Routledge
Taylor & Francis Group

Nutrition and Cancer

ISSN: 0163-5581 (Print) 1532-7914 (Online) Journal homepage: http://www.tandfonline.com/loi/hnuc20

Selective Cytotoxicity of Luteolin and Kaempferol on Cancerous Hepatocytes Obtained from Rat Model of Hepatocellular Carcinoma: Involvement of ROS-Mediated Mitochondrial Targeting

Enayatollah Seydi, Ahmad Salimi, Hamid Reza Rasekh, Zhaleh Mohsenifar & Jalal Pourahmad

To cite this article: Enayatollah Seydi, Ahmad Salimi, Hamid Reza Rasekh, Zhaleh Mohsenifar & Jalal Pourahmad (2018): Selective Cytotoxicity of Luteolin and Kaempferol on Cancerous Hepatocytes Obtained from Rat Model of Hepatocellular Carcinoma: Involvement of ROS-Mediated Mitochondrial Targeting, Nutrition and Cancer, DOI: 10.1080/01635581.2018.1460679

To link to this article: https://doi.org/10.1080/01635581.2018.1460679

Published online: 25 Apr 2018.

NUTRITION AND CANCER
https://doi.org/10.1080/01635581.2018.1460679

Taylor & Francis
Taylor & Francis Group

Selective Cytotoxicity of Luteolin and Kaempferol on Cancerous Hepatocytes Obtained from Rat Model of Hepatocellular Carcinoma: Involvement of ROS-Mediated Mitochondrial Targeting

Enayatollah Seydi[a,b], Ahmad Salimi[c], Hamid Reza Rasekh[d], Zhaleh Mohsenifar[e], and Jalal Pourahmad[d]

[a]Research Center for Health, Safety and Environment, Alborz University of Medical Sciences, Karaj, Iran; [b]Department of Occupational Health Engineering, Alborz University of Medical Sciences, Karaj, Iran; [c]Department of Pharmacology and Toxicology, School of Pharmacy, Ardabil University of Medical Sciences, Ardabil, Iran; [d]Department of Pharmacology and Toxicology, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran; [e]Ayatollah Taleghani Educational Hospital, Faculty of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ABSTRACT

To evaluate the cytotoxicity effects of luteolin (LUT) and kaempferol (KAE) via reactive oxygen species (ROS) mediated mitochondrial targeting on hepatocytes obtained from the liver of hepatocellular carcinoma (HCC) rats. In this study, HCC induced by diethylnitrosamine (DEN) and 2-acetylaminofluorene (2-AAF). In the following, rat liver hepatocytes and mitochondria were isolated and tested for every eventual apoptotic and anti-HCC effects of LUT and KAE. The results of MTT assay showed that LUT and KAE were able to induce selective cytotoxicity in hepatocytes of HCC group in a dose- and time-dependent manner. Treatment of mitochondria from hepatocytes of HCC group with LUT and KAE were accompanied by loss of mitochondrial membrane potential (MMP) and mitochondrial swelling and release of cytochrome c ($P < 0.001$) via reactive oxygen species (ROS) generation before cytotoxicity ensued. LUT and KAE also increased activation of caspase-3 ($P < 0.001$ and $P < 0.01$, respectively). Flow-cytometry analysis indicated that the mode of cell death induced by these flavonoids were mostly apoptosis. Importantly, LUT and KAE were nontoxic for healthy hepatocytes and mitochondria. Therefore, we suggest that LUT and KAE are a good candidate for the complementary therapeutic agent against HCC.

ARTICLE HISTORY
Received 30 July 2017
Accepted 27 November 2017

Introduction

Hepatocellular carcinoma (HCC) represents a major form of primary liver cancer in adults. It is the second and sixth leading cause of cancer-related death in males and females, respectively (1,2). Currently, surgical resection and local ablative therapies are adopted when liver transplantation is not accessible, and recurrence is the main reason of death after surgical treatment for this cancer (1,2). Another therapy for HCC treatment is chemotherapy, but it has been shown that the HCC has a high resistance to some treatments, including chemotherapy (3). The treatment of this cancer is still a big challenge in medicine. So finding an effective natural medicine which has anti-HCC effect is of great significance undoubtedly (1,2).

Plant polyphenols gain a remarkable consideration in treating various types of cancers such as colon cancer (4). Flavonoids comprise a large group of plant secondary, they are widely dispersed throughout the plant kingdom and are commonly found in natural sources such as fruits, vegetables, and certain beverages (5).

Several researches have indicated that these natural compounds are available in medical and edible plants, and epidemiological research suggests that these natural compounds play an important role in the prevention of carcinogenesis (6–9). Flavonoids are known to suppress tumor cell growth that is mediated via the induction of apoptosis signaling in various tumor cell lines (10,11). Some studies suggested that flavones and flavonols showed cytotoxicity in vitro to several human cell lines, such as colon, prostate, and human cervical carcinoma cells (12). Luteolin (LUT) as a flavone and Kaempferol (KAE) as a flavonol, are common dietary flavonoids can be found in a variety of vegetables, fruits, and medicinal herbs.

CONTACT Jalal Pourahmad j.pourahmadjaktaji@utoronto.ca Prof. of Toxicology and Pharmacology, Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, 14155–6153, Iran.

Color versions of one or more of the figures in the article can be found online at www.tandfonline.com/hnuc.

LUT and KAE have been shown to have many biological activities including anti-cancer. The anti-cancer effect of LUT and KAE has been studied in vitro and could induce tumor cells apoptosis (13–17). It was also showed that KAE is much less toxic to normal cells in comparison to standard chemotherapy drugs (18). The functions and the cytotoxicity mechanisms of LUT and KAE on hepatocytes and mitochondria isolated from HCC rat model by DEN and 2-AAF were not completely reported till now. This study focused on the apoptotic effect of LUT and KAE on hepatocytes and mitochondria obtained from the liver of HCC rats and the detailed mechanism.

Materials and Methods

Animal Studies

Male Sprague-Dawley rats and their standard feed pellets were obtained from Institute Pasteur (Tehran, Iran). All animals were kept under the constant environmental temperature of 22°C, and 12 h light/dark cycles with free access to feed and distilled water. The protocol for this study was approved by the Animal Care and Use Committee (ACUC), Faculty of Pharmacy, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

The animals were divided into two groups. Group A: Control animals received intraperitoneal injections (i.p.) of corn oil. Group B animals were administered with single dose DEN (200 mg/kg body weight) intraperitoneally (i.p.). Then, 14 days after DEN administration, HCC was promoted with dietary 2-AAF (0.02%, w/w) for 14 days (19). In our previous studies, HCC was confirmed via the histopathological evaluations, determinations of levels liver injury markers and liver alpha-fetoprotein (AFP); as a cancer-specific marker (20–22).

