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RESEARCH ARTICLE

Selective Toxicity of Apigenin on Cancerous Hepatocytes by Directly Targeting their Mitochondria

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ARTICLE HISTORY

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Abstract: ***Background*:** hepatocellular carcinoma (HCC) is the third cause of mortality due to cancer throughout the world.

***Objective*:** The main goal of the current research was to evaluate the selective toxicity of apigenin (APG) on hepatocytes and mitochondria obtained from the liver of HCC rats).

***Method*:** In this research, HCC induced by a single dose of diethylnitrosamine (DEN); 200 mg/kg, i.p, and 2-acetylaminofluorene (2-AAF) (0.02%, through dietary) for 14 days. For confirmation of HCC, histopathological evaluations and determination of serum concentrations of liver toxicity enzymes and specific liver cancer marker; alpha-fetoprotein (AFP) were performed. Then, cancerous and non- cancerous hepatocytes were isolated by using the collagen perfusion method. Eventually, mitochondria isolated from HCC and normal hepatocytes were tested for every eventual toxic effects of APG.

***Results*:** After confirmation of HCC, the results of this research showed that APG (10, 20 and 40 μM) increased mitochondrial parameters such as, mitochondrial membrane potential (MMP), reactive oxygen species (ROS) level, mitochondrial swelling and cytochrome c expulsion only in cancerous hepatocytes. Apoptotic effect of APG on HCC cells was confirmed by caspase-3 activation and Annexin V-FITC and PI double staining analysis.

***Conclusion*:** These results propose the eligibility of the flavonoid APG as a complementary therapeutic agent for patients with hepatocellular carcinoma.

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Keywords: Apigenin, apoptosis, hepatocytes, mitochondria, hepatocellular carcinoma.

INTRODUCTION

Among the most common malignancies hepatocellular carcinoma (HCC) possesses the fifth place and one of the deadliest cancers throughout the world [1]. The main risk factors for this cancer include the hepatitis viral infection (HBV and HCV), food additives, alcohol, aflatoxin B_1, toxic industrial chemicals, air and water pollutants [2]. The metabolism of ingestion materials is performed in the human liver and therefore is vulnerable to carcinogenic assault. On the other side, the liver tolerance is high.In particular, in patients for whom cancer could not be detected in early stage [3].

Despite being a potentially medicinal type of therapy used in the treatment of HCC including chemotherapy, liver resection and transplantation, these treatment methods have a poor tolerance and low efficacy and poor subsequent survival rate, respectively. So, the determination of best choice therapy with good potency and efficacy is urgently needed [4].

Plant flavonoids are a known family of plant metabolic compounds [5]. Epidemiologic researches show an anti-cancer effect of high dietary intake of flavonoids and other natural food product [6]. It is assumed that natural food compound contain products that have protective effects against diseases (including various cancers). These effects also independent of those known nutrients and micronutrients [7]. Various flavonoid compounds due to differences in chemical structure, and pharmacokinetic parameters such as absorption, distribution have a different effect on human health [5, 7]. Apigenin (APG), a flavone, is found in natural product food such as fruits [8]. It has been shown that APG has a different functions and biological properties, including anti-inflammatory, anti-cancer, and anti-oxidant properties [9].

The results of *in vitro* studies on different cancer cell lines showed that APG has inhibited the growth of cancer cell *via* inducing cell cycle arrest and apoptosis [10]. Moreover, APG is reportedly non-mutagenic, low and ignorable of toxicity compared with other flavonoids [11]. Thus, APG may be a good candidate compound for anticancer drugs [12]. In the several studies, animal models are commonly used to assess the anti-cancer effects of natural compounds, particularly the polyphenols [13]. The compounds such as diethylnitrosamine (DEN) and 2- acetylaminofluorene (2-AAF) are commonly used in cancer research to create models of cancer, especially liver cancer [14].

In our study, we aimed to determine the selective apoptotic effects of APG on hepatocytes and mitochondria obtained from the liver of HCC rats in comparison with those of untreated normal rats. So far there is no report regarding the anti-cancer or apoptotic effects APG has on HCC liver hepatocytes.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (120–130 g), fed a standard chow diet and given water *ad libitum*, were used in all experiments. Rats were purchased from Institute Pasteur (Tehran, Iran). All rats were kept in a controlled room temperature of 20◦C–25◦C and humidity 50%–60% and were faced to 12 h light/dark cycles. The animal treatment in this study was according to the ethical guidelines issued by the Committee of Animal Experimentation of Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Experimental Method

Briefly, the rats were divided into two groups and 5 rats were placed in every group. Group A was an untreated normal group (Fig. **1**). In this research, HCC was induced by DEN; as a single dose 200 mg/kg, i.p, and 2-AAF (0.02%, through dietary) for 14 days (group B) [14]. The body weight was recorded every week for 15 weeks. Afterward, the blood samples were permitted to coagulate and centrifugation for 10 min at 1000×g and 4 ◦C to access serum for evaluation of Alpha-Fetoprotein (AFP), alanine transaminase (ALT), Aspartate transaminase) AST), and Alkaline phosphatase (ALP). At the end of the estimated HCC induction process (week 15), 5 rats were sacrificed with an overdose of barbiturate. The tissue samples from liver both groups were harvested and were kept for histological evaluation. If HCC confirmed by the histological analysis, mitochondria were then isolated from liver hepatocytes of the remaining animals (A and B groups).

Alpha-Fetoprotein (AFP) Assay

AFP concentration levels on animal's serum were assayed using the method of Taha *et al*., [14].

Biochemical Assessments

The activities of ALT, AST and ALP on animal's serum were evaluated using the method of Taha *et al*., [14].

Histopathological Analysis

Histopathological examination was assayed by using the method of Taha *et al*., [14].

