Original Article

Apoptotic effects of acacetin in human colon cancer HT-29 and HCT 116 cells

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ABSTRACT

Aim: Acacetin is a natural flavone compound, which is found in several plants as Robinia pseudoacacia and is demonstrated to have anticancerogenic activities in many types of cancer (e.g., human nonsmall cell lung cancer, and prostate). Colorectal carcinoma (CRC) is one of the serious health problems and is a complex disease. We intended to find a more effective new candidate for the treatment of colon cancer, and hence, we designed this study to investigate the effects of acacetin on CRC (HT-29, HCT 116) *in vitro*.

Methods: The study was carried out with the methods that determine for apoptosis (WST-1, Caspase 3/BCA, Annexin V).

Results: Acacetin showed antitumor and apoptosis-inducing effects in the CRC cell lines.

Conclusions: Acacetin was effective on CRC cell lines, besides no lethal effect on healthy lung cells (MRC-5).

KEY WORDS: Anticancer, apoptosis, caspase, colorectal, flavonoid

INTRODUCTION

Colorectal carcinoma (CRC) is one of the most common cancer and also one of the main death causes.[1] Its incidence increases with age, and more men than women are affected.[2] In general, the diagnosis of this cancer arises late because of the accelerated metastasis formation. One of the main obstacles in accomplishing a more effective treatment is very rapid spreading of cancer cells through bloodstream.[3] Chemoprevention is the cost-effective and practical approach with a huge potential in cancer prevention. It can be defined as the use of artificial/organic agents to abolish the beginning phases of cancer development or disrupt the promotion of malignant cells to metastatic cancer.[4]

Recently, much research has been performed to obtain knowledge about natural compounds with potential anticancer activity. Various agents derived from plants have been providing a great source for new ideal candidates of cancer therapy.[5] Characteristics of flavonoids, such as safe and easily obtainable, having no substantial harmful effect to healthy cells, making them ideal alternatives for new therapeutics.[6] Acacetin (5,7-dihydorxy-4'-methoxyflavone), is an O-methylated flavone which is found in the plant such as Robinia pseudacacia, as a cancer therapeutics.[7] Acacetin contributes anticancerogenic actions against many kinds of cancer cells including gastric carcinoma, prostate cancer, and T-cell leukemia and also has antiproliferative, anti-inflammatory effects.[8] Acacetin inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression by repressing PI3K/Akt/Ikk, and mitogen-activated protein kinase signaling pathways,[9] coordinates mitogen-activated protein kinases signaling comprising MLK3/MKK3/6 and p38. In our research, we investigated the effects of acacetin in different concentrations and at different times on CRC cell lines using methods that determine for apoptosis (WST-1, Caspase 3/BCA, Annexin V). Our results suggest that acacetin possesses anticancer activity.

MATERIALS AND METHODS

Cell culturing components

The CRC lines HT-29, HCT 116, and healthy lung fibroblastic cell line MRC-5 were obtained from ATCC® (American Type Culture Collection,

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Manassas, VA, USA). The HT-29 and HCT 116 cell line was cultured in McCoy's 5A medium containing 1% penicillin/streptomycin and 10% fetal bovine serum and also DMEM medium was used for culturing MRC-5 cell lines in the condition of at 37°C in 5% CO_2.

Cytotoxicity

After acacetin treatment on HT-29, HCT 116, and MRC-5 cell lines, WST-1 cell proliferation assay was performed to find its role in cell viability. Increasing concentrations of acacetin were obtained on 1×10^4 cells/well, then incubated at 37°C in 5% CO_2 for 24, 48 and 72 h. When the incubation was completed, 10 µl of WST-1 was treated on cells to measure color change levels. Multiscan ELISA reader (Thermo Fisher Scientific™, Germany) was used at 450 nm wavelengths for color development.

Analysis of caspase-3 enzyme activation

The changes of caspase-3 enzyme activity in cancer cells were examined, which is one other important apoptosis sign by using a caspase-3 colorimetric assay kit (BioVision™ Research Products, USA). The chromophore p-nitroanilide (pNA) after cleavage from the fluorogenic substrate DEVD-pNA was measured, spectrophotometricly. After incubation of reaction mix and cells, the mixture was read under 450 nm wavelengths on an Elisa reader (Thermo Electron Corporation Multiskan®Spectrum, Finland). The absorbance values are established to protein concentrations and detected using a Bradford assay.

Phosphatidylserine exposure and cell permeability

It is a well-known outcome that when cell death occurs by apoptosis, phosphatidylserine (PS) components change its location through the cell surface. This information makes PS as an early apoptotic marker for cell death. For this purpose, the level of PS can be distinguished using the green fluorescent dye Annexin V-FITC (BD Pharmingen™, Germany). After cells and dyes were plated in 250 µl buffer and incubation cells, mixture were analyzed immediately using flow cytometry.

