Research Article

Diarylheptanoid–myricanone isolated from ethanolic extract of *Myrica cerifera* shows anticancer effects on HeLa and PC3 cell lines: signalling pathway and drug–DNA interaction

Avijit Paul¹, Sreemanti Das¹, Jayeeta Das¹, Asmita Samadder¹, Kausik Bishayee¹, Ratan Sadhukhan², Anisur Rahman Khuda-Bukhsh¹

1. Cytogenetics and Molecular Biology Laboratory, Department of Zoology, University of Kalyani, Kalyani-741235, India
2. Department of Biochemistry and Biophysics, University of Kalyani, Kalyani-741235, India

OBJECTIVE: To test if myricanone (C₂₁H₂₄O₅), a cyclic diarylheptanoid, has anticancer effects on two different cancer cell lines HeLa and PC3. The present study was conducted with a note on the drug-DNA interaction and apoptotic signalling pathway.

METHODS: Several studies like cytotoxicity, nuclear damage, annexin-V-fluorescein isothiocyanate (FITC)/propidium iodide (PI)-labelled apoptotic assay and cell cycle arrest, immunoblot and reverse transcriptase-polymerase chain reaction (RT-PCR) were used following standard protocols. Circular dichroism (CD) spectroscopy was also done to evaluate whether myricanone effectively interacted with DNA to bring about conformational changes that could strongly inhibit the cancer cell proliferation.

RESULTS: Myricanone showed a greater cytotoxic effect on PC3 cells than on HeLa cells. Myricanone promoted G₀/G₁ arrest in HeLa cells and S phase arrest in PC3 cells. Nuclear condensation and annexin V-FITC/PI studies revealed that myricanone promoted apoptotic cell death. CD spectroscopic data indicated that myricanone had an interaction with calf thymus DNA that changed DNA structural conformation. RT-PCR and immunoblot studies revealed that myricanone activated the apoptotic signalling cascades through down-regulation of transcription factors like nuclear factor-κB (NF-κB) (p65), and signal transducers and activators of transcription 3 (STAT3); cell cycle regulators like cyclin D1, and survivin and other signal proteins like Bcl-2 and up-regulation of Bax, caspase-9 and caspase-3.

CONCLUSION: Myricanone induced apoptosis in both types of cancer cells by triggering caspase activation, and suppression of cell proliferation by down-regulation of NF-κB and STAT3 signalling cascades, which makes it a suitable candidate for possible use in the formulation of therapeutic agent for combating cancer.

KEYWORDS: *Myrica*; diarylheptanoids; plant extracts; apoptosis; NF-κB; STAT3 transcription factor; signal transduction

DOI: 10.3736/jintegrmed2013057


Received June 11, 2013; accepted August 1, 2013.

Open-access article copyright © 2013 Avijit Paul et al.

Correspondence: Prof. Anisur Rahman Khuda-Bukhsh; Tel: +91-33-25828768; E-mail: prof_arkb@yahoo.co.in; khudabukhsh_48@rediffmail.com

1 Introduction

Cellular inflammation is strongly associated with many types of cancer. Nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) are the key transcription factors in inflammatory pathways that play major roles in tumorigenesis.
NF-κB has emerged as a ubiquitous factor involved in the regulation of inflammatory responses, apoptosis and cell proliferation\[6\]. In its resting stage, NF-κB resides in the cytoplasm as a heterotrimer consisting of p50, p65, and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-alpha (IκBα). On activation, the IκBα protein, an inhibitor of NF-κB, undergoes phosphorylation, ubiquitination, and degradation. P50 and p65 are then released to be translocated to the nucleus, to bind specific DNA sequences present in promoters of various genes, and initiate transcription\[3\]. Besides NF-κB, another transcription factor STAT3, in response to certain inflammatory stimuli like interleukin-6 (IL-6), undergoes sequential tyrosine phosphorylation, nuclear translocation and gene transcription. NF-κB and STAT3 not only play a pivotal role in immune response and inflammatory reactions, but also regulate the expression of many anti-proliferative genes like Bcl-xL, Bcl-2, cyclin D1, survivin, etc\[4\].

