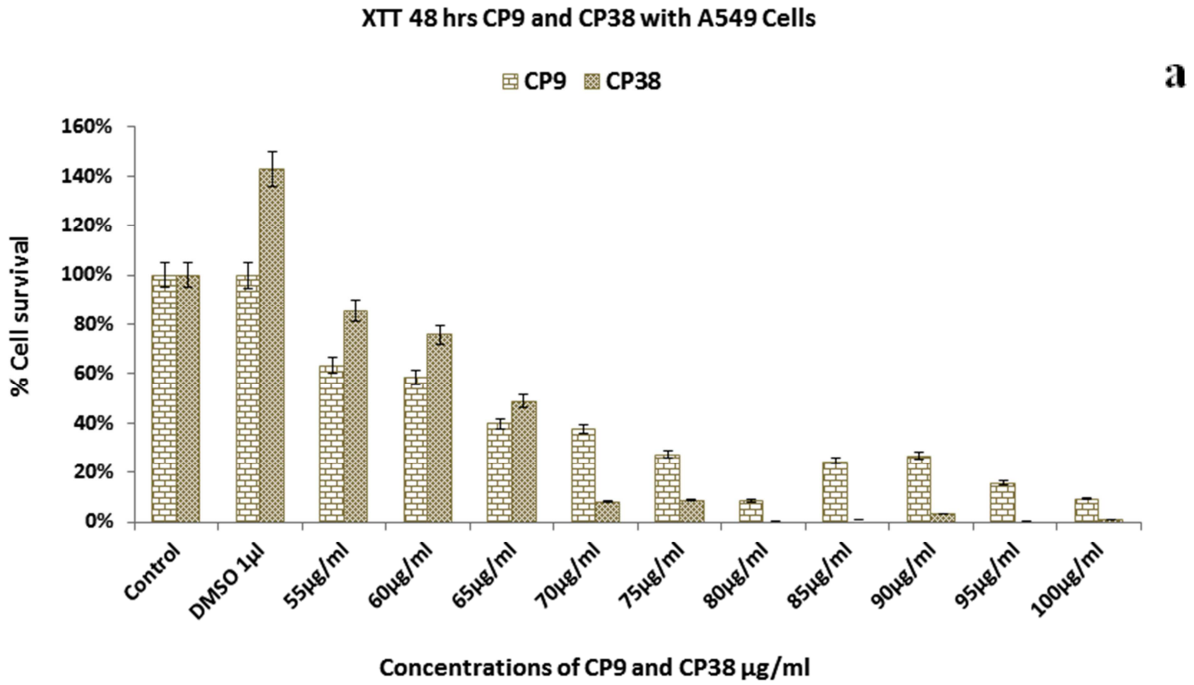


Figure 3. Cytotoxic effect of coumaperine derivatives a) CP-9 and 38; b) CP-32 against L428 cell proliferation. L428 cells were incubated for 48 h in triplicate with different concentrations of coumaperine derivatives. Cell survival was measured by a tetrazolium-formazan XTT assay kit. Control cells represent 100% survival. The average and standard deviation of three independent experiments are shown.



