Anti-proliferative and Apoptotic Effects of morin in human Leukemia cell lines (HUT-78)

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Abstract

Cancer is one of the main cause of mortality in the world which appears by the effect of enviromental physico-chemical mutagen and carcinogen agents. Lymphoblastic leukemia is one the prevalence cancer in human. Chemotherapy and radiotherapy improve remission of the disease but some probability relaps is observed between 20-30% patients. Tumorgenesis and tumor progression are strongly associated with abnormal apoptosis. A number of natural antitumor drugs exert their therapeutic effect by inducing or promoting apoptosis [1]. In the last years, many studies have been performed on the anticancer effects of flavonoids. In cancer therapies, natural compounds have been considered as effective inhibitor agents. There is evidence to support the concept that 2',3,4',5,7 penta hydroxyl flavone (morin) has a great potential to develop into novel cancer preventative agent [2].

Aim

To investigate the effect of morin on apoptosis of human leukemia cells (Nalm-6 and HUT-78).

Material and methods

In this study human chronic lymphocytic leukemia (HUT-78) and human leukemia pre B-cells (Nalm-6) were cultured in RPMI 1640[Sigma], supplemented with 10% fetal bovine serum (FBS), penecilin-streptomycin and L-glutamine. The cultures were incubated at 37°C, 5% CO2 and then inhibitory effect of morin on their proliferation was measured by MTT assay. Cell cycle progression monitored by Sub-G1 apoptosis assay using flow cytometry and Hoechst staining method.

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Results
MTT assay showed that morin inhibited proliferation of Nalm-6 and HUT-78 cell lines in dose-dependent manner significantly (P<0.05, P<0.01, P<0.001). The cell cycle assay using flowcytometry analysis indicated DNA content on the basis of DNA binding to (Propidium iodide) PI. Nalm-6 and HUT-78 cells showed low DNA staining in apoptotic cells. Hoechst staining also showed significant cell death in both cell lines.

Conclusion
The results demonstrated that morin induced apoptosis and Sub-G1 phase formation in leukemia Nalm-6 and HUT-78 cells, therefore it has a potential as an anticancer agent.

Keywords: morin, Nalm-6 and HUT-78 cell line, Anticancer, MTT assay, Sub-G1 assay, apoptosis.

1 Introduction

In recent years, The morbidity and mortality of cancer still reaches a high plateau and is a major public health problem world wide [1] Morin is a flavonoid and ubiquitously occurring and widely consumed as secondary metabolites. There are reports that indicate morin possess a wide variety of physiological activites [3, 4, 5] Very recently, identifying active components from food, vegetables, fruits with apoptosis inducing activity against cancer cell lines and has been emphasized that apoptosis is considered as a primary mechanism for chemoprevention of cancer. Thus, The studies devoting to assess that mechanisms of action of cancer cells appear to be remarkable importance [6, 7, 8, 9].

Many studies report that a high diet in fruits and vegetables lowers the incidence of cancer [10, 11] It has been reported that various fruit and vegetable extracts, particularly grape extract are capable of inhibiting the proteasome activity and this inhibition is associated with tumor cell apoptosis [12]. Morin is an antioxidant that protects various human cells, like myocytes, endothelial cells, hepatocytes, and erythrocytes against oxyradicals damage [13, 14]. Recent studies have suggested that morin may protect free-radical-induced damage of DNA by a mechanism other than solely direct free-radical scavenging [15]. Several in vitro studies have investigated that flavonoids cause inhibition of cellular transformation and proliferation [15, 16] Flavonoids are known to suppress tumor cell growth that is mediated by different type of cell cycle arrest and induction of apoptosis in several tumor cell lines [17, 18, 19].

Apoptosis, or programmed cell death, has an essential role in controlling cell number in many developmental and physiological processes. Apoptosis is impaired in many human tumors, suggesting that disruption of apoptotic function contributes substantially to the transformation of a normal cell into a tumor cell. Apoptosis is an important phenomenon in chemotherapy-induced tumor-cell killing. Components of the apoptosis signaling cascade including caspases [20, 21] along with several other triggers and regulators such as FAS ligand (Fasl) and Bcl-2 family members, are among the most promising targets for pharmacological modulation of cell death [22, 23].

We investigated the effect of morin on Hut-78 and Nalm-6 Human Leukemia cells. Such as a cytotoxicity effect (MTT assay), apoptosis (Hoechst 33258 staining), cell cycle progression (sub-G1 assay).