Isolation of Mitochondria from Rat Hepatocytes

At the end of the estimated HCC induction process (week 15), rats were anesthetized with Ketamine (80 mg/kg, i.p.) and xylazine (5 mg/kg, i.p.) and then rat liver hepatocytes were isolated using the two-step collagenase liver perfusion technique. After isolation of hepatocytes from HCC and normal groups were categorized using flow cytometry. The differential centrifugation (5 min at 760 × g for the first stage and 20 min at 8000 × g for the second stage) were used for isolation of mitochondria from hepatocytes (23–26). In this study, for the determination of mitochondrial toxicity parameters, the mitochondria were suspended in corresponding buffers, respectively. All tests were carried out three times.

Determination of Cytotoxicity

The hepatocytes obtained from the normal and HCC cells (1×10^4/well) were placed in 96-well plates and treated with 2.5–100 μM concentrations of LUT and KAE for 48 h (Cells were maintained in RPMI 1640, supplemented with 10% FBS and antibiotics (50 U/ml of penicillin and 50 μg/ml streptomycin). After treatment, MTT (5 mg/ml in RPMI 1640) reagent was added to each well. After 4 h, the reaction was stopped by addition of 100 μl of DMSO. The absorbance at 570 nm of the solubilized MTT products was measured with an ELISA reader. The process was repeated in triplicate to confirm accuracy. The IC_{50} was calculated by the Logit method (27).

Determination of Caspase-3 Activity

Caspase-3 activity was assayed by using the Sigma's caspase-3 assay kit (Sigma-Aldrich, Taufkirchen, Germany) and the concentration of the p-nitroaniline released from the substrate at 405 nm used for caspase-3 activity was assayed (28,29).

Quantification of Apoptosis

Apoptotic cell detection was performed using FITC conjugated annexin-V and PI. The hepatocytes were treated with 12 μM of LUT and 30 μM KAE for 24 h. After treatment with LUT and KAE, the hepatocytes were pelleted down by centrifugation at 2000 rpm at 4°C. The hepatocytes were resuspended in 0.5 ml of binding buffer (Annexin V binding buffer containing 100 mM HEPES/NaOH, 150 mM NaCl, and 2.5mM $CaCl_2.2H_2O$; pH = 7.4) and again centrifuged. Pallets were dissolved in same buffer containing FITC-Annexin V at the final concentration of 5 μM and PI at 10 μM. Cells were incubated in dark for 15 min and then analyzed by flow cytometry (27).

Measurement of Intracellular ROS

The intracellular generation of ROS was measured by flow cytometry using DCHF-DA. Briefly, the normal and HCC hepatocytes (1×10^6 cells) treated with LUT and KAE at the indicated concentration 12 μM and 30 μM for 24 h. After that, the hepatocytes washed twice with PBS and were stained with 10 μM DCFH-DA for 30 min at 37°C in the dark. The intracellular ROS production was detected by the flow cytometric assay (30).

Measurement of MMP

In this study, loss of MMP was determined by flow cytometry. Briefly, hepatocytes obtained from normal and HCC group were exposed to LUT (12 μM) and KAE (30 μM) for 24 h, and the MMP was measured directly using 10 μM Rh 123. The samples (10^4 events) were analyzed by flow cytometry at an excitation and emission wavelengths of 488 and 530 nm, respectively. The double peak for the Rh 123 fluorescence indicates a redistribution of part of the dye into the cytosol and the double peak in HCC hepatocytes compared with a peak of control untreated hepatocytes (30).

Determination of Mitochondrial Swelling

Mitochondrial swelling was measured spectrophotometrically as a decrease in absorbance at 540 nm. Isolated mitochondria from liver hepatocytes were resuspended in the swelling buffer, which contained 70 mM sucrose, 230 mM mannitol, 3 mM HEPES, 2 mM Tris-phosphate, 5 mM succinate, and 1 μM of rotenone to a final protein concentration of 1000 μg/ml and incubated at 37°C with LUT and KAE at concentration of 35 μM (IC_{50}). Also, $Cacl_2$ is used as a positive control. The absorbance was measured at 540 nm at 15 and 30 min (20).

Cytochrome c Release

In this study, the cytochrome c release was measured using the Quantikine Rat/Mouse Cytochrome c Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, Minn.) (25).

Statistical Analysis

All results in this study are presented as mean ±SD. The statistical analyses were performed using the GraphPad Prism software (version 5). Assays were performed three times. Statistical significance (set at $P < 0.05$) was carried out by using the one-way and two-way ANOVA test.

Results

LUT- and KAE-induced Cytotoxicity

As shown in Figure 1; the HCC hepatocytes viability was decreased by different concentrations of LUT and KAE after 48 h treatment. The results of the cytotoxicity showed that LUT was more active than KAE with an estimated IC_{50} value of 12 μM, compared with KAE with IC_{50} near to 30 μM. Also, no significant decrease in viability was observed in the normal hepatocytes treated with all applied concentration of LUT and KAE compared with the corresponding control group.

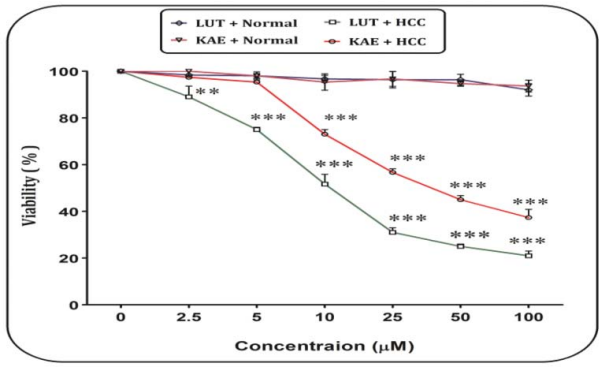

Figure 1. Cytotoxicity assay. Cytotoxic effects of luteolin and kaempferol on hepatocytes from normal group and comparative effects of luteolin and kaempferol at 0–100 μM on the cytotoxic of HCC hepatocytes. The cells were treated with specified concentrations of respective two flavonoids for 48 h, and cytotoxic effects were determined by MTT assay. The results were reported as the mean ± SD ($n = 3$). The stars show that values were significantly different from the corresponding control ($^{**}P < 0.01$ and $^{***}P < 0.001$).

LUT- and KAE-induced Activation of Caspase-3

The caspase-3 activity in hepatocytes was determined 24-h after the addition of LUT (12 μM) and KAE (30 μM). The enzymatic activities of caspase-3 in hepatocytes obtained from the HCC group were significantly increased after 24 h of incubation with LUT and KAE at applied concentrations (12 and 30 μM). Furthermore, our results showed that caspase-3 activity in the hepatocytes from normal group remained unchanged after 24 h of incubation with LUT (12 μM) and KAE (30 μM) (Figure 2).

LUT- and KAE-induced Apoptosis

To figure out the mode of cell death, the double labeling method, using annexin V and PI was utilized to determine apoptotic % versus necrotic cells % 24 h following addition of LUT (12 μM) and KAE (30 μM) in hepatocytes. As shown in Figure 3 the total apoptosis % (including early apoptotic plus late apoptotic hepatocytes) for HCC hepatocytes 39.83 ± 6.10% following LUT (12 μM) addition and 9.32 ± 3.92% after KAE (30 μM) treatments, which was significantly different from those of normal hepatocytes. Our results indicated that LUT is more potent than KAE at inducing apoptosis on HCC hepatocytes.

