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Anticancer effect of calycopterin via PI3K/Akt and MAPK signaling pathways, ROS-mediated pathway and mitochondrial dysfunction in hepatoblastoma cancer (HepG2) cells

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Abstract

Calycopterin is a flavonoid compound isolated from *Dracocephalum kotschyi* that has multiple medical uses, as an antispasmodic, analgesic, anti-hyperlipidemic, and immunomodulatory agents. However, its biological activity and the mechanism of action are poorly investigated. Herein, we investigated the apoptotic effect of calycopterin against the human hepatoblastoma cancer cell (HepG2) line. We discovered that calycopterin-treated HepG2 cells were killed off by apoptosis in a dose-dependent manner within 24 h, and was characterized by the appearance of nuclear shrinkage, cleavage of poly (ADP-ribose) polymerase and DNA fragmentation. Calycopterin treatment also affected HepG2 cell viability: (a) by inhibiting cell cycle progression at the G2/M transition leading to growth arrest and apoptosis; (b) by decreasing the expression of mitotic kinase cdc2, mitotic phosphatase cdc25c, mitotic cyclin B1, and apoptotic factors pro-caspases-3 and -9; and (c) increasing the levels of mitochondrial apoptotic-related proteins, intracellular levels of reactive oxygen species, and nitric oxide. We further examined the phosphorylation of extracellular signal-related kinase (ERK 1/2), c-Jun N-terminal kinase, and p-38 mitogen-activated protein kinases (MAPKs) and found they all were significantly increased in HepG2 cells treated with calycopterin. Interestingly, we discovered that treated cells had significantly lower Akt phosphorylation. This mode of action for calycopterin in our study provides strong support that inhibition of PI3K/Akt and activation of MAPKs are pivotal in G2/M cell cycle arrest and apoptosis of human hepatocarcinoma cells mediated by calycopterin.

Keywords

Apoptosis; Calycopterin; HepG2; Mitochondria dysfunction; PI3K/Akt; ROS

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Conflict of interest The authors declare that there are no conflicts of interest.

Introduction

Apoptosis, or programmed cell death has been described as a mechanism by which cells undergo a natural dying process to control cell proliferation or in response to DNA damages [1, 2]. These apoptotic and cell death program are used to develop novel therapies against cancer cells using agents with cytotoxic properties as chemotherapy and radiation therapy [3–5]. Two main core pathways that established in apoptosis are; (a) the cell death receptor-mediated extrinsic pathway and the mitochondrial-mediated intrinsic pathway. This extrinsic pathway is triggered by extracellular membrane anchored death receptors binding such as Fas (and other similar receptors such as tumor necrosis receptor 1 and its relatives) with its extracellular ligand, Fas-L, and sequential caspase activations. (b) The intrinsic pathway that leads to apoptosis under the control of mitochondria and its associated molecules. In both cases, these pathways merge and result in cell death. Extracellular or intracellular signals alter mitochondrial membrane permeability and cytochrome c release into the cytosol which is an apoptotic signal. Cytochrome c recruits Apaf-1, pro-caspase-9 and forms the apoptosome, which is a downstream trigger of the caspase 9/3 signaling cascade, that is established as the main process of cell death by apoptosis [6, 7].

Natural compounds have been a fertile source of potential chemotherapeutic and chemoprevention agents, and they have received great attention because they are considered to be safer due to lower risk of mutagenicity in non-cancerous cells [8]. Calycopterin, 5,4'-dihydroxy-3,6,7,8-tetramethoxyflavone (Fig. 1), is a flavonoid and immunoinhibitory agent can be purified from *Dracocephalum kotschyi*, and widely used in Asia as medicine for multiple indications. The inhibitory effect of *D. kotschyi* extract on the lectin-induced cellular immune response was originally described [9, 10]. Compounds such as calycopterin to have dual effect depending on cell type and cellular milieu and environment are described [11, 12]. Resveratrol and celastrol are two examples of such compounds for having neuroprotective and anticancer properties in different settings. Resveratrol is neuroprotective in Alzheimer's disease (AD) models [11] and other neurodegenerative diseases models [13, 14] or cytotoxic in adenocarcinoma cells [15], where celastrol is neuroprotective in neurodegenerative diseases like, motor neuron disease or Lou Gehrig's diseases [16], AD [17] and has cytotoxic or anticancer effect in several cancer cells [18, 19]. We recently showed calycopterin is neuroprotective in PC-12 cells consistent with neuroprotective property of this natural compound. In the PC12 cell study, calycopterin protects PC12 cells against H₂O₂-induced apoptosis and decrease ER stress-associated factors, attenuating inflammatory responses and modulating the level of CREB phosphorylation and Nrf2 pathway [20, 21]. We now discovered the cytotoxic property of calycopterin in HepG2 cells and unravel a mechanism for its cytotoxicity via mitochondrial dysfunction and PI3K/Akt and MAPK signaling.

