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Synthetic racemates of abyssinone I and II induces apoptosis through mitochondrial pathway in human cervix carcinoma cells

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ABSTRACT

Abyssinones I and II are prenylated flavanones existing in plant *Erythrina abyssinica* showing diverse biological activities including anticancer activities. We synthesized racemic mixtures of these flavanones from corresponding chalcones and herein we report for the first time the molecular mechanisms of cell death, anti-proliferative effect and ability to induce apoptosis in human cervical carcinoma (HeLa) cells. Cytotoxicity was assessed by MTT assay to determine LD50 for prenylated chalcones and their corresponding flavones. Abyssinones promoted apoptosis by up regulation of p53 and Bax, along with down regulation of Bcl-2. Apoptosis induction was mediated through mitochondrial pathway releasing cytochrome c and Apaf-1 into cytosol; associated with activation of caspase-3. Further they were able to decrease the expression of cell proliferation markers PCNA and cyclin D1 indicating anti proliferative activity. These observations demonstrate that abyssinones trigger apoptosis via mitochondrial pathway by activation of caspase-3 and disrupts cell cycle.

1. Introduction

Carcinogenesis is the result of an imbalance in tissue homeostasis. The rate of replication and cell death are balanced in a stable tissue. In certain circumstances the sustained rate of cell replication can exceed the rate of apoptosis, resulting in hyperplasia which in itself does not imply tumor development [1,2]. Therefore cancer is a multifactorial disease that requires multi target therapeutic approach. Numerous natural compounds, drug cocktails in combination with synthetic and semisynthetic compounds are used to enhance therapeutic efficacy towards multi drug therapy.

Flavonoids are phenolic compounds having diverse biological activity. Many flavonoids have shown anti-tumour activity against various cancer cell lines and in xenograft models affecting the overall process of carcinogenesis by several mechanisms. In particular, it counter acts oxidative stress and induce apoptosis contributing towards prevention of cancer onset and development [3]. Modulation of redox potential by flavonoids in cancer cells, affects signal transduction [4], activation of redox-sensitive transcription factors [5] and expression of specific genes that influence cell proliferation and apoptosis [6,7].

Flavonoids are used in cancer treatment strategy to increase the efficacy of drugs and to reduce the side effects [8]. Studies have shown that members of flavonoids family exhibit a wide spectrum of anticancer mechanisms [9] and most of the studies based on molecular mechanisms are conducted in isoflavanones [10]; not on abyssinones.

Abyssinones are prenylated flavonoids isolated from East African medicinal plant *Erythrina abyssinica* [11] widely used as folk medicine for treating malaria and syphilis [12]. Prenylated flavanones are known to have chemopreventive and aromatase inhibitory activity [13]. Thus abyssinones gained importance and hence scientists started synthesizing these molecules to study their biological activities. Moriaty et al. synthesized abyssinone II and reported their aromatase inhibitory activity [14]. However, the molecular mechanisms involved in initiation of cell death, the targets and the anticancer action of abyssinones are not completely understood.

Abbreviations: MTT, 3-(4, 5-dimethylthiazol-2yl-)-2, 5-diphenyl tetrazolium bromide; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; PVDF, polyvinylidone difluoride; SDS–PAGE, sodium dodecyl sulfate–poly acrylamide gel electrophoresis; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; TBST, tris buffered saline tween-20, JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide).

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Apoptosis or programmed cell death is a key factor for onset and development of cancer. The molecular mechanism of apoptosis are mediated through intrinsic or extrinsic pathway which are tightly regulated by the activation of aspartate specific cysteine proteases known as caspases [15]. Extrinsic pathway is activated through death receptor present on cell surface once specific ligand binds to it, ultimately leading to release of cytochrome c from mitochondria. Cytochrome c interacts with apoptosis protease-activating factor (Apaf-1), and procaspase-9 to form a supramolecular complex called the apoptosome. The apoptosome, in turn activates caspase-3 through autocatalysis of caspase-9 culminating in apoptosis [16]. Bcl-2 family proteins are very important in mitochondria-dependent apoptotic pathway as it tightly regulates the stability of mitochondrial membrane, and oligomerization in response to apoptotic signal, resulting in release of cytochrome c [17]. The intrinsic pathways activated due to intracellular stress also activate proteins like Bax resulting in apoptosis independent of surface bound receptors. In present study we have chosen certain apoptotic markers to study the potency of abyssinones in inducing apoptosis in cervical cancer cell line HeLa.

p53 is a tumour suppressor protein that plays important role in cellular response to stress and differentiation [18]. Such signals lead to phosphorylation of p53, which translocates to nucleus and brings about consequent target gene expression such as Bax. Expression of Bax is important for mitochondrial dependent apoptosis [19]; hence we choose p53 as one of the important marker to study apoptotic effect of abyssinones. Further we also analyzed effect of abyssinones on cell cycle and studied expression of important cell cycle regulating markers like PCNA (proliferating cellular nuclear antigen) and cyclin D1 in cervical cancer cell lines [20].

In our earlier studies we have shown cytotoxic activity in breast cancer cell lines from series of chalcones and corresponding flavanones synthesized in our laboratory. [21]. Further these compounds were studied for their binding energies with aromatase and other enzymes of steroidogenesis *in silico* [22]. In present study we synthesized racemates of abyssinone I and II as established from our earlier methods, we further demonstrated molecular mechanisms of apoptosis induced by these abyssinone in cervical cancer cell line.

