Punicalagin reduces cancer cell proliferation in human HepG2 cellsby modulating the expression of PI3K/Akt signaling molecules

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Background: Hepatocellular carcinoma (HCC), the major primary malignant tumor of the liver, is one of the most life-threatening human cancers in the world, resulting in almost one million deaths every year. Currently available treatment options cause serious adverse effects. Considering the limited treatment options and dismal prognosis for HCC, chemoprevention has been considered the best strategy to reduce its current morbidity and mortality. In this regard, recently naturally occurring polyphenols are receiving increased attention because of their promising efficacy in several cancer models.

Aim: The current study was aimed at assessing the effect of punicalagin on PI3K/Akt signalling Mechanisms in HepG2 cells *in vitro.*

Methods: The Human Liver cancer cells (HepG2) were obtained from National Centre for Cell Science, Pune, India and were grown in culture flasks, separately containing DMEM medium, respectively supplemented with 10% FBS under 5% CO2, 95% air at 37°C. Upon reaching confluence, the cells were trypsinized and passaged. After 80% confluency, HepG2 cells were treated with different concentrations of punicalagin (50, 75, 100 and 150µg/ml) and cell viability was assessed by MTT assay. Antioxidant enzymes such as super oxide dismutase (SOD), Catalase (CAT) were assessed by sandwich-ELISA methods. Gene expression analysis of PI3K, Akt, and cytochrome-C were analyzed by Real Time PCR analysis and the data were analysed using one-way-ANOVA.

Results: Punicalagin treatment to HepG2 cells significantly ($p<0.05$) reduced proliferation of cells at the concentrations of 75, 100 and 150mg/ml. Antioxidant enzymes such as SOD and CAT were found to be improved in drug treated HepG2 cells. It also potentially downregulated mRNA expression of PI3K, Akt and Cytochrome-C in liver cancer cells ($p<0.05$).

Conclusion: Our current study clearly indicates that punicalagin potentially reduced the growth of the HepG2 cells by modulating the expression of PI3K/Akt signalling molecules facilitating antioxidant enzymes. Hence, punicalagin could be considered as one of the naturally occurring polyphenols for the treatment of liver cancer.

Key words: Punicalagin, liver cancer, apoptosis, poly phenols.

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INTRODUCTION

Cancer a major cause for death refers to uncontrolled cell growth due to genetic and epigenetic changes resulting in formation of neoplasms or tumor. An estimate suggests that about 9.6 million deaths were recorded on the basis of cancer in the year 2018, ranking second leading cause for death (Ferlay et al., 2015). Based on the increased incidence rate of cancer it is estimated that by 2020 16 million cases will be reported (Wang et al., 2018) and further increases upto 12.8 percentage by 2025 (Sathishkumar et al., 2020). More than 11 million people are diagnosed with

cancer and this rate has been estimated to increase to 16 million by 2020. Cancer deaths are most commonly related to lung (1.76 million deaths), colorectal (862 000 deaths), stomach (783 000 deaths), liver (782 000 deaths) and breast (627 000 deaths) (Saini et al., 2020).Cause for occurrence of cancer includes, lifestyle changes (like tobacco chewing, alcohol consumption and cigarette smoking); exposure to harmful chemicals, radiations, pollutants,toxins; and biological agents like certain strains of virus (human papillomavirus, Epstein-Barr virus, hepatitis B and C etc). Growth of cancer cells, in general, requires continuous oxygen and blood supply. As the cancer cells grow and proliferate they form a lump or tumor. This tumor grows in size and at a point of time the tumor blocks the blood vessels that supply the necessary nutrients to them. In order for further survival they undergo a process named as angiogenesis
(formation of new blood vessels) thus there is no hindrance in nutrients supply. Therefore as cancer progression takes place the cancer affected epithelial cells undergoes transformation to form mesenchymal cells. This transfer of mesenchymal cells, makes the cancer cells moreinvasive and promote their entry into blood vessels and lymphatic system. Upon entry into the circulation these cancer cells reach different organs in the body and start to multiply in the secondary site. This process of migration to secondary site is known as metastatic stage, referringto spread of cancer to distant regions. Inflammation is one of the major underlying pathogenesis of cancer. Inflammation acts as a double ended sword, they play a positive role in the amelioration of cancer and also a negative role in the progression of cancer. This is due to the lack of reversal of inflammation so that the inflammation persists for a long period of time. Thus chronic inflammation releases number of cytokines, chemokines, adhesion molecules and enzymes (Singh et al., 2019).