Materials and methods

Materials

Calycopterin, 5,4'-dihydroxy-3,6,7,8-tetramethoxyflavone was purified from *D. kotschyi* Boiss and the chemical structure was confirmed in our laboratory as reported previously [20]. MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide),

dimethylsulfoximine (DMSO), and propidium iodide (PI) were purchased from Sigma (St. Louis, MO, USA). Anti-cell division cycle 25 homolog C (cdc25c), anticyclin B1, anti-Bcl-, anti-Bax, anti-pro-caspase 3, anti-pro-caspase 9, anti-poly ADP-ribose polymerase (PARP), anti-extracellular signal-regulated kinase 1/2 (ERK1/2), anti-c-Jun N-terminal kinase (JNK), anti-p38 MAPK, anti-Akt, anti-phospho-ERK1/2 (Thr202/Tyr204), anti-phospho-JNK (Thr183/Tyr185), anti-phospho-p38 MAPK (Thr180/Tyr182), anti-phospho-Akt (Ser473), and anti-phospho-phosphatidylinositol-3-kinase (PI3K) (Tyr458/Tyr199) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Benzyloxycarbonyl–Val–Ala–Asp–fluoromethyl ketone (z-VAD-fmk), LY294002, U0126, SP600125, and SB203580 were purchased from Promega (Madison, WI, USA). DMEM cell cultures, fetal bovine serum (FBS) were purchased from Gibco (Gaithersburg, MD, USA). All other reagents were commercial products of the highest available purity grade.

Cell culture and treatment

The human hepatocarcinoma (HepG2) cell line was purchased from National Cell Bank of Iran (NCBI), Pasteur Institute of Iran (Tehran, Iran), and maintained in DMEM medium supplemented with 10 % fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. These cells were kept at 37 °C in a humidified atmosphere containing 5 % CO₂. Calycopterin was dissolved in DMSO to make a stock of 100 mM and further diluted to final concentrations of 10–200 µM with serum-free culture medium. The amount of DMSO added to the cell culture was less than 0.1 % in all cases.

Cell viability assay

Cell viability was determined using the MTT assay. Briefly, 2.5×10^4 cells were seeded in 96-well plates at 37°C with 5 % CO₂ for overnight incubation and treated with appropriate concentrations of calycopterin for 24 h. The cells were then incubated with a serum-free medium containing MTT at a final concentration of 0.5 mg/ml for 4 h. The dark formazan crystals formed were dissolved in DMSO, and the absorbance was measured at 570 nm.

Quantification of apoptotic cells by flow cytometry

The extent of apoptosis was measured through annexin-V-FITC/PI apoptosis detection kit (Invitrogen, CA, USA) according to the manufacture's instruction. Calycopterin-treated cells for 24 h were harvested, washed twice with PBS, gently re-suspended in annexin-V binding buffer and incubated with annexin-V-FITC/PI in dark for 10 min and analyzed by flow cytometry using FloMax software. The fraction of cell population in different quadrants was analyzed using quadrant statistics.

Cell cycle analysis

HepG2 cells were plated at a density of 1×10^5 cells/well in six-well plate and incubated for 24 h to resume exponential growth. Cells were exposed to different concentrations of calycopterin for 24 h and whole cells were harvested and fixed with 70 % ethanol for 1 h at 4 °C. Fixed cells were washed in phosphate-buffered saline (PBS). Cells were then incubated with 1 U/ml of RNase A (DNase free) and 5 µg/ml of propidium iodide for 30 min at 4 °C in the dark. A FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ,

USA) was used to analyze the cell cycle distribution. The amount of subG1 and G2/M phase cells was determined as a percentage of the total number of cells.

Analysis of DNA fragmentation

The fragmented DNA was extracted with the apoptotic DNA ladder kit (Invitrogen, CA, USA) per the manufacturer's instructions. DNA samples were analyzed by electrophoresis (1 h at 80 V/30 mA) on a 1.2 % agarose gel containing 0.5 mg/ml ethidium bromide.

AO/EB and DAPI staining assay

Acridine orange/ethidium bromide (AO/EB) double fluorescent dyes were used to qualitatively observe apoptotic cells. AO can emit a green fluorescence if it passes through the complete cell membrane and embeds in nuclear DNA, while EB can mark nuclear DNA of damaged cells and emit a red–orange fluorescence [20]. Calycopterin-treated cells were added into 60 µl AO/EB (100 µg/ml AO: 100 µg/ml EB = 1:1) and then immediately observed and photographed by an inverted fluorescence microscope (Zeiss, Germany). For DAPI staining, calycopterin-treated HepG2 cells were fixed with 4 % paraformaldehyde for 10 min and stained with 1 µg/ml DAPI for 5 min, followed by observation under a fluorescence microscope.