2. Materials and methods

2.1. Reagents

DMEM, trypsin–EDTA, MTT, DMSO, TRIzol, oligodT, trizma base, BSA, protease inhibitor cocktail, NBT/BCIP, triton X-100, tween 20, propidium iodide, RNase A, JC-1 dye (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbo-cyanine iodide), primary antibodies, viz., β Actin, Cytochrome C, APAF-1, BCL-2 Bax and secondary antibodies viz., anti-mouse, anti-sheep, anti-rabbit were procured from Sigmaaldrich, USA. Primary antibodies Caspase-3 and p53 were purchased from Imgenex and anti-Cyclin D1 was purchased from MerckMillipore. FBS was procured from Life Technologies, agarose from Lonza, M-MuLV reverse transcriptase and dNTPs from Merck GeNei, DNase I and *Taq* DNA polymerase from New England Biolabs.

2.2. Cell culture

Human cervical cancer cell line (HeLa) was obtained from NCCS, Pune, India. Cells were maintained in Dulbecco's modified Eagle's medium with high glucose (DMEM) supplemented with 10% FBS, incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Estimation of LD_{50}

Effect of racemic abyssinones I and II on cytotoxicity of human cervical cancer cells was analyzed using 3-(4, 5-dimethylthiazol-2yl-)-2, 5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells were seeded into a 96-well plate at a density of 1×10^4 cells per well and incubated at 37 °C for 24 h. Fresh media containing different concentrations of the compound dissolved in DMSO were added and incubated for 24 h. The vehicle controls received equivalent DMSO. 20 μl of MTT (5 mg/mL) was then added to each well and the cells were incubated for 2 h. Formazan crystals were dissolved by adding 100μL of DMSO. The absorbance was measured at 570 nm in an ELISA plate reader (Spectramax, Molecular Devices, USA) and LD_{50} was determined. All assays were repeated more than 9 times and statistical analysis was performed using Sigmastat software.

2.4. Protein extraction and western blotting

1.5×10^6 cells were seeded into a 25 cm^2 culture flask and incubated overnight. Subsequently, media containing different compounds were added to the cells and incubated further for 24 h, washed with ice-cold PBS. For whole cell lysate, cells were lysed and solubilized in RIPA buffer containing protease inhibitor cocktail. To obtain separate cytosolic and nuclear fraction, the cells were harvested using a cell scraper in ice-cold PBS and centrifuged at 1000 rpm for 2 min at 4 °C. The cell pellet was resuspended in chilled hypotonic buffer solution (20 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1X protease inhibitor cocktail) and incubated for 15 min at 4 °C. 25 μl of 10% NP40 was added, vortexed for 10 s and centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was used as cytoplasmic fraction. The pellet was re-suspended in 50 μl of cell extraction buffer (100 mM Tris–Cl, pH 7.4, 2 mM Sodium orthovanadate, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% Glycerol, 0.1% SDS, 1 mM Sodium fluoride, 0.5% Deoxycholate. 20 mM Tetrasodium diphosphate, 1X protease inhibitor cocktail) for 30 min at 4 °C with vortexing at 10 min

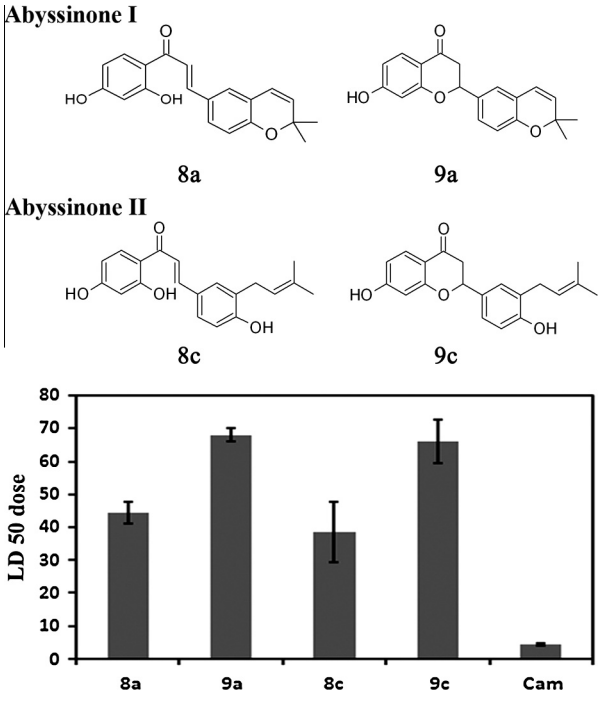

Fig. 1. Effect of abyssinone on the growth of HeLa cells. Cytotoxic activity was performed with increasing concentration of abyssinones (0–100 μM) for 24 h. Cell viability was measured using MTT assay. The experiment was independently repeated for more than nine times. Values shown are mean ± SD of LD_{50} dose of 8a, 9a, 8c and 9c along with standard camptothecin. A significant difference between treated and controls was $p < 0.001$ or $p < 0.05$ for chalcones and flavanones.