Statistical analysis

Results are expressed as the mean, standard error of the mean. Analysis of data was performed using one-way ANOVA.

RESULTS

Concentration- and time-dependent inhibitory effect of acacetin on colorectal carcinoma cells

Due to the determination of antiproliferation effects for acacetin on HT-29, HCT 116, and MRC-5 healthy cells, the cells were incubated with increasing concentrations (5, 10, 25, 50, and 100 µM) of acacetin for 24, 48 and 72 h using WST-1 cell proliferation assay. According to the results of the WST-1 assay, decreased cell proliferation level was found in the CRC cell line as compared with controls ($^*P < 0.05$). However, there were no important changes in the healthy cell line.

Investigation of acacetin effect on caspase-3 enzyme activity

The HT-29, HCT 116, and MRC-5 cells were treated with increasing concentrations of acacetin for 48 h, and analysis of changes in caspase-3 enzyme activities was made to find the relation for apoptotic way. Compared with the untreated cells, there was a 1.02-fold increase in caspase-3 activity in response to 48 h treatment with 5 µM, and a 1.12-fold decrease in response to 48 h treatment with 10 µM on HT-29 cells ($^*P < 0.05$) [Figure 1]. Furthermore, there was 1.53-fold decrease in response to 48 h incubation with 10 µM and 1.29 fold decrease in caspase-3 activity in response to 48 h incubation with 25 µM on HCT 116 cells. There was no change in the caspase-3 activity of MRC-5 cells under the same conditions.

Quantification of apoptosis by FITC Annexin V/PI double staining

FITC Annexin V/PI double staining was applied to HT-29 and HCT 116 CRC cells and MRC-5 healthy lung fibroblastic cells, which had been exposed to 5 µM and 10 µM acacetin for 48 h on HT-29 cells and had been exposed to 10 µM and 25 µM acacetin for 48 h on HCT 116 cells. The results demonstrated that 48 h incubation of HT-29 and HCT 116 cells with acacetin increased apoptotic cell death when compared with the healthy group ($^*P < 0.05$) [Figures 2 and 3]. Nevertheless, there were no changes in the MRC-5 cell line.

DISCUSSION

The many number of new development on cancer therapy has been improved, however struggling to cope with cancer still a challenge. CRC is one of the major causes of cancer-related death.

It is estimated that one-third of all cancer deaths could be avoided through appropriate dietary modifications.[10] Dietary vegetables and fruits consist of bioactive, nonnutrient, plant compounds called phytochemicals. Many kinds of phytochemicals can affect cancer-related cell signal transduction by the suppression of special enzymes such as different kinds of protein kinases.[11] Many phytochemicals also have been shown cytotoxic effect on different kinds of cancer cells without the side effects of other anticancerogenic agents.[12] Acacetin is one of these phytochemicals, which is called flavonoids that have antiproliferative effects of various cancer cells. It works as an anticancerogenic effect by stopping the cell cycle and stimulating the apoptotic pathways.

Apoptotic and necrotic effects of acacetin on HT-29 and HCT 116 cells have not been addressed before. In this study, we performed cytotoxicity, caspase-3 activity, and apoptotic effect of acacetin on CRC cell lines for the first time.

Shim *et al.* have worked with acacetin activity on MCF-5 breast cancer cells, and their findings suggested that acacetin-stimulate apoptosis by increasing generation of

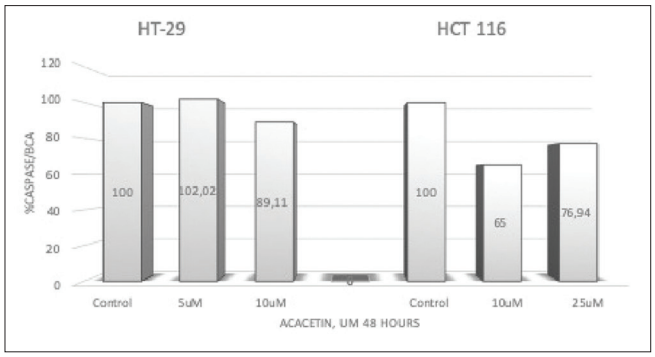


Figure 1: Caspase-3 enzyme activity in response to different concentrations on HT-29 and HCT 116 cell line

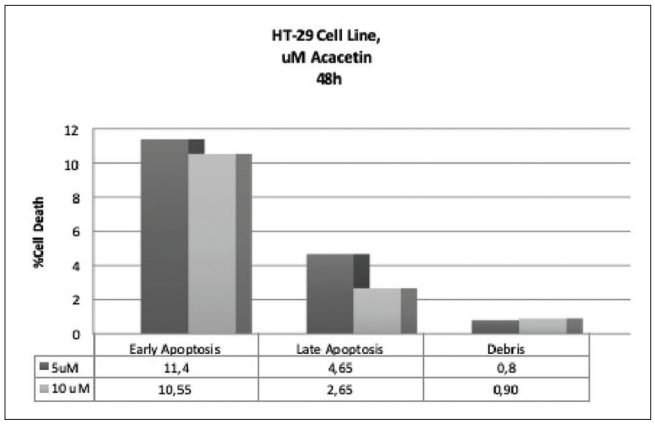


Figure 2: Acacetin caused the apoptosis resulting in the translocation of phosphatidylserine on HT-29 cells

