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Inhibition of cell proliferation and induction of apoptosis in K562 human leukemia cells by the derivative (3-NpC) from dihydro-pyranochromenes family

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Leukemia is a particular type of cancer characterized by the failure of cell death or disability in differentiation of hematopoietic cells. Chronic myelogenus leukemia (CML) is the most studied kind of this cancer. In this study, anti-cancer effect of dihydro-pyranochromenes derivatives were investigated in the human leukemia K562 cells. These compounds were found to be active cell proliferation inhibitors using MTT assay. Among these compounds, 3-NpC was determined as stronger compound with IC50 value of 100±3.1 μM and was chosen for further studies. Induction of apoptosis was analyzed by AO/EtBr staining, DNA fragmentation assay, Annexin V/PI double staining and cell cycle analysis. Furthermore, Western Blot analysis showed that treatment of the cells with 3-NpC led to up-regulation and activation of caspase-3. The results of this investigation clearly indicated that dihydro-pyranochromenes derivatives induce apoptosis in the K562 cell line. This information signalizes also that these compounds may prepare a new therapeutic approach for the treatment of leukemia.

Key words: apoptosis, chronic myeloid leukaemia, dihydro-pyranochromenes, K562 cells.

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INTRODUCTION

Chronic myeloid leukemia (CML) is a myelo-proliferative disease of pluripotent hematopoietic progenitor cells and is associated with the Philadelphia (Ph) chromosome, which is a product of a reciprocal translocation between chromosomes 9 and 22 (t [9; 22][q34; q11]). The molecular consequence of this translocation is the generation of the BCR-ABL1 oncogene which arises out of the conjugation among the breakpoint cluster region gene (Bcr) on chromosome 22 and the Abelson kinase (Abl) gene on chromosome 9. This oncogene encodes the chimeric Bcr-Abl protein with fundamental tyrosine kinase activity. The Bcr-Abl tyrosine kinase activity leads to uninhibited cell proliferation and considerably reduced apoptosis, and therefore starts the malignant development of pluripotent stem cells in the bone marrow (Calabretta & Perrotti, 2004; Quinta's-Cardama & Cortes, 2009). Apoptosis is a kind of cell death that is used by multicellular organisms to prevent of uncontrolled cell proliferation and dispose of unwanted cells. Apoptosis is originally characterized by morphological changes such as membrane blebbing, DNA fragmentation, chromatin condensation, nuclear fragmentation, cell shrinkage and apoptotic bodies formation (Kerr *et al*., 1994; Taylor *et al*., 2008). Apoptosis is performed by the activation of a group of cysteine proteases called "caspases" that cleave proteins during cell death. According to the function of caspases, they are grouped into two biologically different subfamilies. One subfamily intercede the initiation (caspases-8, -9) or execution (caspases-3, -6 and -7) of the apoptotic program (initiator and executioner caspases). Members of the second subfamily (caspases-1, -4, -5, -11,-12, and -14) are involved in the inflammatory processes (Earnshaw, 1999). The caspases can be activated through a large number of stimuli via extrinsic and/or intrinsic pathways. Each pathway needs particular initiating signals to start an energy-dependent cascade of molecular events and activates its own initiator caspase (8, 9 and 10), which will activate in turn the executioner caspase-3/7 and will be followed by characteristic cytomorphological features (Elmore, 2007; Hengartner, 2000). Apoptosis resistance is the reason for concern in developing effective chemotherapeutic agents in various cancers. In recent years, many conventional methods were examined in the treatment of human leukemia and new compounds were investigated to find out agents that have less side effects and more anti-tumor activity. Among different agent we can refer to pyrano-chromene family derivatives.