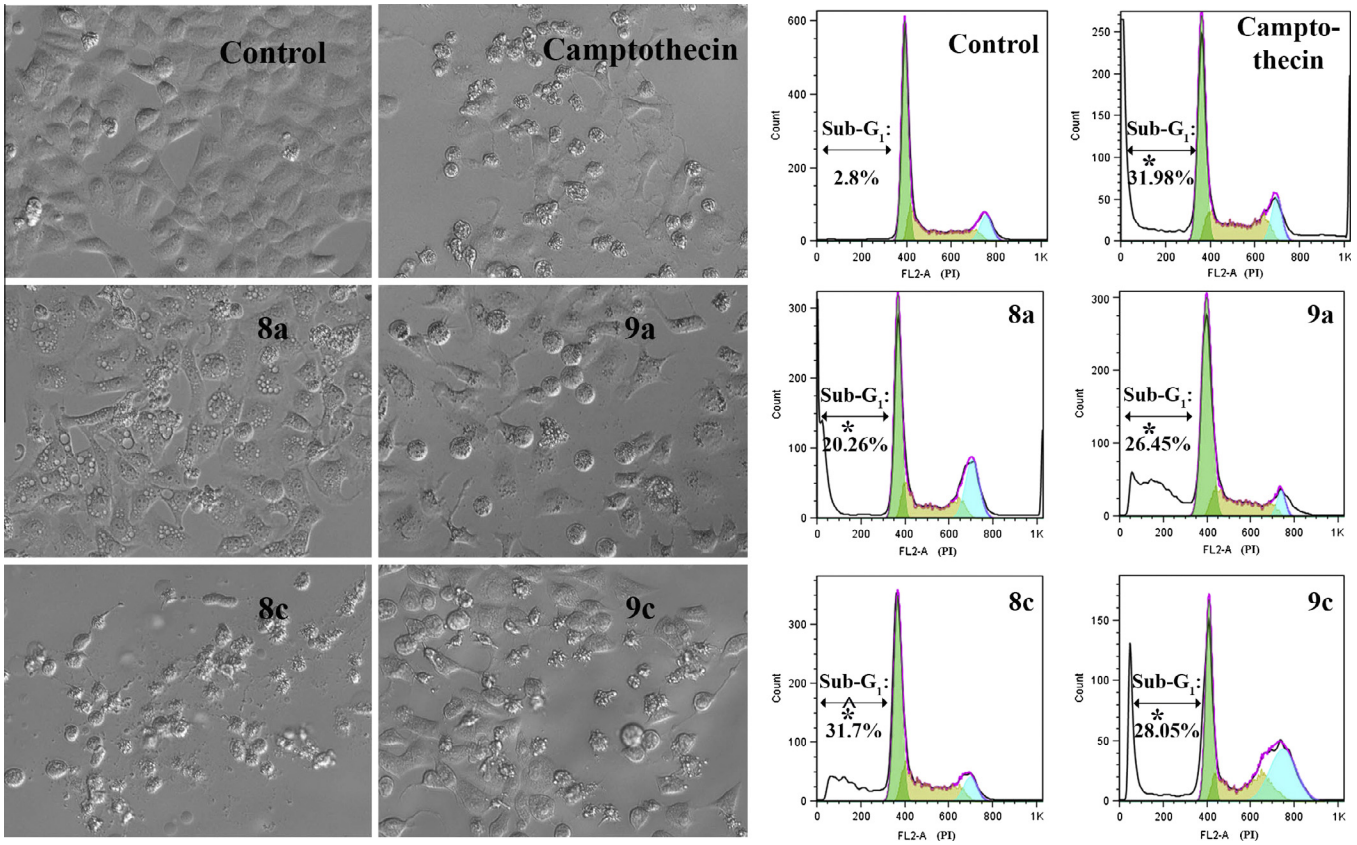

Fig. 2. Flow cytometric analysis of HeLa cells after abyssinone treatment. Cells were treated with half maximum dose (LD_{50}) of chalcone and flavanone form of abyssinone. Representative phase contrast images show apoptotic bodies after 24 h of treatment. HeLa cells were harvested and stained with propidium iodide to perform flow cytometry using 5×10^5 cells to observe the cell distribution. The experiment was independently repeated for three times and percentage of cells in sub G1 was compared with control ($p < 0.001$).

intervals, further centrifuged for 30 min at 14,000 rpm at 4 °C. The supernatant was taken as nuclear fraction. Total protein was quantified by Bradford assay. Equal amounts of soluble protein were resolved on SDS–PAGE (8% or 12%) and electrophoretically transferred to PVDF membrane. The membrane was probed overnight with primary antibody of interest. After washing with TBST, the membranes were incubated in corresponding alkaline phosphatase conjugated secondary antibody for 4 h, washed and the protein bands were visualized using NBT/BCIP substrate.

Normalization was performed using the amount of actin as reference protein in each condition. Protein levels of p53, Bcl-2, Bax, cytochrome c, caspase-3, cyclin D1 and Apaf-1 were quantified in treated samples and compared against that of control using UVIBandMap software. The experiments were carried out thrice and statistical analysis was performed using sigmastat software.