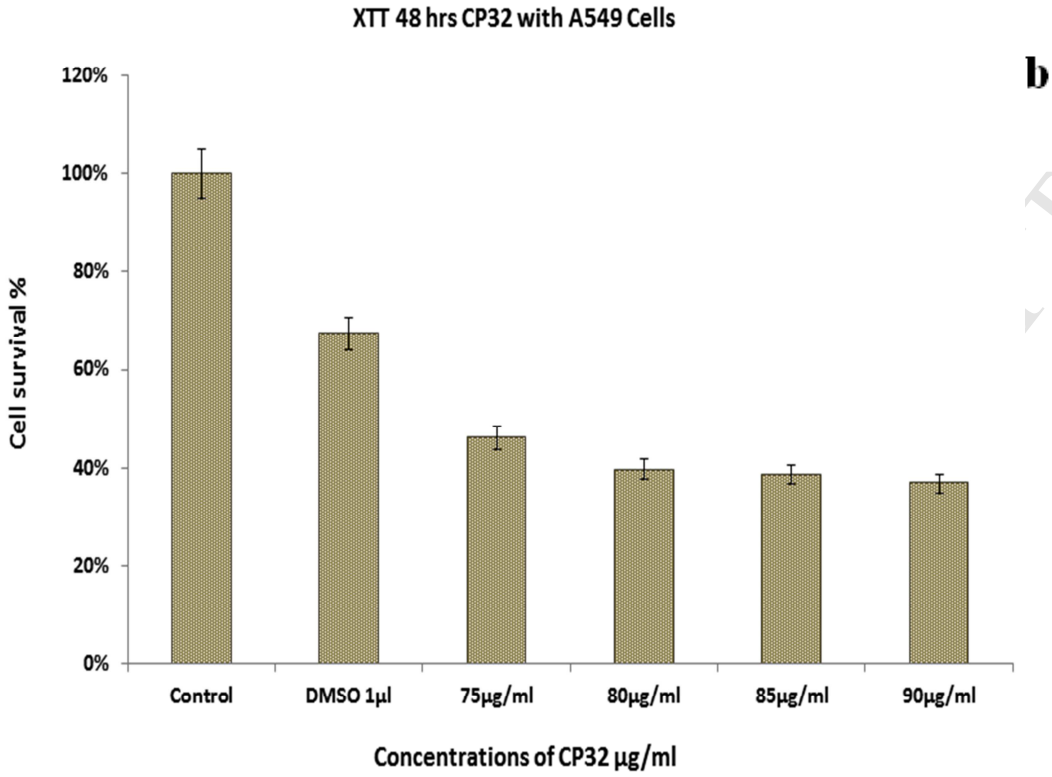


Figure 4. Cytotoxic effect of coumaperine derivatives a) CP-9 and 38; b) CP-32 on A549 cell proliferation. A549 cells were seeded and incubated for 24 h to form a monolayer. Then the cells were incubated for 48 hrs in triplicate with different concentrations of coumaperine derivatives. Cell survival was measured by a tetrazolium-formazan XTT assay kit. Control cells represent 100% survival. The average and standard deviation of three independent experiments are shown.

3.4 Effect of Coumaperine derivatives CP-9, CP-32 and CP-38 on the inhibition of A549 cell migration

In the cell migration assay, the vehicle (DMSO) treated cells mostly closed the wound after 48 h incubation, in contrast to coumaperine derivatives treated cells, where wound closure was prevented (**Figure 5a-c**) at the concentration of 5µg/mL, 10µg/mL and 5µg/mL of CP-9, CP-32,

and CP-38 respectively which clearly shows that these compounds inhibited A549 cell migration in culture.