Chromene (Benzopyran) is a heterocyclic ring system in which a benzene ring and a pyran ring are fused together. It is an important structural component in natural compounds and is available in natural alkaloids, anthocyanins, tocopherols and flavonoids. Different natural and synthetic derivatives of chromene have important activities such as anti-cancer, anti-proliferative, anti-vascular, anti-microbial, anti-oxidant, anti-viral, TNF-α inhibitor, anti-fungal, anti-oxidant, anti-spasmolytic, estrogenic, anti-helminthic, anti-HIV, anti-tubercular, anti-inflammatory, herbicidal, analgesic, anti-anaphylactic, anti-Alzheimer, anti-leukemic and anti-convulsant activity (Mohammadi Ziarani *et al*., 2011; Thomas & Zachariah, 2013). According to the Kemnitzer and coworker studies, the 4-aryl-4H-chromenes were found to inhibit tubu-

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Abbreviations: 3-NpC, 2-amino-4-(3-nitrophenyl)-5-oxo-4, 5-dihydro pyrano]3, 2-c[chromene-3-carbonitrile; Bcr-Abl, breakpoint cluster region gene-Abelson kinase; FBS, fetal bovine serum, CML, Chronic myelogenus leukemia; MTT, 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; PI, propidium iodide; Caspases, cysteine-aspartic proteases; AO/EtBr, Acridine Orange /Ethidium Bromide

lin polymerization via binding to colchicine binding site and leading to a cell cycle arrest and apoptosis. Recently, anti-cancer effects of the 4-aryl-4H-chromenes have been reported in several cell lines (Kemnitzer *et al*., 2004; Kemnitzer *et al*., 2008). In the present study, anti-cancer effects of synthetic derivatives from dihydro-pyranochromene family on K562 cell line as experimental model for CML have been investigated (Fig. 1). These compounds were detected as potent apoptosis inducing agents but the mechanism of action of these compounds are not clear, yet.

MATERIALS AND METHODS

Materials. The cell culture medium (RPMI 1640), fetal bovine serum (FBS) and penicillin–streptomycin were purchased from Gibco BRL (life technologies, Paisley, Scotland). K562 cell line was obtained from Pasteur Institute, Tehran, Iran. The cell culture plates were obtained from SPL (Korea). Ethidium bromide (EtBr), acridine orange (AO), proteinase K were obtained from Sigma. MTT assay kit, dimethylsulfoxide (DMSO), RNase, Annexin-V FITC Apoptosis kit, propidium iodide (PI), Cell Extraction Buffer and ECL kit were purchased from Roche (Germany). Anti- caspase-3 and anti-β actin, were purchased from Alexis Biochemicals.

General procedure for the preparation of the dihydro-pyranochromenes compounds. The synthesis of the dihydro-pyranochromenes was achieved by the three-component condensation of an aromatic aldehyde, malononitrile and 4-hydroxycoumarin in the presence of 10 mol % catalyst. The reaction was carried out in aqueous ethanol (1:1, H_2O–EtOH) at room temperature using diammonium hydrogen phosphate ($(NH_4)_2HPO_4$) as catalyst or at reflux using S-proline as catalyst to afford products including including 2-amino-4-phenyl-5-oxo-4, 5-dihydro pyrano-[3, 2-c]chromene-3-carbonitrile (4-PC), 2-amino-4-(4-cyanophenyl)-5-oxo-4, 5-dihydro pyrano-[3, 2-c]chromene-3-carbonitrile (4-CNpC), 2-amino-4-(4-hydroxyphenyl)-5-oxo-4,5-dihydro pyrano-[3, 2-c]chromene-3-carbonitrile (4-HpC), 2-amino-4-(4-nitrophenyl)-5-oxo-4, 5-dihydro pyrano-[3,2-c]chromene-3-carbonitrile (4-NpC), 2-amino-4-(4-nitrophenyl)-5-oxo-4, 5-dihydro pyrano[3, 2-c]chromene-3-carboxylate (c-4-NpC), 2-amino-4-(3-nitrophenyl)-5-oxo-4, 5-dihydro pyrano]3, 2-c[chromene-3-carbonitrile (3-NpC) in high yields. The structure of synthesized compound was confirmed by IR, ^{1}H NMR, ^{13}C NMR, as well as Mass spectrometry (Abdolmohammadi & Balalaie, 2007).

Cell culture. The human K562 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, Penicillin (100 U/ml) and Streptomycin (100 μg/ml) at 37ºC and 5% CO_2.