Linkage between cancer and inflammation is indicated by numerous lines of evidence: transcription factors NF-κB and STAT3 are activated by most cancer risk factors; an inflammatory condition precedes most cancers; chemotherapeutic agents and γ-irradiation activates NF-κB and leads to chemo-resistance and radio-resistance; most gene products linked to inflammation, survival, and proliferation are regulated by NF-κB and STAT3\[5\]. Thus, agents causing suppression of pro-inflammatory pathways may provide opportunities for both prevention and treatment of cancer.

*Myrica cerifera* (Myricaceae), commonly known as “wax myrtle” or “bayberry,” is one such plant whose roots are used as an astringent, fruits and leaves to treat dental problems, stomach-ache, constipation, and as a skin cleanser\[6\]. *Myrica* contains several organic compounds including diarylheptanoids (myricanone), terpenoids, flavonoids, tannins and phenols\[7\]. Recently, several chemotherapeutic agents have been identified from natural sources; plant extracts shown to block NF-κB pathway include curcin, resveratrol, etc. Pharmacologically, these agents are quite safe, cost effective and with least side-effects, making them suitable for therapeutic purposes. Curcinum has been reported to down-regulate both NF-κB and STAT3 pathways\[8\]. Many diarylheptanoids have been shown to have anti-inflammatory effects\[9,10\] and induced apoptosis by tumor necrosis factor-related apoptosis inducing ligand, activation of death receptor, nuclear damage and cell death in various cancer cells\[10\]. To our knowledge, anticancer potential of myricanone in relation to the inflammatory pathways is not yet clearly established, particularly in respect of the frequency of expression of NF-κB and STAT3 in cervical and prostate cancer cells.

Therefore, our primary objectives were to investigate the effect of myricanone, a diarylheptanoid isolated from ethanolic extract of *Myrica cerifera*, on the induction of apoptosis in prostate (PC3) and cervical cancer (HeLa) cells lines, and to determine the target of action and to track down the NF-κB and STAT3 signalling cascades in both the cancer cell lines.

2 Materials and methods

2.1 Chemicals and reagents

Dulbecco’s modified Eagle’s medium (DME), fetal bovine serum (FBS), penicillin-streptomycin-neomycin (PSN) antibiotic, trypsin and ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco BRL (Grand Island, NY, USA). Tissue culture plastic wares were obtained from BD Bioscience (USA). Propidium iodide (PI), fluorescein isothiocyanate (FITC) acridine orange, 4’,6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and all other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All organic solvents used were of high-performance liquid chromatography grade.

2.2 Isolation of bioactive fraction and identification of myricanone from crude ethanolic extract of *Myrica cerifera*

Ethanolic extract of *Myrica cerifera* is available in the market as a homeopathic mother tincture and was purchased from HAPCO (B.B. Ganguly Street, Kolkata, India). Myricanone was isolated from the extract of *Myrica cerifera* by adopting the methods of Matsuda et al\[11,12\] with slight modifications. Briefly, the extract was evaporated at 30 °C. The dried extract (22 g) was dissolved in methanol and the methanol-soluble part was partitioned in an *n*-butanol:H₂O (1:1 in volume ratio) solvent system. The *n*-butanol soluble part was subjected to ordinary phase silica gel (230-400 μm mesh) column chromatography. The column was then eluted sequentially with chloroform:methanol (9:1 v/v) solvent system and 4 fractions (F₁, F₂, F₃, and F₄) were collected (200 mL each), of which, the F₂ showed the maximum anti-cancer potentials on initial trials, while the others had relatively less potentials. For this, F₂ fraction was further subjected to ordinary phase silica gel (230-400 μm mesh) column chromatography. The column was then eluted sequentially with *n*-butanol:H₂O (1:1 in volume ratio) solvent system and 5 sub-fractions (F₂₁, F₂₂, F₂₃, F₂₄, and F₂₅) were collected (200 mL each). Then we further purified it by preparative thin layer chromatography and characterized through Fourier transform infrared spectroscopy (FTIR) and mass spectroscopy.