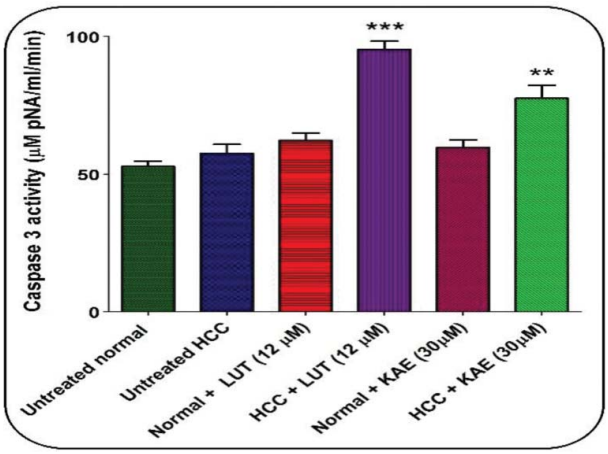

Figure 2. Induction of caspase-3 activity. Following treatment with LUT (12 μM) and KAE (30 μM) for 24 h, caspase-3 activity was analyzed by Sigma-Aldrich kit. The kit determines is producing pNA that is released from the interaction of caspase-3 and AC-DEVD-pNA (peptide substrate). The results were reported as the mean $\pm$ SD ($n = 3$). The stars show that values were significantly different from the corresponding control ($^{**}P < 0.01$ and $^{***}P < 0.01$).

LUT- and KAE-induced ROS Formation

Further, ROS generation assayed by fluorescent probe, DCFH-DA, and monitored by flow cytometry. In the hepatocytes isolated from HCC group, the potency of ROS generation was: LUT > KAE. LUT and KAE-induced elevation in ROS production only in the hepatocytes isolated from HCC group. Figure 4 (D and F) shows LUT (12 μM) and KAE (30μM) increased DCF fluorescence intensity (H_2O_2 production) as the DCF peak shifted rightward on the *x*-axis. The mean of DCF fluorescence increased from 147.74 (Untreated HCC) to 397.43 (LUT plus HCC), and 147.74 (Untreated HCC) to 341.40 (KAE plus HCC). These outcomes confirm the effect of LUT and KAE on mitochondrial ROS formation. Increased cellular ROS formation is always an upstream event for apoptosis signaling.

LUT- and KAE-declined MMP

There is growing evidence that changed mitochondrial function is linked to apoptosis and a declining MMP is associated with mitochondrial dysfunction. Hence, in the next step, we assayed the effect of LUT (12 μM) and KAE (30 μM) on the MMP using the fluorescent probe Rh 123 and monitored its alteration using flow cytometry. The double fluorescence peak observed was indicative of redistribution of Rh 123 into the cytosol and the collapse of the MMP and depolarization of the mitochondrial membrane. As displayed in Figure 5D and F, the significant ($P<0.001$) collapse of the MMP was observed only in the hepatocytes isolated from HCC group after treatment with IC_{50} of two mentioned flavonoids for 24 h.

LUT- and KAE-induced Mitochondrial Swelling

The results of this study showed that LUT more than KAE significantly ($P < 0.05$) decreased the mitochondrial swelling at 15 and 30 min of incubation only in the mitochondria isolated from liver hepatocytes of the HCC group (Figure 6B). In our study, we used $Cacl_2$ (50 μM) as a positive control (MPT inducer) in the mitochondrial swelling assay.

LUT- and KAE-induced Cytochrome c Release

The process of apoptosis may involve the release of cytochrome c from the mitochondria; this eventually induces apoptosis by activation of the caspases cascade such as caspase-3. Treatment of hepatocyte obtained from HCC group with LUT (12μM) and KAE (30 μM) for 24 h induced significant ($P < 0.05$) cytochrome c release into the cytosol. Furthermore, there was no significant cytochrome *c* release in the hepatocytes from the normal group after 24 h of incubation with LUT (12 μM) and KAE (30 μM) (Figure 7A). Significantly, the pretreatment of LUT and KAE-treated mitochondria with the MPT inhibitors like cyclosporine A (CsA, 5 μM) and antioxidants such as butylated hydroxyl toluene (BHT, 5 μM), inhibited cytochrome c release as compared with LUT and KAE-treated group ($P < 0.05$) (Figure 7B). These results confirm the direct role of oxidative stress and MPT pore opening in LUT (12 μM) and KAE (30 μM) induced cytochrome c release in HCC hepatocytes.

Discussion

HCC is a type of cancer of the liver (primary liver cancer) with very high mortality and a poor prognosis. HCC is an important health problem and also studies have shown that apoptosis signaling is impaired in the HCC (31,32). The products with naturally origin resource have been traditionally accepted as treatments because of the popular opinion that they induce low adverse effects. LUT and KAE are important members of the flavonoids family that are present in fruits and vegetables (10,14). Studies show that flavonoids have a cytotoxic effect on several cancer cell lines, including prostate, lung, and colon cancers, but no research so far reported the selective cytotoxicity of LUT and KAE on hepatocytes and mitochondria obtained from HCC. In this study, we tested the cytotoxic effects of LUT and KAE on hepatocytes from the HCC rat model. It was shown that two