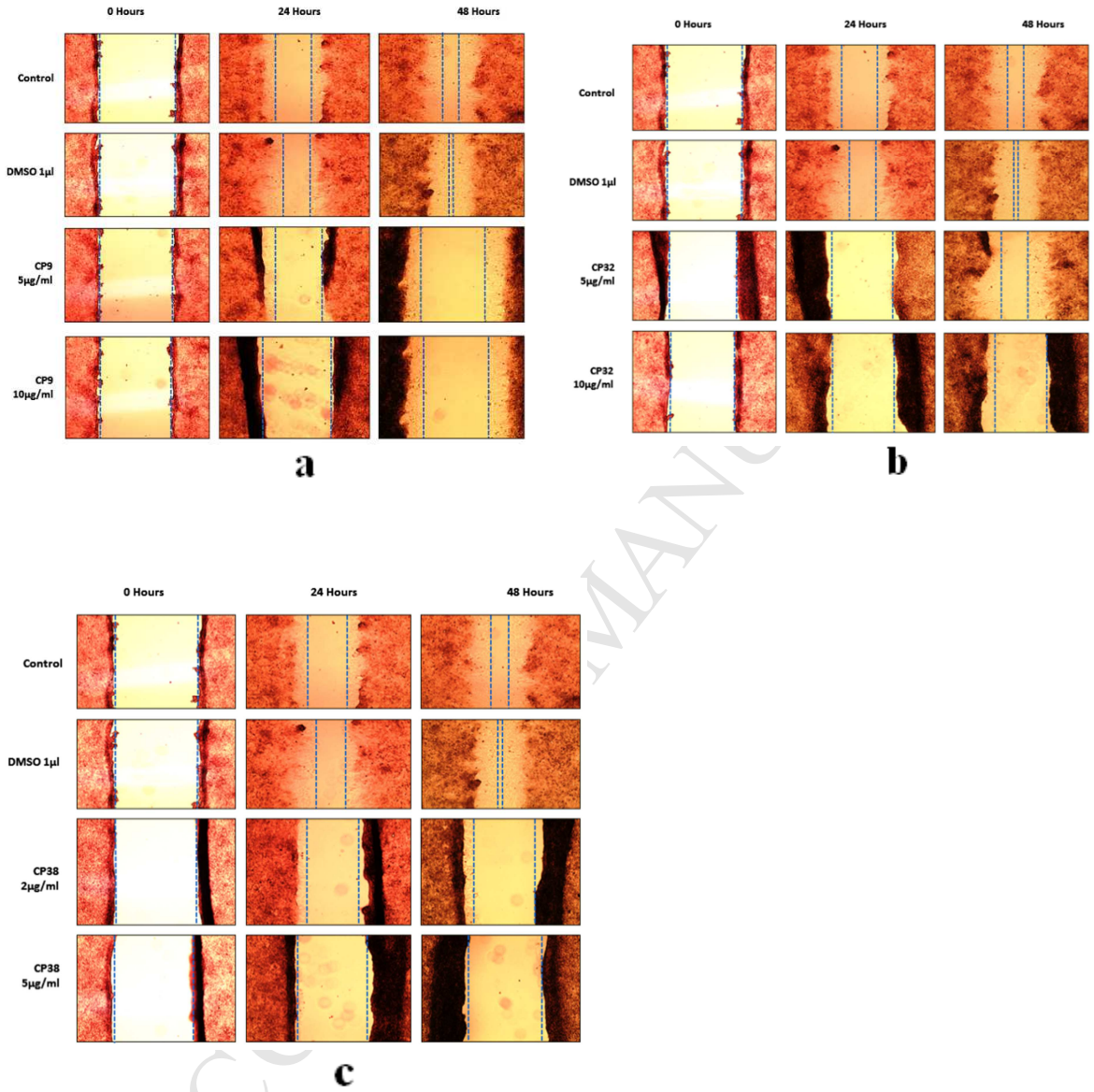
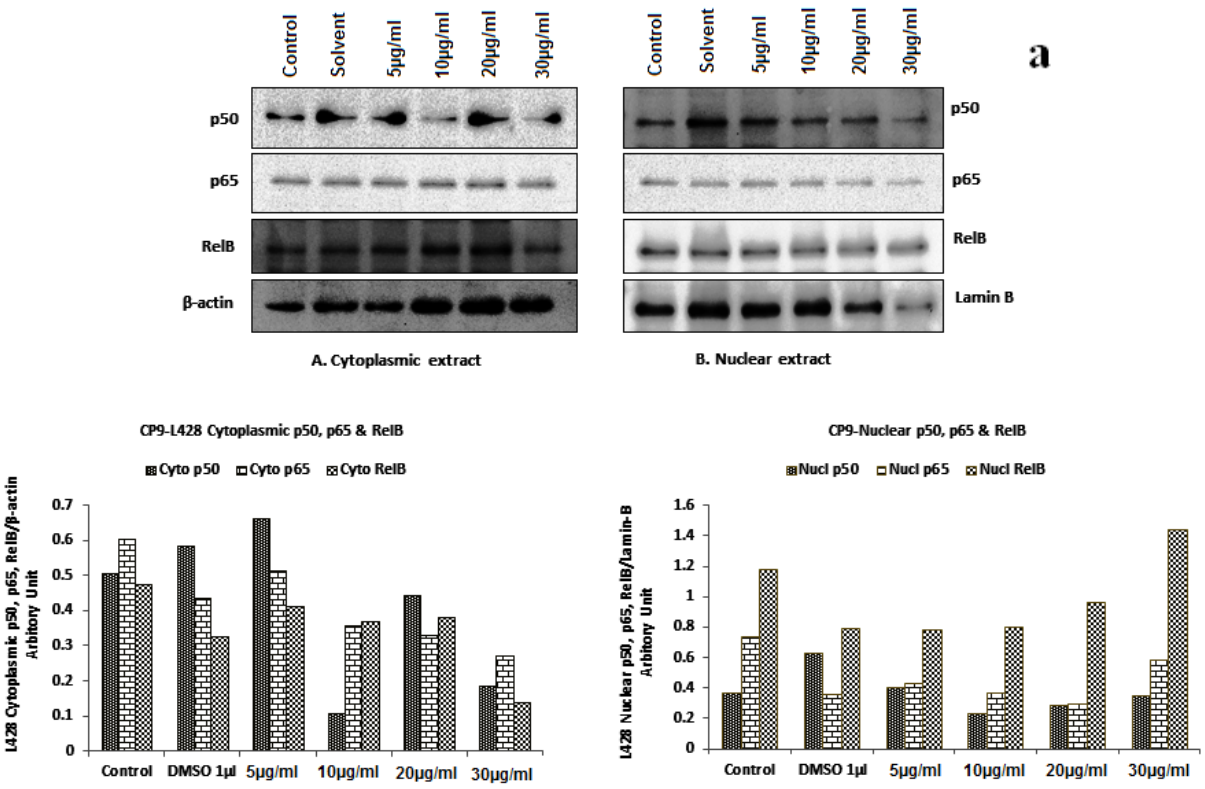


