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ORIGINAL ARTICLE

Sugiol (12-hydroxyabieta-8,11,13-trien-7-one) targets human pancreatic carcinoma cells (Mia-PaCa2) by inducing apoptosis, G2/M cell cycle arrest, ROS production and inhibition of cancer cell migration

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Summary

Purpose: *Plants produce a diversity of molecular scaffolds with tremendous pharmacological potential. In the present study we evaluated the anticancer activity of the plant-derived natural product sugiol. We also evaluated its effects on apoptosis-related key proteins, cell cycle phase distribution, reactive oxygen species (ROS) and mitochondrial membrane potential (MMP).*

Methods: *Cell viability was evaluated by MTT assay while clonogenic assay was done to determine the effects of sugiol on the cancer cell colony formation. Flow cytometric measurements were carried out in order to assess the effects of sugiol on cell cycle progression, apoptosis, MMP and ROS generation.*

Results: *Sugiol reduced the cell viability of Mia-PaCa2 human pancreatic cancer cells in a concentration-dependent manner. The IC_{50} of sugiol on the cell line was 15 μM. The anticancer activity of sugiol was found to be ROS-mediated alterations in MMP, ultimately favoring apoptosis as determined by the annexin V/propidium iodide (PI). Additionally, sugiol caused cell cycle arrest in G2/M phase of the cell cycle and upregulated the expression of Bax, with concomitant downregulation of Bcl-2 expression in comparison to the untreated cells. It also inhibited the migratory capacity of Mia-PaCa2 cells at the IC_{50} concentration.*

Conclusion: *In conclusion our results indicate that sugiol is a potent anticancer molecule and may prove essential in pancreatic cancer therapy.*

Key words: *apoptosis, cell migration, flow cytometry, pancreatic cancer, sugiol*

Introduction

Plants produce a tremendous diversity of compounds categorized as primary and secondary metabolites [1]. The plant secondary metabolites have great importance in pharmacology and always fascinated scientists to venture into their biological activities. Among plant secondary metabolites, terpenes form one of the largest and diverse classes [1]. Several studies have reported that monoterpenes prevent the proliferation of different cancers [2] and have been shown to possess anticancer activities against many human cancers like breast, liver, skin, lung, colon, prostate, and pancreatic carcinomas [3]. Due to their diverse molecular scaffolds they exhibit the capacity to interact with a number of cellular enzymes and other molecular entities explaining thus their activities [4].

One of the important and rare diterpene is sugiol which has recently been reported to exhibit

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great pharmacological potentials. Several biological activities have been reported for this molecule [5] which include antiinflammatory, antioxidant, antimicrobial and anticancer properties [6,7]. However, the anticancer activity of sugiol has not been studied thoroughly. The present study therefore ventured to evaluate the anticancer activity of sugiol against Mia-PaCa2 human pancreatic cancer cell line. Pancreatic cancer is considered as one of the leading causes of death across the globe ranking fourth in United States alone [6]. The treatment regimens for pancreatic cancer primarily depend on the disease stage. Moreover, the symptoms of pancreatic cancer generally appear at advanced disease stages [7]. Therefore, pancreatic cancer is generally detected at an advanced stage when it has already spread to other organs. Owing to these factors, only 15–20% of patients undergo surgery and the outcomes are very poor with 5-year survival rate up to only 5% [8]. The upsurge of drug resistance, limited treatment options and the associated side effects with the existing drugs has driven extensive researches for the development of substitute options for the treatment of pancreatic cancer. The present study was designed to investigate the anticancer effects of sugiol on human pancreatic carcinoma cells (Mia-PaCa 2) and examine its effects on the migration of these cells.

Methods

Reagents, chemicals and cell culture

Sugiol, RNase A triton X-100, and dimethylsulfoxide (DMSO) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). The fluorescent probes DCFH-DA, DiOC6, 4'-6-diamidino-2-phenylindole (DAPI), fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine and antibiotics were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). The human pancreatic cancer cell lines Mia-PaCa2 was procured from Cancer Research Institute of Beijing, China, and it was maintained in RPMI-1640 medium (Gibco-Invitrogen) supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/mL), streptomycin (100 mg/mL), and 10 % FBS.