2.3 Preparation of the drugs

The desired amount of myricanone was mixed with culture medium (DME) and the mixture was sonicated with probe sonicator (Sonics, USA). The solution was...
diluted with DMEM to final concentrations up to 50 µg/mL for use in subsequent experiments.

2.4 Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were collected from fresh human whole blood by following standard Ficoll/Hypaque gradient centrifugation method[13]. The isolated PBMCs were then cultured as primary culture for 24 h in standard DMEM for cell viability assessment.

2.5 Cell culture

Human prostate cancer cell line (PC3), human cervical cancer cell line (HeLa) and immortal human keratinocyte line (HaCaT) (procured from National Centre for Cell Science, Pune, India) and PBMCs were grown in DMEM supplemented with 10% FBS and 1% antibiotic (PSN) at 37 °C and in 5% CO₂ with 95% humidity.

2.6 Cell viability assay

Cultured cells as well as PBMCs were treated with different doses of myricanone ranging from 10 to 50 µg/mL. After 48 h of treatment, percentage of cell viability was assessed using MTT assay and the half inhibitory concentration (IC₅₀) value was determined. The intracellular formazan crystals formed were solubilized with acidic isopropanol and the absorbance of the solution was measured at 595 nm. Percentage viability was calculated as (optical density (OD) of drug-treated sample/OD of control sample) × 100%.

2.7 Treatment types

On the basis of MTT results, three different doses (D1 10 µg/mL, D2 20 µg/mL and D3 30 µg/mL) of myricanone were selected for further experiments. Control groups received only media without any myricanone treatment. Myricanone-treated cells were incubated for 48 h throughout all the experiments.

2.8 Interaction of myricanone with calf thymus DNA determined by circular dichroism spectra

Determination of interaction of myricanone with calf thymus DNA (CT-DNA) by circular dichroism (CD) spectra was performed as previously described[14]. Briefly, the interaction was checked on naked CT-DNA (1.0 mmol/L) with 20 µg/mL myricanone, using untreated CT-DNA as control. CD spectra showed changes in the structure of DNA, which were monitored in the region of 200 to 450 nm, using 1 cm path length cells through spectropolarimeter (JASCO J720, Japan). Analysis of the CD spectra was done by using Origin 8 software.

2.9 Assessment of cellular morphology

Cells were seeded in 6-well plates (1 × 10⁵ cells per well) with the above indicated concentrations of myricanone. After 48 h, the cells were observed under an inverted phase contrast microscope (Axioskop plus 2, Zeiss, Germany) and photographed with respect to control cells.

2.10 Fluorescence microscopy

Control and myricanone-treated cells were stained with 10 µg/mL of DAPI for 10 min at 37 °C. The stained cells were imaged using a fluorescence microscope (Leica, Germany).

2.11 DNA fragmentation assay

DNA of control and treated cells were extracted by standardized phenol-chloroform method and DNA gel electrophoresis was performed in 1% agarose gel[15].

2.12 Flow cytometric analysis of early and late apoptosis by annexin V-FITC/PI staining

In order to evaluate apoptosis and necrosis, externalization of phosphotidylserine during apoptosis and leakage from necrotic cells was observed by annexin V-FITC/PI dual staining using standard protocol[19]. Differentiation of cell population was done on a flow cytometer (FACS Calibur, BD Bioscience) and was assessed as: (a) viable cancer cells (annexin –ve; PI –ve), (b) early apoptotic cancer cells (annexin +ve; PI –ve), (c) late apoptotic cancer cells (annexin +ve; PI +ve), and (d) necrotic cells (PI +ve). Samples were analyzed in Cylogic (v.1.2.1) software and determination was based on mean fluorescence intensity of 10 000 events.

