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Apoptotic mechanisms of myricitrin isolated from *Madhuca longifolia* leaves in HL-60 leukemia cells

Monaj Kumar Sarkar
SASTRA Deemed University: Shanmugha Arts Science Technology and Research Academy
Amrita Kar
SASTRA Deemed University: Shanmugha Arts Science Technology and Research Academy
Adithyan Jayaraman
SASTRA Deemed University: Shanmugha Arts Science Technology and Research Academy
Karthi Shanmugam
SASTRA Deemed University: Shanmugha Arts Science Technology and Research Academy
Vadivel Vellingiri (vadivel@carism.sastra.edu)
SASTRA Deemed University: Shanmugha Arts Science Technology and Research Academy
https://orcid.org/0000-0002-2709-433X
Santanu Kar Mahapatra (santanu@scbt.sastra.edu)
SASTRA Deemed University: Shanmugha Arts Science Technology and Research Academy

Short Report

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Abstract

Background: Myricitrin, a naturally occurring flavonoid in *Madhuca longifolia*, possesses several medicinal properties. Even though our earlier work revealed its role against the proliferation of acute myelogenous leukemia cells (HL-60), its molecular mechanisms have not yet been revealed. The current study aims to explore the molecular mechanisms of myricitrin (isolated from an ethnomedicinal drug *Madhuca longifolia*) to induce apoptosis in HL-60 cells.

Methods and Results: Treatment with IC-50 dose of myricitrin (353 µM) caused cellular shrinkage and cell wall damage in HL-60 cells compared to untreated control cells. Myricitrin treatment reduced the mitochondrial membrane potential (22.95%), increased DNA fragmentation (90.4%), inhibited the cell survival proteins (RAS, B-RAF, & BCL-2) and also induced pro-apoptotic proteins (p38, pro-caspase-3, pro-caspase-9 and caspase-3) in the HL-60 cells.

Conclusions: The present study provides scientific evidence for the apoptosis caused by myricitrin in HL-60 leukemia cells. Hence, the phytochemical myricitrin could be considered as a potential candidate to develop an anticancer drug after checking its efficacy through suitable pre-clinical and clinical studies.

Introduction

Flavonoids are plant as secondary metabolites, chemically defined by their typical structure which consists of diphenylpropanes (C6-C3-C6) and carry two different aromatic rings containing three oxygenated heterocyclic carbons [1]. Myricitrin, a 3-O-rhamnoside of myricetin, is a flavonoid synthesized in several edible plants, including Chinese bayberry (*Myrica rubra*) [2 – 6]. Myricitrin is used as a flavor modifier in edible foods and dairy products in Japan [7]. Myricitrin is listed as “safe” by several committees such as U.S. Flavor and Extract Manufacturer Association (USFEMA) and the Joint Expert Committee on Food Additives (JECFA) [7].

Myricitrin showed anti-bacterial activity [4], anti-allergic effect [3], antioxidant and anti-diabetic activities [8], and anti-allodynic property [1]. It reduces inflammation [9], inhibits nitric oxide production in normal cells [2], functions as myeloperoxidase inhibitor [10] and protects against atherosclerosis [11]. Myricitrin exhibited anti-proliferative activity against prostate cancer PC-3 cells [12], azoxymethane-induced premalignant lesions [13] and ovarian cancer cells [14]. Our previous study revealed the anti-leukemic activity of myricitrin isolated from *Madhuca longifolia* leaf in HL-60 cells by inducing reactive oxygen species (ROS), cell membrane damage, and increasing lipid peroxidation [6]. However, the detailed mechanisms of the anti-proliferative action of myricitrin needs to be investigated. Hence, the present work aims to reveal the apoptotic mechanisms of myricitrin through *in vitro* studies in HL-60 cells.