ISOLATION OF MITOCHONDRIA FROM RAT HEPATOCYTES

In our study, the isolation of rat hepatocytes from liver both groups were isolated using the two-step collagenase liver perfusion technique [15]. Then, to determine the cell membrane lysis the trypan blue uptake was used. After isolation of hepatocytes from both groups, cancerous and non-cancerous hepatocytes were categorized using flow cytometry. In the next step, mitochondria from both groups are prepared from hepatocytes (30 $\times 10^6$ cells) [15b]. The hepatocytes are afterwards pelleted (300 g for 3min) and re-suspended in 10 ml of Solution A (0.01M tricine, 0.25M sucrose, 10mM NaH_2PO_4, 1mM EDTA, 2mM $MgCl_2$ at pH= 8). Finally, the differential centrifugation (5min at 760× g for the first stage and 20 min at 8000 × g for the second stage) were used in isolation of mitochondria from hepatocytes [16]. For the determination of ROS generation, mitochondrial swelling and MMP, the mitochondria were suspended in corresponding buffer, respectively. The concentrations of APG (10, 20 and 40 µM) were selected based MTT assay (1/2 IC_{50}, IC50 and 2 IC_{50}).

SUCCINATE DEHYDROGENASE (SDH) ACTIVITY ASSAY

In this study, the alteration of SDH or mitochondrial complex II activity by APG (0-100µM was measured by reduction of MTT [17].

DETERMINATION OF ROS LEVEL

Isolated mitochondria from control and HCC hepatocytes were placed in respiration buffer. Following this step, DCFH (Fluorescent probe) was utilized for the mitochondrial ROS level at the EXλ= 488 nm and EMλ=527 nm [15b].

MEASUREMENT OF CHANGES IN MMP

Isolated mitochondria from normal and HCC hepatocytes were placed in MMP buffer. In the next step, Rhodamine 123 (Rh 123, a cationic fluorescent dye) MMP used for the MMP assay at the EXλ= 490 nm and EMλ=535 nm [15b].

MITOCHONDRIAL SWELLING ASSAY

In this experiment, isolated mitochondria from normal and HCC hepatocytes were suspended in swelling buffer and incubated with 10, 20 and 40 μM of APG. Finally, absorbance was assayed at 540 nm [15b].

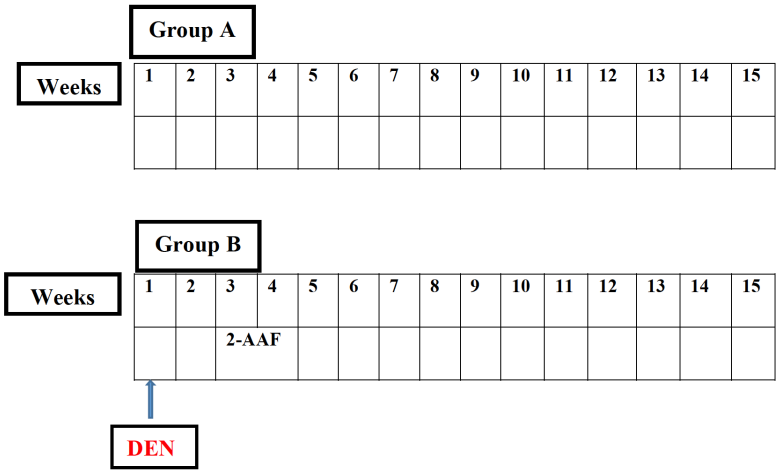

Fig. (1). Experimental method of this study. The animal were separated into two experimental groups; A (normal group), B (HCC group). Group A rats served as control animals and was fed a standard diet until end of the experimental duration (15 week). HCC induced by diethylnitrosamine (DEN); at a single dose 200 mg/kg, i.p, and 2-acetylaminofluorene (2-AAF) (0.02%, through dietary) for 14 days and then were on the standard diet until end of experimental time (15week).

CYTOCHROME C (CYT C) TEST

The release of cytochrome c by APG was assayed by the Quantikine Rat/Mouse Cytochrome c Immunoassay kit provided by R & D Systems, Inc. (Minneapolis, Minn.).

HEPATOCYTES VIABILITY

Cytotoxicity was determined by MTT test. Hepatocytes from normal and HCC cells were exposed to 2.5-100 μM concentration of APG (made up with the RPMI 1640) for 48h. In the end, absorbance to the each group was determined at 570 nm in a microplate reader [18].

ROS ASSAY BY FLOW CYTOMETRY

In this study, ROS level was observed by flow cytometry using DCHF-DA[19]. Hepatocyte from normal and HCC groups were treated with APG (10 μM). Following this step, ROS production in 6, 12 and 24 h, respectively, were measured [20].

MMP ASSAY *VIA* FLOW CYTOMETRY

After treatment with a concentration of APG (10 μM) for 24h, the hepatocytes cells obtained from normal and the HCC group were incubated with Rh123 (1 mg/ml in DMSO) for 30 min at 37 ℃ and washed 3 times with PBS. The hepatocytes groups were both examined by flow cytometry technique at the EXλ= 488 nm and EMλ=530 nm.

DETERMINATION OF CASPASE-3

The caspase-3 activity in both groups were assayed by using the Sigma's caspase-3 assay kit (Sigma- Aldrich, Taufkirchen, Germany) [21]. The concentration of the p-nitroaniline released from the substrate at 405 nm used for assay caspase 3 activity [22].

QUANTIFICATION OF APOPTOSIS

For evaluation of apoptosis by flow cytometry, the Annexin V-FITC detection kit was used. The cells were placed at 6-well plates and treated with APG (10 μM) for 6, 12 and 24 h. The % of alive, dead, and apoptotic cells were assayed [18].

STATISTICAL ANALYSIS

The all results in this study are shown as mean ±SD. The statistical analyses were carried out by the Graph Pad Prism software (version 5) using one-way and two-way ANOVA followed by Tukey and Bonferroni post hoc tests, respectively. All measurements were performed triplicate. Statistical significance was set at $P< 0.05$.

RESULTS

Alteration of Body and Liver Weights by the DEN/2-AAF Regime

In our study, Table **1A** shows the body (final) and liver weights of control (A) and HCC group (B) of rats. The average body weight of normal group (A) of rats (282.33 ± 13.65 g) was significantly ($P < 0.05$) higher than the HCC group (B) rats (215.33 ± 4.72 g). Moreover, in the HCC group the average liver weight of rats (13.58 ± 0.62 g) after 15 weeks were significantly ($P < 0.05$) higher than of normal group (9.66 ± 1.04 g).