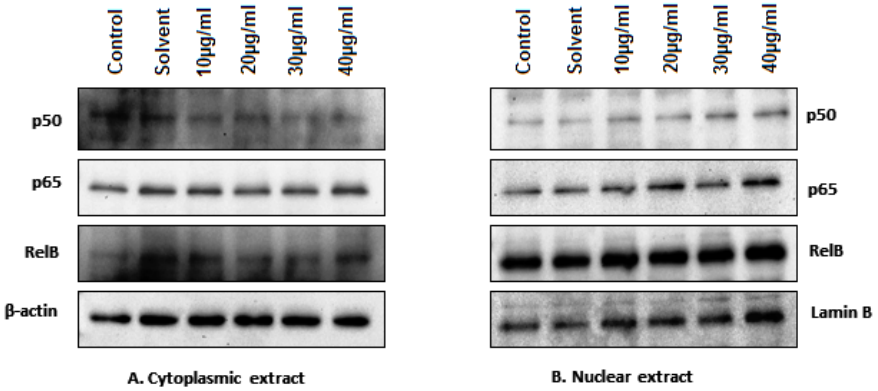
Figure 5. Wound-scratch assay: Effect of coumaperine derivatives a) CP-9, b) CP-32 and c) CP-38 on the A549 cell migration. A549 cell monolayers were "scratched" and were grown in the absence (control) or presence of coumaperine derivatives (2-10 µg/ml) respectively for 48 h. Coumaperine derivatives prevented the cells from closing the wound (Representative of three independent experiments).

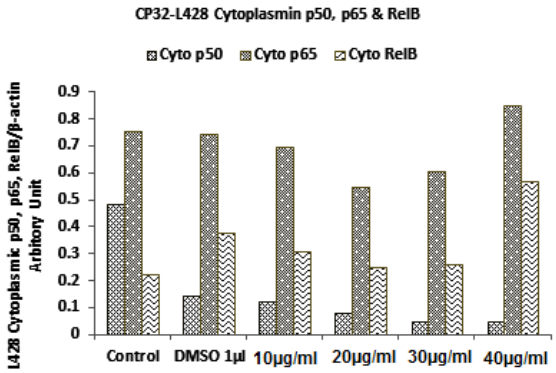
3.5 Western Blot Analysis: Monitoring the expression of NF-κB subunits (p50, p65 and RelB) on coumaperine derivatives (CP-9, CP-32, CP-38) treated cytosolic and nuclear fraction.

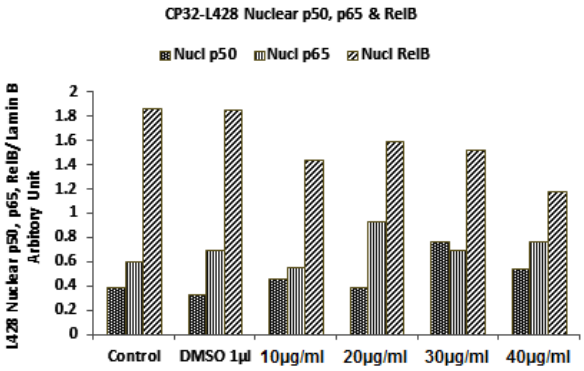
The degree of expression of proteins such as p50, p65 and RelB, both in cytosolic and nuclear extracts of coumaperine derivatives treated and untreated L428 cells were determined by western blot. The results show that the NF-κB subunits p50 and p65 were diminished mainly in the nucleus upon coumaperine derivatives CP-9 and CP-38 treatment and the dose response was observed starting from 2.5 µg/ml to 10 µg/ml (**Figures 6a, c**) whereas CP-32 did not show prominent reduction of these proteins (**Figure 6b**).



b


Control
Solvent
10µg/ml
20µg/ml
30µg/ml
40µg/ml
p50
p65
RelB
β-actin
A. Cytoplasmic extract
Lamin B
B. Nuclear extract


CP32-L428 Cytoplasmin p50, p65 & RelB
Cyto p50
Cyto p65
Cyto RelB
L428 Cytoplasmic p50, p65, RelB/β-actin Arbitory Unit
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
Control
DMSO 1µl
10µg/ml
20µg/ml
30µg/ml
40µg/ml


CP32-L428 Nuclear p50, p65 & RelB
Nucl p50
Nucl p65
Nucl RelB
L428 Nuclear p50, p65, RelB/Lamin B Arbitory Unit
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
Control
DMSO 1µl
10µg/ml
20µg/ml
30µg/ml
40µg/ml