Cell viability assay. The K562 cells (1×10^5 cells/well) were seeded in 12 well cell culture plates for 24 h prior to treatment, and then treated with different concentrations of the investigated dihydro-pyranochromenes and Cell viability was assessed using the MTT assay (Mosmann 1983). After culturing for 24 h, different concentrations of the compounds were added to each well for 24, 48 and 72 h. After the incubation periods, 10 μL of MTT (final concentration, 0.5 mg/mL) was added to each well, and then the plates were incubated in the dark for 4 h at 37ºC. Afterwards, 100 ml of 0.04 N HCl in isopropanol was added to immediately dissolve the formazan crystals, and the absorbance values were determined at 570 nm using a microplate reader (Elx 800 Microplate Reader, Bio-TEK).

Morphological study of apoptotic cells. For morphological analysis, the cells were seeded in 24-well plates at 1×10^5 cells/well and treated with 100 μM of 3-NpC (stronger compound) for 72 h. Morphology of apoptosis was evaluated by AO/EtBr staining using fluorescence microscopy (Zeiss Axoscope 2 Plus, Germany). Cells were washed with cold PBS and adjusted to a cell density of 1×10^5 using phosphate-buffered saline (PBS). 10 μl of the cells were then put on a glass slide and mixed with AO/EtBr solution (1:1, v/v) in a final concentration of 100 μg/mL (Mahdavi & Yazdanparast, 2007).

DNA fragmentation assay. The 3-NpC treated K562 cells were harvested after 24, 48 and 72 h and washed twice with PBS. 100 ml lytic solution (10 mM Tris-HCl, 10 mM EDTA, 0.5% Triton X-100) was added and mixed well. 10 ml of 10 mg/ml RNase A for 1 h at 37ºC and 10 ml of 20 mg/ml proteinase K for 2 h at 50ºC was also added. DNA was extracted by phenol/ chloroform/isoamyl alcohol (25:24:1) to eliminate the proteins and lipids. Next, it was washed with ethanol and resuspended in TE buffer. Finally, Aliquot of each sample was loaded into the 2% agarose gel including ethidium bromide and electrophoresed for 2 h (Mahdavi *et al*., 2011).

Flow cytometric assessment of apoptosis in K562 cells. To confirm the results of previous analysis, apoptotic cells were quantified by annexin V/PI double staining method with analyzing of phosphatidylserine on the outer surface of apoptotic cell membranes. The treated and untreated K562 cells were harvested after 72 h and washed twice with PBS. Then, 10^5 cells were resuspended in 100 μL binding buffer (1x) and stained with 5 μL FITC-conjugated Annexin V and 10 μL PI, simultaneously. Afterward, it was incubated for 15 min in the dark, and then analyzed by flow cytometry (Partec Pas, Germany) (Moosavi *et al*., 2007).

Cell cycle analysis. The K562 cells (1×10^5 cells/well) were cultured 24 h prior to treatment and treated with 100 μM of 3-NpC for 72 h. After harvesting, the cells were washed twice with PBS, suspended in ethanol (70%) and stored at –20ºC until analysis. Afterward, 50 μg/ml propidium iodide containing 20 μg/ml RNase was added and incubated at room temperature for 2 h ,and the cells were then analyzed by flow cytometry (Nicoletti *et al*., 1991). Cell cycle distribution was analyzed by Cell Cycle Software (Partec Pas, Germany) and percentage of the cells in each phase was determined.

Immunoblot analysis. The K562 cells were treated with 100 μM of 3-NpC, harvested after 48 and 72 h and lysed by loading dye containing, 1% Triton X-100, 1% SDS, 50 mM Tris-Cl (pH 6.8), 100 mM NaCl, 10% glycerol, β-mercaptoethanol and Bromophenol blue. Concentration of protein in each sample was determined using Lowry's method (Lowry *et al*., 1951). Equal quantities of protein (40–50 μg) were loaded on 12% SDS-polyacrylamide gel electrophoresis (PAGE), and were then transferred to PVDF membranes. Transfer of proteins was evaluated by ponceau-red staining. Non-specific binding sites of membranes were blocked by incubation in the blocking buffer (TBS buffer containing 0.1% Tween-20 and 5% milk) for 1 h at room temperature. After overnight incubation with primary anti-bodies (anti-caspase-3 and anti-actin) at 4ºC, the membranes were incubated by HRP conjugated secondary anti-bodies for 1 h. Finally, the proteins were detected by ECL (enhanced chemiluminescence) detection system (ThermoScientific) and intensities of the bands were quantified with the NIH ImageJ software.