MTT cell viability assay

The cytotoxic effect of sugiol against the pancreatic cancer cell line Mia-PaCa2 was determined using MTT assay as previously described [8] and was expressed as IC_{50}. Cancer Mia-PaCa2 cells were placed at a density of 2×10^5 cells/well into 12-well plates and treated with sugiol 7.5-60 µM for 48 hrs. Afterwards, cell viability was estimated as previously reported [8]. Since, sugiol exhibited lower IC_{50} against Mia-PaCa2 cell line, further studies were carried out on this cell line.

Colony formation assay

For clonogenic assay, Mia-PaCa2 cells at the exponential growth phase were harvested and counted with a hemocytometer. Seeding of the cells was done at 200 cells per well and incubation for 48 hrs followed to allow the cells to attach. Then different doses (0, 7.5 15 and 30 µM) of sugiol were added to the cell culture. After treatment, the cells were again kept for incubation for 6 days, washing was done with phosphate buffered saline (PBS) and methanol was used to fix colonies and then stained with crystal violet for about 30 min before being counted under light microscope.

Estimation of cell cycle distribution of Mia-PaCa2 cells

The cells were placed in 12-well plates at a density of $2x10^5$ cells/well and sugiol was added to the cells at 0, 7.5, 30 and 60 µM concentrations, followed by 24-h incubation. DMSO was used as control. For estimation DNA content, PBS was used to wash the cells and fixed in ethanol at -20°C. This was followed by re-suspension in PBS holding 40 µg/ml PI RNase A (0.1 mg/ml) and Triton X-100 (0.1%) for 30 min in the dark at 37°C. Afterwards, analysis was carried out by flow cytometry as previously reported [8].

Reactive oxygen species and mitochondrial membrane potential

Mia-PaCa2 cells were seeded at a density of 2×10^5 cells/well in a 12-well plate, kept for 24 hrs and treated with 0, 20, 40 and 80 µM sugiol for 72 hrs at 37°C in 5% CO_2 and 95% air. Thereafter, the cells treated with different concentrations of sugiol were collected, washed twice with PBS and re-suspended in 500 µl of DCFH-DA (10 µM) for ROS estimation and DiOC6 (1 µmol/l) for MMP estimation at 37°C in the dark for 30 min. The samples were then analyzed instantly using flow cytometry as previously reported [9].

Flow cytometric analysis of cell apoptosis

Human pancreatic cancer Mia-Paca cells at a density of 2×10^5 cells/well were platted in 6-well plates and exposed to 0, 7.5, 15 and 30 µM sugiol for 48 hrs. For estimation of apoptotic cell populations FITC-Annexin V/PI Apoptosis Detection Kit was used following the manufacturer's instructions (Beijing Biosea Biotechnology, China).

Cell migration assay

Cell migration assay was carried out by Boyden chamber assay with some modifications. Cells at the density of 5×10^4 cells/well were suspended in 2% FBS medium and placed in the upper chamber of 8 µm pore size transwells. Afterwards, medium supplemented with 10% FBS was added to the lower chamber. This was followed by 48-h incubation. On the upper surface of the membrane, non-migrated cells were removed, while on the lower surface of the membrane the migrated cells were fixed in methanol (100%) and stained with Giemsa. The cell migration was estimated by counting the number of migrated cells under a microscope.

Western blot analysis

After treatment with various concentrations of sugiol, cells were harvested and lysed in lysis buffer (20 mM HEPES, 350 mM NaCl, 20% glycerol, 1% Nonidet P 40, 1 mM MgCl2, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 1 mM PMSF, protease inhibitor cocktail, and phosphatase inhibitor cocktail). Out of the total protein samples a 20 µg aliquot was separated on 10% SDS-PAGE gel. The gel was then transferred to nitrocellulose membranes, blocked with 5% bovine serum albumin (BSA) and probed with a primary antibody. This was followed by probing with the required secondary antibody. Finally, the signal was perceived with WEST-SAVE Up™ luminal-based ECL reagent (ABFrontier, Korea).

Statistics

All experiments were carried out in triplicate and the values were expressed as mean ± SD. The values were considered significant at p*<0.01, p**<0.01 and ***p<0.0001. The statistical analysis was carried out by Graph pad prism 7.