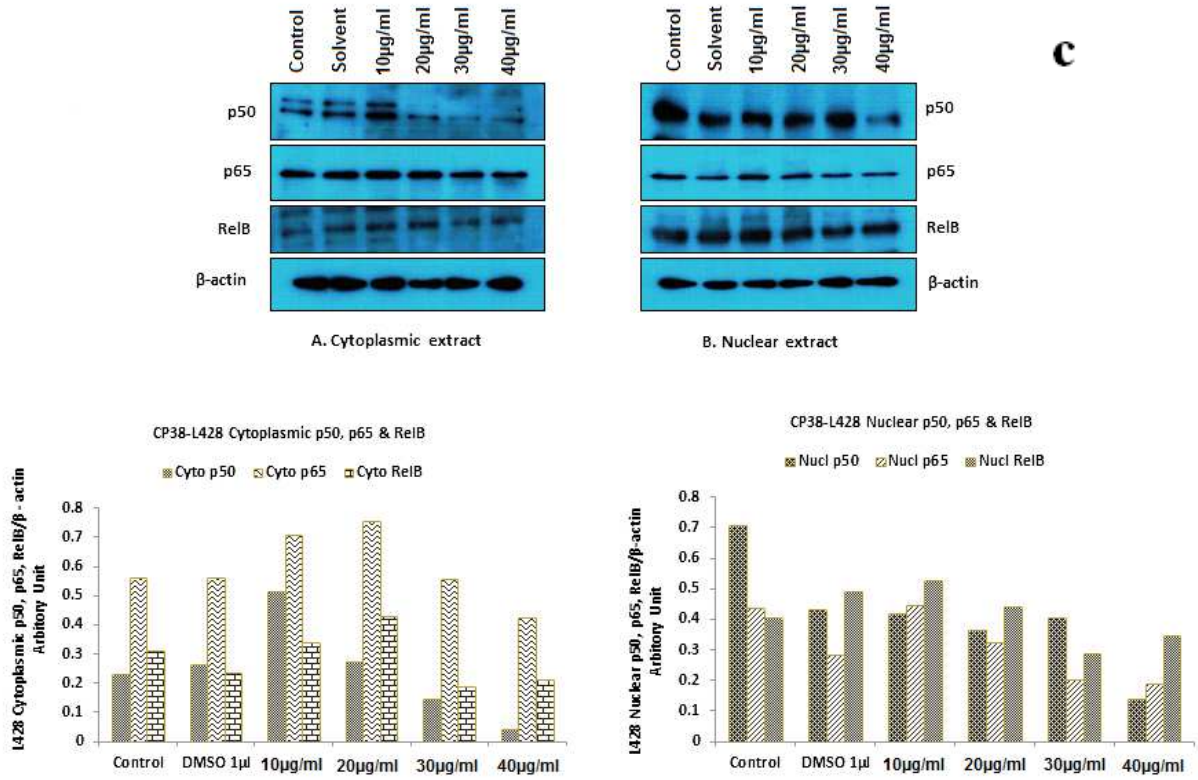


Figure 6. Western-blot analysis monitors the expression of NF-κB proteins, p50, p65 and RelB, both in cytosolic and nuclear extracts: Coumaperine derivatives a) CP-9, b) CP-32 and c) CP-38 treatment decreases the quantity of nuclear NF-κB subunits. Western blot of the NF-κB subunits in L428 cells that were incubated with coumaperine derivatives at different concentrations or with vehicle (DMSO; Control) for 2 h. Cytosolic and Nuclear extracts were prepared and western blots were run with antibodies against p50, p65 and Rel B. Anti-β-actin and Lamin B was used as loading control (Representative of at least three independent experiments).

The active compounds inhibited only the classical NF-κB pathway but not the alternative one (RelB)

3.6 Confocal Microscopic Studies: Nuclear localization of NF-κB subunits (p50 and p65) in L428 cells treated with CP-9 and CP-38

Further support to the western blot results comes from nuclear localization studies, the immuno-cytochemical comparison of treated and untreated L428 cells with CP-9 and CP-38 was carried out. Interestingly, the coumaperine derivative CP-9 and CP-38 treated cells shows the depletion of constitutive NF-κB subunits from the nuclei of most treated cells in a dose dependent manner. The depletion of NF-κB subunits was found to be at the concentrations of 10 µg/ml and 30µg/ml of CP-9 and CP-38 respectively and was comparable to that of control cells (**Figure 7a-b**).