2.13 Analysis of cell cycle

After 48 h of myricanone treatment, cells were suspended in phosphate-buffered saline (PBS) and fixed by addition of 70% ice cold methanol. The fixed cells were harvested, and washed with PBS and RNase (1 µg/µL) was added to the samples and the cells re-suspended in PI staining (10 µg/mL). The fluorescence was measured at 585 nm. Flow-cytometric data were analyzed using Cylogic (v.1.2.1) software.

2.14 RNA extraction and quantitative reverse transcriptase-polymerase chain reaction analysis

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as previously described[16]. The sequences of the forward and reverse primers used for specific amplifications are shown in Table 1.

2.15 Immunoblot analysis

Western blot analysis was done by using anti-NF-κB, STAT3, phosphor-specific STAT3 (Tyrosine 705), Bax, Bcl-2, caspase-9, and caspase-3 antibodies. Alkaline phosphatase-conjugated secondary antibodies were used for this purpose. Bound antibodies were developed by 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium and the band intensity was measured using ImageJ software (ImageJ software NIH, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the house-keeping gene.

2.16 Statistical analysis

Data were expressed as the means of values of three independent experiments. No statistical method was used for data analysis.
3 Results

3.1 Characterization of chromatographic fraction

FTIR and mass spectroscopic data revealed that F2.2 fraction was found to be rich in myricanone (C21H24O5, MS m/z: 356(M-Na)) which was selected for further study. The mass spectra of the purified myricanone have been shown in Figure 1. The FTIR data represent peak at 3 317/cm for OH group, 2 854/cm for CH2 group, 1 713/cm for C=O group, 1 505/cm for aromatic CH group and aliphatic CH3 group of the purified myricanone.

3.2 Cell viability and cytotoxicity

The cytotoxicity of myricanone was evaluated on PBMCs as well as HeLa and PC3 cells by using MTT assay. The results indicated that myricanone significantly inhibited the proliferation of both cell lines in a dose-dependent manner when incubated for 48 h. Myricanone showed very little cytotoxic effect on both HaCaT (95.92% viability) and PBMCs (89.27% viability) even at its highest dose (50 µg/mL). The highest dose of myricanone (50 µg/mL) allowed 31.12% HeLa and 24.95% PC3 cells to remain viable, killing the rest. Data on percentage of cell viability in HeLa and PC3 cells treated with different doses of myricanone at 48 h have been furnished in Figure 2. The IC50 values of myricanone in HeLa and PC3 cells were 29.6 µg/mL and 18.4 µg/mL respectively, from where it

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequences</th>
</tr>
</thead>
</table>
| NF-κB       | Fwd 5′-GCAGCCTATCACCAACTCT-3′  
             | Rev 5′-TACTCCCTTCTTCTACCAACC-3′ |
| STAT3       | Fwd 5′-CCTGGGCAATGGAGCTGCTTTG-3′  
             | Rev 5′-CGACATTGGAAATCGTGGTTA-3′ |
| IL-6        | Fwd 5′-AACGATGATGCAGGCTTTGGA-3′  
             | Rev 5′-GACATTGGAAATCGTGGTTA-3′ |
| Cyclin D1   | Fwd 5′-GCCAGATGAGCCGATGAGGC-3′  
             | Rev 5′-CCTTCAGGGCCTGTTTCG-3′ |
| Survivin    | Fwd 5′-CTGATTTGGCCACAGTGGTT-3′  
             | Rev 5′-CTTCAGCTCCCTCTCTCTC-3′ |
| Bcl-2       | Fwd 5′-TGACTCTCCGATCAGGAGGG-3′  
             | Rev 5′-CTTCAGCCATCCGAGGACC-3′ |
| Bax         | Fwd 5′-AGTAACATGGACGTGCAGAGG-3′  
             | Rev 5′-ATGGTCTGATAGTCCGAG-3′ |
| Caspase-3   | Fwd: 5′-AGGGGGCTATTAGGAGCAG-3′  
             | Rev: 5′-TACACGGGCTTTTTTCGG-3′ |
| GAPDH       | Fwd 5′-CCCACTAACTCAGTTAGGG-3′  
             | Rev 5′-CCTTCAGGGAATGTTTCG-3′ |

NF-κB: nuclear factor-κB; STAT3: signal transducer and activator of transcription 3; IL-6: interleukin-6; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Table 1 The primer sequences used for the study of reverse transcriptase-polymerase chain reaction

Figure 1 Mass spectroscopic data and chemical structure of myricanone
could be assumed that myricanone was more effective to PC3 at much lower concentration than to HeLa cells.