Liver is the major metabolic and detoxification center of body. The building blocks of liver are the hepatocytes. Liver is involved in the carrying out critical functions namely maintaining the metabolism of macronutrients (such as carbohydrates, proteins and lipids), maintaining the blood volume, assisting the immune system, regulation of signaling molecules responsible for the growth, detoxification of foreign agents and drugs, homeostasis of cholesterol level (Treftset al., 2017). They play an essential role in various physiological function of the body. Liver cancer is the second leading cause for cancer related death in the developing countries and sixth leading cause of cancer in developed countries (Li&Wang, 2016). The major type of liver cancer is hepatocellular carcinoma, cancer arising from the epithelial cells of the liver (80-85%) and the second type is cholangiocarcinoma, cancer arising from the cholangiocytes (10-12%) (Gelband et al., 2015). The incidence of liver cancer is influenced by a number of risk factors namely, hepatitis C virus (HCV), hepatitis B virus, aflatoxins, alcohol, obesity, fluke worm infection and diabetes (Li&Wang, 2016).

Punica granatum, pomegranate, is a healthy and nutritional fruit with a lot of medicinal properties. It is generally found in Asian, Southwest America, Arizona, Mexico, California and Africa countries (Abdollahzadeh et al.,2011). They are noted for a number of pharmacological and biological properties such as anti-inflammatory, anti-cancer, diabetes management,antioxidant, and wound healing activity (Venusova et al., 2021). These properties are exhibit because of *Punica granatum* rich phytochemicals. Punicalagin is the major tannin compound isolated from the peels of pomegranate. The main components of Punicalagin are ellagic acid (EA), gallic acid dimers, gallagic acid and EA dimers (Oudane et al., 2018). A number of studiesshowed the invitro biological activity of Punicalagin, namely stimulation of apoptosis in promyelocytic leukemia cells, colon cancer cell lines and glioma cells, arresting the cancer cell growth and proliferation, and regulating the inflammatory signaling (Bialonska et al., 2010).

Thus in our study we have hypothesized that punicalagin is a potential tannin component that has a positive role in combating the cell proliferation and cell growth of cancer cell in hepatocellular carcinoma via the PI3K/AKT signaling.

Materials and methods

The Human Liver cancer cells (HEPG2) will be obtained from National Centre for Cell Science, Pune, India and will be grown in culture flasks, separately containing DMEM medium, respectively supplemented with 10% FBS under 5% CO2, 95% air at 37°C. Upon reaching confluence, the cells will be trypsinized and passaged.

Passaging of cells

The medium from the T-75 culture flask was aspirated, rinsed with 5 ml of PBS and aspirated quickly. Trypsin-EDTA solution (2 ml) was added and incubated at room temperature (in the laminar hood) for 1 min. Then the trypsin-EDTA solution was aspirated quickly and the flask was incubated in CO2 incubator for 1-3 min. HEPG2 cells will be passaged by adding 1 ml of FBS containing medium, to the flask and the cells were transferred to 1ml centrifuge tube for centrifugation. Following centrifugation the supernatant were removed, and the pelletted cells were resuspended with fresh growth medium. The cell suspension was then transferred to sterile T-75 flasks and the volume of medium was made-up to 10 ml with growth medium/flask.