Results

Sugiol effects on the viability of Mia-PaCa2 pancreatic cancer cells

For evaluation of the antiproliferative activity of sugiol (Figure 1) against Mia-PaCa 2 cancer cells, different concentrations of sugiol were used ranging from 0-60 µM. The results indicated that sugiol exerted its antiproliferative activity in a dose-dependent manner and significantly reduced the viability of Mia-PaCa2 cancer cells (Figure 2). Sugiol was found to exhibit an IC_{50} of 15 µM against the Mia-PaCa2 cancer cells. Moreover, treatment of cells with sugiol caused significant reduction in the colony formation. Again, the effect of sugiol on colony formation was concentration-dependent (Figure 3).

Sugiol caused G2/M cell cycle arrest of Mia-PaCa2 cells

It was observed that the percentage of Mia-PaCa2 cells was considerably increased in the G2 phase of the cell cycle at 0 to 30 µM of sugiol concentrations, causing G2/M cell cycle phase arrest (Figure 4). Additionally, the populations of Mia-PaCa2 cells in G2 phase were significantly increased at a dose of 7.5 µM, however the most evident effects of sugiol on Mia-PaCa2 cell cycle were observed at the highest test concentration of 30 µM. Thus, sugiol-induced G2/M increase of Mia-PaCa2 cancer cells was dose-dependent.

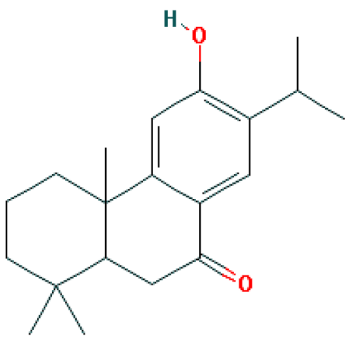

Figure 1. Chemical structure of sugiol.

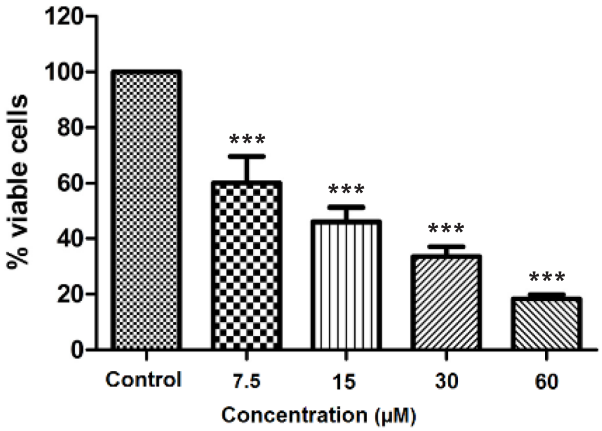

Figure 2. Effect of indicated doses of sugiol on the viability of Mia-PaCa2 cancer cells. All experiments were carried out in triplicate and expressed as mean ± SD. ***p<0.0001.

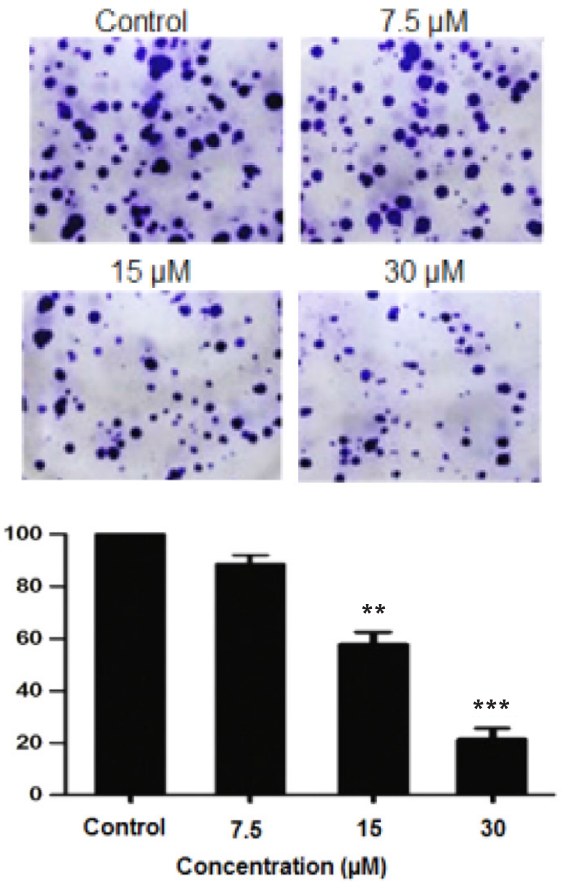

Figure 3. Effect of indicated doses of sugiol on colony forming potential of Mia-PaCa2 cancer cells at indicated doses. All experiments were carried out in triplicate and expressed as mean ± SD. p**<0.01 and ***p<0.0001.