2.5. Flow cytometry analysis

HeLa cells were seeded into 25 cm^2 culture flasks at a density of 1.5×10^6 and incubated for 24 h, later treated with different concentrations of abyssinones for 24 h. The cells were harvested by trypsinization and washed twice with PBS. For cell cycle analysis, cells were fixed gently in ice-cold 70% ethanol and incubated at 4 °C for overnight and then re-suspended in PBS with 0.1 mg/ml RNase A and incubated at 37 °C for 30 min. The cell cycle phase and presence of apoptotic nuclei was determined by propidium iodide (PI) staining. Flow cytometry analyses were performed using a FACSCalibur instrument (Becton Dickinson, USA) and data was analyzed using FlowJo software.

HeLa cells were seeded into 25 cm^2 culture flask at a density of 1×10^6 and treated with different concentrations of abyssinones for 24 h. The cells were harvested by trypsinization and cell pellet was washed twice with PBS. To determine mitochondrial potential, the cells were re-suspended in PBS and incubated with pre-warmed JC-1 dye at 37 °C for 10 min and subjected to flow cytometry in Guava EasyCyte HT (Merck Millipore). The percentage of cells fluorescing red or green were determined using GuavaSoft1.1.

2.6. Mitochondrial staining

HeLa cells were cultured on coverslips and treated with different abyssinones for 12 h. Post treatment they were washed with PBS, stained with pre-warmed JC-1 dye in dark at 37 °C for 15 min. The coverslips were then washed with PBS, mounted on slides using Vectashield mounting medium and observed under a fluorescence microscope.

2.7. RNA extraction and reverse transcriptase-PCR analysis

1.5×10^6 HeLa cells were seeded into a 25 cm^2 culture flask. After overnight incubation, cells were treated with different derivatives of abyssinones for 24 h. Total RNA was extracted from the cells using TRIzol reagent as suggested by the manufacturer. The obtained RNA was treated with DNase I to eliminate DNA contamination. The purity and integrity of the RNA were checked spectroscopically and on denaturing agarose gel electrophoresis before carrying out the analytical procedures. Using oligo (dT) 5 μg of RNA