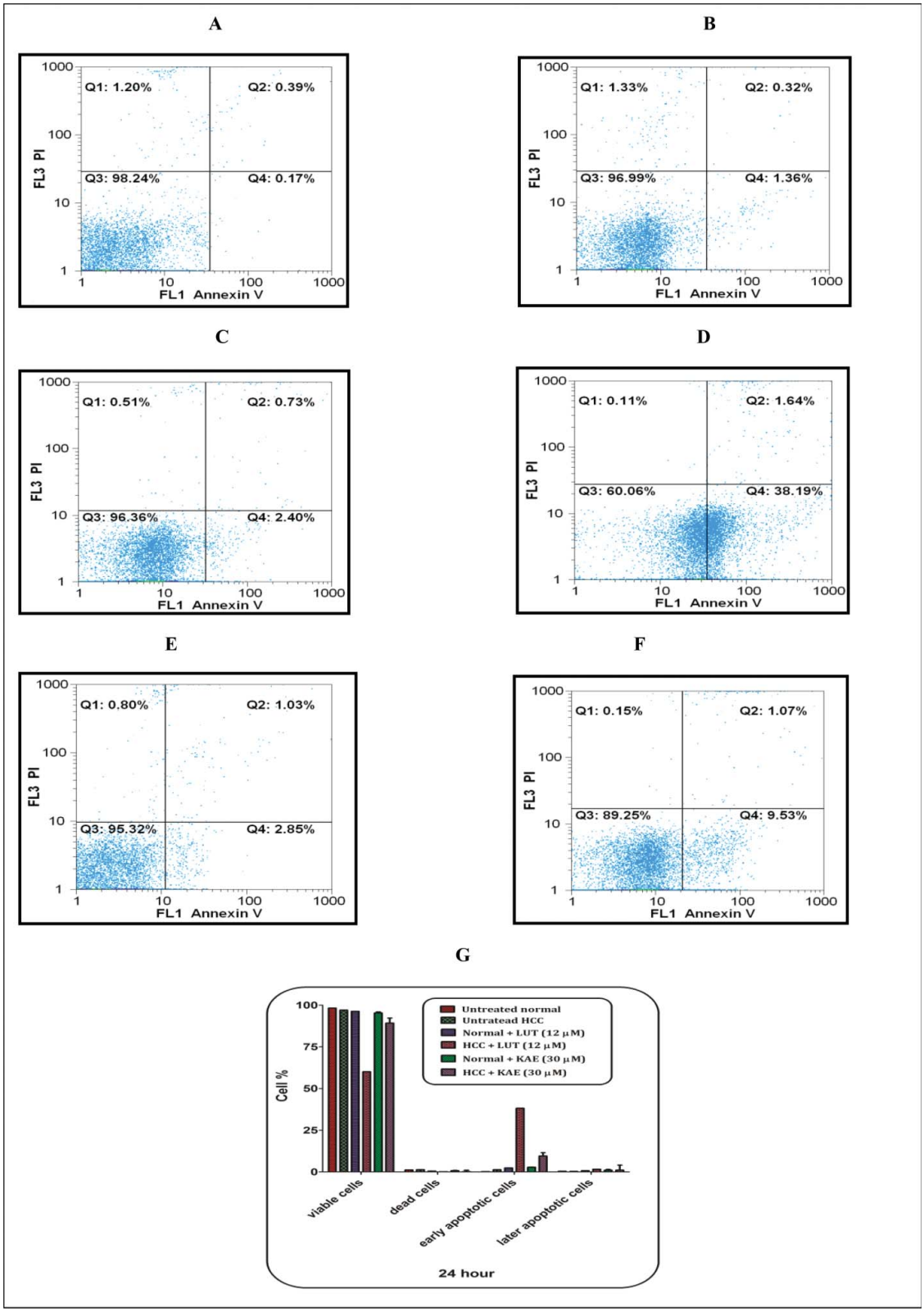

Figure 3. Apoptosis induction. Detection of apoptosis by LUT (12 μM) and KAE (30 μM) in the hepatocytes isolated from normal and HCC groups using annexin V/PI staining. Hepatocytes were incubated with LUT (12 μM) and KAE (30 μM) for 24 h. (A) Untreated normal; (B) untreated HCC; (C) normal plus LUT (12 μM); (D) HCC plus LUT (12 μM); (E) normal plus KAE (30 μM); and (F) HCC plus KAE (30 μM). (G) Four different cell populations were detected after the Annexin V/PI staining of hepatocytes. Q1; the necrotic dead cells, Q2; the ruptured apoptotic bodies representing the late apoptosis, Q3; the viable cells, Q4; the apoptotic cells representing the early apoptosis). The results were reported as the mean $\pm$ SD ($n = 3$).

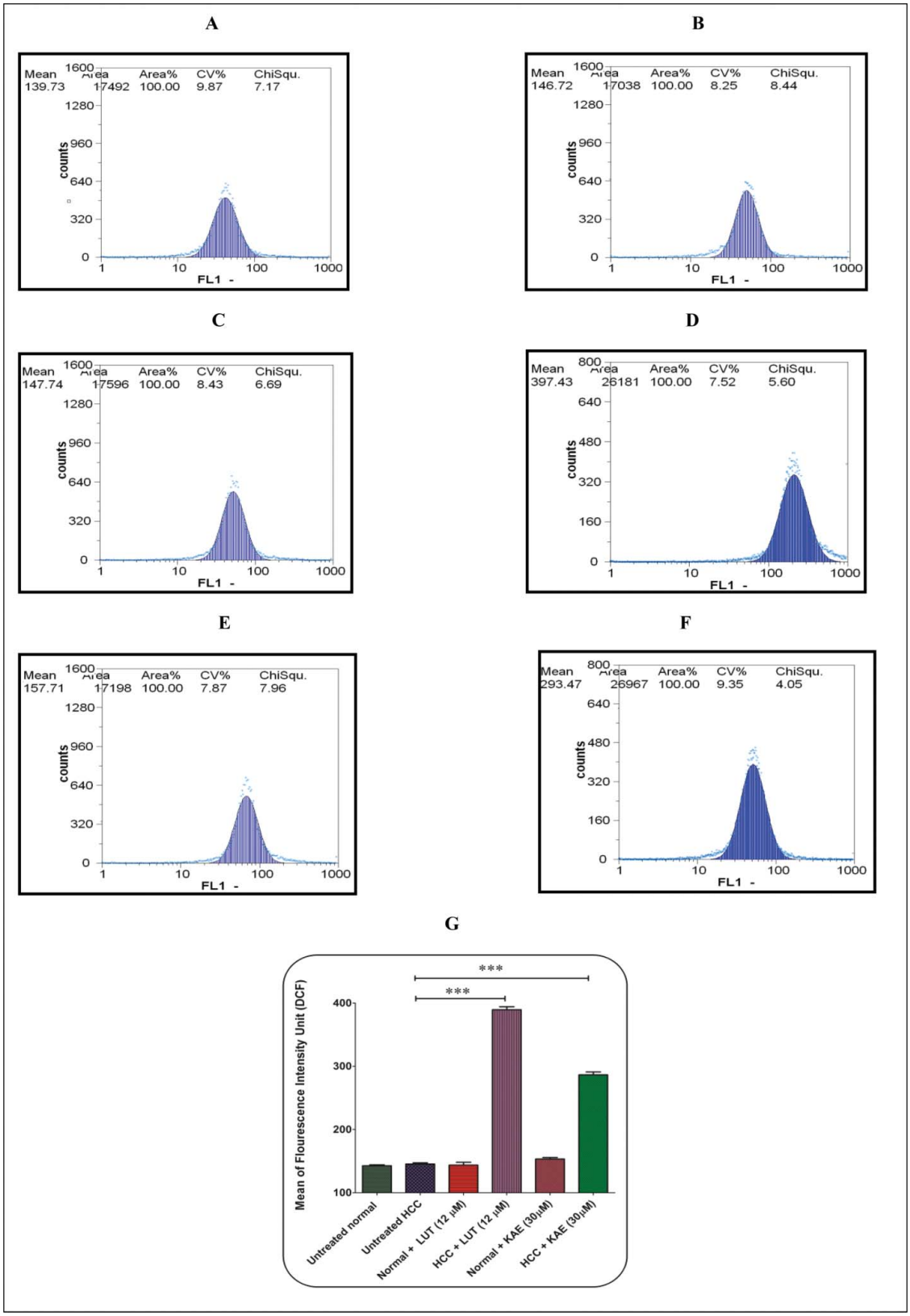

Figure 4. Determination of ROS level. Induction of ROS generation by LUT (12 μM) and KAE (30 μM) in the hepatocytes isolated from normal and HCC groups. Hepatocytes were incubated with LUT (12 μM) and KAE (30 μM) for 24 h. (A) Untreated normal; (B) untreated HCC; (C) normal plus LUT (12 μM); (D) HCC plus LUT (12 μM); (E) normal plus KAE (30 μM); and (F) HCC plus KAE (30 μM). D and F show increased DCF fluorescence intensity (H_2O_2 production) as the DCF peak shifts right ward on the *x*-axis. FL1: the fluorescence intensity of DCF. (G) Mean of fluorescence intensity (DCF). The results were reported as the mean $\pm$ SD ($n = 3$). The stars show that values were significantly different from the HCC control (untreated HCC) group ($^{***}P < 0.01$).