Sugiol augmented the accretion of ROS and reduced the MMP level

Mia-PaCa2 cells were exposed to different concentrations of sugiol and the levels of ROS and MMP were evaluated. A considerable upsurge in intracellular ROS and a significant reduction of MMP levels were observed in the sugiol-treated Mia-PaCa2 cells as compared to the control. Sugiol treatment considerably augmented the ROS levels at 7.5, 15, and 30 μM as compared to the control (Figure 5). Furthermore, sugiol considerably reduced MMP level up to around 38% in 48 hrs at 100 μM concentration in Mia-PaCa2 cells compared to the control (Figure 5).

Sugiol induced apoptosis in Mia-PaCa2 cells

The effect of sugiol on cell cycle arrest was carried out at different concentrations ranging from 7.5 to 30 μM. The apoptotic cell populations increased from 4.2% in the control to 37.6% at 80 μM concentration (Figure 6). To assess whether sugiol triggers apoptosis in Mia-PaCa2 cells via

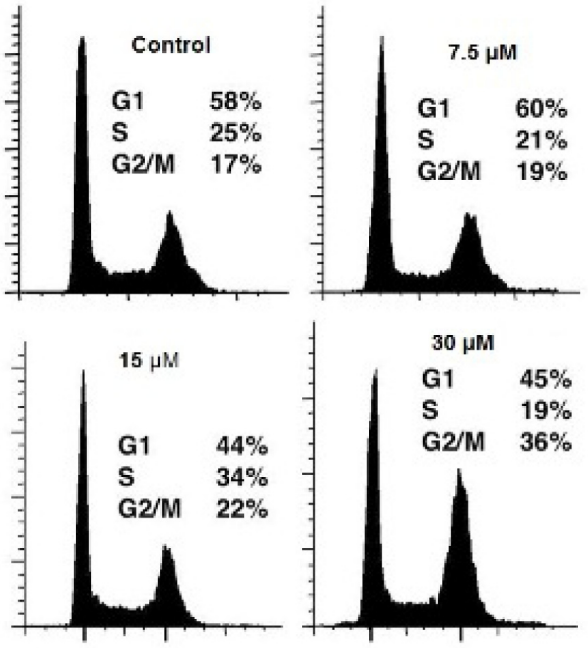

Figure 4. Effect of indicated doses of sugiol on cell cycle arrest as determined by flow cytometry. The Figure shows that sugiol triggered G2/M cell cycle arrest in Mia-PaCa 2 cancer cells in a concentration-dependent manner. All experiments were carried out in triplicate.

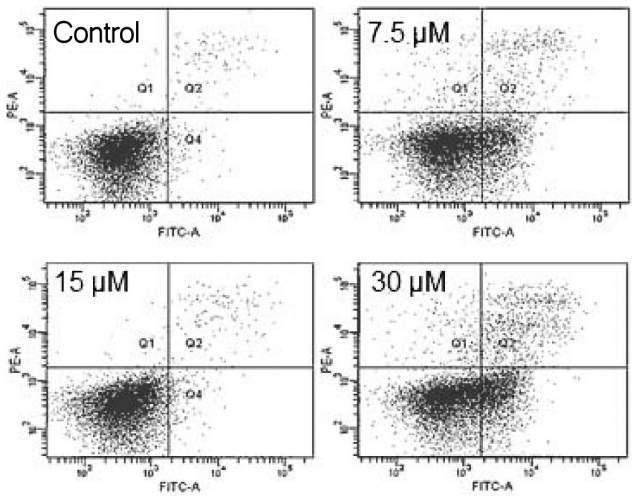

Figure 5. Induction of apoptosis by sugiol at indicated doses as depicted by annexin V/PI flow cytometry. The experiments were carried out in triplicate. The Figure shows that apoptotic populations increased in a concentration-dependent manner.