3.3 Morphological changes

The morphological changes of HeLa and PC3 cells treated with D1, D2 and D3 were also observed with respect to control. Results revealed the morphological changes (cell shrinkage and blebbing) were typical of apoptosis. Exposure to D1, D2 and D3 for 48 h caused the majority of cells to shrink, round up and detach from the culture dish (Figure 3).

3.4 Drug-CT-DNA interaction demonstrated through CD spectral analysis

CD spectrum of CT-DNA showed a positive band at 275 nm due to base stacking and a negative CD band at 248 nm due to change in helicity, which are characteristics of a typical B form DNA. Myricanone did not have a CD spectrum of them when they were free in solution. In case of drug added to CT-DNA, a positive CD band appeared at

Figure 2 Analysis of cytotoxic and apoptotic potentials of myricanone

Cells were cultured in presence of various concentrations of myricanone (10-50 µg/mL) for 48 h. Cell proliferation was measured by MTT assay. IC50 value of myricanone on HeLa and PC3 cells were found to be 29.6 µg/mL and 18.4 µg/mL, respectively. Data are expressed as mean ± standard error of mean, n=3.

HaCaT: immortal human keratinocyte line; PBMC: peripheral blood mononuclear cell; HeLa: human cervical cancer cell line; PC3: human prostate cancer cell line; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC50: half inhibitory concentration.

Figure 3 Morphological changes of control and D1-, D2-, and D3-treated HeLa and PC3 cells for 48 h of myricanone treatment (Phase contrast microscopy, ×200)

Myricanone showed greater apoptotic effect on both HeLa and PC3 cells in a dose-dependent manner. In higher doses, more apoptotic round-shaped, cellular blebbing, and shrunk cells were observed in both cells.

D1: 10 µg/mL; D2: 20 µg/mL; D3: 30 µg/mL.
272 nm and negative CD band at 242 nm, which showed an increase in CD molar ellipticity. This observation is an indicator of a strong binding of drug (myricanone) to the CT-DNA and bringing conformational change in CT-DNA (Figure 4).

3.5 Fluorescence microscopic observation by DAPI staining

Myricanone-induced DNA damage in HeLa and PC3 cells was confirmed by DAPI staining. The control cells did not take positive staining with DAPI and showed no cell with visible chromatin condensation. However, with different drug treatment, cells with chromatin condensation appeared to increase in number. The D3-treated cells showed the maximum chromatin condensation (Figure 5).

3.6 DNA fragmentation assay

DNA fragmentation was also tested by agarose gel electrophoresis which indicated a significant increase in inter-nucleosomal DNA fragmentation of HeLa cells and PC3 cells. DNA laddering, a characteristic of apoptotic DNA was observed in all the treated cells with respect to their respective control cells (Figure 6).

3.7 Apoptosis study by annexin V-FITC/PI assay

To confirm apoptosis, flow cytometric analysis of HeLa and PC3 cells was performed using dual stain annexin V-FITC/PI. After treatment of myricanone for 48 h, both early and late apoptotic cells were significantly increased in PC3 cells, where as in case of HeLa cells late apoptotic cells were significantly increased in a concentration-dependent manner. The percentage of necrotic cells was negligible in both cell types (Figure 7).

3.8 Cell cycle analysis

Flow cytometric analyses revealed that myricanone induced cell cycle arrest at different stages in HeLa and PC3 cells. In case of HeLa cells there was an increase in cell populations in G0/G1 stages with consequently decrease in G2/M stages of the cell cycle after 48 h of drug treatment where as in case of PC3 arrest occurred at S stage (Figure 8).