Materials And Methods

Materials

Myricitrin was isolated from the leaves of *Madhuca longifolia* (Voucher number BSI/CDM/273) as described in our previous paper [6] (Supplementary Figure 1). HL-60 cells were purchased from NCCS, Pune, India and maintained in a sterile humidified CO_2 incubator at 37^o C using RPMI 1640 media (Himedia) supplemented with 10% fetal bovine serum (FBS-Himedia) and 1% antibiotic solution (Himedia).

Cell morphological changes

Changes in the morphology of HL-60 cells upon treatment with myricitrin were studied using light microscopy (Olympus BX43) as described by Lee et al. [15]. IC-50 doses of myricitrin (353 µM) and doxorubicin (82 µM) were used to treat Hl-60 cells based on our previous report [6]. The untreated and treated cell suspensions were incubated in a sterile CO_2 incubator at 37◦C for 24 h. Then, the suspension was centrifuged at 2000 rpm for 8 min, the cell pellet was washed twice with sterile PBS and re-suspended in PBS. Then, 10 µL of cell suspension was placed on a sterile slide and observed for morphological changes under 40 x magnification.

Mitochondrial membrane potential (MMP)

The effect of myricitrin on the MMP of HL-60 cells was measured by following the method of Dash et al. [16]. HL-60 cells (1 x 10^6 cells in 950 µL) were seeded in a sterile 6-well plate, treated with 50 µL of IC-50 doses of myricitrin (353 µM) and doxorubicin (82 µM) and kept in a CO_2 incubator at 37◦C for 24 h. After treatment, the cell suspension was centrifuged at 2000 rpm for 8 min, the pellet was collected and washed twice with PBS. Further, the cell pellet was re-suspended in 300 µL of PBS, mixed adequately with 1 µM of Rhodamine-123 (Rh-123) and incubated in the dark at 37◦C for 30 min. Then, the fluorescent intensity was measured by spectrofluorometer (Biotek, synergy H1 multi-mode plate reader) using excitation (493 nm) and emission (522 nm) wavelengths. Based on the results, the MMP was calculated and expressed on a percentage basis.

DNA fragmentation assay

DNA fragmentation in myricitrin-treated HL-60 cells was determined by TUNEL assay using APO-Direct kit (BD Pharmingen, Cat. No. 556381) [17]. HL-60 cells (2 x 10^6 cells in 950 µL) were treated with an IC-50 dose of myricitrin (353 µM) for 24 h in a sterile CO_2 incubator at 37◦C. Then, the DNA fragmentation was analyzed in a flow cytometer (Beckman Coulter, CyAn ADP, Miami, FL, USA) using green (for dUTP-FITC incorporated in fragmented DNA) and red (for PI binding to DNA) filters and the results were processed through Kaluza software (Version 2.1, Beckman coulter).

In silico docking study

A molecular docking study was conducted with Schrödinger software (Schrödinger Release 2020-4: Glide, Schrödinger, LLC, New York) to analyze the interaction of myricitrin with the leukemia protein targets. The molecular structure of myricitrin was drawn using MarvinSketch (V19.13) and prepared using the LigPrep

module implemented in Schrödinger. The energies of the structure were optimized using OPLS_2005 force-field. All possible ionization states were generated between the biological pH ranges of 5 - 9. The chiralities of the input structure were retained during the ligand preparation. Crystal structure of target proteins such as H-RAS (2CL7), N-RAS (3CON), K-RAS (4OBE), B-RAF (4MBJ), and BCL2 (6QGG) were obtained from the protein data bank (PDB) and used to analyze the binding affinity of myricitrin. The crystal structures were processed using the Protein Preparation Wizard implemented in Schrödinger. Protein preparation briefly includes the addition of missing hydrogen atoms, correction of metal ionization states, enumeration of bond orders to hetero groups, removal of co-crystallized water molecules, determination of optimal protonation states for histidine residues, optimization of hydrogen bond network of proteins, and minimization of relaxing in strained bonds. All crystal structures were co-crystallized with either a known inhibitor or endogenous ligand molecule. A 3D grid box for each protein was positioned by keeping the co-crystallized ligand as the centre, which covers all the crucial binding pocket amino acids. The prepared ligand was docked against each protein using Glide extra precision docking mode (Glide XP). OPLS3 force-field was used to score the docked complexes. The molecular interactions of the docked complex were visualized and analyzed using the molecular graphics system PyMOLV1.8 (Schrodinger, LLC).