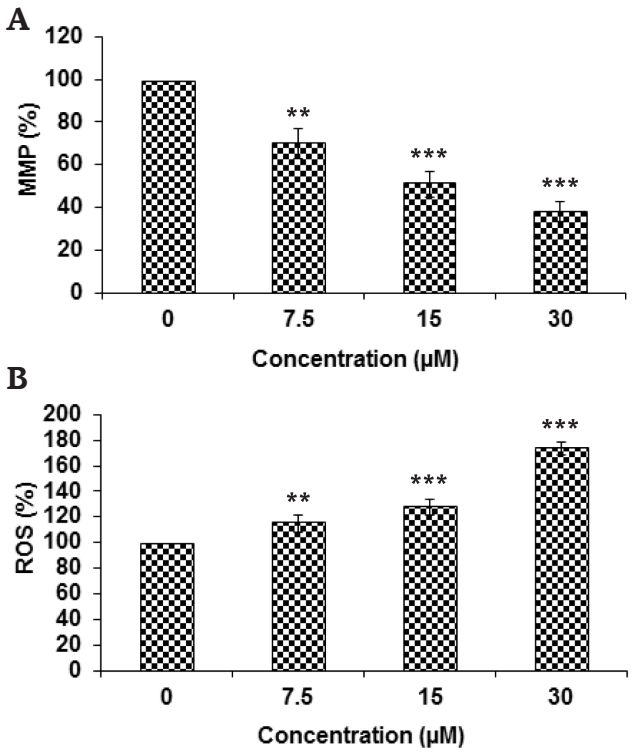

Figure 6. Effect of indicated doses of sugiol on **(A)** ROS generation and **(B)** mitochondrial membrane potential. The Figure shows that MMP decreased and ROS increased in Mia-PaCa2 cells as the concentration of sugiol increased. All experiments were carried out in triplicate and expressed as mean ± SD. p**<0.01 and ***p<0.0001.

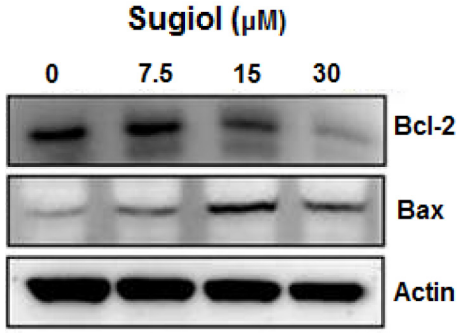

Figure 7. Effect of sugiol on the expression of Bax and Bcl-2 determined by western blot analysis. The Figure shows that the expression of Bcl-2 decreased and that Bax increased with increasing concentrations of sugiol. All experiments were carried out in triplicate.

the mitochondrial pathway, we investigated the expression levels of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 through western blot analysis. The results revealed that that sugiol upregulated the expression of Bax, with concomitant downregulation of Bcl-2 expression in comparison to the untreated cells kept as control (Figure 7). Taken together, the results indicate that mitochondria and Bcl-2 family members are associated with sugiol-triggered cell apoptosis in Mia-PaCa2 cells.

Sugiol inhibited migration of Mia-PaCa2 cells

We next examined if sugiol can inhibit the migration of Mia-PaCa2 cancer cells at the IC_{50} concentration at different time intervals by wound healing assay. The results of the assay showed that sugiol reduced the migratory capacity of Mia-PaCa2 as observed by Boyden chamber assay, while the control cells showed fairly good capacity to migrate, as depicted in Figure 8.

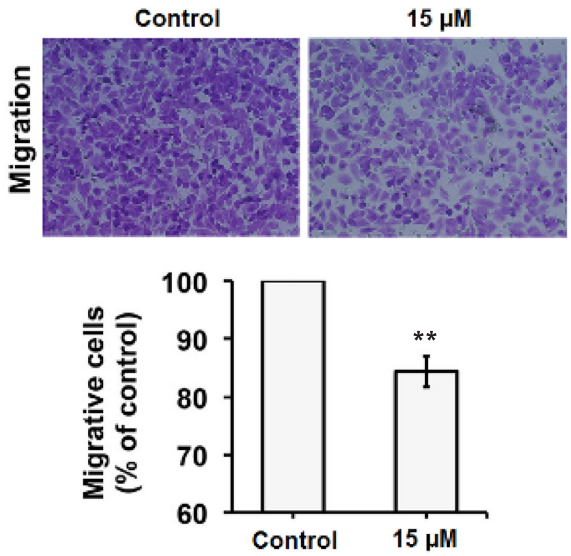

Figure 8. Cell migration inhibitory potential of sugiol at IC_{50} concentration (15 μM). The Figure shows that sugiol at 15μM decreased the migration of Mia-PaCa 2 cells. All experiments were carried out in triplicate and expressed as mean ± SD. p**<0.01.