3.9 Assessment of mRNA and protein level expression of myricanone-induced apoptosis

Members of the Bcl-2 family of proteins are known to
be critical regulators of the apoptotic pathway. Caspases play crucial roles in apoptosis of myricanone-treated HeLa and PC3 cells. RT-PCR and Western blot analysis revealed that the expression of Bax, caspase-9 and caspase-3 was up-regulated and Bcl-2 expression was down-regulated in both cell lines (Figure 9). Western blot analysis was performed for the study of cleaved caspase-3, the active form of caspase-3, in both the cell lines treated with myricanone. Caspase-3 cleavages were observed in both myricanone-treated cell lines (Figure 9B). GAPDH was taken as the housekeeping gene.

3.10 Effect of myricanone on inflammatory pathways through RT-PCR and Western blot analysis

Expression level of STAT3 in HeLa as well as PC3 cells was found to be significantly suppressed at mRNA and protein level and inhibit STAT3 phosphorylation was inhibited when treated with myricanone. Also mRNA expression of IL-6 was found to be down-regulated. Likewise, NF-κB expression was also suppressed at mRNA and protein levels after myricanone treatment, when compared to untreated control in both HeLa and PC3 cells (Figure 10). The mRNA expression levels of cyclin D1 and survivin were also down-regulated in both cell lines (Figure 11). GAPDH was taken as the housekeeping gene.

4 Discussion

The aim of modern medicines is to develop drugs that are safe, efficacious, and affordable. Recently, researchers have been exploring various plant extracts, particularly those which are known to have some medicinal value, providing valuable information for their possible use in therapeutic formulation of anti-cancer drugs.

In our present work, we investigated the cytotoxic effects of myricanone, a diarylheptanoid extracted from *Myrica cerifera*, which could suppress the proliferation of PC3 and HeLa cells by interfering with NF-κB and STAT3 inflammatory pathways. Myricanone demonstrated cytotoxicity against PC3 and HeLa cells in a dose-dependent manner. It was interesting to note that myricanone had very negligible cytotoxic effect on...
both HaCaT (normal epithelial cells) and PBMCs, thus showing its preference for cytotoxic effects on cancer cells, which is a welcome feature. From the drug-CT-DNA interaction study, our results also demonstrated that the drug interacted with DNA to bring about DNA conformational changes that could be linked up to their apoptotic phenomenon.

Apoptosis is a tightly regulated mechanism that can be easily distinguished by its diverse alterations to the cells, including chromatin condensation, DNA fragmentation, cytoplasmic shrinkage, and the formation of apoptotic bodies\(^1\). Myricanone showed cytotoxic effects on both PC3 and HeLa cells. From morphological studies we observed cytoplasm shrinkage, membrane blebbing, and formation of apoptotic bodies in both cell lines. Chromatin condensation and DNA damage were observed through DAPI staining and apoptotic laddering of DNA in both cell lines. Apoptosis was further confirmed by annexin-V/FITC staining in both cell lines. These observations therefore provided clear evidence of the apoptotic potential of myricanone in various types of cancer cells.

Myricanone induced G\(_0\)/G\(_1\) and S phase accumulation in HeLa and PC3 cells, respectively and increased Bax expression, leading to activation and cleavage of caspase-3.

![Figure 8](image8.png)

**Figure 8** Flow cytometric analysis of the cell cycle in myricanone-treated HeLa and PC3 cells with respect to untreated cells

In HeLa cells, arrest occurred at G\(_0\)/G\(_1\) stage whereas in PC3 cells arrest occurred at S phase. D\(_1\): 10 µg/mL; D\(_2\): 20 µg/mL; D\(_3\): 30 µg/mL.