Western blotting analysis

The effect of myricitrin on the expression of cell signaling pathway proteins (H-RAS, N-RAS, K-RAS, RAF, p38, BCL-2, Pro-caspase-9, Pro-caspase-3, Caspase-3 and BAX) in HL-60 cells was studied using the western blotting technique. HL-60 cells (4 x 10^6 cells) were seeded in a sterile 6-well plate, treated with an IC-50 dose of myricitrin (353 µM) and incubated in a humidified CO_2 incubator for 24 h. After incubation, the cells were collected by centrifugation, washed with PBS and treated with 100 µL of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mg/mL leupeptin, 1 mM PMSF, 0.1 % CHAPS, pH 7.4). Then the protein concentration of the cell lysate was measured using the Bradford reagent. Cell lysate (50 µg of protein) with loading dye was subjected to 10% SDS polyacrylamide gel electrophoresis. Then, the separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane and blocked with 3% BSA. PVDF membrane with protein bands was then incubated with primary antibodies from Santa Cruz (USA) such as H-RAS (SC-35), N-RAS (SC-31), K-RAS (SC-30), RAF (SC-271929), Pro-caspase-9 (56073), Pro-caspase-3 (31A1067), p-38 (SC-7973), BCL-2 (SC-7382), BAX (6A7) and GAPDH (SC-365062) followed by gentle washing with TBST & TBS. Then, the membrane was treated with horse-radish peroxidase (HRP) conjugated secondary antibodies (Sigma Aldrich, A0545) for 2 h. The target proteins were visualized using an ECL detection kit (Biorad clarity max, Catalog No. 1705062) through Gel Documentation system (Biorad Chemidoc) and processed with Image-J software (Version 1.48).

Statistical analysis

For the MMP experiment, the results were expressed as mean with standard deviation. Statistical analysis was carried out by one-way analysis of variances (ANOVA) followed by Tukey’s post hoc test

using Graph Pad Prism 5.0. Values marked with *** $p < 0.05$ are considered statistically significant as compared to untreated control.

Results And Discussion

Cell morphology

Apoptosis is an essential feature of cell death, which mainly causes damage to the cell wall and the release of cellular debris. Even though cancer cells show resistance to apoptosis because of their cell wall rigidity, chemotherapeutics can alter the morphology of cancer cells. Cellular morphological changes in HL-60 cells in response to myricitrin treatment were observed using upright light microscopy and the results exhibited shrinkage and damage of the cell walls (Supplementary Figure 2). Myricitrin-treated sample illustrated more damages and cell wall ruptures compared to untreated control cells. Thus, the cytotoxic potential of myricitrin through altering the cell wall rigidity of leukemia cells is revealed. Several cellular enzymes can be released due to myricitrin-induced morphological changes in HL-60 cells, as we noticed the release of lactate dehydrogenase in our previous study [6]. Myricitrin is a 3-O-α-L-rhamnopyranoside of myricetin and the rhamnose sugar might increases the water solubility of myricetin and thus enhances its bioavailability. In agreement with our results, rhamnose-containing phytochemicals like ursolic acid and betulic acid saponins exhibited cytotoxic potential against human colorectal adenocarcinoma cells [18]. In addition, L-rhamnose alone revealed an anti-cancer effect in Ehrlich carcinoma-bearing mice [19]. Hence, the presence of the rhamnose component might facilitate/improve the anti-proliferative activity of myricitrin.