Effect of DEN/2-AAF Regime on Serum AST, ALT and ALP and Specific HCC Marker

Rats treated with DEN/2-AAF to induce hepatocellular carcinoma showed significant ($P < 0.05$) increase in serum AST, ALT and ALP as well as serum AFP concentrations (Table **1B**).

Histopathology

The histopathological findings on hepatic slices from two groups (A and B) are shown in Fig. (**2A-C**). In the hepatic slices from normal group rats, there was normal liver parenchyma with the typical architecture characterized by central vein, granulated cytoplasm, and small uniform nuclei (Fig. **2A**). Hepatic sections of the HCC group exhibited atypical architecture with hyperchromatic nuclei and a significant variation in nuclear size were also binucleated (Fig. **2B** and **C**).

Cytotoxicity Effect of APG on Hepatocyte

The cytotoxicity effect of APG on hepatocytes isolated from untreated control and HCC groups were determinate after incubation in culture medium including APG at concentrations of 2.5, 5. 10, 25, 50 and 100 μM for 48 h. As shown in Fig. (**2E**), APG significantly reduced cell viability in hepatocytes obtained from HCC rats in a concentration -dependent manner; But only little decrease was remarked on cytotoxicity effect at normal group hepatocytes following the addition of the highest dose of APG (100 μM) (Fig. **2D**).

Effect of APG on SDH Activity

The effect of APG (0, 5, 10, 20, 50 and 100μM) on mitochondrial SDH activity was measured by the MTT test, using mitochondria isolated from liver hepatocytes of both A and B groups following

Table 1. **(A) Effect of DEN/2-AAF regime on final body and liver weights. All results were reported as the mean ± SD. Five rats were placed in each group. (B) Effects of HCC inducing DEN/AAF regimen on the liver injury enzyme and AFP in serum of the test and control animals. All results were reported as the mean ± SD. Five rats were placed in each group.**

A

Liver Weight (g)	Final Body Weight (g)	Group
9.66± 1.04	282.33± 13.65	**Untreated control group (A)**
13.58± 0.62*	215.33± 4.72*	**HCC group (B)**

B

AFP (IU/L)	ALP (IU/L)	AST (IU/L)	ALT (IU/L)	Group
0.46±0.05	628±10	86±17	97±15	**Untreated control group (A)**
2.86± 0.32*	772± 38*	680± 67*	789± 54*	**HCC group (B)**