Cell viability assay

Cell viability was assayed using a modified colorimetric technique that is based on the ability of live cells to convert MTT, a tetrazolium compound into purple formazan crystals by mitochondrial reductases (Mosmann, 1983). Briefly, the cells (1 ×104/well) were exposed to different concentrations of punicalagin HEPG2 cells for 24 or 48 h. At the end of the treatment, 100 l of 0.5 mg/ml MTT solution was added to each well and incubated at 37 ◦C for an hour. Then the formazan crystals formed was dissolved in dimethyl sulfoxide (100 l) and incubated in dark for an hour. Then the intensity of the color developed was assayed using a Micro ELISA plate reader at 570 nm. The number of viable cells was expressed as percentage of control cells cultured in serum-free medium. Cell viability in control medium without any treatment was represented as 100%. The cell viability is calculated using the formula: % cell viability = [A570 nm of treated cells/A570 nm of control cells]×100.

Assessment of antioxidant enzymes (SOD)

Activity of superoxide dismutase was assessed using RayBioTech Human SOD enzyme-linked immunosorbent assay (ELISA) kit (RayBioTech Life Inc, Georgia, USA). Every assay was carried out twice. The kit used a sandwich ELISA method, in which the sample's SOD binds to a polyclonal antibody that is specific to SOD and is coated on a microplate. SOD that has been attached to the plate is detected by a secondary, biotinylated SOD-specific antibody. At 450 nm, the intensity was measured. The final readings were calculated in pg/ml and a set of standards was loaded alongside. A trained and calibrated professional carried out the procedure

Assessment of antioxidant enzymes Catalase (CAT)

Activity of catalase was assessed using RayBioTech Human Catalase enzyme-linked immunosorbent assay (ELISA) kit (RayBioTech Life Inc, Georgia, USA). Every assay was carried out twice. The kit used a sandwich ELISA method, in which the sample's CAT binds to a polyclonal antibody that is specific to CAT and is coated on a microplate. CAT that has been attached to the plate is detected by a secondary, biotinylated CAT-specific antibody. At 450 nm, the intensity was measured. The final readings were calculated in pg/ml and a set of standards was loaded alongside. A trained and calibrated professional carried out the procedure.

Quantitative RT-PCR

mRNA expression levels of PI3K, AKT and cytochrome c were examined using Real-Time PCR. The total RNA was isolated by using Tri Reagent slandered protocol. Total RNA (2µg) from each sample was reverse transcribed using commercial manufacturer's protocol. Thereverse transcriptase kit was provided by Eurogentec (Seraing, Belgium). The cDNA was createdusing 2 g of total RNA. The list of primer sequences are mentioned in as well as the house keeping gene as given below. The genes were amplified using a real-time PCR system(Stratagene MX 3000P, CA, USA) under the following reaction conditions: 40 cycles of 95°Cfor 30 s, 59-60°C for 30 s, and 72°C for 30 s. Using the melt and amplification curves as a guide,relative quantification was created. The following primers were used in this study.Akt-FW-5'--ATGAGCGACGTGGCTATTGT-3'; RW-5'--GAGGCCGTCAGCCACAGTCT-3'; Cytochromec–FW-5'-AGGGAGGCAAGCACAAGACTG-3', RW-5'-TCCATCAGTGTATCCTCTCCC-3'; GAPDH-FW-5'-GCACCGTCAAGGCTGAGAA-3'; R: 5'-AGGTCCACCACTGACACGTTG-3'.

Results

Cell viability test

Fig 1. represents the effect of punicalagin on cell viability in HepG2 cells. Each bar represents mean±S.E.M of 3 individual observations. a-compared to control; b-compared to punicalagin 100µg treated cells. Significance was considered at the levels of $p<0.05$.