2 Material and Methods

2.1. Cell line and cell culture
HuT-78 cells were derived from peripheral blood of a 50-years old Caucasian male patient with sezary syndrome. The cells exhibit features of mature T-cell line with inducer/helper phenotype and release T-cell growth factor (IL-2) [24]. Nalm-6 cell established from the peripheral blood of a 19-year-old man with
acute lymphoblastic leukemia (ALL) in relapse in 1976 [25]. Both of them were cultured in RPMI 1640 with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C in 5% CO₂ incubator.

2.2. Cell viability assay
To determine cell viability a microculture tetrazolium technique (MTT) assay was performed according to the method previously described by Arung et al (R-11), with minor modifications. The MTT assay provides quantitative measurement of viable cells by determining the amount of formazan crystals produced by metabolically active cells.

Nalm-6 or HuT-78 cells were seeded into 96-well plate (5-7×10³ cells per well). After 24 h incubation morin in different concentrations were added to each well and incubated for 48 hours, followed by incubation with 5 mg/ml MTT for 4h. The supernatant was removed after centrifugation, finally 100 μL of DMSO was added to each well.

The absorbance of cells was measured at 570 nm with Eliza reader. The data were analyzed with Tukey Test measured by one way ANOVA.

3 Flow cytometric analysis of sub-G₁ apoptosis
The cells were separated into three treatment groups as stated for the cell viability assay above. The cells were analyzed for sub-G₁ apoptosis using the method described by Sandra et al (R-12) Cellular DNA content was determined by flow cytometric analysis of PI-labeled cells. HuT-78 and Nalm-6 cells were grown to exponential phase, seeded at density of 10⁶ cells per well into 24-well plate and incubated for 24 h then treated with 3 concentration of morin and incubated for 48 h, followed by incubation the cells were harvested and fixed in ice-cold 70%. ethanol, stored at 4°C washed with PBS (phosphate buffered saline) (PH.7.2), treated with 25 μg/mL RNase at 37°C for 15 min, and stained with 50μg/mL propidium iodide (PI) for 20 min. The PI fluorescence of individual nuclei was measured using a FACS caliber apparatus.

4 Hoechst 33258 and staining
Cells in the process of apoptosis would show significant morphological changes in the nuclear chromatin, which can be revealed by Hoechst 33258 staining. Cells (1×10⁶/mL) were seeded in the 6-well plates and incubated at 37°C for 24 h in RPMI 1640 medium containing 10% FBS and 1% penestrep. The medium was changed and the cells were incubated for 48 h prior to treatment with 10μg/mL and 50μg/mL morin. After treatment the cells were washed twice with PBS and then fixed for 30 min at 4°C with 300 μL 3% paraformaldehyde in PBS.

After fixation the cells were collected at 1800 rpm in a centrifuge, and then washed three time with PBS and centrifuged. The cells were stained with 300μl bis-benzimide (Hoechst 33258) at a concentration of 1mg/mL for 30 min in dark and room temperature. Finally, the cells were washed once with PBS (1mL) and then resuspend in 300μL PBS.

The stained nuclei were viewed by fluorescence microscopy. Appropriate excitation filters were used for detection of Hoechst 33258 (365 nm excitation and 420 nm emission).

The IC50 (medium inhibition concentration) is the concentration of toxic compound that reduces the biological activity by 50%. The IC50 value was obtained from the MTT assay and the data were analyzed with SPSS software. Differences were considered to be statistically significant at p<0.05, p<0.01 and p<0.001.
5 Result

5.1. Vital capacity test
To investigate the potential effect of Morin on proliferation of HuT-78 and Nalm-6 cells, the cells were exposed to 0-100\(\mu\)g/mL of morin for 48h. Fig 1, 2 show that morin induces cell death in dose-dependent manner. Further, exposure to morin was associated with cell shrinkage as detected in phase-contrast microscopy (Fig 3, 4.) IC\(_{50}\) was reached at a concentration of 10\(\mu\)g/mL in both cell lines.