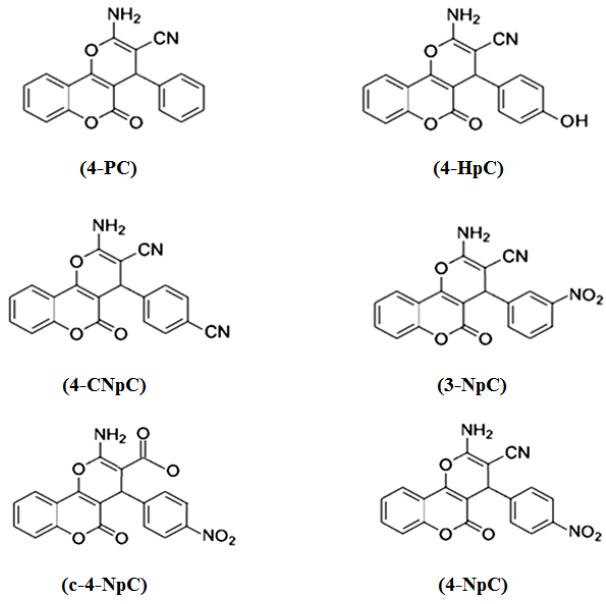

Figure 1. Structure of the investigated dihydro-pyranochromenes derivatives.

Statistical evaluation. Data are represented as mean ±S.D. of three independent tests and statistically analyzed by Student's *t*-test. Differences were considered meaningful at $p<0.05$.

RESULTS

MTT assay was performed for evaluation of the effect of the investigated dihydro-pyranochromenes on the viability of the K562 cells. The cells were treated with different concentrations of the compounds for 24, 48 and 72 h. The results of MTT assay showed that all compounds reduced the cell viability, in a dose- and time-dependent manner (all the data not shown). As shown in Table 1 after exposure for 72 hours, the IC50 value of 4-PC, 4-CNpC, 4-HpC, 4-NpC, c-4-NpC and 3-NpC for K562 cells were 300±4.5, 260±5.2 240±3.8 220±4.7 140±2.6 and 100±3.1 μM, respectively. As shown in Table 1, the 3-NpC was more toxic in comparison with other compounds and was chosen for other studies. In K562 cells exposed to 140 μM of 3-NpC for 72 h, the cell viability decreased approximately 65–70% compared with control (Fig. 2).

Table 1. IC50 values of investigated compounds after 72 h.
The IC50 value of 3-NpC for K562 cells was 100±3.1 μM and it was more toxic in comparison with other compounds.

Compound	IC50 (μM)
4-PC	300±4.5
4-CNpC	260±5.2
4-HpC	240±3.8
4-NpC	220±4.7
c-4-NpC	140±2.6
3-NpC	100±3.1

Each value represents the average of triplicate measurements ±S.D.

The morphology of K562 cells treated with 100 μM of the 3-NpC was investigated by phase contrast microscopy (Zeiss, Germany). It was found that control cells were in a round form, while the treated cells aggregated after 24 h, and apoptotic bodies were formed by parts of the condensed cytoplasm and nucleus after 48–72 h (Fig. 3). These data confirmed the occurrence of apoptosis in the treated cells.