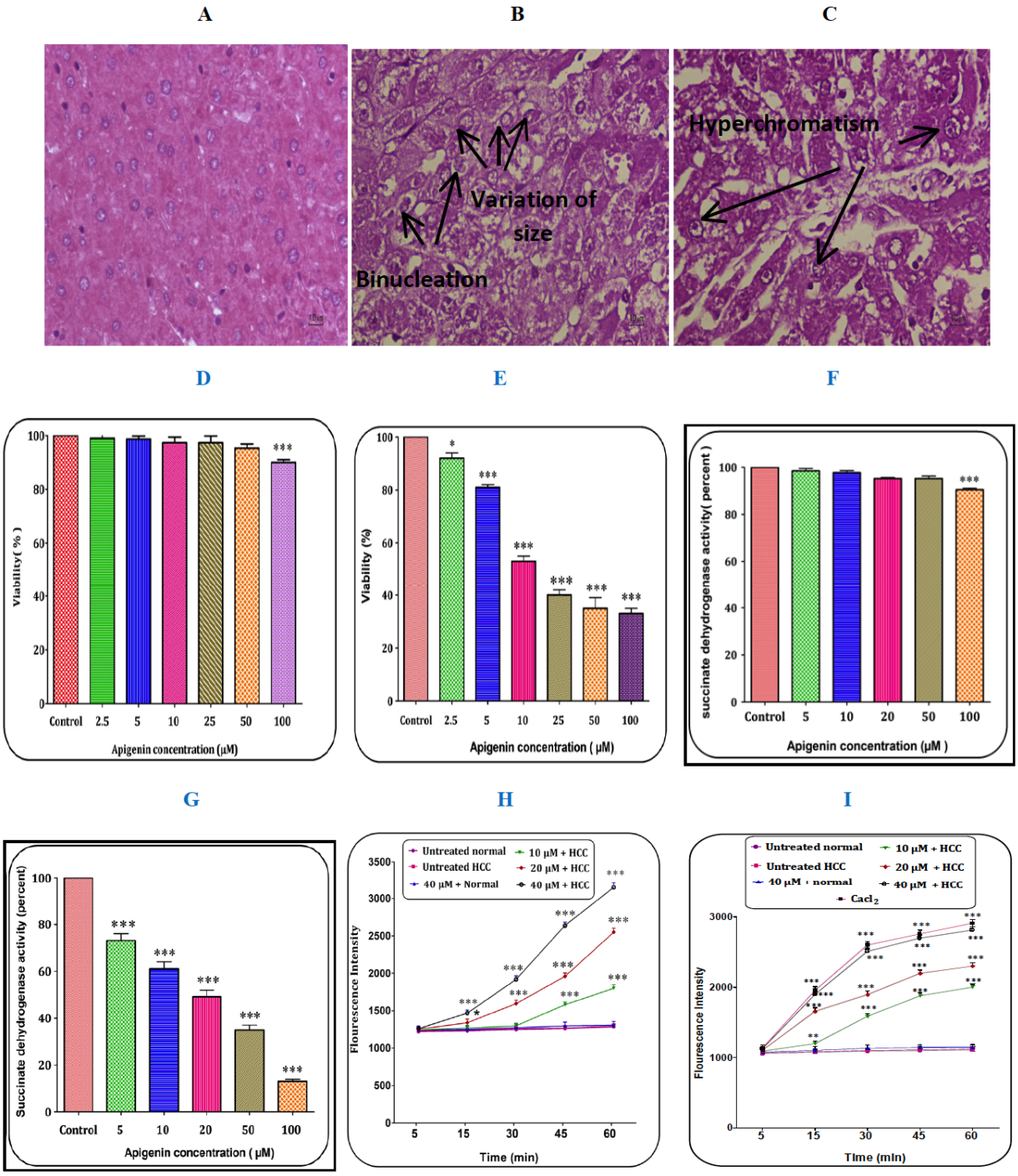

Fig. (2). Histopathological analysis. **(A)** Liver section from the normal group shows normal cellular architecture (H&E; 40×magnification). **(B and C)** Liver sections from the HCC group show areas of aberrant hepatocellular phenotype with variation in nuclear size, hyperchromatism, binucleation, and irregular sinusoids (H&E; 40×magnification). **(D-E)** Effect of APG (2.5–100 μM, for 48 h) on hepatocytes viability. All results were reported as the mean ± SD (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$: significantly different from the vehicle only group (APG concentration="0"). **(F-G)** The effect of APG concentrations on the SDH activity in the liver mitochondria obtained from hepatocytes of normal **(F)** and HCC groups **(G)**. All results were reported as the mean ± SD (n = 3). $^{***}P < 0.001$ compared with control mitochondria (APG concentration="0"). **(H)** Effect of APG on mitochondrial ROS level. The results showed that different concentrations of APG (10, 20 and 40 μM) were increased ROS level in the mitochondria HCC group (at various time intervals within 1h of incubation). Values presented as mean ± SD (n = 3). *, *** Significant difference in comparison with corresponding untreated HCC group ($p<0.05$, $p<0.001$, respectively). **(I)** Evaluation of the decline of MMP. The results showed that different concentrations of APG (10, 20 and 40 μM) were induced the collapse of MMP in the mitochondria HCC group (at various time intervals within 1h of incubation). Values presented as mean ± SD (n = 3).**, *** Significant difference in comparison with corresponding untreated HCC group ($p<0.01$, $p<0.001$, respectively).

60 min of incubation. Our results showed a significant concentration-dependent decrease in the SDH activity ($P<0.05$) (Fig. **2G**) only in the HCC rats group. But in the normal rats group only the highest concentration of APG (100μM) could induce significant decrease in the mitochondrial enzyme activity ($P<0.05$) (Fig. **2F**).

Effect of APG on Mitochondrial ROS Level

As displayed in Fig. (**2H**), various concentrations of APG (10, 20 and 40 µM) induced significant H_2O_2 formation ($P< 0.05$) in the mitochondria from HCC group. Lower concentration of APG (10µM) significantly raised H_2O_2 the generation only at 45 and 60min time points, whereas the (20 µM) concentration significantly increased H_2O_2 formation at the earlier time of 30min as well as at 45 and 60 min. However the highest concentration of APG (40µM) significantly increased H_2O_2 formation at all-time points of 5, 15, 30, 45 and 60 min compared with their corresponding unexposed HCC mitochondria. Also, various concentrations of APG (10, 20 and 40 µM) did not induce significant H_2O_2 formation within 60min of incubation in the mitochondria from normal group.

Effect of APG on MMP

It was shown that APG concentrations (10, 20 and 40 µM) significantly ($p<0.05$) decreased the MMP in the mitochondria from HCC group compared with those of their corresponding unexposed HCC group. On the other hand, APG at the above mentioned concentrations (10, 20 and 40 µM) did not induce significant ($p<0.05$) MMP within incubation time (60 min) in the mitochondria from normal rats group compared with those of their corresponding untreated normal group (Fig. **2I**).

ALTERATIONS IN MITOCHONDRIAL SWELLING BY APG

The results in Fig. (**3A**), various concentrations of APG (10, 20 and 40 µM) significantly increased the mitochondrial swelling within the incubation time (1 hour) only in the mitochondria of HCC group. Addition of same concentrations of APG did not induce mitochondrial swelling in the mitochondria from normal group.

The decrease in absorbance at 540 nm is indicative of mitochondrial swelling.

ALTERATIONS IN CYTOCHROME C (CYT C) EXPULSION BY APG

As shown in Fig. (**3B**), APG (20 μM) caused the significant expulsion of cyt c only in the mitochondria from HCC group.

Pretreatment of APG (20 µM)-treated mitochondria from the HCC group accompanied by cyclosporine A (CsA), as a MPT inhibitors like, and butylated hydroxyl toluene (BHT), as a antioxidants,