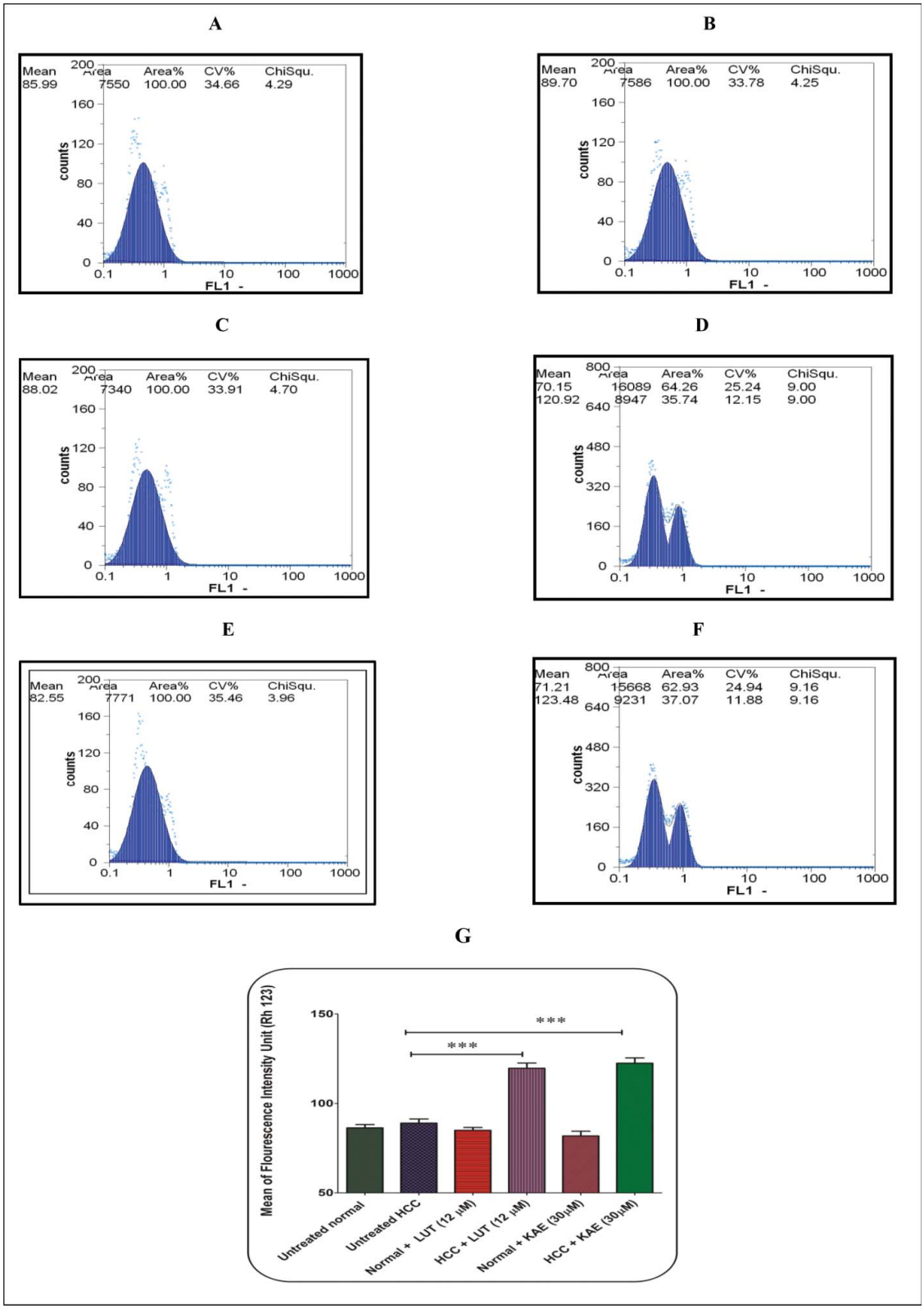

Figure 5. MMP assay. Effects of LUT (12 μM) and KAE (30 μM) on the MMP in the hepatocytes isolated from normal and HCC groups. Hepatocytes were incubated with LUT (12 μM) and KAE (30 μM) for 24 h. (A) Untreated normal; (B) untreated HCC; (C) normal plus LUT (12 μM); (D) HCC plus LUT (12 μM); (E) normal plus KAE (30 μM); and (F) HCC plus KAE (30 μM). The double peak for the Rh123 fluorescence indicates a redistribution of part of the dye into the cytosol. (G) Mean of fluorescence intensity (Rh 123). The results were reported as the mean $\pm$ SD ($n = 3$). The stars show that values were significantly different from the HCC control (untreated HCC) group ($^{***}P < 0.01$).

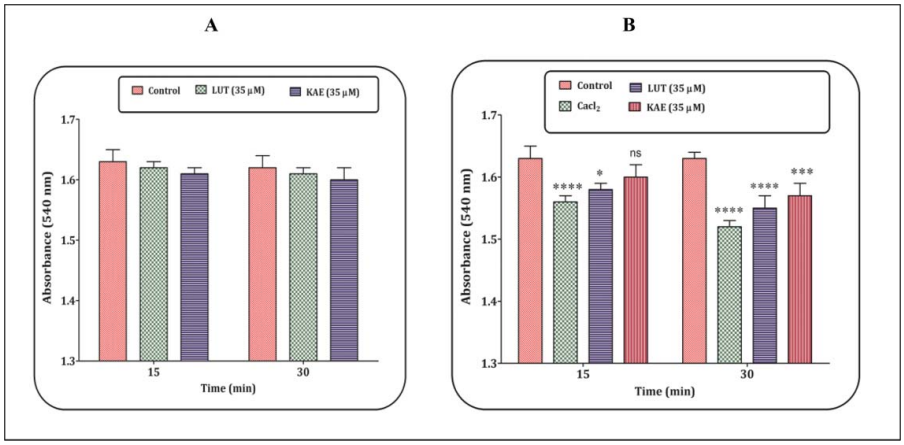

Figure 6. Determination of mitochondrial swelling. Effects of LUT (35 μM) and KAE (35 μM) on the mitochondrial swelling at within 30 min of incubation in the mitochondria obtained from normal (A) and HCC hepatocytes (B). The results were reported as the mean ± SD ($n = 3$). The stars show that values were significantly different from the corresponding control ($^{*}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$).

flavonoids were able to induce selective cytotoxicity in HCC hepatocytes in a dose-dependent manner, and the order of potency of two flavonoids was; LUT > KAE. Comparing their structural characteristics, it was suggested that the cytotoxic effects of LUT and KAE on HCC hepatocytes might be dependent on the position and substitutions of hydroxyl groups in the compounds core molecule. Furthermore, to explain the anti-HCC function of LUT and KAE and its related mechanism, a series of tests were carried out. In this research, our results are in full agreement with the previously published cytotoxic effect of LUT and KAE against transformed cells (33,34).