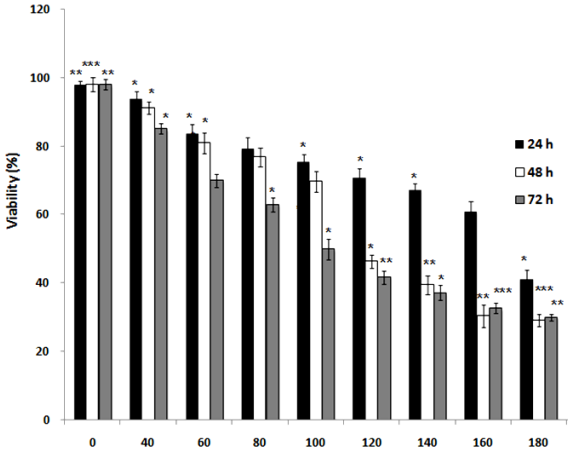

Figure 2. Effect of the 3-NpC on cell viability of the K562 cells.
The cells were subjected to indicated concentrations of the 3-NpC for 24, 48 and 72 h. Cell viability was assessed by MTT test and presented as percent of the corresponding controls. The results are the means of three independent experiments] (***) $P<0.001$, (**) $P<0.01$, (*) $P<0.05$).

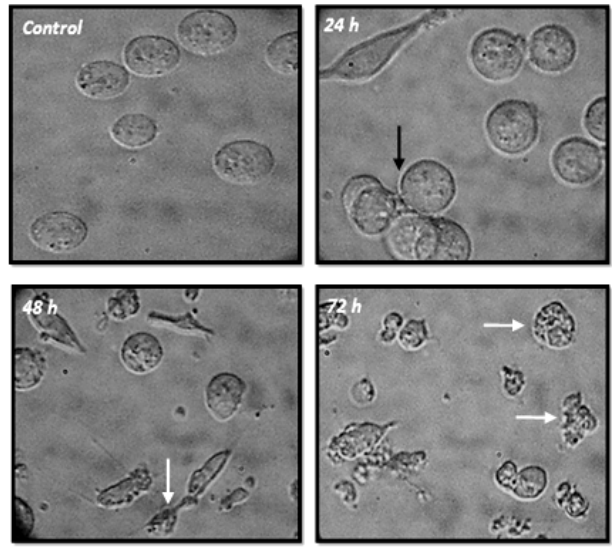

Figure 3. Morphological changes of K562 cells treated with 100 μM 3-NpC.
Images of the K562 cells were taken by phase contrast microscope. After 24 h of exposures to 3-NpC, the cells aggregated (black arrow) and after 48 and 72 h, cytoplasm and nucleus were condensed (white arrow), whereas control cells were found in a round form.