Measurement of mitochondrial membrane potential (MMP)

The mitochondrial membrane potential (MMP) was measured using the mitochondria-specific lipophilic cationic fluorescence dye JC-1. As a monomer, JC-1 is capable of selectively entering the mitochondria. Under normal conditions, JC-1 aggregates within the mitochondria and emits red fluorescence, but when the MMP collapses during apoptosis, JC-1 emits green fluorescence. The ratio of red to green JC-1 fluorescence reflects the change in MMP. Cells were seeded into 96-well polystyrene culture plates and treated with different concentrations of calycopterin. After 24 h, 100 µl of medium was removed from each well and 100 µl of 5 µg/ml JC-1 was added to the cells for 1 h. Finally, cells were washed twice with PBS, and then analyzed by fluorescence microplate reader (Molecular Devices Co., CA, USA).

Detection of intracellular ROS, NO production, and intracellular GSH level

Cellular ROS was measured with 2',7'-dichlorodihydro-fluorescein diacetate (H2DCFDA; Sigma). For this assay, the cells were plated in 6-well polystyrene culture plates at a density of 1×10^5 cells/ml. After 24 h, 10 µM H2DCFDA was added to the wells for 30 min at 37 °C. Then, cells were washed twice with PBS and immediately evaluated using a fluorescence microscope. The production of NO was expressed by the concentration of nitrite in the supernatant of the cell cultural medium and was determined by the Griess reagent. The culture supernatant was incubated with the same volume of the Griess reagent (0.1 % (w/v) *N*-(1-naphthyl)-ethylenediamine and 1 % (w/v) sulfanilamide in 5 % (v/v) phosphoric acid) for 10 min at room temperature. Then, the absorbance of the chromophoricazo-derivative molecule was measured at 540 nm with an ultraviolet spectrometer (UV-2550, Shimadzu Corporation, Kyoto, Japan). The amount of nitrite was calculated using a sodium nitrite standard curve. The cellular level of reduced glutathione

(GSH), another index reflecting cellular reducing power, was measured by staining cells with NDA. GSH is oxidized to oxidized glutathione (GSSG) under the stress of ROS. Cells were stained with 200 μ M NDA at 37 °C for 30 min, the level of GSH was determined by measuring the fluorescence intensity by flow cytometer with the FL-1 filter and an excitation wavelength of 480 nm.

Isolation of mitochondrial and cytosolic fractions

Mitochondrial and cytosolic fractions were prepared as described previously [21] with minor modifications. Generally, HepG2 cells were pretreated with calycopterin for 24 h, then, re-suspended in solution containing buffer A (10 mM 4-(2-hydroxyethyl)-piperazine ethane sulfonic acid (HEPES), 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 68 mM sucrose, 220 mM mannitol, 0.1 % BSA, supplemented with protease inhibitors (1 mM phenyl methyl sulfonyl fluoride (PMSF), 2 μ g/ml aprotinin, and 0.1 mM leupeptin) for 30 min in ice to incubate. After that, cells were triturated by a 27 gage syringe (25–40 strokes) to become homogeneous, and then centrifuged at 200 $\times$ g for 2 min to omit unbroken cells. Supernatants phase (500 μ l) was then transferred to a new tube and centrifuged at 7,000 $\times$ g for 15 min to pellet all the mitochondria. The supernatants centrifuged at 16,000 $\times$ g for 30 min and collected as cytosolic fractions. Mitochondrial fraction was subsequently solubilized in TBSTDS (10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.5 % sodium deoxycholate, 0.05 % SDS, 0.02 % NaN₃, 0.0004 % NaF) containing protease inhibitors. These steps were performed at 4 °C and then mitochondrial and cytosolic fractions stored at –70 °C till use.

SDS-PAGE and Western blot analysis

HepG2 cells were initially seeded at a density of 1×10^6 in 100-mm² dishes. After treatment with selected doses of calycopterin for the indicated times, adherent cells were collected for protein extract preparation. Briefly, treated and control cells were lysed with RIPA buffer, and then nuclear and cytoplasmic extracts were separated using NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific, Waltham, MA, USA). Equal amounts of lysate (based on the protein contents) were then separated using SDS-PAGE, blotted onto polyvinylidene di-fluoride membranes, reacted with specific primary antibodies, and then visualized with appropriate conjugated secondary antibodies. Immunoreactive bands were detected using the ECL method and visualized on Kodak Bio-MAX MR film.