Discussion

Pancreatic cancer is considered as one of the most disastrous malignant tumors worldwide. The treatment regimens for pancreatic cancer primarily depend on the disease stage. Moreover, the symptoms of pancreatic cancer generally appear at advanced disease stages [6,7]. Therefore, this malignancy is generally detected at an advanced stage when it has already spread to other organs. Owing to these factors, only 15–20% of patients undergo surgery and the outcomes are very poor with 5-year survival rate up to 5% only [8]. The sharp increase in the incidence of pancreatic cancer [10], lack of proper therapy and the severe side effects associated with the existing drugs has made it necessary to search for new and more effective molecules. Since natural products have minimum toxicity, they are being considered as potential anticancer agents. In the current study, sugiol was evaluated against the pancreatic cancer cell line Mia-PaCa2 for its potential anticancer activity and the results indicated that this molecule exhibits significant anticancer activity against these cells. The cytotoxic effect of sugiol was found to be dose-dependent and the IC_{50} of sugiol was 15 μM against Mia-PaCa2 cell line. Moreover, sugiol also reduced the colony forming tendency of these cells, suggesting that it is a potent cytotoxic agent. One of the reasons for apoptosis might be the observed capacity of sugiol to cause cell cycle arrest as it induced G2/M phase increase of Mia-PaCa2 cancer cells in a dose-dependent manner. Cell cycle arrest and apoptosis are known to be essential mechanisms for normal cell growth and proliferation. Apoptotic cell death is triggered when explicit checkpoints are arrested during cell cycle [11]. Consistent with this, several anticancer agents cause cell cycle arrest and have been found to be clinically effective for cancer treatment [12]. In addition, drugs with apoptosis-inducing properties may minimize potential drug resistance. Our results indicated that cells treated with sugiol underwent apoptosis in a dose-dependent manner. Although apoptosis is triggered via different routes, the mitochondrial pathway is the crucial signalling pathway in the induction of apoptosis. It is well established that Bcl-2 family proteins are frequently the main players in the mitochondrial apoptotic pathway and the antiapoptotic and proapoptotic protein members of Bcl-2 protein family control apoptosis by regulating the mitochondrial membrane permeability [13]. While Bcl-2 is a strong antiapoptotic protein, Bax is inducer of apoptosis. Bax is present in the outer membrane of the mitochondria, facilitating the discharge of cytochrome C. In the current study involvement of the mitochondrial apoptotic pathway in sugiol-induced cell death was first detected by the observed reduction in the MMP. This was further strengthened by the changes observed in the Bcl-2 and Bax expression levels, since mitochondrial malfunction is often due to MMP loss and discharge of cytochrome C into the cytoplasm. Protein expression analysis revealed that sugiol caused considerable downregulation of Bcl-2 expression and upregulation of Bax protein, therefore ultimately favoring

apoptosis. Additionally, it was also observed that the sugiol induced intracellular ROS alterations in Mia-PaCa-2 pancreatic cells in a dose-dependent manner. The results suggest that sugiol may trigger apoptosis through ROS-mediated reduction in MMP. Our results are in agreement with previous studies [10,14,15] wherein a number of anticancer agents induce apoptosis in cancer cells by generating high levels of intracellular ROS [14]. Sugiol also inhibited the cell migration of Mia-PaCa2 cells as evidenced from the Boyden chamber assay. Cell migration is the key feature of cancer progression and metastasis [15] and suppression of cell migration may prove essential in suppression of metastasis. This may ensure comparatively longer patient survival. Therefore, the potential of sugiol to suppress migration of Mia-PaCa2 cancer cells indicates that it may prove as an efficient molecule in inhibiting the metastasis of cancer cells [16,17].

Finally, we conclude that sugiol exhibits significant anticancer activity against pancreatic Mia-PaCa2 cancer cell line. The anticancer activity is due to its capacity to induce ROS-mediated alteration in MMP, mitochondrial apoptosis and cell cycle arrest, paving the way for *in vivo* evaluation of this molecule against pancreatic cancer.

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Conflict of interests

The authors declare no conflict of interests.

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