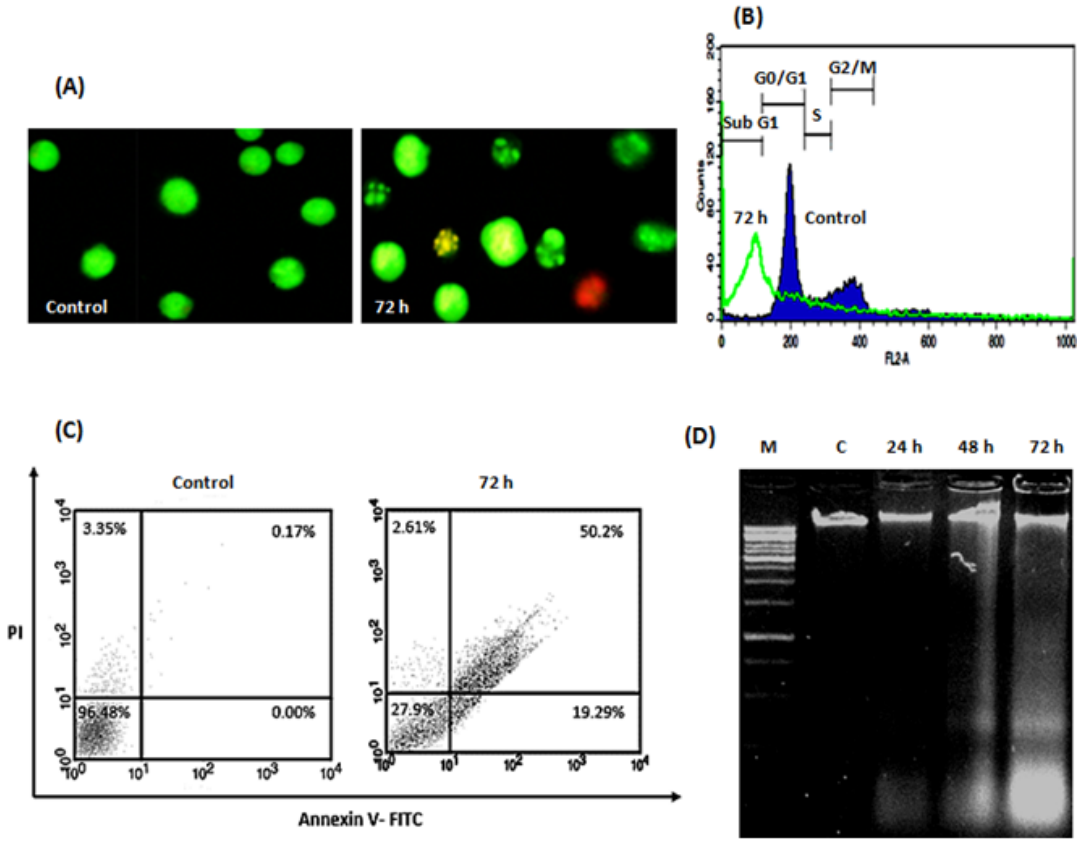

Figure 4. Induction of apoptosis by 3-NpC in K562 cells.
(**A**) Fluorescence micrograph of the K562 cells. After treatment with 3-NpC (100 μM) for 72 h, the cells were harvested and were stained with AO/EtBr. 3-NpC induced condensation and fragmentation of the nuclei (Magnification, 100×). (**B**) Cell cycle analysis of K562 treated-cells by flow cytometry at 72 h after treatment with 100 μM of the 3-NpC. Accumulation of the cells in Sub-G1 peak indicated occurrence of apoptosis. (**C**) Apoptosis was followed by Annexin-V/PI double staining method. After 72 h, untreated and 3NC-treated K562 cells were harvested and analyzed. Flow cytometric analysis showed increased percentage of apoptotic cell. (**D**) DNA fragmentation after 24, 48 and 72 h treatment of the K562 cells with 100 μM of 3-NpC. Agarose gel electrophoresis detected DNA fragmentation pattern.

We also studied the nuclear morphology of the cells using AO/EtBr double staining and the fluorescence microscopy technique. The cells were subjected to 100 μM of 3-NpC for 72 h. Fluorescence microscopy analysis showed that in control cells, the nucleus was big and round without condensation or fragmentation, while the treated cells showed chromatin condensation and fragmentation, emphasizing the happening of apoptosis (Fig. 4A). The live cells appeared green in color with unscathed nuclei, while the early apoptotic cells showed condensed nuclei and were in green color with bright green dots in their nuclei, and the late apoptotic cells appeared in orange color (Fig. 4A).

In addition, DNA fragmentation assay with agarose gel electrophoresis demonstrated a laddering pattern of DNA during treatment with 100 μM of the 3-NpC (Fig. 4D).

Exposure of phosphatidylserine on the surface of the cell is a marker of apoptosis. Annexin V/PI double staining method confirmed apoptosis of K562 treated-cells. The cells in the lower left quarter show viable cells, in the lower right quarter show early apoptotic cells, in the upper left quarter show necrotic cells and in the upper right quarter show late apoptotic cells. Flow cytometric analysis revealed that the apoptotic cell percentage in the control cells from 0.17% increased to 69.31% after 72 h of treatment (Fig. 4C). These results indicated the occurrence of apoptosis in K562 treated-cells.

Distribution of the cells in different phases of cell cycle was studied by flow cytometry. It detected that 3-NpC significantly increased the number of cells in the sub-G1 phase. The cell cycle distribution of control K562 cells were among sub-G1, G0/G1, S and G2/M phase by almost 3.62%, 43.44%, 11.75% and 23.17%, respectively. After treatment of the K562 cells for 72 h, the cell cycle distribution was 49.26%, 22.35%, 10.17% and 9.74% at sub-G1, G0/G1, S and G2/M phase, respectively (Fig. 4B and Table 2). Therefore, appearance of sub-G1 peak (mostly dead cells) after treatment of K562 cells with 100 μM 3-NpC confirmed the happening of apoptosis.