Statistical analysis

All results shown represent mean $\pm$ SD from triplicate experiments performed in a parallel manner unless otherwise indicated. Statistical analyses were performed using GraphPad (San Diego, CA, USA) with an unpaired, two-tailed Student's *t* test. All comparisons are made relative to untreated controls and significance of difference in means measured at **p* < 0.01 and ***p* < 0.001.

Results

Calycopterin reduces HepG2 cancer cell viability

The effect of calycopterin on cell viability of HepG2 cells was investigated by the MTT assay. Calycopterin treatment reduced cell viability significantly in a dose-dependent manner (50, 100, 150, and 200 μM) to 45.12, 40.15, 39.80, and 38.17 % of total, ($p < 0.001$), respectively (Fig. 2). The maximum inhibition of cell growth and survival (~61 %) was observed in cells treated with calycopterin at 200 μM .

Calycopterin induces cell death in HepG2 cells

In order to evaluate whether the cytotoxic effect of calycopterin upon HepG2 cells was due to its effect on cell cycle and apoptosis, we further investigated the effects of calycopterin on cell cycle and on inducing programmatic cell death. Morphological changes, DNA fragmentation, and phosphatidyl serine externalization were analyzed. Calycopterin treatment (50 and 100 μM) for 24 h, caused chromatin condensation dose dependently (Fig. 3a). Nuclear fragmentation and apoptotic bodies formation observed in HepG2 cells treated with calycopterin that consequently could be the cause of significant decrease in cell viability (Fig. 3b). Genomic DNA laddering is a hallmark of apoptosis that we found was apparent in HepG2 cells incubated with calycopterin at 10, 25, 50, and 100 μM for 24 h (Fig. 3c). Apoptotic cell death was further determined by annexin-V staining of phosphatidyl serine exposure on the cell surface, and assessed by flow cytometry (Fig. 3d). This method assesses the total number of cells that have exposed phosphatidyl serine during apoptosis and/or lost plasma membrane integrity. As detected by annexin-V(+), PI(-) % cells using flow cytometry, calycopterin led to a concentration-dependent externalization of phosphatidylserine, a hallmark of apoptosis. Annexin-V(+)/PI(-) and annexin-V(+)/PI(+) represent the cells in early and late stages of apoptosis/necrosis, respectively. The lower left quadrant in Fig. 3 shows intact cells, the lower right quadrant shows early-apoptotic and the upper right quadrant shows late apoptotic or necrotic cells. We compared calycopterin (50 μM)-treated HepG2 cells with vehicle-treated controls and expressed the percentages of late apoptotic cells that was increased from 0.87 to 37.91 % (Fig. 3d). These results show major hallmarks of apoptotic cell death, and demonstrate that calycopterin induces apoptosis in Hep G2 cells.

Calycopterin induces G2/M phase cell cycle arrest

To further examine the relation of growth inhibition with cell cycle arrest and apoptosis, HepG2 cells were treated with 10–100 μM calycopterin for 24 h and analyzed using fluorescence-activated cell sorting. We found calycopterin-treated HepG2 cells go through cell arrest in a dose-dependent manner at the G2/M phase. The S phase was significantly decreased in calycopterin-treated HepG2 cells (Fig. 4). An increase in the G2/M phase from 34.29 to 49.23 % ($p < 0.01$) and a decrease in the S phase from 29.54 to 18.95 % ($p < 0.01$) (Table 1) was found. Calycopterin did not influence the G0/G1 phase, however. These data are consistent with G2/M phase arrest.

Calycopterin inhibits the expression of key mitotic proteins; Cyclin B1, cdc2, and cdc25c

Important mitotic cyclin/kinase/phosphatase proteins related to the G2/M phase are cyclin B1, cdc2, and cdc25c. The G2/M phase is controlled by a complex formed cyclin B1 and cdc2 and this complex is regulated by cdc25c [22, 23]. Since our results show that calycopterin can induce G2/M phase arrest in HepG2 cells (above), we evaluated the expression of cyclin B1, cdc2, and cdc25c. We found that protein levels of cyclin B1, cdc2, and cdc25c decreased in a dose-dependent manner (Fig. 5a–d).

Calycopterin activates caspase 3, caspase 9 and induces cytochrome c release in HepG2 cells

To further unravel the mechanism of apoptotic signaling in calycopterin-treated HepG2 cells, caspase activation, and cytochrome c release was investigated. These are known to be frequently involved in a chemically induced apoptotic signaling pathway [24] and the resultant activation of caspase cascade involving caspase-9 and caspase-3, leading to PARP degradation [25]. We found the levels of cytochrome c released from mitochondria increased dose dependently in the presence of calycopterin in the 50 and 100 μM doses (Figs. 6a, 7a, b). Consistent with cytochrome c release, cleaved caspase-9, and caspase-3 levels were increased at these two doses. PARP is a downstream target of active caspase-3 during induction of apoptosis, we found an increase in PARP cleavage [expressed as cleaved/un-cleaved poly (ADP-ribose) polymerase] (Fig. 6).