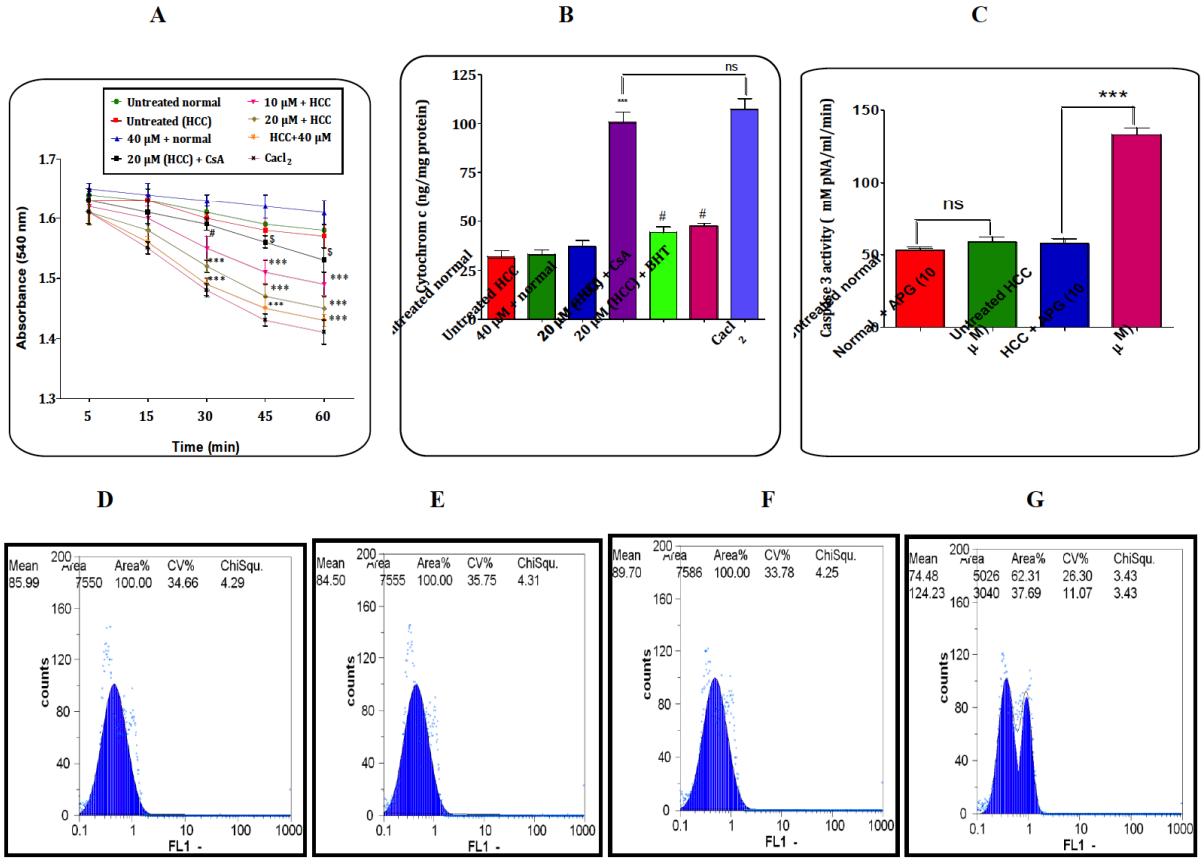

Fig. (3). (A) The effect of APG (10, 20 and 40 µM) on mitochondrial swelling. The mitochondrial swelling in both groups were measured within 1 h of incubation. All results were reported as the mean ± SD (n = 3). *** Significant difference in comparison with corresponding untreated HCC group ($p<0.001$). $ significant difference in comparison with untreated HCC plus APG (20µM) ($p<0.001$). **(B)** Cytochrome c expulsion assay by using corresponding rat/mouse cytochrome c ELISA kit. Increased cytochrome c expulsion after addition of a concentration of APG (20 µM) in the mitochondria obtained from hepatocytes of HCC group but not normal groups. Also, Pretreatment of APG (20 µM)-treated mitochondria from HCC group accompanied by CsA and BHT inhibited cyt c expulsion as compared with APG -treated HCC group. Values presented as mean ± SD (n = 3). ***Significant difference in comparison with untreated normal and untreated HCC groups ($p<0.001$). # Significant difference in comparison with HCC plus APG (20 µM) ($p<0.05$). **(C)** Evaluation of caspase 3 activity by using Sigma-Aldrich kit. Caspase-3 activation in the both HCC and untreated control rat hepatocytes following the exposure to APG (10 µM). HCC and control rat hepatocytes were incubated in RPMI 1640 medium. All results were reported as the mean ± SD (n = 3). *** Significant difference in comparison with corresponding untreated HCC group ($p < 0.001$). **(D-G)** MMP assay by flowcytometry. APG significantly decline the MMP ($\Delta\Psi m$) in the hepatocytes obtained from HCC group. The double peak for the Rh123 fluorescence indicates redistribution of part of the dye into cytosol. **D**) Untreated normal group (24h); **E)** Untreated HCC group (24h); **F)** Untreated normal plus APG (10 µM) (24h); **G)** Untreated HCC plus APG (10µM) (24h).

inhibited cyt c expulsion as compared with APG -treated HCC group. These results confirm the direct action of oxidative stress process and MPT pore opening in cyt c expulsion.

Effect of APG Treatment on Caspase-3 Activation

In this study, caspase-3 activity was significantly raised in hepatocytes isolated from only HCC rats group when incubated with APG (10 µM), but not in those of obtained from the normal rats group (Fig. **3C**).

APG Causes Dissipation of MMP in HCC Hepatocytes

Rhodamine 123 cathionic fluorescent probe was used to evaluate the changes in the MMP (ΔΨm), which accumulates in the mitochondrial inter-membrane apace and redistributes into the cytosol following the dissipation of mitochondrial membrane potential and membrane depolarization. The double fluorescence peak observed was indicative of redistribution of rhodamine 123 into the cytosol and collapse of the ΔΨm and depolarization of the mitochondrial membrane. As shown in Fig. (**3G**), the significant collapse of the ΔΨm was observed only in the hepatocytes isolated from HCC rats group (B) 24 h after exposure with APG (10 µM).

Effect of *In Vitro* APG on Hepatocytes ROS Level

In our study, concentration of APG (10µM) caused significant ROS (H_2O_2) formation in hepatocytes isolated from HCC hepatocytes *in vitro* using flow cytometry assay (Fig. **4**). APG - induced elevation in H_2O_2 production in hepatocytes isolated from