It was reported that these compounds can act, under certain conditions, as pro-oxidants (35). The pro-oxidant condition depends on the total number of OH groups in a flavonoid molecule and involvement of free transition metal ions in oxidation processes and concentration of flavonoids (36). In this research, we found that LUT and KAE selectively induced increased ROS generation in the hepatocytes only from the HCC group. The order of capability of two flavonoids at increasing ROS in HCC hepatocytes was; LUT > KAE. These results suggest that LUT (12 μM) in lower concentration compared to KAE (30 μM) can increase ROS generation and induce oxidative stress. This suggests that the position of the hydroxyl

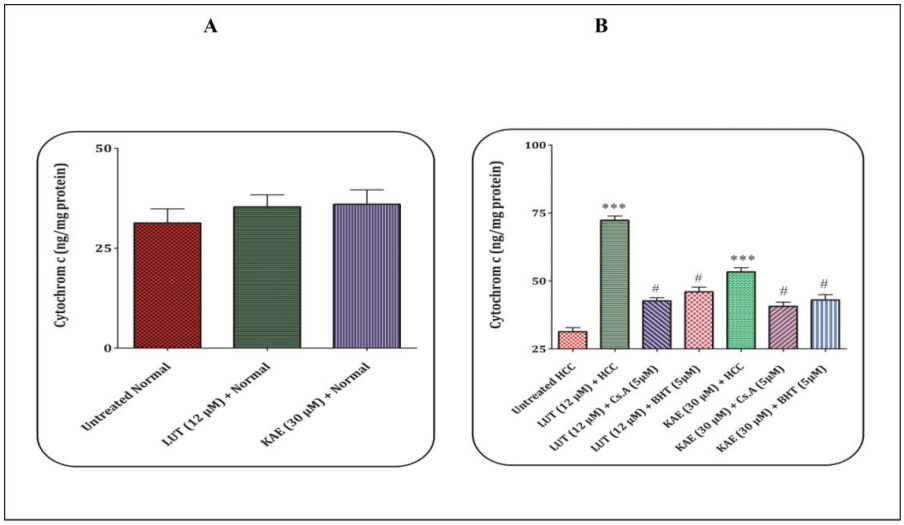

Figure 7. Cytochrome c expulsion. LUT (12 μM) and KAE (30 μM) on the cytochrome c expulsion in the normal (A) and HCC (B) mitochondria isolated from both groups. As shown in this figure, pretreatment of with BHT (5 μM) and CsA (5 μM) significantly ($P < 0.05$) inhibited cytochrome c expulsion in the HCC mitochondria. The amount of expelled cytochrome c in cytosol was assayed using Rat/Mouse cytochrome c ELISA kit. The results were reported as the mean ± SD ($n = 3$). The stars show that values were significantly different from the corresponding control ($^{***}P < 0.001$). $^{\#}$Significant difference in comparison with LUT (12 μM) plus HCC and KAE (30 μM) plus HCC ($P < 0.001$).

group and concentration may influence the ROS generation. Many studies have shown that several mitochondria-targeted drugs have a selective potency to kill cancer cells (oxidation therapy) with no effect on normal cells in preclinical and clinical testing. It has been shown that cancer cells in comparison with normal cells are more vulnerable to irreversible damages induced by oxidative stress and subsequent apoptosis. Recently many researchers in anti-cancer drug development used the differences between the mitochondria of cancerous and normal cells to find a mean to kill cancer cells selectively (37). Furthermore, several reports showed that ROS are involved in triggering apoptosis signaling in cancer cells. Specifically, it was reported that the drug-caused cancer cell apoptosis has resulted from increased ROS generation in targeted tumor cells (38). Our results from the parameters of mitochondrial toxicity showed that LUT- and KAE-induced significant alteration in the mitochondrial swelling. This alter is a result of increased generation of ROS.

On the other hand, some evidence has shown that ROS might increase decline in MMP during drug-caused cancer cell apoptosis (38). The decrease of MMP ($\Delta\Psi_m$) after stimulus disrupts outer mitochondrial membrane, and therefore leads to cytochrome c release from mitochondria and induces apoptosis (39). We found that after adding LUT and KAE to the hepatocytes and mitochondria isolated from the HCC group, the MMP and cytochrome c was decreased and increased, respectively. In agreement with other published studies, a decline of MMP and increase of ROS generation and as well as cytochrome c release were recognized as expected tumor cell response to LUT and KAE (33,34,40). Our results also confirmed that LUT and KAE can decrease MMP and trigger cytochrome c release into the cytosol in HCC hepatocytes. However, LUT and KAE did not induce any expulsion of cytochrome c and decrease of MMP in mitochondria obtained from normal hepatocytes. Interestingly, pretreatment of HCC mitochondria with CsA and BHT inhibited LUT and KAE induced expulsion of cytochrome c, indicating that the mitochondrial permeability transition caused by LUT and KAE is permeability transition pore complex mediated. Furthermore, the release of cytochrome c contributes to the activation of caspase-3, a final mediator of apoptosis (39). The order of potency for two flavonoids at decreasing of MMP and subsequent cytochrome c release from mitochondria into cytosol was: LUT > KAE in the hepatocytes isolated from HCC group.

To investigate the involvement of caspases cascade in apoptosis pathway, we detected the activity of caspase-3 in LUT and KAE treated hepatocytes obtained from HCC group. Our results showed that LUT and KAE could activate caspase-3. This suggests LUT and KAE can induce apoptosis through a mitochondria-dependent pathway. The order of LUT and KAE to activate caspase-3 was: LUT > KAE in the hepatocytes isolated from HCC group. Apoptosis plays an important role in regulating cell number in numerous developmental and physiological and pathological conditions (1). In the etiology of most malignant tumors defect in apoptosis signaling has been reported. So, investigating drugs that induce apoptosis in tumor cells has become a target for the development of anticancer drugs (41–44). Mitochondrial targeting is the main gateway for design and development of anti-cancer drugs causing tumor cell apoptosis (1).

Our results showed that LUT- and KAE-induced apoptosis only in hepatocytes isolated from HCC but the not normal group. The induction of apoptosis is subsequent to the decrease of MMP, cytochrome c expulsion, and caspase-3 activation. The order of efficacy of LUT and KAE at inducing apoptosis was: LUT > KAE in the hepatocytes from HCC group. Our results showed that ROS-mediated MMP collapse is the initiating step in the LUT- and KAE-induced apoptosis in the hepatocytes obtained from HCC group.