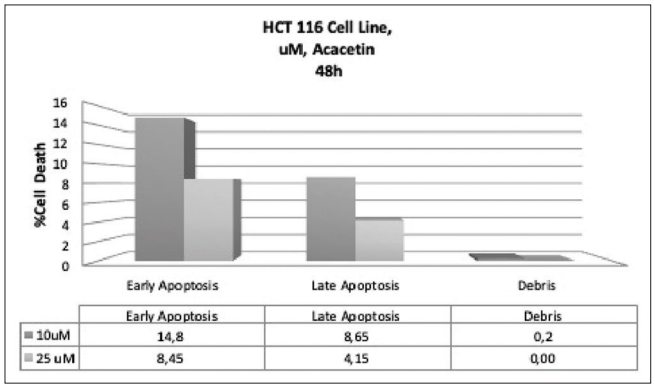


Figure 3: Acacetin caused the apoptosis resulting in the translocation of phosphatidylserine on HCT 116 cells

ROS and caspase 8 activation causing to a reduction of mitochondrial membrane potential and the release of AIF and cytochrome c. They also found that acacetin plays a role in the activation of caspase-7 cascade that occurs in caspase-8 and 9 stimulation, so as resulted in apoptosis.[13]

The PI3-K/Akt signaling pathway is also an important cancerogenesis. Jung *et al*. showed that acacetin is a powerful inhibitor of PI3-K and has a suppression effect of melanoma cell growth. Chien *et al*. also reported acacetin restrain the proliferation of nonsmall cell lung cancer cells, A549.[14] Furthermore, they performed that acacetin does not lead to the formation of harmful effects on healthy lung fibroblast WI-38 cells.

According to our cytotoxicity test, 5 μM and 10 μM doses were the most effective doses, and 48 h was determined the optimum time for the HT-29 cells, 10 μM and 25 μM doses were the most effective doses, and 48 h was also determined the optimum time for the HCT 116 cells.

The caspase 3 activation cascade is one of the major kinases for a number of apoptotic mechanisms. We showed acacetin-triggered caspase-3 activation in HT-29 cells at 5 μM. In addition to these data, our results showed decrease effect of caspase activation with the incubation 10 μM and 25 μM acacetin over 48 h in HT-29 cells and HCT 116 cells. Therefore, we suggested acacetin stimulated apoptosis in a concentration and time-dependent manner, which could be associated with other caspase activities or alternative apoptotic pathways.

Our results showed correlation with studies of Watanabe *et al*.,[15] they found that inhibition of acacetin to the growth of the human T cell leukemia cell lines by caspase-3 activity. We also obtain results about acacetin-induced activation of caspase-3 in HT-29 cells with at incubation of 5 μM concentration of acacetin. Oppositely, we found that decreasing the influence of acacetin on caspase-3 activity in HCT 116 cells. These different result of different types of colon cancer cell lines depends on the distinctive characteristic features of the cells itself.

Pan *et al*. also investigated that caspases played any role in cell death mechanisms stimulated by acacetin. They found acacetin activated apoptosis in a time and dosage-dependent manner in AGS cells via caspase-3 activation but not caspase-1. Similarly, we found out acacetin treatment to HT-29 cells promoted caspase-3 activity, while the enzyme activity inhibited in HCT 116 cells.

Our findings show that our study produced reasonable cell death results for apoptosis, which is very important control mechanisms for cancerogenesis. As a supportive data of our results that we indicated, any apoptotic effect of acacetin was shown on MRC-5 cell line which was our healthy control. We suggested that acacetin possesses selectivity between normal and cancer cells.

The empirical results reported herein should be considered in light of some limitations. They are, therefore subject to biases and confounding that may have influenced our experiments. To progress with the *in vivo* studies for the finding of new treatment agents for CRC, different biochemical analyses for such as (1) detection of activation of other caspases, (2) determination of alternative apoptotic pathways with the appropriate cell biology experiments also need to be done.

Despite the aforementioned limitations, we believe our findings are relevant. Our study could accurately be described as the foundation for future studies.

As a result of acacetin's apoptotic effects on colon cancer in this study, acacetin might be used as an anticancer treatment agent in the future. It might be an important step in the development of a new targeted therapy in colon cancer. In addition of these, it also provides a different perspective for the cancerogenesis mechanism with the next experimental designs. In light of these data, our next purpose is to examine the underlying molecular pathway of acacetin in signaling transduction.

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Conflicts of interest

There are no conflicts of interest.

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