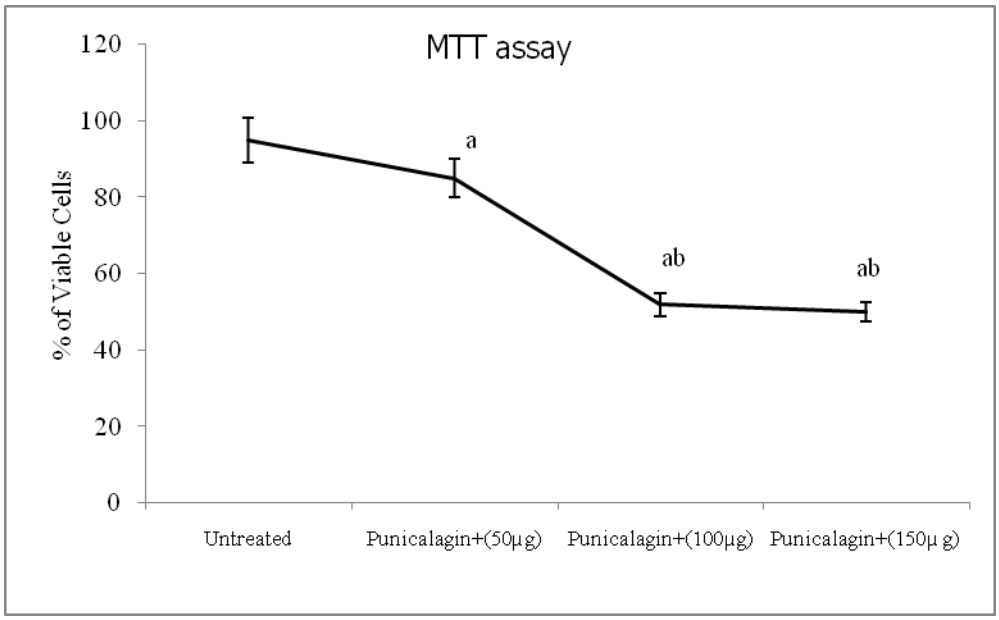

Figure 1. Effect of punicalagin on cell viability in HepG2 cells.

Fig 2. represents the effect of punicalagin on SOD activity in HepG2 cells. Each bar represents mean±S.E.M of 3 individual observations. a-compared to control; b-compared to punicalagin 100µg treated cells. Significance was considered at the levels of $p<0.05$.

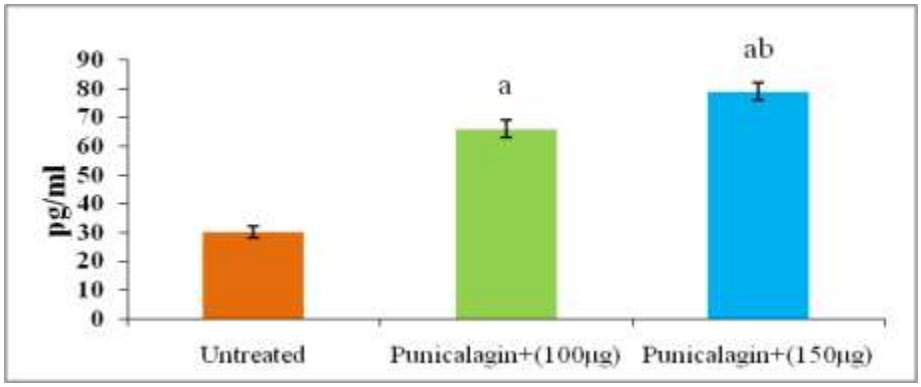

Figure 2 Effect of punicalagin on SOD activity in HepG2 cell

Increase in Superoxide dismutase levels in HepG2 cells by Punicalagin:

Fig 3. Represents the effect of punicalagin on catalase activity in HepG2 cells. Each bar represents mean±S.E.M of 3 individual observations. a-compared to control; b-compared to punicalagin 100µg treated cells. Significance was considered at the levels of $p<0.05$.