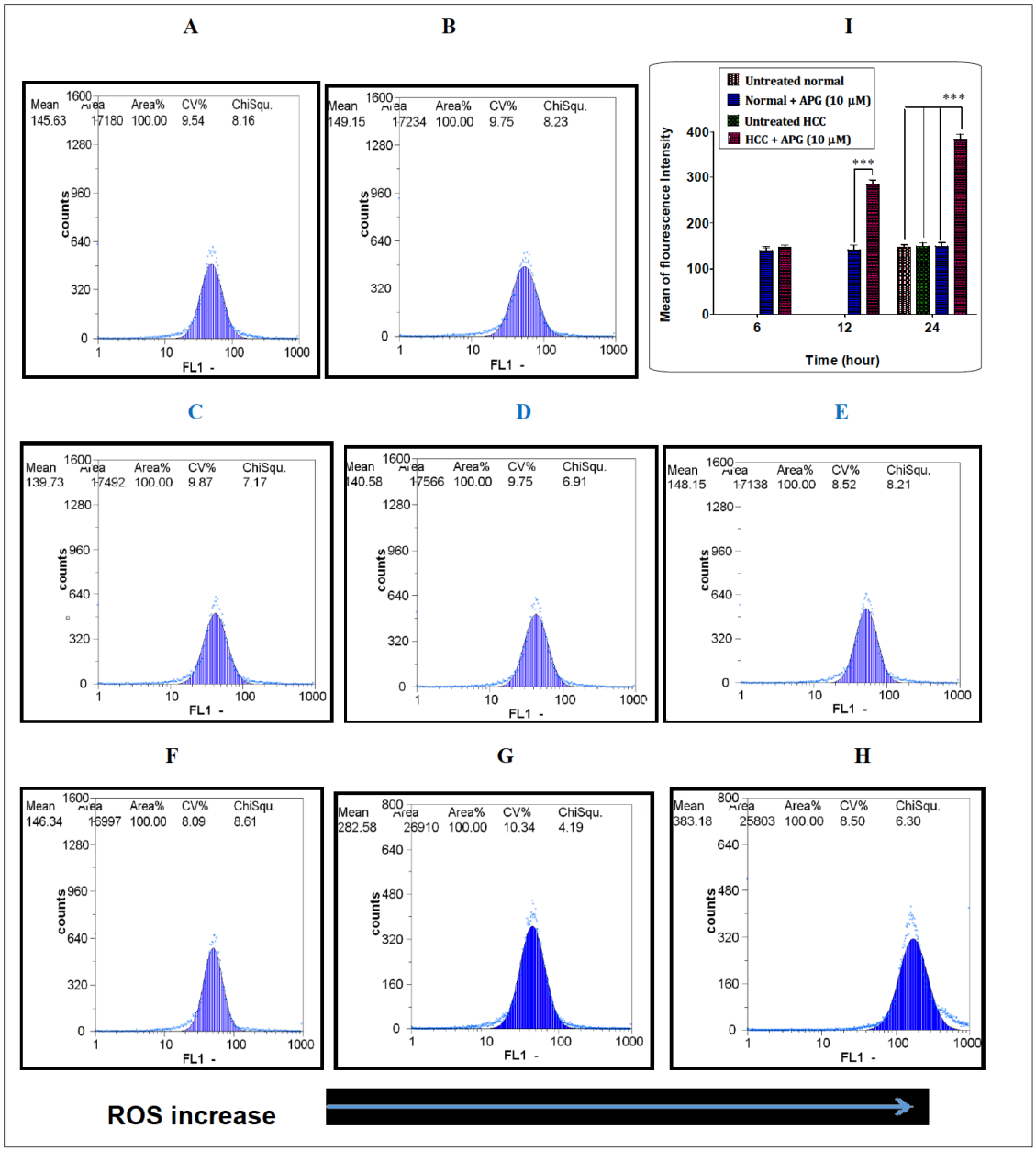

Fig. (4). ROS measuremeant by flowcytometry. ROS level after the addition of APG (10 µM) at of 6, 12 and 24h time points. **A)** Untreated normal (24h); **B)** HCC normal (24h); **C)** Untreated normal plus APG (10µM) (6h); **D)** Untreated normal plus APG (10 µM) (12h); **E)** Untreated normal plus APG (10 µM) (24h); **F)** Untreated HCC plus APG (10 µM) (6h); **G)** Untreated HCC plus APG (10µM) (12h); **H)** Untreated HCC plus APG (10 µM) (24h); **I)**. F-H 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. $^{***}P<0.001$; compared with untreated normal and untreated HCC and untreated normal plus APG (10 µM).