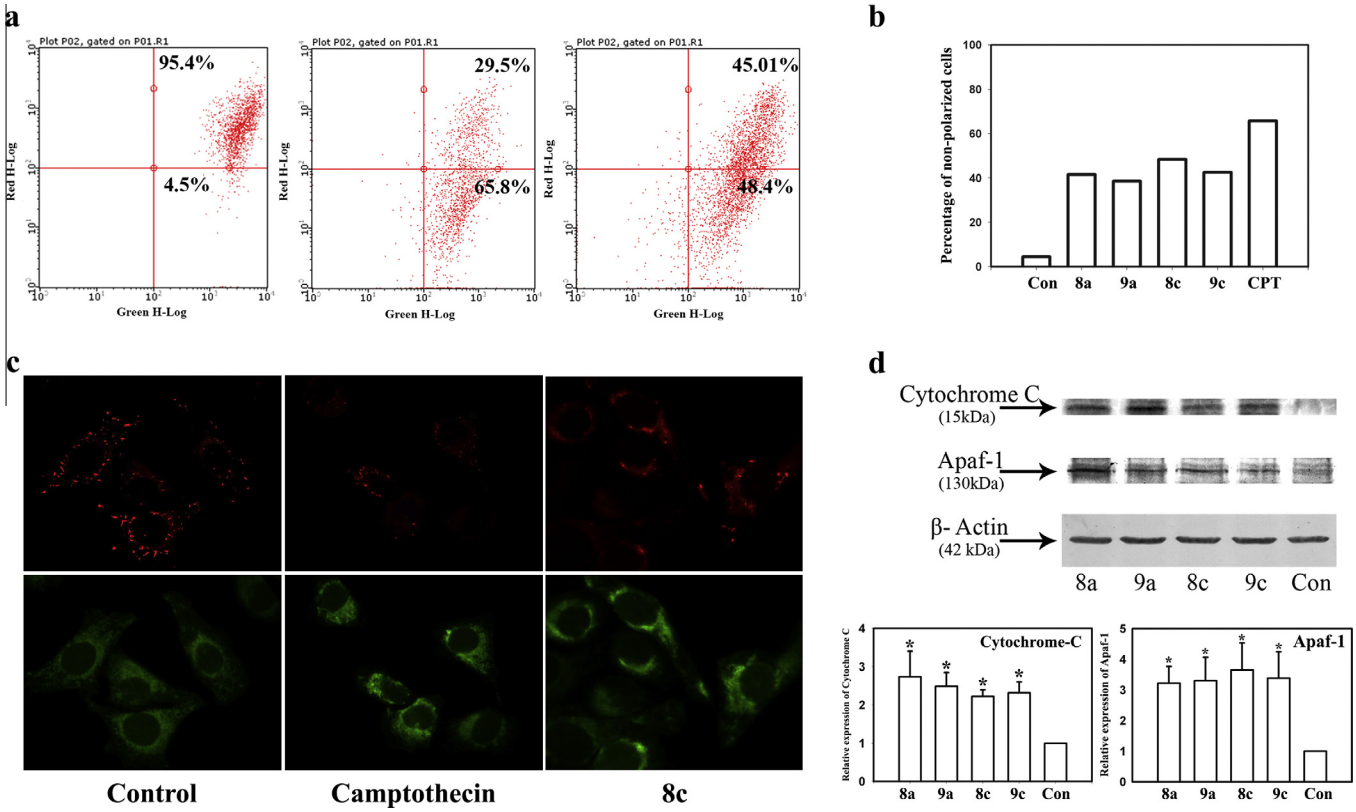

Fig. 3. Release of cytochrome c and Apaf-1 to cytosol was induced by abyssinone. (a) HeLa cells were treated with chalcones (8a and 9a) and flavanones (8c and 9c) for 24 h. Cells were then harvested and changes in mitochondrial membrane potential were detected using JC-1 in Guava EasyCyte system, (b) Percentage of non-polarized cells in different treatment groups is represented in the graph. (c) HeLa cells grown on coverslips were treated with flavonoids and camptothecin followed by staining with JC-1 dye. Cells showing differential mitochondrial membrane potential were captured under fluorescence microscope. (d) The cytosolic fraction of cell lysate was subjected to western blotting to observe the released cytochrome c and Apaf-1 as seen in the treated samples. Values are mean ± SD of three independent experiments where (*) represent significant difference by $p < 0.001$.

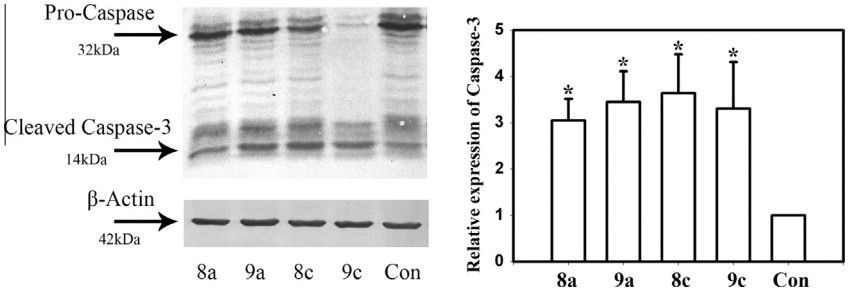

Fig. 4. Effect of abyssinones on caspase-3 in HeLa cells by western blot analysis. Cytosolic fraction prepared from HeLa cells treated with abyssinones for 24 h were subjected to western blot analysis. Blots were probed with antibody specific to Procaspase-3 and cleaved caspase-3. A representative blot is given from triplicate experiments. Statistical analysis of expression level of cleaved caspase indicated significant difference between treated and control by $p < 0.001$.

was reverse transcribed into cDNA using Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase (Merck-GeNei, India).

The cDNA for PCNA, cyclinD1 and GAPDH were amplified by PCR with specific primers: PCNA forward primer 5′-TGTAAACCTGCAGAGCATGG-3′ and reverse primer 5′-TCACTCCGTCTTTTGCACAG-3′; cyclin D1 forward primer 5′-CCCTCGGTGTCCTACTTCAA-3′ and reverse primer 5′-TGGCATTTTTGGAGAGGAAGT-3′; GAPDH forward primer 5′-CCACCCATGGCAAATTCCATGGCA-3′ and reverse primer 5′-CTCAGACGGCAGGTCAGGTCCACC-3′. The PCR products were electrophoresed on 1.5% agarose gel along with 100 bp DNA molecular weight markers and stained with ethidium bromide. The gel image was captured using the UVI Pro Platinum system and densitometric analysis was performed using GAPDH as internal control in UViBandMap software. The optical density was expressed as relative expression of the mRNA considering control as 100% and those of the treated samples were transformed to percentages of the control. Data represented are repetition of three sets analyzed using sigmastat software.

3. Results

3.1. Abyssinones reduces the viability of HeLa cells

The effect of abyssinones on viability of HeLa cells were studied by MTT assay at various concentrations of abyssinones in five different experiments. LD_{50} was calculated by plotting graph using sigma plot software. The data represented in Fig. 1 is mean and

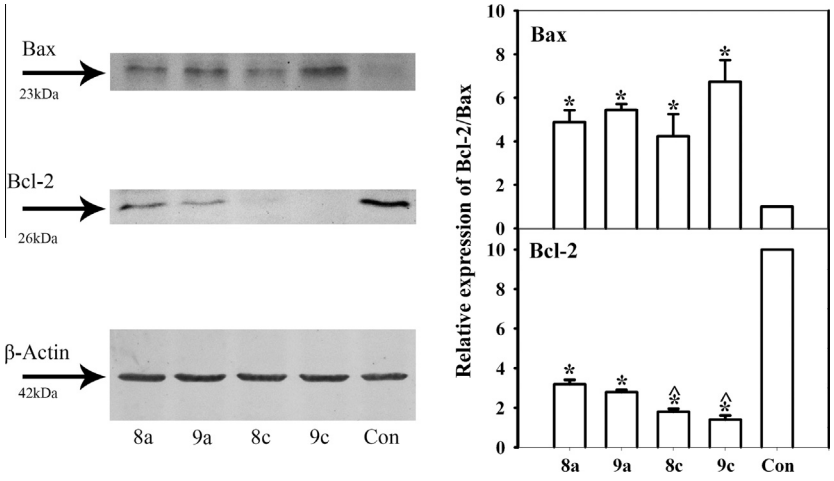

Fig. 5. Abyssinonone induced mitochondrial potential is regulated by Bcl-2 proteins. Cytosolic fraction was extracted from HeLa cells treated with chalcone and flavanone form of abyssinone for 24 h. Bcl-2 and Bax protein expression was measured by western blot analysis. Densitometric analysis was performed to quantify levels of Bcl-2 and Bax expression to evaluate the effect of abyssinone on Bcl-2/Bax ratio. Values represented are mean ± SD obtained from three independent experiments. A significant difference indicated between treated and control groups $p < 0.001$ (*). Abyssinone II significantly down regulated Bcl-2 compared to abyssinone I $p < 0.05$ (^).