Substantial evidence supports the idea that Bcl-2 family members regulate the release of mitochondrial proteins [24, 26, 27]. The Bcl-2 family can be divided into two subgroups, pro-death members and anti-death members. Among the pro-death members, Bak and Bax have been categorized as the last gateway of cytochrome c release, and their homo-oligomerization on the mitochondrial membrane is essential for release, while the anti-apoptotic Bcl-2 members prevent mitochondrial protein release by interacting with and inhibiting both Bak and Bax [24]. In the present study, we found that calycopterin treatment significantly increased the level of the pro-apoptotic protein Bax (Fig. 6b). In contrast, the level of the anti-apoptotic protein Bcl-2 decreased (Fig. 6b). Since an increased ratio of Bax/Bcl-2 can exacerbate cytochrome c release thereby increasing apoptosis, our data consistently demonstrate that calycopterin-induced apoptosis takes place via the mitochondrial pathway (intrinsic apoptotic pathway) and cytochrome c- dependent activation of the caspase cascade, including caspase-9 and caspase-3.

Calycopterin induces mitochondrial dysfunction in HepG2 cells

Changes in mitochondrial membrane potential (ψ) are one of the early events leading to mitochondrial functional alterations. Therefore, we sought to investigate the role of the mitochondrial-mediated apoptotic pathway in calycopterin's anticancer activity. Incubation of HepG2 cells with calycopterin (10–100 μM) for 24 h, resulted in a dose-dependent decrease in the mitochondrial membrane potential (Fig. 7a). The dose-dependent decrease of the OD₅₈₅/OD₅₃₈ ratio indicated that the mitochondrial membrane potential had collapsed (Fig. 7a). Calycopterin (50 μM), caused a significant release of cytochrome c from mitochondria to the cytosol fraction (more than 5 fold) as compared to the vehicle-treated controls (Fig. 7b).

Calycopterin induces ROS, NOS production, and GSH depletion in HepG2 cells

—The generation of intracellular ROS and depletion of GSH is reported to be associated with mitochondria, its MMP and cell apoptosis [28]. We examined the levels of ROS and GSH in HepG2 cells treated with calycopterin. ROS was monitored by the oxidation sensitive fluorescent dye DCFH-DA. Dose-dependent increase in DCFH-DA fluorescence was detected in calycopterin-treated cells (Fig. 8a). Rapid generation of ROS, up to threefold faster than the control, was detected only 2 h after incubation with calycopterin (data not shown), and this fluorescence intensity was increased tenfold after 24 h incubation with calycopterin. It caused a significant decrease in the intracellular GSH content after 24 h incubation (Fig. 8b). Similarly, calycopterin treatment at 50 and 100 μ M significantly enhanced the level of nitrites in HepG2 cell culture supernatant ($p < 0.01$). The level of nitrites in 50 and 100 μ M-treated cells was 72.6 and 86.6 times than that in control cells (data not shown). To verify the relationship between ROS generation and cell toxicity, we examined the effects of ROS scavengers, NAC (a well-known antioxidant and GSH precursor), and catalase (a H_2O_2 -scavenging enzyme), on calycopterin-induced cell death. The cells were treated with 1 mM NAC or 2,000 U/ml catalase for 30 min, and then the change of MMP and cell toxicity was determined. As shown in Fig. 8c, both NAC and catalase inhibited the loss of MMP and protected cells from calycopterin cytotoxicity and significantly reduced apoptosis as indicated by DAPI staining (Fig. 8d).

Calycopterin acts via PI3K/Akt and MAPKs pathways in HepG2 cells

The phosphorylation of Akt is related to protection of cells from apoptosis [29]. Calycopterin markedly dephosphorylated PI3K/Akt at 10, 25, 50, and 100 μ M, but increased the phosphorylation of ERK1/2. Phosphorylation of JNK and p-38 MAPK was significantly increased at 50 and 100 μ M (Fig. 9). These data suggest that calycopterin modulated the PI3K/Akt and MAPKs pathways in HepG2 cells. To unravel the possible roles and effects of PI3K/Akt and MAPKs in calycopterin-induced apoptosis, we examined the changes in cell cycle and protein level due to G2/M cell cycle arrest and apoptosis in the presence or absence of specific inhibitors for PI3K (LY294002), ERK1/2 (U0126), JNK (SP600125), p38-MAPK (SB203580), and caspases (z-VAD-fmk). We found SP600125, and SB203580 significantly reversed calycopterin-induced apoptosis, but there was no significant change in the G2/M phase cells. Pre-treatment with U0126 prominently reduced G2/M and subG1 phases in HepG2 cells (Fig. 10). The pan-caspase inhibitor z-VAD-fmk restored the calycopterin-induced pro-caspase-3 and cleaved PARP levels. In addition, LY294002 enhanced calycopterin on suppression of pro-caspase-3 and increase of cleaved PARP (Fig. 10). These results support the conclusion of Akt, JNK, and p38 MAPK pathway are directly related to the induction of apoptosis, and the ERK1/2 pathway is involved in the G2/M cell cycle arrest mechanism caused by calycopterin in HepG2 cells.