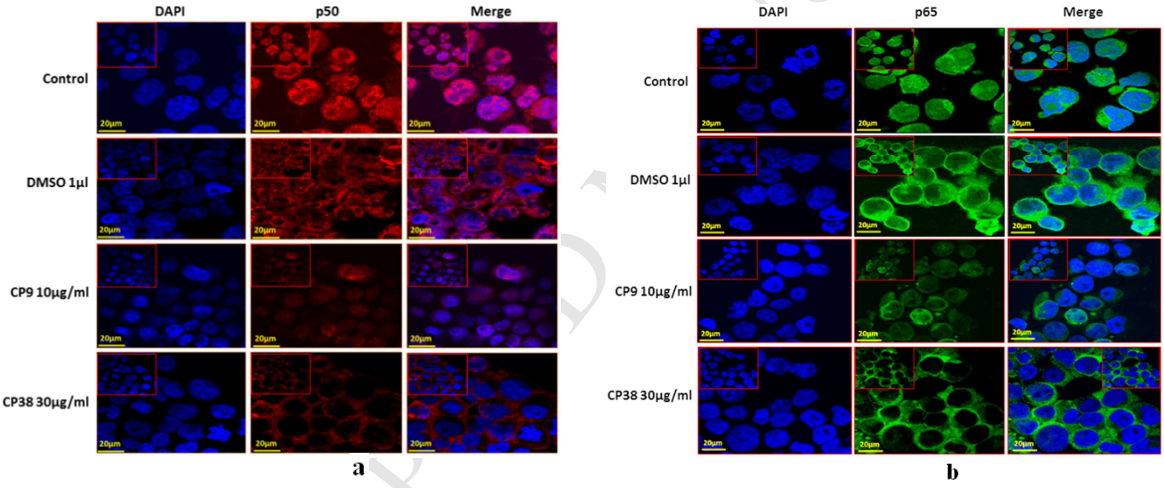


Figure 7. Confocal microscopic image: Coumaperine derivatives CP-9 and CP-38 treatment depletes the nuclear NF-κB subunits a) p50 and b) p65. L-428 cells treated with 10 µg/ml of CP-9 and 30 µg/mL of CP-38 were cyto-centrifuged (Shandon Cytospin 4) at 900 rpm for 5 min and fixed in 8% formalin for 20 minutes. Then the cells were incubated with antibodies against p65 and p50, followed by their respective fluorescent secondary antibodies, and the nucleus were stained with DAPI and the signals were detected using Olympus FV1000-IX81 confocal microscope.

Discussion

With the synthesized compounds in hand, we decided to study their cytotoxic effect against ~~cancer cell lines– Hodgkin's lymphoma derived~~ L428 ~~cells~~ and ~~lung adenocarcinoma derived~~ A549 cell and their NF-κB inhibition pathway. Piperine is known to exhibit myriad biological activities including, anti-inflammatory and anti-tumor activities [14], arises in part due to its antioxidant activity, correlated to the presence of phenolic moiety, Michael acceptor unit and conjugated double bonds. Piperlongumine, an another important pepper alkaloid present in *Piper longum* L., is found to be selectively toxic to cancer cells *in vitro* and *in vivo* [36], also possess phenolic moiety, Michael acceptor unit (two) and conjugated double bonds. Hence, it is anticipated that the coumaperine and its derivatives possessing the aforementioned structural features may also show anti-inflammatory and anti-cancer activity.

Several pepper alkaloids are shown to be a potent NF-κB inhibitor. Piperlongumine and several of its analogs have been found to effectively inhibited (IC_{50} = 1.76 µM (0.56 µg/ml), 1.89-6.06 µM (0.6–1.92 µg/ml) respectively) constitutive expression of NF-kB in A549 cells [37]. Further, piperlongumine effectively inhibited DNA binding activity of NF-κB in A549 cells in concentration dependent manner with maximum inhibitory activity at 25 µM (7.92 µg/ml) [37]. There are plethora reports in which piperine is shown to inhibit NF-κB activation in several cell lines. In RAW 264.7 cells, piperine (at 100 µg/ml), significantly reduced the LPS-mediated NF-κB activation (by ~75%) in a luciferase reporter gene assay [16]. It also significantly inhibited (at 100 µg/ml) IL-β1 induced NF-κB activation (by ~90%) in human OA chondrocytes [22].