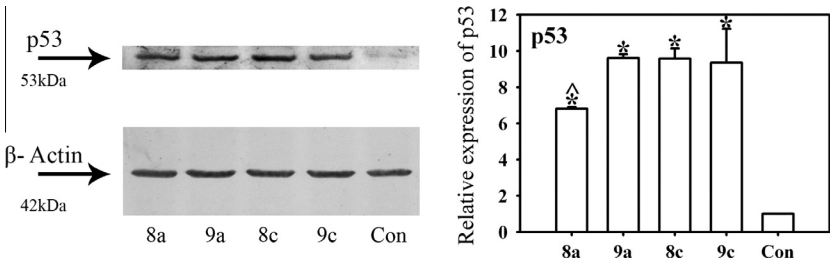

Fig. 6. Abyssione induced p53 expression in HeLa cell lines. Hela cells were treated with LD_{50} dose of chalcone and flavanone form of abyssinones for 24 h. Cell lysate prepared from above cells were subjected to western blot analysis for p53. There was significant increase in p53 level compared to control as represented by histogram of three independent experiments $p < 0.001$. Among abyssinones, '8a' induced significantly less p53 expression compared to others $p < 0.05$.

SD for LD_{50} of chalcone (denoted as No. 8) and flavanone (denoted as 9) of corresponding abyssinone I and II. Chalcone which is the precursor of abyssinone I (8c) was most effective compared to other compounds. The above sets of compounds were further evaluated for apoptotic and antiproliferative activity.

3.2. Abyssinones induced apoptosis by altering cell cycle

To determine whether abyssinone-induced cytotoxicity involves alterations in cell cycle progression, the DNA content was analyzed by flow cytometry. There was significant increase in percentage of cells in the sub-G1 phase of cell cycle in treated samples by 2–3 folds; '8c' being highest as compared to control and its flavanone '9c' followed by '9a' (Fig. 2). The induction of apoptosis by abyssinones was substantiated by microscopic visualization. The cells showed typical characteristic of apoptosis such as shrunken nucleus, apoptotic bodies and peripheral clumping (Fig. 2).

3.3. Loss of mitochondrial potential results in release of cytochrome c

Cells harvested after treating with abyssinones and stained in JC-1 showed lowered mitochondrial potential. Untreated cells exhibited red fluorescence due to JC-1 aggregates which changed to green upon treatment due to monomeric form of JC-1. Compound '8c' exhibited highest percentage of cells (48%) with green fluorescence compared to other flavanoids. Exposure to abyssinone for 24 h in HeLa cells caused disruption of mitochondrial transmembrane potential resulting in release of cytochrome c. The released cytochrome c along with dATP binds to Apaf-1 to form an oligomeric apoptosome (Fig. 3). Further this apoptosome mediates cytochrome c-dependent autocatalytic activation of procaspase-9 leading to the activation of caspase-3 and apoptosis. Western blot analysis performed for both cytochrome c and Apaf-1 from cytosolic fraction prepared from HeLa cells treated with abyssinone for 24 h shows increased expression indicating caspase-3 mediated apoptosis (Fig. 4).

3.4. Altered Bcl-2 to Bax ratio in abyssinone treated HeLa cells

Release of cytochrome c is tightly regulated by members of Bcl-2 family. To study whether abyssinone induced apoptosis as a result of altered expression of pro or anti-apoptotic proteins, Bax and Bcl-2 respectively; we performed western blot analyses for Bax and Bcl-2 (Fig. 5). There was significant decrease in Bcl-2 protein expression, concomitantly, the level of Bax proteins increased significantly. Bax/Bcl-2 ratio increased 2-fold when cells were treated at LD_{50} dosage of abyssinone for 24 h, favoring apoptosis (Fig. 5). However within Abyssinone I and II the chalcone and flavanone form of I potentially down regulated Bcl-2.