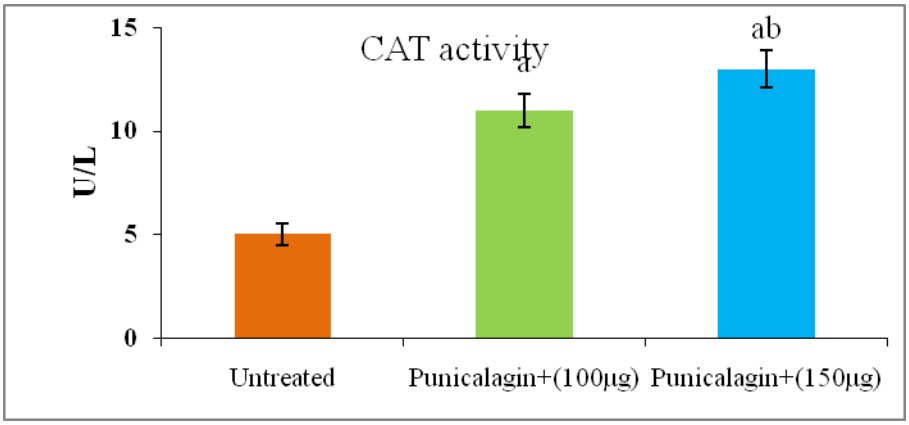

Figure 3 Effect of punicalagin on catalase activity in HepG2 cells

Increase in Catalase activity in HepG2 cells by Punicalagin:

Fig 4 A & B Down regulation of PI3K mRNA expression

Fig 4 A & B. Represents the effect of punicalagin on PI3K mRNA expression in HepG2 cells. Each bar represents mean±S.E.M of 3 individual observations. a-compared to control; b- compared to punicalagin 100µg treated cells. Significance was considered at the levels of $p<0.05$.

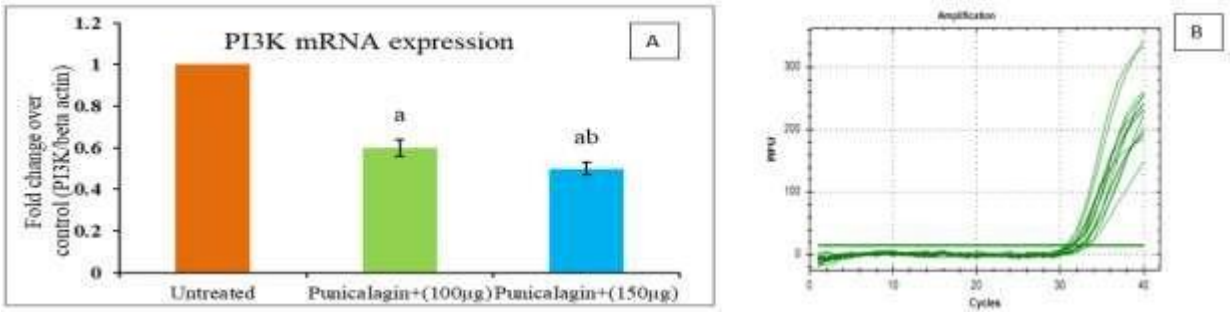

Figure 4 Effect of punicalagin on PI3K mRNA expression in HepG2 cells

Fig 5 A &B. Down regulation of AKT mRNA expression

Fig 5 A &B. Represents the effect of punicalagin on Akt mRNA expression in HepG2 cells. Each bar represents mean±S.E.M of 3 individual observations. a-compared to control; b- compared to punicalagin 100µg treated cells. Significance was considered at the levels of $p<0.05$.