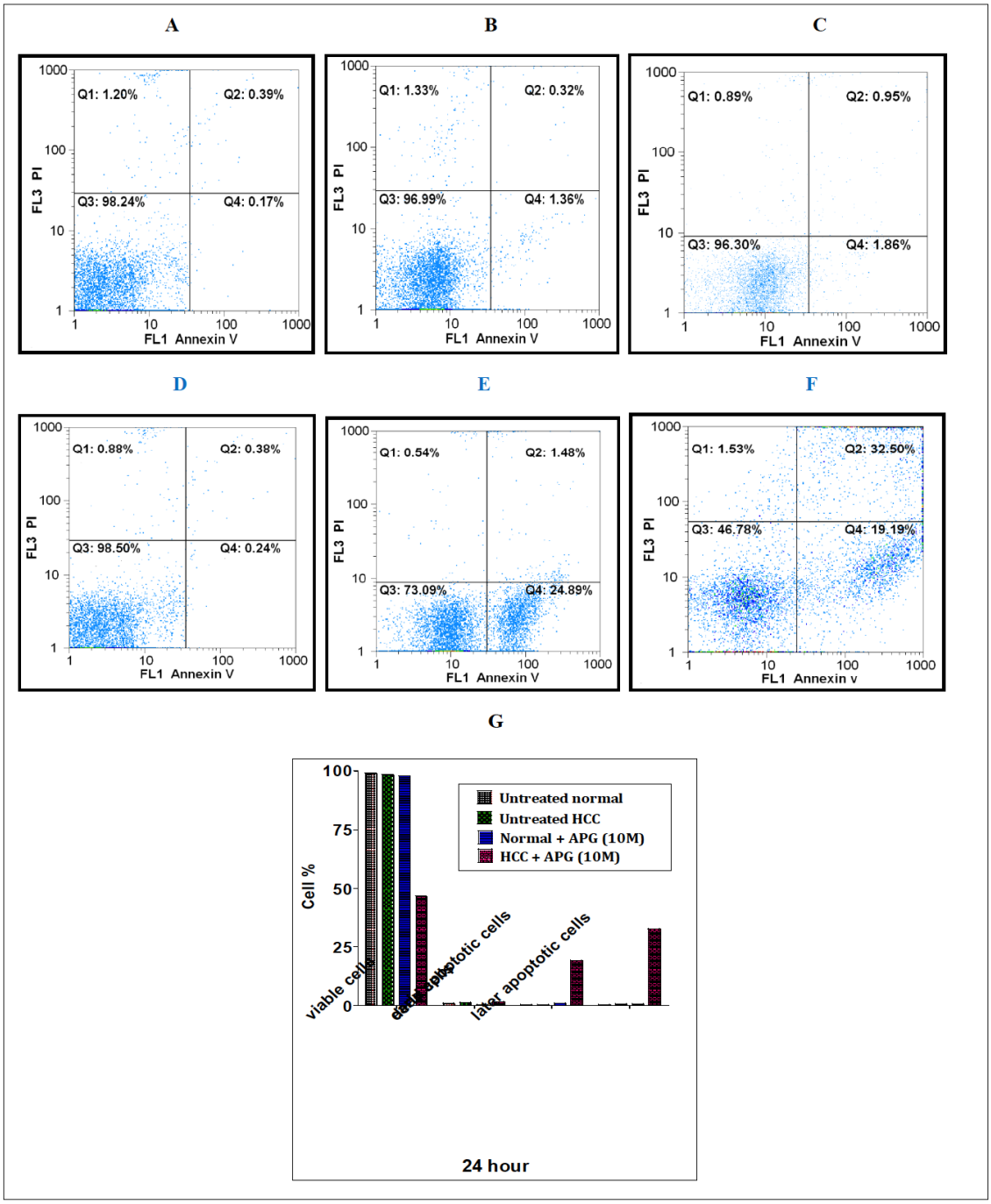

Fig. (5). Induction of apoptosis by APG (10μM) in the hepatocytes isolated from normal and HCC groups. Hepatocytes were incubated with APG (10 μM) for 0, 12 and 24 hours. **A)** Untreated normal (24h); **B)** Untreated HCC (24h); **C)** Untreated normal plus APG (10μM) (24h); **D)** Untreated HCC plus APG (10μM) (6h); **E)** Untreated HCC plus APG (10μM) (12h); **F)** Untreated HCC plus APG (10μM) (24h);**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).

HCC group was compared with the control normal and control HCC groups. Fig. (**4F-H**) shows APG (10μM) induced increased DCF fluorescence intensity (H_2O_2 production) as the DCF peak shifted rightward on the x-axis.

Effect of the APG on Cell Apoptosis

The % apoptosis / necrosis caused by APG were quantified using Annexin V-FITC and PI with flow cytometry. Annexin V staining can detect phosphatidylserine and as such could be used for its analysis. The corresponding dot-plots illustrate apoptosis status, which are displayed in Fig. (**5**). The percentage of apoptotic cells (both early and late apoptosis) treated with APG (10 μM) at 12 and 24 hour time points were 25.43% ± 2.4% and 1.54%± 0.9% (12h), 19.19% ± 2% and 32.5%± 3% (24h), respectively (Fig. **D-F**).

DISCUSSION

Today, a variety of cancers as a global health problem have been reported, which are also the second cause of death after

cardiovascular disease [23]. The results of the studies show that the incidence of HCC is rising and the disease remained as one of the important subjects for liver transplantation. Two common types of hepatitis, including HBV and HCV are the main risk factors for HCC [24]. Experimental induction of HCC in rodents by DEN [25], due to the discovery of compounds with potential anti-cancer effect, is one of the best animal disease models for HCC [26] with similar imitation of this malignancy tumor in humans [27]. APG is a flavonoid with non-mutagenic activity that is found in natural food, such as a variety of fruits and vegetables [28]. Several studies were reported to possess anti-cancer activity through various mechanisms by APG; including induction of cell cycle arrest and apoptosis [29] inhibition of mutagenesis [30] and suppression of signal transduction [31].

In our study, the considerable decrease in appetite and food intake in HCC group cause weight loss which could be an indirect suggestion of the decreasing hepatic function, and raise in the liver weight of the animals in HCC group, which could be assigned to the induction of tumors in the liver following DEN/2-AAF treatment [32]. Some studies have also confirmed these data and reported that the treated rats with this regime resulted in a marked decrease of final body weight and increase in liver weight [33].

The results of this study also confirmed that damage to the liver is caused by DEN/2-AAF at/after week 15 of the HCC induction protocol. Raise of serum alpha-fetoprotein in the HCC rats might probably result from the toxic effects of DEN/AAF treatment, which induced genetic changes in the rodent liver cells [34], the reason why DEN/AAF treated animals could develop hepatocellular carcinoma [32].

The histopathological results in this study were supported by biochemical results obtained experimental animals. The hepatocytes showed variation in nuclear size and hyperchromatic nuclei. These results were all in agreement with the findings of previous investigates. This is attributed to metabolic activation of DEN in the rat liver and reactive metabolites generated which are recognized to damage hepatocytes [14, 32].

Mitochondria play important roles in cellular metabolism and death process. The alterations in genome, function, and structure of this organelle have been related to cancer. In many studies, due to these specific alterations, the mitochondria has been suggested as a promising target for cancer therapy [15b].

Several studies have shown that the mitochondria were differences in tumor tissue than in normal tissue, such as grow faster, fewer and smaller and also had the morphological transformed. It has also been observed that mitochondria from liver tumours are more fragile than those of normal liver [32].

In this study, we assayed the effect of APG on hepatocyte viability (24 h) obtained from HCC rats at various concentrations (2.5–100 μM). APG significantly decreased the viability of HCC hepatocytes at all concentrations using MTT assay. This finding was quite in agreement with previous investigations regarding the capability of APG at decreasing cell viability in the various cancer cells [7]. Besides, APG at the concentrations 5-100 μM also significantly reduced the activity of SDH in the mitochondria obtained from cancerous but not cancerous hepatocytes, confirming selective and direct action of this flavonoid on tumor cell mitochondria [35].

Natural compounds such as flavonoids can act, as a double-edged sword under certain conditions, as pro-oxidants and so, increase the oxidation of other compounds. These important circumstances depend 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]. It has been previously shown that biological effects of flavonoids (Particularly anticancer effect) may be mediated *via* pro-oxidant action. In cancer, the regulatory mechanisms of cell growth and proliferation moderated *via* flavonoids is widely complex: at low level of ROS can stimulate the cell division and the promotion of tumor growth, and a high level of ROS is cytotoxic to the cell and can cause apoptosis. Cancer cells exhibit a higher and more persistent oxidative stress level compared to normal cells, rendering tumor cells more vulnerable to being killed by drugs (some flavonoids) that raise ROS levels [36].

Our results both fluoremetric and flowcytometric techniques showed that APG raised ROS production only in the hepatocytes and mitochondrial isolated from the HCC group BUT NOT untreated control group. This is also in agreement of another published work indicated that ROS may act a key role in apoptosis signaling caused by APG in the ANA-1 cells [37].