The synthesized compounds were initially screened for their NF-κB inhibitory activity using luciferase reporter gene assay with various concentrations (results summarized in **Table 1**). The

simplest coumaperine derivative, CP-9 without any ~~substituted~~ substituent on the aromatic ring ~~(CP-9)~~, exhibit~~ed~~ 71.9% inhibition at 10 µg/ml, introduction of –OH or -OMe group at the *para* position (CP and CP-38) lead to marginal decrease in the activity, 68.4% and 69.7% inhibitions respectively at 10 µg/ml. However, replacing –OMe with –*O*cyclopentyl group decrease~~d~~ the activity significantly (69.7 to 57.6%), suggesting a bulkier hydrophobic group is not acceptable at this position. Incorporation of more than one -OMe group with respect to *para* methoxy group *viz* dimethoxy in the case of CP-10 and trimethoxy groups, as in the case of CP-50 and CP-27 decreases the activity, suggesting, unsubstitution (CP-9) or monosubstitution (CP-38) is optimal for the desired activity. Replacing electron donating methyl group in CP-38 (-OMe) with electron withdrawing acetyl (–Ac) group as in CP-158 (-OAc) leads to marginal improvement in the activity (69.7 to 71.4%). Further, the effect of electron donating substituent was studied using

-$N(CH_3)_2$ group. To our surprise, we observed excellent inhibitory activity (95.5% at 10 µg/ml), suggesting, a stronger electron donating substituent brings beneficial activity.

Next, we turned our attention to the influence of number of olefinic double bonds, and incorporated an additional olefinic double bond to CP-38, CP-32 and PIP, which respectively gave the compounds CP-102, CP-146 and PIP-155. All the three compounds, CP-102, 146 and PIP-155 (tri-olefin conjugated) showed lesser activity than their di-olefin conjugated counter parts (**Table 1**). Thus, increasing the number of olefinic double bond from di to tri-olefin conjugation decreases the activity. Taken together, unsubstitution/monosubstitution (at *para* position) and di-olefin conjugated coumaperine derivatives showed better inhibition of NF-κB in a luciferase reporter gene assay. Based on the above inhibition study, the compounds, CP-9, CP-

32 and CP-38 were chosen for further biological studies including, inhibition of cell proliferation (L428 and A549 cells) and cell migration (A549 cells) and NF-κB inhibition pathway.

In order to study the effect of coumaperine derivatives on cell proliferation CP-9, CP-32 and CP-38 were incubated with cancer cell lines L428 and highly resistant A549. All the compounds inhibited cell proliferation markedly in a dose dependent manner (**Figure 3 and 4**). Against L428 cells, CP-32 (IC50: 0.39 µg/ml) exhibited better inhibition ~~of cell proliferation~~ than CP-38 (IC50: 16.85µg/ml) and CP-9 (IC50: 43.25µg/ml). The difference in inhibitory activity may be correlated to electron donating ability of substituent on the aromatic moiety. CP-32 with stronger electron donating group $–N(CH_3)_2$ showed better inhibitory activity than CP-38 and CP-9 with relatively weaker electron donating groups, $-OCH_3$ and –H respectively ($-N(CH_3)_2$ > -OMe > H). Against A549 cells, the simple coumaperine derivative, CP-9 (with no substituent on the aromatic moiety) exhibited better inhibition (IC50: 57.15 µg/ml) ~~of cell proliferation~~ than CP-32 (IC50: 69.1µg/ml) and CP-38 (IC50: 63.2µg/ml) with electron donating substituents $-N(CH_3)_2$ and –OMe respectively. Thus, inhibitory activity of coumaperine derivatives, CP-9, CP-32 and CP-38 against A549 cells is just reverse to L428 cells. In other words, inhibitory activity of coumaperine derivatives with a weaker electron donating substituent (on the aromatic moiety) showed better inhibitory activity than coumaperine derivatives with a stronger electron donating substituent (-H > $-OCH_3$ > $-N(CH_3)_2$).

CP-9, CP-32 and CP-38 were then evaluated for inhibition of A549 cell migration. The coumaperine derivatives were added to the monolayer of A549 ~~Human Lung carcinoma cells~~ and scratched to study their ability to inhibit cell migration, CP-9 and CP-38 exhibited better inhibition ~~of cell migration at lower concentration~~ (5 µg/ml) than CP-32 (10 µg/ml) (**Figure 5**).

The above results indicate that the coumaperine derivative with a weaker electron donating substituent (CP-9, -H and CP-38, -OMe), showed better inhibition ~~against A549 cell migration~~ than a stronger electron donating substituent (CP-32, -$N(CH_3)_2$).