Discussion

Apoptosis is a form of programmed cell death that is characterized by a variety of morphological features, including changes in the plasma membrane such as loss of membrane asymmetry and attachment, cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation [4]. It is critically important to develop new drugs that not

only significantly induce apoptosis in tumor cells but also have minimum or no side effects. In this report, we reported of substantial evidence that calycopterin modulates apoptosis in HepG2 cells, via the proteolytic activity of PARP, caspase activation, DNA fragmentation, morphological changes, and cell viability of HepG2 cells. Our investigation on extrinsic or intrinsic apoptotic pathways, appears to be the target of calycopterin. We found that calycopterin-induced HepG2 apoptosis is caspase-dependent and it significantly increased the expression of cleaved caspase-3 (Fig. 6). Meanwhile, in apoptotic cells, activated Bax is translocated, as well as integrated into mitochondrial membranes [30]. The overexpression of Bax and low expression of Bcl-2 eventually leads to mitochondrial damage, depolarization, MMP collapse, cytochrome c release, and caspase-3 activation through either homologous dimerization or the promotion of mPTP formation in the inner and outer mitochondrial membranes [28]. Experiments on this study consistently show that calycopterin significantly reduced anti-apoptotic Bcl-2 and increased pro-apoptotic Bax, leading to the loss of MMP and the release of cytochrome c from mitochondria membrane in HepG2 cells suggesting that mitochondrial dysfunction is involved in calycopterin-induced apoptosis.

The program cell death induced by calycopterin on HepG2 cells was associated with an early elevation of intracellular ROS. Incubation with calycopterin for 2 h, caused increase in ROS levels up to threefold, and continued to increase with time, reaching almost tenfold after 24 h incubation. Simultaneously, this excessive ROS generation led to a significant decrease of intracellular GSH, a main non-protein antioxidant in the cell, disabling them to clear intracellular ROS. Our data are consistent with other studies and show that calycopterin caused significant reduction of intracellular GSH in HepG2 cells. The experiments with antioxidants NAC and catalase in this study confirmed this as both NAC and CAT inhibited the cytotoxicity of calycopterin associated with suppressed ROS generation and GSH depletion. These data support the basis that calycopterin induce a decrease of MMP in HepG2 cells. In addition, relevant to the timing of ROS burst and MMP depolarization, the ROS burst occurred before intracellular MMP depolarization. This is supported by the evidence from experiments with HepG2 co-incubated with calycopterin and antioxidant NAC or catalase. Both NAC and catalase blocked the MMP depolarization completely. These results demonstrate that the ROS burst was a pre-requisite for MMP collapse and cell death induction by calycopterin.

It is important to note that apoptosis is accompanied with a reduction of the G1 phase cells and an increase in the G2/M phase cells, signifying a cell cycle arrest in G2/M phase. Therefore, our findings are in agreement with previous studies showing that an increase in the number of G2/M phase cells is clearly related to apoptosis [31, 32]. A recent study showed that a mulberry extract containing caffeoyl quinic acid and kaempferol derivatives, induced apoptosis and G2/M arrest in HepG2 cells [33]. In our experiment, we found calycopterin decreased the percentage of G1 phase and increased G2/M phase cells which could be due to an apoptotic response of HepG2 cells. As caspases play a pivotal role in the apoptotic cell death [34], we provide evidence that calycopterin regulated activation of caspases. Our data suggest that a calycopterin decrease of pro-caspase-3, -9, and an increase of caspase-3 which are integral part of the intrinsic apoptotic signaling pathway. We further showed that the activity of caspase-3 and cleavage of PARP were abolished by the pre-