Conclusion

In conclusion, our results showed that LUT and KAE selectively induced apoptosis signaling via the mitochondrial-dependent pathway in hepatocytes from the HCC rat model by targeting upstream events including the increase of ROS generation, the decrease of MMP, swelling of mitochondria, the release of cytochrome c and finally enhancing the activity of caspase-3 in cytosol. Also, the change of MMP can lead to burst of ROS, which in turn will aggravate the loss of MMP. Finally, for future studies we suggest that more precise mechanistic and clinical trials studies be conducted to evaluate the anticancer effects of LUT and KAE.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by the Shahid Beheshti University of Medical Sciences, Grant no. (1392-1-94-11832, 2013).

References

1. Ding S, Hu A, Hu Y, Ma J, Weng P, et al.: Anti-hepatoma cells function of luteolin through inducing apoptosis and

cell cycle arrest. *Tumor Biol* **35**, 3053–3060, 2014. doi:10.1007/s13277-013-1396-5.
2. Khare S, Zhang Q, and Ibdah JA: Epigenetics of hepatocellular carcinoma: role of microRNA. *World J Gastroenterol: WJG* **19**, 5439, 2013. doi:10.3748/wjg.v19.i33.5439.
3. Jin F, Wang Y, Li M, Zhu Y, Liang H, et al.: MiR-26 enhances chemosensitivity and promotes apoptosis of hepatocellular carcinoma cells through inhibiting autophagy. *Cell Death Dis* **8**, e2540, 2017. doi:10.1038/cddis.2016.461.
4. Pandurangan AK, Sadagopan SKA, Dharmalingam P, and Ganapasam S: Luteolin, a bioflavonoid, attenuates azoxymethane-induced effects on mitochondrial enzymes in BALB/c mice. *Asian Pacific J Cancer Prevent* **14**, 6669–6672, 2013. doi:10.7314/APJCP.2013.14.11.6669.
5. Lopez-Lazaro M: Distribution and biological activities of the flavonoid luteolin. *Mini Rev Med Chem* **9**, 31–59, 2009. doi:10.2174/138955709787001712.
6. Bonofiglio D, Giordano C, De Amicis F, and Lanzino M, Ando S: Natural products as promising antitumoral agents in breast cancer: Mechanisms of action and molecular targets. *Mini Rev Med Chem* **16**, 596–604, 2016. doi:10.2174/1389557515666150709110959.
7. Kou X, Kirberger M, Yang Y, and Chen N: Natural products for cancer prevention associated with Nrf2–ARE pathway. *Food Sci Human Wellness* **2**, 22–28, 2013. doi:10.1016/j.fshw.2013.01.001.
8. Tanaka T, Makita H, Ohnishi M, Murakami A, Koshimizu K, et al.: Chemoprevention of oral and colon carcinogenesis by natural compounds protocatechuic acid, 10-acetoxychavicol acetate, s-methyl methane thiosulfonate, and hesperidin from vegetables and fruits. In: *Food Factors for Cancer Prevention*. Springer Nature, Wien, Austria, 1997, pp. 194–199.
9. Zhou Y, Li Y, Zhou T, Zheng J, Li S, et al.: Dietary natural products for prevention and treatment of liver cancer. *Nutrients* **8**, 156, 2016. doi:10.3390/nu8030156.
10. Lee E-J, Oh S-Y, and Sung M-K: Luteolin exerts antitumor activity through the suppression of epidermal growth factor receptor-mediated pathway in MDA-MB-231 ER-negative breast cancer cells. *Food Chem Toxicol* **50**, 4136–4143, 2012. doi:10.1016/j.fct.2012.08.025.
11. Lee H-J, Wang C-J, Kuo H-C, Chou F-P, Jean L-F, et al.: Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/Bak and activation of JNK. *Toxicol Appl Pharmacol* **203**, 124–131, 2005. doi:10.1016/j.taap.2004.08.004.
12. Zhang Q, Zhao X-H, and Wang Z-J: Cytotoxicity of flavones and flavonols to a human esophageal squamous cell carcinoma cell line (KYSE-510) by induction of G 2/M arrest and apoptosis. *Toxicol Vitro* **23**, 797–807, 2009. doi:10.1016/j.tiv.2009.04.007.
13. Huang WW, Chiu YJ, Fan MJ, Lu HF, Yeh HF, et al.: Kaempferol induced apoptosis via endoplasmic reticulum stress and mitochondria−dependent pathway in human osteosarcoma U−2 OS cells. *Mol Nutri Food Res* **54**, 1585–1595, 2010. doi:10.1002/mnfr.201000005.
14. Luo H, Rankin GO, Li Z, DePriest L, and Chen YC: Kaempferol induces apoptosis in ovarian cancer cells through activating p53 in the intrinsic pathway. *Food Chem* **128**, 513–519, 2011. doi:10.1016/j.foodchem.2011.03.073.
15. Ou Y-C, Kuan Y-H, Li J-R, Raung S-L, Wang C-C, et al.: Induction of apoptosis by luteolin involving akt inactivation in human 786-o renal cell carcinoma cells. *Evidence-Based Complement Alter Med* **2013**, 2013. doi:10.1155/2013/109105.
16. Sharma V, Joseph C, Ghosh S, Agarwal A, Mishra MK, et al.: Kaempferol induces apoptosis in glioblastoma cells through oxidative stress. *Mol Cancer Therapeut* **6**, 2544–2553, 2007. doi:10.1158/1535-7163.MCT-06-0788.
17. Wang T-T, Wang S-K, Huang G-L, and Sun G-J: Luteolin induced-growth inhibition and apoptosis of human esophageal squamous carcinoma cell line Eca109 cells in vitro. *Asian Pacific J Cancer Prevent* **13**, 5455–5461, 2012. doi:10.7314/APJCP.2012.13.11.5455.
18. Chen AY, and Chen YC: A review of the dietary flavonoid, kaempferol on human health and cancer chemoprevention. *Food Chem* **138**, 2099–2107, 2013. doi:10.1016/j.foodchem.2012.11.139.
19. Taha MME, Abdul AB, Abdullah R, Ibrahim TAT, Abdelwahab SI, et al.: Potential chemoprevention of diethylnitrosamine-initiated and 2-acetylaminofluorene-promoted hepatocarcinogenesis by zerumbone from the rhizomes of the subtropical ginger (Zingiber zerumbet). *Chemico-Biol Interact* **186**, 295–305, 2010. doi:10.1016/j.cbi.2010.04.029.
20. Seydi E, Motallebi A, Dastbaz M, Dehghan S, Salimi A, et al.: Selective toxicity of Persian Gulf Sea cucumber (Holothuria parva) and sponge (Haliclona oculata) methanolic extracts on liver mitochondria isolated from an animal model of hepatocellular carcinoma. *Hepatitis Monthly* **15** (12), 2015. doi:10.5812/hepatmon.33073.
21. Seydi E, Rasekh HR, Salimi A, Mohsenifar Z, and Pourahmad J: Myricetin selectively induces apoptosis on cancerous hepatocytes by directly targeting their mitochondria. *Basic Clin Pharmacol Toxicol* **119**(3), 249–58, 2016. doi:10.1111/bcpt.12572.
22. Talari M, Seydi E, Salimi A, Mohsenifar Z, Kamalinejad M, et al.: Dracocephalum: Novel anticancer plant acting on liver cancer cell mitochondria. *BioMed Res Int* **2014**, 2014. doi:10.1155/2014/892170.
23. Barogi S, Baracca A, Castelli GP, Bovina C, Formiggini G, et al.: Lack of major changes in ATPase activity in mitochondria from liver, heart, and skeletal muscle of rats upon ageing. *Mechanisms Ageing Develop* **84**, 139–150, 1995. doi:10.1016/0047-6374(95)01640-6.
24. Seglen PO: Preparation of isolated rat liver cells. *Met Cell biol* **13**, 29–83, 1976. doi:10.1016/S0091-679X(08)61797-5.
25. Seydi E, Rasekh HR, Salimi A, Mohsenifar Z, and Pourahmad J: Selective toxicity of apigenin on cancerous hepatocytes by directly targeting their mitochondria. *Anticancer Agents Med Chem* **16**, 1576–1586, 2016. doi:10.2174/1871520616666160425110839.
26. Seydi E, Rasekh HR, Salimi A, Mohsenifar Z, and Pourahmad J: Myricetin selectively induces apoptosis on cancerous hepatocytes by directly targeting their mitochondria. *Basic Clin Pharmacol Toxicol* **119**, 249–58, 2016. doi:10.1111/bcpt.12572.
27. Zhang Q, Zhao X-H, and Wang Z-J: Flavones and flavonols exert cytotoxic effects on a human oesophageal adenocarcinoma cell line (OE33) by causing G2/M arrest and inducing apoptosis. *Food Chem Toxicol* **46**, 2042–2053, 2008. doi:10.1016/j.fct.2008.01.049.
28. Sakahira H, Enari M, and Nagata S: Cleavage of CAD inhibitor in CAD activation and DNA degradation during apoptosis. *Nature* **391**, 96–9, 1998. doi:10.1038/34214.