The results of previous studies showed that ROS play a pivotal role in any stage of the carcinogenesis process. The mechanism of cell growth and proliferation by flavonoids in cancer, is complex, so that ROS in low concentrations of may promote the cell mitosis leading to tumor growth, On the other hand, at high levels, ROS is cytotoxic and the could stimulate apoptosis signaling [38].

Carcinogenesis is a multistage process [39]. Indeed, oxidative damage has been suggested to implement a key role in the promotion of tumorgenesis, in spite of the counteracting effect of the cellular antioxidant defense system. In fact, newly many *in vitro* researches on flavonoids with anti-cancer activity through the induction of oxidative stress have been performed [40]. Fortunately, our results confirmed selectivity and potentiality of APG on HCC tumor cell mitochondria.

Our results obtained by both fluoremetric and flowcytometric techniques showed that APG induced significant decrease in the level of $\Delta\Psi m$ in mitochondria isolated from hepatocytes of the only HCC rat group but not in those of the untreated control rat group. It is well known that mitochondria can play a role in the execution of apoptosis and also in death signal transduction through the MPT pore opening which is consequent to membrane potential collapse and cytochrome c expulsion into the cytoplasm through activation of caspase-3 following apoptosome formation [41]. Another study showed that treatment of A549 cells with APG leads to a decline in the level of $\Delta\Psi m$, expulsion of cyt c and other pro-apoptotic agents from mitochondria before the induction of apoptosis [7, 42].

The mitochondrial swelling and cyt c expulsion as subsequent events following mitochondrial permeability transition were also monitored in this study. As mentioned earlier, APG (10, 20 and 40 μM) induced selective effects on mitochondria obtained from hepatocytes of only HCC rats. The inhibition of cyt c release by the pre-exposure of CsA and BHT in the HCC mitochondria supports the hypothesis that apoptosis signaling induction by APG is a result of oxidative stress process and opening of the MPT pores.

Apoptosis is a process that majorly regulates cell number and proliferation as part of the normal development process. However, cancer cells tend to inactivate the cell cycle control points and the eventual progression of apoptosis. Therefore, the stimulation of apoptosis has been emphasized in a study on anticancer strategies [43]. Previous reports indicate that different flavonoids could activate the proteolytic activity of caspases, support their value as potential anticancer candidates for regulating tumor growth [43].

To test the aforementioned effects of APG, there is a need to check whether APG is able to induce apoptosis in HCC cells. Apoptosis or Type I cell death, which occurs either through intrinsic or extrinsic pathways, requires the activation of the caspases cascade. In this study, the mode of APG- induced apoptosis signaling was examined by the assessment of caspase-3 activity. The incubation of hepatocytes from HCC rats in a medium

containing APG (10μM), resulted in a statistically significant increase in caspase-3 activity in comparison with control hepatocyte. In line with the results of this study, it has already been reported that APG can act as a therapeutic agent by orchestrating a caspase-3 dependent apoptotic pathway [44]. Phosphatidylserine externalization is one of the earliest phenomena in apoptosis, which can be determined by annexin V and PI staining. The incubation of liver hepatocytes obtained from only HCC rats with APG for 6, 12 and 24 h resulted in significant raise in percentage of apoptosis phenotype. In general, the outcome of this study's cellular and mitochondrial experiments suggests that APG –selectively induced apoptosis in HCC rat hepatocytes by directly acting on their mitochondria.

Finally, the results of our study indicated that APG increases the mitochondrial ROS formation through the disruption of MMP only in the mitochondrial isolated from the liver of HCC rats. This process resulted in a decrease in the MMP, alteration of mitochondrial swelling and release of cyt c, which can selectively orchestrate apoptosis signaling in liver hepatocytes of cancerous HCC rats.

The anticancer vision is based on cytotoxic molecules that selectively target mitochondria and induce perturbation of the organelle in only cancerous cells, thereby bypassing upstream pro-apoptotic events that probably are defects in the tumor cell [45]. Most of these anticancer candidate molecules (especially flavonoids, such as myricetin [46], luteolin [47] and kaempeferol [48]) target the mitochondrial permeability transition (MPT) pores either by directly binding to a specific pore molecule or through enhancing of ROS formation resulting from mitochondrial respiratory chain disruption or Ca^{+2} influx. These events lead to mitochondrial membrane permeability transition, mitochondrial swelling, and finally cytochrome c expulsion from the mitochondria and formation of the apoptosome in the cytosol of tumor cell caspase-3 activation [45, 49].

In our current research, it is likely that apigenin can increase ROS generation through disruption in ETC. The increased ROS could oxidize the thiol groups surrounding the MPT pore and change the status of pore into open. These thiol groups originated from mitochondrial GSH, bound to their receptors located around MPT pores. Oxidation of surrounding reduced thiol groups resulted in disulphide bridge formation which induce mechanical pressure on mitochondrial membrane leading to opening of pores. Therefore, collapse of MMP, an event which is subsequent by influx of osmolites into mitochondrial inter-membrane and cause mitochondrial swelling.

Other results also showed that apigenin induced MMP decline and cytochrome c expulsion was prevented by BHT, which is an antioxidant suggesting that MPT pore opening was a subsequent of ROS formation. This effluxes cytochrome c and other apoptogenic proteins from the mitochondria and ignite the apoptosis signaling through the formation of apoptosome complex and caspase-3 activation, in HCC hepatocytes.

CONCLUSION

In this study, it was shown that APG is an effective anti-HCC candidate with very low and ignorable cytotoxicity towards normal liver cells. This research adds evidence that APG could selectively induce apoptosis in HCC hepatocytes and inhibit tumor growth by directly targeting mitochondria. According to our findings, APG can be recommended as a new anti HCC drug candidate.

LIST OF ABBREVIATIONS

2-AAF	=	2-acetylaminofluorene
AFP	=	Alpha-fetoprotein
APG	=	Apigenin
DEN	=	Diethylnitrosamine
HCC	=	Hepatocellular carcinoma
MMP	=	Mitochondrial Membrane Potential
ROS	=	Reactive Oxygen Species

DISCLOSURE

Part of this article (with different compound as anti HCC drug candidate) has been previously published in Basic & Clinical Pharmacology & Toxicology, DOI: 10.1111/bcpt.12572.

CONFLICT OF INTEREST

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