treatment with pan-caspase inhibitor z-VAD-fmk. In addition, pre-incubation with z-VAD-fmk block the calycopterin-induced subG1 and G2/M phase increase. In this study, experiments with calycopterin in HepG2 cells show the inhibition of the constitutive level of PI3K and its downstream target Akt, consistently shown to be involved in apoptosis [35, 36]. Similarly, our investigation indicates that phosphorylation of Akt decreases with the elevation of pro-apoptotic proteins. In the experiments that we inhibited kinases show that PI3K inhibitor LY294002 enhanced apoptosis induced by calycopterin. This confirms that PI3K and Akt are two important kinases involved in apoptotic process. The MAPKs family are characterized and involved in apoptosis in variety of cellular toxicities, such as ultraviolet radiation and many chemotherapeutic agents [37]. Others have reported that p38 MAPK inhibit phosphatase cdc25 that promotes G2/M phase progression by removing inhibitory phosphates from CDK1 [38]. ERK1/2 and JNK are key signal pathways typically associated with responses to mammalian mitogenic activation [39]. Activation of ERK1/2 and JNK promote a G2/M cell cycle arrest [40]. The ratio between the intensity and duration of pro and anti-apoptotic signals regulated by ERK1/2 determines whether cells will survive or undergoes apoptosis [41]. In this study, we show that calycopterin induce that phosphorylation of p38 MAPK, ERK1/2, and JNK in dose dependently. The use of specific PI3K and MAPKs inhibitors prior to adding calycopterin to the HepG2 cells confirmed that PI3K and MAPKs are critical players. Data presented in this article demonstrate the use of a specific PI3K inhibitor inhibited de-phosphorylation of the Akt. ERK1/2 and JNK inhibitors also inhibited phosphorylation of ERK1/2 and JNK. The p38 MAPK inhibitor SB203580 inhibited downstream of the p38 MAPK, however. A study by Kumar and colleagues [42] showed that p38MAPK inhibitor SB203580 can specifically block the level of p38MAPK to phosphorylate its downstream substrate but not its activation by upstream MAPKK. A schematic diagram of describing this is shown in Fig. 11.

In conclusion, our study provides a novel insight into the mechanism of calycopterin-induced apoptosis in HepG2 cells and shows a link between ROS and the cell death process. Our data suggest that calycopterin induce apoptosis of HepG2 cells via apoptotic pathway that involves ROS production, Bcl2 family protein expression, and mitochondria depolarization and MMP disruption. In addition, calycopterin-induced G2/M cell cycle arrest and apoptosis through the de-phosphorylation of Akt and phosphorylation of MAPKs, which affects the levels of mitochondrial apoptotic proteins. Therefore, the natural herbal product calycopterin has therapeutic potential for hepatocellular carcinoma. Further studies are required to investigate and confirm calycopterin's anticancer effect in other cellular models and in vivo.

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Fig. 1.
Chemical structure of calycopterin isolated from *Dracocephalum kotschy*

Fig. 2.

Calycopterin reduces the viability of HepG2 cells. Cells were seeded at a density of 1×10^4 cells/mL in 96-well polystyrene culture plates at 37 °C with 5 % (v/v) CO₂ for 1 day. After 24 h of incubation, cells were incubated with calycopterin at the indicated concentrations for 24 h and then processed and assayed using MTT assay kit. Each value represents the mean $\pm$ SD of three experiments. Very significant at ** $p < 0.001$

Fig. 3.

Calycotriptin induces cell death in HepG2 cells. Cells were treated with calycotriptin at the indicated concentrations for 24 h. **a** cells were stained with AO/EB or DAPI and then were photographed by fluorescence microscopy ($\times 400$ magnification) (*Arrows* point to fragmented nuclei). Condensed nuclei observed in the calycotriptin-treated group shown by *arrows*. **b** The percentage of apoptotic cells in calycotriptin-treated HepG2 cells at indicated concentrations. **c** DNA fragmentation was determined using a DNA ladder extraction kit. **d** Apoptosis was assessed by Annexin-V-PI double staining (*a* untreated cells; *b–d* treated cells with 25, 50, 100 μM calycotriptin, respectively, versus control) significant at $*p < 0.01$

Fig. 4.

Flow cytometry of cell cycle phase distribution. Calycopterin induces G2/M phase arrest on HepG2 cells. Calycopterin added at indicated concentrations, and cells were incubated for 24 h. Cell cycle proportions were determined by flow cytometry after staining with propidium iodide. Statistical analysis of cell cycle phase distribution is shown in Table 1

Fig. 5.