The caspases play a key role in apoptosis. Caspase-3 is an important executioner caspase and is expressed as an inactive pro-enzyme. The activation of caspase-3 is done by an initiator caspase *via* internal cleavages to detach the large and small subunits. Western blot was used to assess protein levels of procaspase-3 and cleavage of caspase-3. As shown in Fig. 5A significant increase in the expression of procaspase-3 and caspase-3 cleavage was seen after 48–72 h of 3-NpC treatment. This increase was approximately 0.4±0.15 and 1.4±0.3 fold after 48 and 72 h, respectively (Fig. 5B). Therefore, the result of this assay also confirmed that cell death is occurring *via* apoptosis.

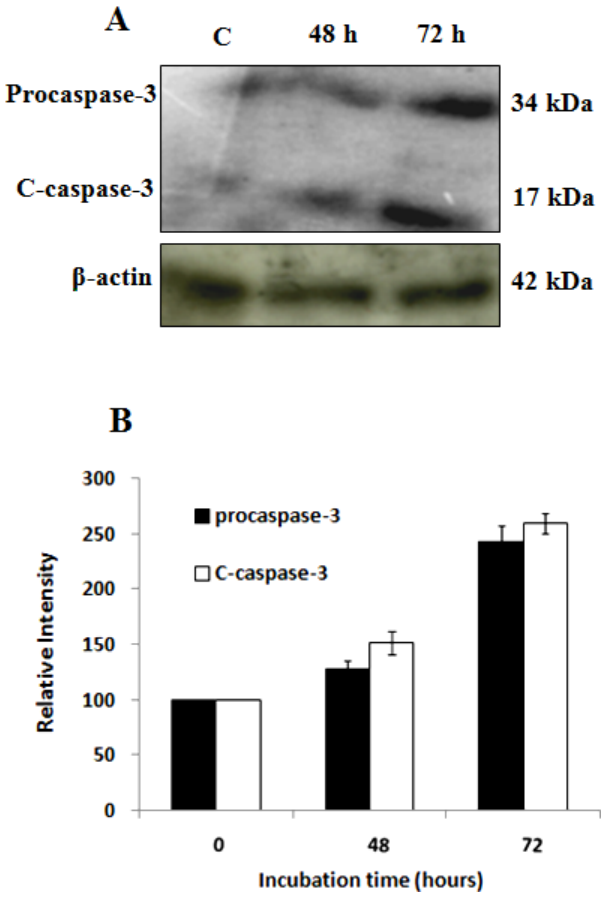

Figure 5. Effect of the 3-NpC on caspase-3 activation in the K562 cell line.
(**A**) Protein expression level of procaspase-3 and C-caspase-3 was determined after 48 and 72 h treatment of the cells with 100 μM of the 3-NpC. β-Actin was used as protein loading control. (**B**) Quantitation of relative changes in expression level of procaspase-3 and C-caspase-3 by ImageJ software and normalized to the intensity of β-actin protein.