Coumaperine derivatives were then studied for inhibition of NF-κB proteins of L428 cells. CP-9, CP-32 and CP-38 were treated with L428 cells and the nuclear and cytoplasmic protein lysates were prepared and expressions of NF-κB subunits were analyzed by western blot. CP-9 and CP-38 suppress significantly the p50 and p65 subunits both in cytosolic and nuclear fractions at the dosage of 10 µg/ml, 30 µg/ml respectively (**Figure 6a, c**). But, CP-32 did not show prominent reduction of these proteins (**Figure 6b**). Suggesting that coumaperine derivatives with a weaker electron donor (-H > -OCH_3 > -$N(CH_3)_2$) may beneficial for inhibition of NF-κB subunits (p50 and p65). In addition to western blot analysis, the inhibition of NF-κB subunits (p50 and p65) by coumaperine derivatives (CP-9, CP-32 and CP-38) was confirmed by nuclear localization using confocal microscopy~~ic images~~. The immuno-cytochemical comparison of treated and untreated L428 cells show~~s~~, depletion of NF-κB subunits such as p50 and p65 from the nuclei of most treated cells in a dose dependent manner (10 µg/ml and 30 µg/ml of CP-9 and CP-38 respectively) and was comparable to that of control cells (confocal microscopic images, **Figure 7**). Which clearly indicates C-P9 and CP-38 inactivates the NF-κB pathway in vitro.

4. Conclusion

Understanding inhibition pathway of NF-κB, a key transcription factor often associated with several diseased states including cancer, by small molecules is crucial for design and development of efficient inhibitors for biological targets. Coumaperine and its derivatives were synthesized and investigated for inhibition of NF-κB pathway and evaluated for cytotoxicity

against cancer cell lines, ~~Hodgkin's lymphoma derived~~ L428 ~~cells~~ and ~~lung adeno carcinoma derived~~ A549 cells. ~~Initial~~ Screening against ~~inhibition of~~ NF-κB reporter gene (of L428 cells) shows~~ed~~, coumaperine derivatives inhibited constitutively expressing NF-κB ~~by a reporter gene assay~~ in a dose dependent manner. ~~The~~ Substituent on the aromatic moiety and number of olefinic double bond influenced the inhibitory activity. CP-32 with a stronger electron donating group (-$N(CH_3)_2$) showed highest inhibitory activity (at 10 µg/ml) among the coumaperine derivatives tested. ~~The~~ Introduction of more than one methoxy group (compare CP-10 with 27 and 50) to the aromatic ring and increasing the number of olefinic double bond (from two to three, compare CP-32, 38 and PIP with CP-146, 102, and PIP-15 respectively) decrease the inhibitory activity. Based on luciferase assay study, CP-9, 32 and 38 were further evaluated for inhibition of L428 and A549 cell proliferation ~~cancer cells (L428 and A549) proliferation~~ and A549 cell migration ~~(A549)~~. They exhibited dose dependent activity. ~~In the~~ Cell proliferation study shows that coumaperine derivatives exhibited complementary inhibitory pattern against L428 and A549 cells. CP-32 with a stronger electron donating showed better inhibition (IC50: 0.39 µg/ml) than simple coumaperine derivative CP-9 (with no substituent) (IC50: 43.25 µg/ml) against L428 cell proliferation. Against A549 cells ~~proliferation,~~ simple coumaperine derivative, CP-9 showed better inhibition (IC50: 57.15 µg/ml) than CP-32 (IC50: 69.1 µg/ml). The cell migration study ~~showed~~ revealed that CP-9 and 38 inhibited A549 cell migration at lower concentration (5 µg/ml) than CP-32 (10 µg/ml). The effect of coumaperine derivatives, CP-9, 32, and 38 on inhibition of NF-κB ~~inhibition~~ pathway was studied *via* western blot and confocal microscopy. CP-9 and CP-38 suppress NF-κB subunits p50 and p65 both in cytosolic and nuclear fractions in ~~a~~ concentration dependent manner, whereas CP-32 did not show prominent reduction of these proteins. CP-9 and CP-38 diminish p50 and p65 proteins significantly at the

dosage of 10 µg/ml and 30 µg/ml respectively. We conclude that coumaperine derivatives, CP-9 and CP-38 strongly inactivate the NF-κB pathway in vitro, with a potential to be used as anti-inflammatory compounds.

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Highlights:

Coumaperine (a minor component of white pepper) rarely investigated for synthesis and biological activities.

Coumaperine (CP) derivatives, synthesized in less number of synthetic steps and biological activities evaluated.

NF-κB, a key transcriptor, often associated with several disease, including cancer.

Coumaperine derivatives (CP-9, 32 and 38) effectively inhibited NF-kB protein of L428 cells and cancer cell (L428 and A549) proliferation.

CP-9 and CP-38 strongly inactivate the NF-κB pathway in vitro, with a potential to be used as anti-inflammatory compounds.