An uptake of 27.32% myricitrin was observed at 120 min and after that, the uptake level decreased in HL-60 cells (Data not shown). Uptake of phyto-compounds by the cell is regulated by the plasma membrane, which is necessary for their cytotoxicity. Limitations of drugs' therapeutic effect might be due to their impermeability to the plasma membrane and poor cellular delivery [20]. The molecular weight, size, high polarity of the molecules, presence of sugar moiety and interactions between phyto-compound and phospholipids of the cell membrane could be responsible for the poor absorption of myricitrin. Reduced uptake of myricitrin after 120 min may be due to either efflux of the phyto-compound by the cells or disturbance of cell membrane fluidity [21]. Once myricitrin entered into HL-60 cells, it induced apoptosis by damaging the cell membrane, altering MMP, causing DNA fragmentation and regulating cell signaling proteins.

MMP

Loss of MMP leads to the release of cytochrome-C from the mitochondrial wall, which results in apoptosis and therefore it is a vital indicator of cellular health [22]. The MMP of HL-60 cells treated with IC-50 dose of myricitrin was measured and the results revealed a significant ($p < 0.05$) loss of MMP in HL-60 cells (22.95%) (Supplementary Figure 3). As myricitrin increased the production of intracellular ROS [6], the free radical mediated damage can cause oxidation of proteins and lipids in the inner wall of mitochondria and it may result in decreased MMP in HL-60 cells. Loss of MMP could increases the

permeability of membrane pores, which are early apoptotic mechanisms followed by swelling and disruption of the mitochondrial membrane. Similarly, *Gymnema montanum* caused apoptosis in leukemia cells by reducing MMP up to 20% in HL-60 cells [23].

DNA Fragmentation

Apoptosis can cause cellular morphological and structural changes, which include shrinkage, cell wall damage, biochemical alterations, chromatin condensation and DNA fragmentation. In TUNEL assay, the reactants penetrate the nucleus, bind with the labeled dUTPs onto the OH moieties of fragmented DNA using TdT enzyme and the DNA fragmentation level was visualized through fluorescence probes. In this study, maximum DNA fragmentation was observed in HL-60 cells treated with myricitrin (90.4%) when compared with untreated control cells (0.1 to 1.9%) (Figure 1 and Supplementary Table 1). DNA fragmentation is an important hallmark of the apoptosis in cancer cells, which results from the damaging of chromatin structure into smaller fragments by the activation of endonucleases [24]. The double-stranded DNA is cleaved by DNA fragmentation factor, which exhibits endonuclease activity at A/T-rich regions of DNA strands in the presence of Mg^{2+}.

Cell signaling study

In silico docking results demonstrated the affinity of myricitrin towards the target proteins. Myricitrin exhibited binding affinity with H-RAS (-7.289 Kcal/mol), K-RAS (-6.309 Kcal/mol), N-RAS (-4.756 Kcal/mol), B-RAF (-10.482 Kcal/mol), and BCL2 (-4.771 Kcal/mol) of HL-60 cells (Supplementary Table 2 & Supplementary Figure 4). Thus, inhibition of the presently investigated cell survival proteins by myricitrin might have induced the apoptotic pathways in HL-60 leukemia cells. These *in silico* findings are in agreement with the results of western blotting analysis of cell signaling proteins, which revealed that myricitrin inhibits the expression of different RAS (K-RAS, N-RAS, and H-RAS) and RAF oncoproteins in HL-60 cells (Figure 2A).

The members of the RAS gene family are K-RAS, H-RAS and N-RAS, which all encode proteins that have a pivotal role in cell survival and proliferation. Different types of human cancers, including leukemia, pancreatic, lung and colorectal cancers, are driven by mutations in RAS genes. When RAS genes mutated, cells grow uncontrollably and evade death signals in addition to giving resistance to cells towards cancer drugs. Nearly 30% of human cancers, including solid tumors and hematologic malignancies, are associated with mutations in RAS genes. Parikh et al. [25] noticed that all RAS proteins can induce myeloid leukemia. Hence, inhibition of different RAS proteins increases the chance of apoptosis in leukemic cells. In the RAS/RAF/MEK/ERK1/2 pathway, H-RAS, K-RAS and N-RAS are small GTP-binding proteins, which can activate RAF protein based on signals from cell surface receptors and thus regulate cells' survival [26]. Blockage of RAS proteins is essential to control cell division and proliferation of leukemia cells, as they are critical components of the RAS/RAF/MEK/ERK signaling pathway. Western blotting results (Figure 2A) indicated that myricitrin inhibits RAS proteins and thus blocks the RAS/RAF/MEK/ERK cell survival pathway. Similarly, other phyto-constituents like tocotrienol inhibited the