DISCUSSION

Dihydro-pyranochromene derivatives are heterocyclic compounds that demonstrate significant biological and pharmaceutical activities (Thomas & Zachariah, 2013; Ghorbani-Vaghei *et al.*, 2011). In recent years, the cytotoxic activities of tetrahydro-pyranochromenes and dihydro-pyranopyran derivatives against various cancer cell lines have been demonstrated. Interestingly, different cells responded differently to the compounds in a cell type dependent manner (Emmadi *et al.*, 2012; Heidary Alizadeh *et al.*, 2010). The discovery and characterization of 4-aryl-4H-chromenes family as potent inducers of apoptosis in T47D and Jurkat cell lines was also reported (Kemnitzer *et al.*, 2004). Cytotoxic activity of the new series of these compounds was also investigated against various tumor cell lines including MCF-7, KB, Hep-G2, MDA-MB-231 and SKNMC (Akbarzadeh *et al.*, 2012). Among these compounds, the 5-(3-methylphenyl) isoxazol-3-yl analog showed the most potent cytotoxic activity against all five human tumor cell lines. In contrast to other studies, these cells did not respond differently to the compounds in a cell type dependent manner. The proposed mechanism for these series of compounds was found to be tubulin destabilizers, binding at or close to the binding site of colchicine (Kemnitzer *et al.*, 2004). In another study, Anti-proliferative activity of the pyrano[3,2-c]benzopyran derivatives were investigated against MCF-7 (breast cancer cell line), A549 (human lung cancer cell line) and Bel7402 (liver cancer cell line) (Mo *et al.*, 2004; Jacquot *et al.*, 2002). Although these compounds showed a significant cytotoxic activity, neither of them exhibited a significant estrogenicity or an affinity for estrogen receptors alpha (hER alpha). Some 2-amino-4-aryl-3-cyano-7-(dimethylamino)-4H-chromenes were also tested against a panel of six human tumor cell lines and the cytotoxic activities of these compounds have been detected (Vosooghi *et al.*, 2010). Substitution of fluoro, chloro and bromo atoms in 2-, 3- and 4-positions of the phenyl ring showed that halogenations of the C3 position resulted in a significant increase of activity and also that substitution of F or Br in 3- position obtained better result (Vosooghi *et al.*, 2010). In the present study, we investigated the anti-cancer effect of dihydro-pyranochromene derivatives on K562 cell line as experimental model for CML. In our investigation, it was observed that dihydro-pyranochromene derivatives decreased the viability of K562 cells in a dose and time dependent manner. It was revealed that 4-PC is less active compound against the compounds that contain any electron-withdrawing groups such as NO_2, OH and CN in the phenyl ring. The 3-NpC was found to be the most active compound with IC50 value of 100±3.1 μM at 72 h, because of substitution of NO_2 group at the 3-position of the phenyl ring. It was found that replacing the 3-CN group (4-NpC) by 3-methyl ester (c-4-NpC) increased the activity of this compound while according to recent studies, replacing the 3-CN group by 3-methyl ester (or 3-ethyl ester) in 4-aryl-4H-chromenes decreased the activity of the compounds (Kemnitzer *et al.*, 2004). Since inhibition of apoptosis is a hallmark of cancers, induction of apoptosis in cancer cells is one of the suitable plans for anti-cancer agent expansion (Kavanagh, 2003). In our study, we used variable assessments for confirming the occurrence of apoptosis in the K562 cells by 3-NpC such as DNA fragmentation assay and morphological studies. Translocation of phosphatidyl serine to the surface of the cell is a sign of apoptosis. To confirm this matter, we used annexin V/PI double staining method. Furthermore, because of close relation between cell cycle and apoptosis, we evaluated sub-G1 phase of the cell cycle in K562 treated-cells. For further evaluation, activation of caspase-3 that plays an important role in the initiation and execution pathways of apoptosis was analyzed.

In conclusion, we reported dihydro-pyranochromenes derivatives as potent apoptosis inducing agents in the K562 cells. We found that 2-amino-4-(3-nitrophenyl)-5-oxo-4, 5-dihydro pyrano]3, 2-c[chromene-3-carbonitrile (3-NpC) is most active in comparison with the other compounds. This compound is good candidate for further pharmacological studies to determine efficient chemotherapeutic for the treatment of human cancer disorders.

Table 2. The percentage of control and 3-NpC treated cells in each phase of the cell cycle.
The percentage of apoptotic (Sub-G1 phase) and non-apoptotic (other phases) cells in each cycle was detected by flow cytometry.

Time	Sub-G1	G0/G1	S	G2/M
Control	3.62±1.21	43.44± 2.34	11.75±1.07	23.17±0.87
72 h	49.26±2.46	22.35±1.65	10.17±0.48	9.74±0.73

Data are reported as means ± S.E.M. of the percent of control cells.

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