<table>
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<th>S.DEV</th>
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<tr>
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Figure 1: Antiproliferative effect of morin on HUT-78 in 48h. (P<0.001***)

<table>
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<tr>
<th>dose</th>
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<th>S.DEV</th>
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<tr>
<td>100</td>
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Figure 2: Antiproliferative effect of morin on Nalm-6 in 48h. (P<0.05*, P<0.01**)
Figure 3: Photomicrograph of HUT_78 treatment by morin in 48h: A) control, B) treatment 10µg/ml, C) treatment 50µg/ml x 20
5.2. Sub-G1 apoptosis
To understand the mechanism of inhibition of morin, The effect of morin on cell cycle progression was analyzed by flowcytometry. In this experiment, 0, 10 and 50 µg/mL of morin were used. Fig. 5 shows that sub-G₁ apoptotic cell content was markedly increased when HuT-78 and Nalm-6 cells were treated with morin. In the absence of the morin, sub G₁ was formed 2.84% (Nalm-6) and 3.64% (HUT-78) (fig. 5-a) whereas a concentration of 10µg/mL resulted on 31.84% (Nalm-6) and 54.27% (HUT-78) of cells being in sub G₁ phase (fig. 5-b). This proportion of cells further increased in 50µg/mL concentration morin, 65.42% (Nalm-6) and 72.32 (HUT-78) (fig. 5-c). Therefore, an increase in the sub-G₁ apoptotic fraction may be the major cause of reduced viability of Nalm-6 and HuT-78.
5.3. Result of Hoechst staining

The nuclei were stained with bis-benzimide (Hoechst 33258) in order to study any nuclear morphology changes. Cells undergoing apoptosis displayed profound structural changes, including a rapid blebbing of the plasma membrane and nuclear disintegration of the nuclei of cells treated with morin. There were more shrinking cells that lost their inhibiting ability in the morin treated group than in the control (fig.6).
6 Discussion

Phenolic compounds constitute one of the main classes of secondary metabolites [26]. They also contribute to the nutritional qualities of fruits and vegetables. Among these compounds, flavonoids constitute one of the most ubiquitous groups of plant phenolics [27]. Flavonoids occur as aglycones, glycosides and methylated derivatives [28]. Flavonoids belong to a chemically heterogeneous group of small molecules with chemopreventive activity [29]. They exert specific cytotoxic activity towards cancer cells which has generated large interest in developing flavonoid-based cytostatics for anti-cancer therapy [30]. Previous studies have demonstrated a significant anti-cancer activity in some natural flavonoids such as apigenin [31], genistein [32], quercetin [33] and luteolin [34]. In other studies, flavone [35], luteolin [36], genistein [37], quercetin [38], and fisetin [39], induced significant apoptosis in Bv-173 cells, while genistin and rutin did not. The apoptosis inducing effect of apigenin was intermediate. morin has a molecular weight less than 500 Da. It has not more than 5 hydrogen donor sites and 10 acceptor sites and log P value under 5. This property indicates its drug likeness/oral bioavailability as suggested by Lipinski’s rule of five [40]. Also morin is an isomere of quercetin, the lead compound on which L Y...
294002 was designed, which has been extensively used both in vivo and in vitro for targeting phosphoinositide-3-kinase (pI3K) in different cancers [41].

In this investigation we have characterized the effect of morin on the proliferation of human leukemia cell lines namely HuT-18 and Nalm-6 (pre-B cell) cells. Primary results demonstrated that morin inhibited growth of HuT-78 and Nalm-6 cells in a concentration range of 10-50μg/mL. Further analysis demonstrated that cultured HuT-78 and Nalm-6 cells, treated with morin exhibited morphological features of apoptosis such as a membrane shrinkage and chromosomal condensation (Fig.6-1) which is supported by additional findings. morin has a cytotoxic effect on HuT-78 and Nalm-6 in a concentration dependent manner, with IC50 10μg/mL of the drug. (Fig. 4-1, 4-2) altered nuclear morphology (Fig. 4-3, 4-4) are consistent with the previous reports of cells undergoing apoptosis. Kerr et al 2008 stated that characteristics of cells undergoing apoptosis include the formation of sharply delineated, uniformly fine granular masses adjacent to the nuclear envelope and cytoplasmic condensation[42] Morphological analysis of sub.G1 apoptosis assay also confirmed that morin induced apoptosis in HuT-78 and Nalm-6 Cells. (Fig. 5-1, 5-2). Apoptosis usually results in typical morphological and biochemical, characteristics, including condensed chromatin in the cells, appearance of apoptotic bodies, presence of hypodiploid peak in F.CM analysis and DNA ladder bands on agarose electrophoresis [43, 44].

Our data in MTT and sub G1 clearly indicates that certain morin has a cytotoxic effects on HuT-78 stronger than Nalm-6.

![Figure 7: Comparing of cytotoxicity effect of morin on both of Nalm-6 and HUT-78](image)

Our results indicated that morin inhibited growth of Hut-78 and Nalm-6 cells and inducing G0/G1 phase arrest in human leukemia cell.

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