![Figure 9](image9.png)

**Figure 9** Effects of myricanone on mRNA and protein level expressions in HeLa and PC3 cells with respect to untreated cells

A: RT-PCR analysis of Bax, Bcl-2 and caspase-3 of control and treated cells. mRNA expression analysis showed up-regulation of Bax, caspase-3 and down-regulation of Bcl-2 activity. B: Expression of Bax, Bcl-2, caspase-9 and caspase-3 of control and treated cells was analyzed by Western blot analysis. Significant up-regulation of Bax, caspase-3, and caspase-9 and down-regulation of Bcl-2 was found in myricanone-treated cells in a dose-dependent manner. The cleavage of caspase-3, which is the active form of caspase-3 was also observed in both cell lines. GAPDH acts as a housekeeping gene. The intensity of the control was normalized to 1, and the intensity of each band from treated cells is compared with the control.

RT-PCR: reverse transcriptase-polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; D1: 10 µg/mL; D2: 20 µg/mL; D3: 30 µg/mL.
Caspases are crucial mediators of apoptosis. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. This correlated with down-regulation of various proliferative and anti-apoptotic gene products, including cyclin D1, survivin and Bcl-2. These effects of myricanone in both cell lines are mediated through suppression of constitutively active NF-κB. Myricanone also inhibited IL-6-induced activation of STAT3 in both cell lines.

Multiple intracellular signalling pathways including NF-κB, STAT3 and caspase interact in the regulation of programmed cell death. Previous reports suggest that over-expression of NF-κB and STAT3 in prostate and cervical cancer cells can be regulated by several natural compounds. These compounds induce the expression of Bcl-2 and Bax in a favorable ratio to prevent apoptosis and also inhibit expression of caspase-9 and caspase-3. By traditional standards, NF-κB is an unconventional therapeutic target. P65, active subunit of NF-κB plays an important role in apoptosis. Whether myricanone can inhibit constitutively activated NF-κB in PC3 and HeLa cell lines has not been previously reported. We therefore probed the possibility that myricanone might inhibit NF-κB and found significant down-regulation of NF-κB in both cell lines. On activation, the IκBα protein undergoes ubiquitinylated degradation and p65 and p50 are then released to be translocated to the nucleus, bind specific DNA sequences present in the promoters of various genes, and initiate transcription. In our study we found significant down-regulation of p65 in both cell lines when treated with myricanone. Thus p65 localization into nuclear transcription region was also inhibited in cells treated with myricanone, supporting the involvement of NF-κB inactivation in myricanone-induced apoptosis.

STAT3 is an oncogenic transcription factor which promotes cell proliferation and survival. STAT3 is persistently phosphorylated in many types of human cancer cell lines or primary tumors, including liver cancer, breast cancer, prostate cancer, and head and neck cancer, and several haematological malignancies. Because IL-6 is known to activate STAT3, we tested whether myricanone could affect STAT3 activation induced by IL-6. We found that expression of IL-6 was down-regulated significantly with concomitant decrease in STAT3 expression level in myricanone-treated cancer cells, thus showing clear evidence of induction of STAT3 by IL-6. Proteins that regulate cell survival, including Bcl-2 are direct targets of STAT3.

Our study also demonstrated that myricanone suppressed several genes that are regulated by NF-κB and STAT3 including the proliferative cyclin D1 and antiapoptotic gene products like survivin and Bcl-2. This implies that suppression of STAT3 and NF-κB activation by myricanone could facilitate apoptosis. Constitutively

---

**Figure 10** mRNA and protein level expressions in myricanone-treated HeLa and PC3 cells with respect to untreated cells
A: RT-PCR analysis of IL-6, STAT3 and NF-κB. Expression analysis revealed significant down-regulation of IL-6, STAT3 and NF-κB expression when treated with myricanone with respect to control cells. B: Expression of STAT3, phosphorylated STAT3 (Tyrosine 705) and NF-κB (p65) of control and treated cells was analyzed by Western blotting. GAPDH acts as a housekeeping gene. STAT3 and NF-κB expression was found to be significantly down-regulated in myricanone-treated cells compared to untreated control cells. The intensity of the control was normalized to 1, and the intensity of each band from treated cells is compared with the control.