29. Shahraki J, Motallebi A, Barekati I, Seydi E, and Pourahmad J: Comparison of cellular and molecular cytotoxic mechanisms of Cochlodinium polykrikoides in isolated trout and rat hepatocytes. *Toxicol Environ Chem* **96**, 917–930, 2014. doi:10.1080/02772248.2014.980132.
30. Salimi A, Roudkenar MH, Sadeghi L, Mohseni A, Seydi E, et al.: Ellagic acid, a polyphenolic compound, selectively induces ROS-mediated apoptosis in cancerous B-lymphocytes of CLL patients by directly targeting mitochondria. *Redox Biol* **6**, 461–471, 2015. doi:10.1016/j.redox.2015.08.021.
31. Fabregat I: Dysregulation of apoptosis in hepatocellular carcinoma cells. *World J Gastroenterol* **15**, 513–20, 2009. doi:10.3748/wjg.15.513.
32. Schattenberg JM, Schuchmann M, and Galle PR: Cell death and hepatocarcinogenesis: Dysregulation of apoptosis signaling pathways. *J Gastroenterol Hepatol* **26**(Suppl 1), 213–9, 2011. doi:10.1111/j.1440-1746.2010.06582.x.
33. Choi AY, Choi JH, Yoon H, Hwang K-Y, Noh MH, et al.: Luteolin induces apoptosis through endoplasmic reticulum stress and mitochondrial dysfunction in Neuro-2a mouse neuroblastoma cells. *Europ J Pharmacol* **668**, 115–126, 2011. doi:10.1016/j.ejphar.2011.06.047.
34. Li W, Du B, Wang T, Wang S, and Zhang J: Kaempferol induces apoptosis in human HCT116 colon cancer cells via the Ataxia-Telangiectasia Mutated-p53 pathway with the involvement of p53 Upregulated Modulator of Apoptosis. *Chemico-Biol Interact* **177**, 121–127, 2009. doi:10.1016/j.cbi.2008.10.048.
35. Stepanic V, Gasparovic AC, Troselj KG, Amic D, and Zarkovic N: Selected attributes of polyphenols in targeting oxidative stress in cancer. *Curr Topics Med Chem* **15**, 496–509, 2015. doi:10.2174/1568026615666150209123100.
36. Procházková D, Boušová I, and Wilhelmová N: Antioxidant and prooxidant properties of flavonoids. *Fitoterapia* **82**, 513–523, 2011. doi:10.1016/j.fitote.2011.01.018.
37. Nogueira V, and Hay N: Molecular pathways: reactive oxygen species homeostasis in cancer cells and implications for cancer therapy. *Clin Cancer Res* **19**, 4309–4314, 2013. doi:10.1158/1078-0432.CCR-12-1424.
38. Leung HW-C, Kuo C-L, Yang W-H, Lin C-H, and Lee H-Z: Antioxidant enzymes activity involvement in luteolin-induced human lung squamous carcinoma CH27 cell apoptosis. *European J Pharmacol* **534**, 12–18, 2006. doi:10.1016/j.ejphar.2006.01.021.
39. Cheng A-C, Huang T-C, Lai C-S, and Pan M-H: Induction of apoptosis by luteolin through cleavage of Bcl-2 family in human leukemia HL-60 cells. *Europ J Pharmacol* **509**, 1–10, 2005. doi:10.1016/j.ejphar.2004.12.026.
40. Bestwick CS, Milne L, and Duthie SJ: Kaempferol induced inhibition of HL-60 cell growth results from a heterogeneous response, dominated by cell cycle alterations. *Chemico-Biol Interact* **170**, 76–85, 2007. doi:10.1016/j.cbi.2007.07.002.
41. Essack M, Bajic VB, and Archer JA: Recently confirmed apoptosis-inducing lead compounds isolated from marine sponge of potential relevance in cancer treatment. *Mar Drugs* **9**, 1580–606, 2011. doi:10.3390/md9091580.
42. Grossman D, and Altieri DC: Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. *Cancer Metastasis Rev* **20**, 3–11, 2001. doi:10.1023/A:1013123532723.
43. Park KI, Park HS, Nagappan A, Hong GE, Lee DH, et al.: Induction of the cell cycle arrest and apoptosis by flavonoids isolated from Korean Citrus aurantium L. in non-small-cell lung cancer cells. *Food Chem* **135**, 2728–35, 2012. doi:10.1016/j.foodchem.2012.06.097.
44. Zhang J, Park HS, Kim JA, Hong GE, Nagappan A, et al.: Flavonoids identified from Korean Scutellaria baicalensis induce apoptosis by ROS generation and caspase activation on human fibrosarcoma cells. *Am J Chin Med* **42**, 465–83, 2014. doi:10.1142/S0192415X14500311.