expression of RAS and RAF proteins in HL-60 cells [27]. In addition to RAS and RAF proteins, myricitrin also inhibited the cell survival protein BCL2, an anti-apoptotic factor, which prevents the activation of BAX protein in the mitochondrial outer membrane and thus inhibits cell death [28].

On the other hand, myricitrin activated the pro-apoptotic factors such as p38, pro-caspase-3 and pro-caspase-9 in HL-60 cells (Figure 2B). The p38 regulates extrinsic and intrinsic apoptotic factors such as BAX, Caspase-9 and Caspase-3 and thus induces apoptosis in HL-60 cells [29]. In agreement with our results, other phytochemicals 3,6-Dihydroxyflavone also reported to activate p-38 in HL-60 cells [30]. Activation of pro-apoptotic proteins by myricitrin might led to the activation of caspases followed by DNA fragmentation and apoptosis. Myricitrin-treated HL-60 cells exhibited the reduction of Pro-caspase-9 & Pro-caspase-3 and expression of Bax and Caspase-3 (Figure 2B). Decreased BCL-2 and increased Bax expressions in myricitrin-treated HL-60 cells indicates mitochondrial-mediated apoptosis. Similarly, syringaresinol inhibited BCL2 and induced the expression of BAX, Pro-caspase-9/3, and thereby resulted in apoptosis in HL-60 cells [31]. Nobiletin induced Pro-caspase-9/3, altered BCL2 & BAX, and also increased p38 and caused MAPK mediated apoptosis in HL-60 cells [32].

Conclusions

Myricitrin is a natural constituent of several plant-based products and is found to play a key role as an anti-proliferating agent against leukemic HL-60 cells. The current work demonstrated experimental evidence for the potential anti-leukemic mechanism of myricitrin against HL-60 cells. Myricitrin induced apoptosis in HL-60 cells by causing cell wall damage, reducing MMP, increasing DNA fragmentation, up-regulating the expression of apoptotic proteins and down-regulating cell survival proteins. Based on the current research outcomes, myricitrin could be considered a potential anti-leukemic candidate for the development of pharmaceutical/nutraceutical formulations after conducting further studies using suitable pre-clinical and clinical models.

Declarations

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Conflicts of interest / competing interests

The authors declare that there are no conflicts of interest.

Ethics approval

This article does not contain any studies with human participants or animal performed by any of the authors.

Consent to Participate (Ethics)

Not applicable as this work does not contain studies with human participants or animals.

Consent to Publish (Ethics)

Not applicable as this work does not contain studies with human participants or animals.

Authors' contributions

Mr. Monaj Kumar Sarkar has performed the *in vitro* experiments, acquisition and analysis of data, and writing the manuscript draft. Ms. Amrita Kar and Mr. Adithyan Jayaraman have provided technical support for *in vitro* experiments especially western blotting and acquisition of data. Mr. Karthi Shanmugam helped to perform *in silico* studies. Dr. Vellingiri Vadivel has contributed in experimental design, supervision, conceptualization, project administration and manuscript writing. Dr. Santanu Kar Mahapatra has conceptualized and designed the experiments, analyzed the data, manuscript writing and contributed reagents/materials/analysis tools.

Availability of data and material

The data and materials of this study are available from the corresponding author upon reasonable request.

Code availability

Not applicable.

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