3.5. Abyssinones activate p53 leading to loss of cell viability

It was observed in earlier result from FACS analysis that treatment of abyssinone induces DNA damage. We further showed that it leads to activation of p53. In order to verify the role of p53, HeLa

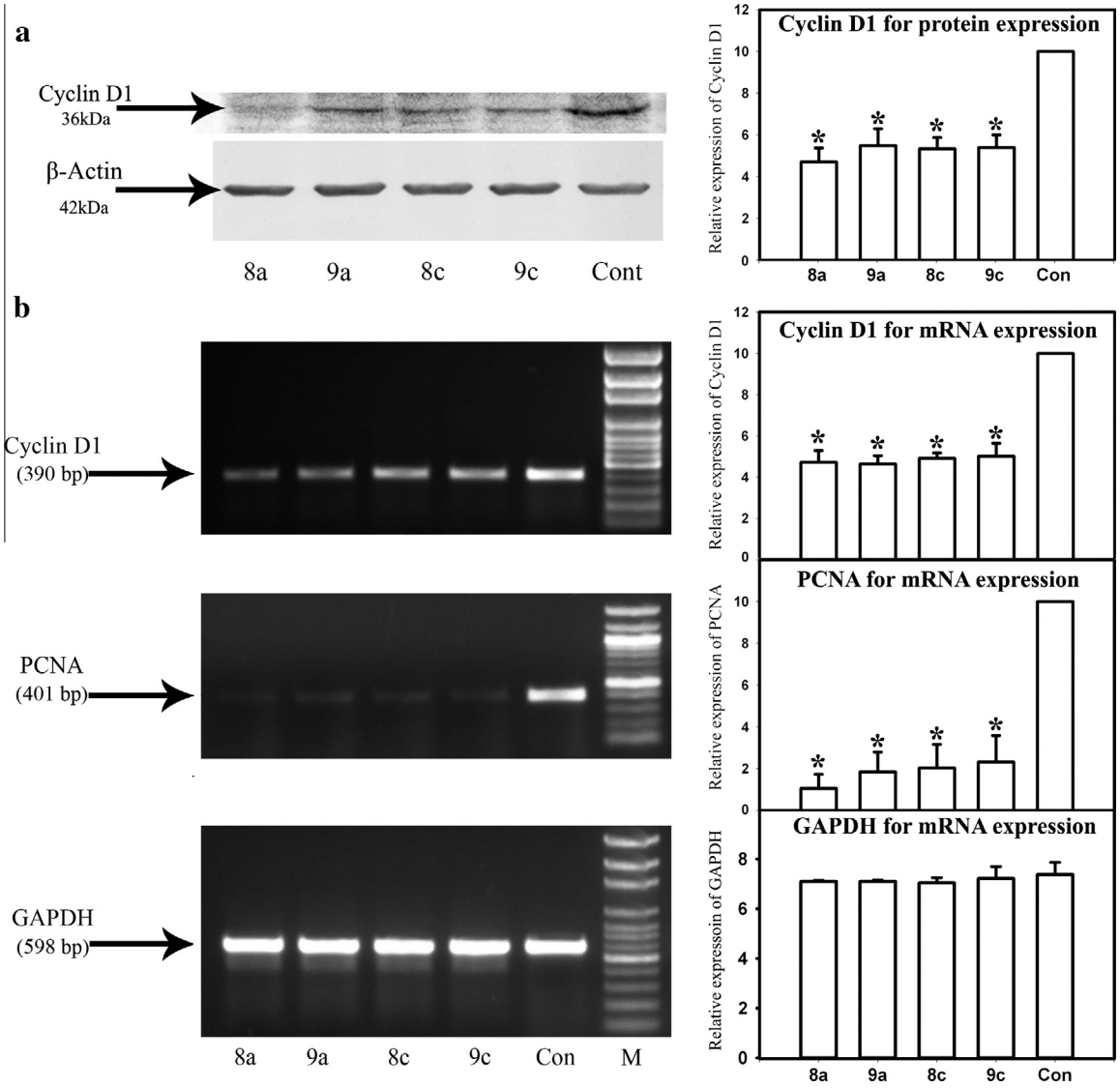

Fig. 7. Effect of abyssinone on proliferation of HeLa cells. (a) Western blot was done using cell lysate after treating cells with abyssinone for 24 h and probed with Cyclin D1 antibody. Densitometric analysis was done to show the significant difference in the expression compared to β-Actin as control. Data shown are mean ± SD of three individual experiments $p < 0.001$. (b) RNA was isolated from HeLa cells treated with abyssinone at LD_{50}. It was reverse transcribed to cDNA and sqRT-PCR analysis was performed using primers specific to PCNA, cyclin D1 and internal control gene GAPDH.

cells were treated with abyssinones for 24 h and lysate was subjected to western blot analysis (Fig. 6). There was increase in p53 expression after treating HeLa with abyssinone I and II indicating its association with mitochondria mediated apoptosis leading to DNA damage. '8a' was less effective in up-regulating p53 expression compared to others, indicating differential response among abyssinones.

3.6. Decreased expression of cell proliferation markers

Cell multiplication is controlled by the balance between proliferation and programmed cell death. In order to understand the link between apoptotic related proteins and proliferation markers, we examined the expression levels of PCNA and Cyclin D1. We observed that abyssinone not only induced apoptotic markers, but also reduced expression of cell proliferative markers like PCNA and cyclin D1 in HeLa cells after 24 h of treatment (Fig. 7). This was further substantiated by decrease in cyclin D1 protein expression (Fig. 7) and increase in sub-G1 phase in cell cycle (Fig. 2).