Calycopterin alters the expression of cell-cycle-related mitotic proteins cyclin B, cdc 25c, and cdc 2. The expression of proteins was assessed by immunoblotting. **a** Representative blots are shown. Densitometric analysis of the effect of calycopterin on the expression of cyclin B1 (**b**), cdc 2 (**c**), and cdc 25c (**d**). Each value represents the mean $\pm$ SD of three experiments; bars (significant at $*p < 0.01$, and very significant at $**p < 0.001$ versus control group)

Fig. 6.
a Calycopterin alters the expression of apoptosis-related proteins caspase 9 and, 3 and cleavage of PARP. The expression of proteins was assessed by immunoblotting. **b** Representative image of immunoblots for Bcl-2 and Bax (western blotting analysis). β-actin as a house-keeping protein is used for loading control. The *graph* shows the ratio of Bax/Bcl-2 values. Densitometry analysis shows the mean ± SD of four independent experiments. Very significant at ** $p < 0.001$

Fig. 7.

a Loss of mitochondrial membrane potential (ψ_m) after calycopterin treatment in HepG2 cells for 24 h. The ratio of *red* to *green* JC-1 fluorescence reflects the change in mitochondrial membrane potential. **b** Representative images of immunoblots for cytochrome c (Western blotting analysis). The graph shows the ratio of cytochrome c (in cytosol and mitochondria) values. The results are expressed as the mean $\pm$ SD of three separate experiments. Significant at $*p < 0.01$ and very significant at $**p < 0.001$ versus control

Fig. 8.

Calycopterin induces ROS generation, cause GSH depletion and mitochondria membrane potential (MMP) disruption in HepG2 cells. **a** A graph showing the effect of calycopterin on cellular ROS production. After treatment with calycopterin for 24 h, cells were incubated with 10 μM DCFH-DA for 30 min and then immediately assayed. **b** A graph showing the effect of calycopterin on the cellular GSH level. **c** Histograms showing the effects of exogenous NAC and catalase (CAT) on the calycopterin-induced ROS generation, GSH depletion, MMP disruption, and cell viability of HepG2 cells. Cells treated with calycopterin for 24 h with or without pre-treatment with 1 mM NAC or 2,000 U/ml catalase. **d** Catalase and NAC protected cells from apoptotic effect of calycopterin determined by DAPI staining ($\times 100$, magnification). Data represent mean $\pm$ SD of three independent experiments; significant at $*p < 0.01$, and very significant at $**p < 0.001$

Fig. 9.

Regulation of PI3K/Akt and MAPKs by calycopterin. HepG2 cells were treated at the indicated concentrations of calycopterin for 24 h. Whole cell lysates were prepared from these cells and subjected to Western blot analysis. For phosphorylated Akt and MAPKs, the protein levels were normalized to the respective total Akt and MAPKs, and they are shown relative to the value for untreated control cells. Data represent mean $\pm$ SD of three independent experiments; significant at $*p < 0.01$

Fig. 10.

The effects of PI3K/Akt and MAPKs inhibitors on calycop-terin-induced G2/M cell cycle arrest and apoptosis in HepG2 cells. **a** HepG2 cells were pretreated with the indicated concentrations of PI3K and MAPKs inhibitors: LY294002, SP600125, U0126, and SB203580 for 2 h with 50 μ M of calycop-terin for 24 h. For phosphorylated Akt and MAPKs, the protein levels were normalized to the respective total Akt and MAPKs. **b** HepG2 cells were pretreated with indicated concentration of MAPKs inhibitors: SP600125, U0126, and SB203580 and pan-caspase inhibitor z- VAD-fmk with 50 μ M of calycop-terin for 24 h. **c** HepG2 cells were pretreated with pan-caspase inhibitor z- VAD-fmk and PI3K inhibitor LY294002 for 2 h with calycop-terin for 24 h. Data represent the mean $\pm$ SD of three replicate independent experiments, significant at $*p < 0.01$

Fig. 11. Possible cellular mechanisms involved in calycoperin-induced apoptosis in HepG2 cells. Calycoperin triggers ROS and NO production, which induces mitochondrial oxidative/nitrative stress and inhibits the PI3K/Akt pathway, and the subsequent release of cytochrome c followed by caspase-3 activation and apoptosis. The PI3K/Akt pathway indicated by thick arrows promotes ROS and NO generation and is involved in mitochondria-mediated apoptosis via increasing Bax and decreasing Bcl-2 protein expression

Table 1

Flow cytometry analysis in HepG2 cells treated with calycopterin

Percentage (mean $\pm$ SD) of HepG2 cells at each cell cycle phase after treatment with various concentrations of calycopterin at 24 h			
Calycopterin (μ M)	G0/G1 %	S %	G2/M %
0	36.17 $\pm$ 1.39	29.54 $\pm$ 0.50	34.29 $\pm$ 0.97
10	39.47 $\pm$ 2.31	24.95 $\pm$ 2.25	35.58 $\pm$ 0.57
25	38.85 $\pm$ 2.88	21.98 $\pm$ 1.74	39.17 $\pm$ 1.49
50	35.30 $\pm$ 2.89	20.47 $\pm$ 2.48	41.23 $\pm$ 0.67
100	31.48 $\pm$ 3.06	18.95 $\pm$ 1.21	49.23 $\pm$ 2.84

Results are representative of three replicates of independent experiments