NF-κB: nuclear factor-κB; STAT3: signal transducer and activator of transcription 3; IL-6: interleukin-6; RT-PCR: reverse transcriptase-polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; D1: 10 µg/mL; D2: 20 µg/mL; D3: 30 µg/mL.

**Figure 11** RT-PCR analysis of cyclin D1 and survivin of control and treated cells
Myricanone-treated cells showed significant down-regulation of cyclin D1 and survivin expression when compared with untreated control cells. GAPDH acts as a housekeeping gene. The intensity of the control was normalized to 1, and the intensity of each band from treated cells is compared with the control.

RT-PCR: reverse transcriptase-polymerase chain reaction; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; D1: 10 µg/mL; D2: 20 µg/mL; D3: 30 µg/mL.
active STAT3 has been implicated in the induction of resistance to apoptosis, possibly through the expression of Bcl-2 and cyclin D1\textsuperscript{[24,25]}. Previous reports\textsuperscript{[26,27]} have revealed that natural active compounds play a key role in modulating the expression of proliferative and anti-apoptotic gene products through regulation of STAT3 or NF-κB or both. Our present study confirms the role of myricanone in inducing apoptosis through action on certain survival gene products.

5 Conclusions

Overall, these findings clearly demonstrate that myricanone could induce apoptosis in HeLa and PC3 cells through suppression of proliferative NF-κB, and STAT3 pathways and caspase activation where effect of myricanone was more pronounced in PC3 than HeLa cells. Collectively, the ability of myricanone to suppress NF-κB and STAT3 activation, inhibit IL-6 signalling, down-regulate the expression of cyclin D and Bcl-2 provides a sound basis for enhancing treatment efficacy and reduce toxicity. Overall, these findings suggest that myricanone showed greater effects on PC3 cells than on HeLa cells, which may be due to the drug effects more pronounced on NF-κB and STAT3 levels in PC3 cells. Thus, from the above findings it can be suggested that myricanone has considerable anti-cancer potentials against prostate and cervical cancer cells \textit{in vitro}, indicating its possible future use in formulation of a successful therapeutic agent for combating cancer.

6 Acknowledgements

This work was partially supported by a grant sanctioned to Prof. A.R. Khuda-Bukhsh, Department of Zoology, University of Kalyani, India, by Boiron Laboratories, Lyon, France. We are also thankful to Dr. Utpal Ghosh, Department of Biochemistry and Biophysics, University of Kalyani, India, by Boiron Laboratories, to Prof. A.R. Khuda-Bukhsh, Department of Zoology, University of Kalyani, India, by Boiron Laboratories, to Prof. A.R. Khuda-Bukhsh, Department of Zoology, Lyon, France. We are also thankful to Dr. Utpal Ghosh, Department of Biochemistry and Biophysics, University of Kalyani, India, for permitting us to use their laboratory for conducting a part of the work.

7 Conflict of interests

The authors declare that they have no competing interests.

REFERENCES


Submission Guide

Journal of Integrative Medicine (JIM) is a PubMed-indexed, peer-reviewed, open-access journal, publishing papers on all aspects of integrative medicine, such as acupuncture and traditional Chinese medicine, Ayurvedic medicine, herbal medicine, homeopathy, nutrition, chiropractic, mind-body medicine, Taichi, Qigong, meditation, and any other modalities of complementary and alternative medicine (CAM). Article types include reviews, systematic reviews and meta-analyses, randomized controlled and pragmatic trials, translational and patient-centered effectiveness outcome studies, case series and reports, clinical trial protocols, preclinical and basic science studies, papers on methodology and CAM history or education, editorials, global views, commentaries, short communications, book reviews, conference proceedings, and letters to the editor.

● No submission and page charges  ● Quick decision and online first publication

For information on manuscript preparation and submission, please visit JIM website. Send your postal address by e-mail to jcim@163.com, we will send you a complimentary print issue upon receipt.

Editors-in-Chief: Wei-kang Zhao & Lixing Lao. ISSN 2095-4964. Published by Science Press, China.