4. Discussion

Chemopreventive approach in cancer treatment has gained lot of interest to reduce increasing incidence of cancer and as line of treatment along with radiation therapy. Flavonoids are non toxic apoptotic agents and acts as cell growth inhibitors in many cancer cell lines. Many flavonoids perturb cell cycle, G1 or G2/M cell cycle arrest and induce apoptosis. Dietary flavanoid like quercetin and chalconoid, isoliquiritigenin depolarizes mitochondrial membrane potential releasing cytochrome c to cytosol leading to upregulation of p53 and caspase activity [23,24]. Abyssinones exhibit diverse biological activity and the most important one is aromatase inhibition. In our earlier study we have reported cytotoxic activity of synthetic abyssinones as racemic mixture [21]. Studies on breast cancer cell line indicated that prenylated chalcone and flavanones were having better inhibitory activity compared to their non-prenylated counterparts [25]. In present study we have chosen 9a and 9c along with their chalcone precursors 8a and 8c to demonstrate the molecular mechanisms initiating apoptosis.

Abyssinone II has been reported to show good anti tubercular and anti bacterial activity; mechanistic studies have shown that it hyperpolarizes the bacterial membrane potential and inhibits important cellular molecules of biosynthesis [26].

Mitochondria are known to play central role in apoptosis wherein both extrinsic and intrinsic pathways in apoptosis converge to trigger cell death [27]. It is also believed that any imbalance in expression levels of Bcl-2 and Bax proteins will disrupt the outer membrane of mitochondria. Genistein is a naturally occurring isoflavanoid having anticancer effect in multiple cell lines. It sensitizes HeLa cells inhibiting cell proliferation and activates different caspases, increasing Bax expression suggesting apoptosis is mediated through both extrinsic and intrinsic pathways [28]. Similar studies have been done in many naturally occurring and synthetic isoflavanoids. We observed that abyssinones up-regulated apoptotic protein Bax and down regulated anti apoptotic protein Bcl-2. There was decrease in Bcl-2/Bax ratio indicating abyssinone might modulate mitochondrial permeability releasing cytochrome c from mitochondria [29]. Our results indicated that abyssinones are able to induce changes in mitochondrial membrane potential releasing cytochrome c and Apaf-1 (Fig. 3). Apaf-1 is a protein present in inter membrane space of mitochondria released into cytoplasm. Since Bcl-2/Bax ratio is a key factor in regulating the mitochondrial membrane potential, abyssinones induced apoptosis in HeLa cells by altering its ratio releasing cytochrome c and apaf-1 from mitochondria. Further we observed cleavage of procaspase-3 to activated caspase-3; the culminating marker of apoptosis in both pathways [30,31]. Chalcone '8c' showed maximum cell death (29.3%) followed by another chalcone '8a' (27%) after treatment with 30 and 40 μM for 24 h. However flavanones '9c' and '9a' were able to induce cell death at 60–70 μM after 24 h of treatment in HeLa cells (Fig. 2). Further, gene expression studies also demonstrated that chalcones were more effective than flavonone in inducing apoptosis as shown by western blots of apoptotic markers. Thus, prenylated chalcones were more effective in inducing apoptosis indicating potential anticancer agent for human cervical cancer therapy.

p53 plays important role in regulating the cellular signaling pathways involved in cell differentiation. It induces expression of apoptotic markers that indicates activation of both death receptor and mitochondrial apoptotic pathways. During stress p53 is phosphorylated and translocated to nucleus for target gene expression [32]. Many studies have been reported to determine molecular mechanism of flavanoids induced cell death. Quercetin suppressed viability in HeLa cells inducing changes in cell cycle and mitochondrial apoptosis through p53 dependent mechanism [33]. Liquiritigenin a flavanone from Glycyrrhizae induced apoptosis via mitochondrial pathway up-regulating p53, releasing cytochrome c and elevating caspase-3 activity in HeLa cells [32]. Abyssinones were able to induce p53 expression indicating its influence in regulation of cell cycle and pro-apoptotic genes such as BAX [34]. Further there was reduction in cell cycle proliferating markers such as cyclinD1 and PCNA in HeLa cells contributing towards induction of p53 after 24 h treatment. High expression level of PCNA in proliferating malignant tumor cells used as positive biomarker in cancer [35,36]. Cleaved caspase-3 is the activated form of procaspase-3 that act as a lethal protease in the ultimate step of apoptotic pathway [37]. In present study due to changes in mitochondrial membrane caused by abyssinone increase in p53 and Bax expression resulted in cleavage of caspase-3 activity indicating culmination of apoptosis.

These results demonstrated that abyssinones exhibit anticancer effect on HeLa cells probably mediated through change in mitochondrial potential releasing cytochrome c and Apaf-1. The disruption of mitochondrial membrane potential may also lead to increased Bax and decreased Bcl-2 expression. Further, induction of p53 by abyssinone might also lead to increased Bax expression thereby affecting Bcl-2/Bax ratio and ultimately inducing apoptosis through caspase-3. It inhibited growth of HeLa cells as indicated by down regulation of cell cycle markers cyclinD1 and PCNA. The observations from present study show the importance of prenylated flavonoids in apoptotic mechanism of HeLa cells and suggest that abyssinones can be potentially promising chemo preventive agent against cervical cancer. In conclusion we demonstrated that racemates abyssinones may have cytotoxic effects at higher dosage hence there is less scope for chemotherapeutic potential. However, our study indicates that it can act as chemopreventive agent as it is a potential anti-proliferative and anti apoptotic agent.

Acknowledgments

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