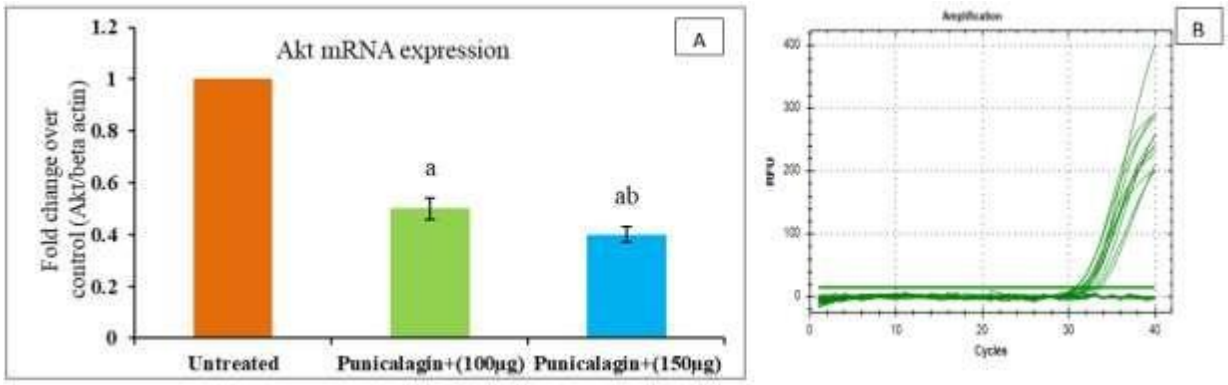

Figure 5 Effect of punicalagin on Akt mRNA expression in HepG2 cells

Fig 6 A&B. Down regulation of Cytochrome c mRNA expression:

Fig 6 A&B. represents the effect of punicalagin on cytochrome-C mRNA expression in HepG2 cells. Each bar represents mean±S.E.M of 3 individual observations. a-compared to control; b-compared to punicalagin 100µg treated cells. Significance was considered at the levels of $p<0.05$.

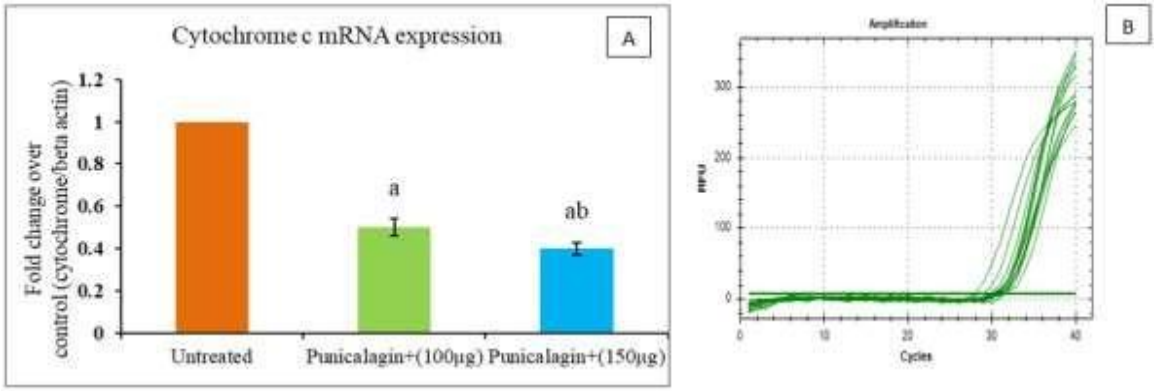

Figure 6 Effect of punicalagin on cytochrome-C mRNA expression in HepG2 cells

Discussion

HepG2 cell lines are analogs of human hepatocytes that are commonly used for investigations relating liver. These cell lines are majorly used for studying hepatotoxicity, detoxification process, metabolic process etc. the characteristic features of HepG2 cells is that they are non- tumorigenic cells but possess high ability of proliferation (Donato et al., 2015). More over their morphological features are likely to represent epithelial cells that have the potential to carry out different functions similar to hepatocytes. The MTT assay was done to reveal the viability of the cancer cell both treated and untreated groups. In this study the results of MTT assay revealed thatthe control cells showed 100% viability whereas upon treatment with 100 and 150 µg/ml of punicalagin, there was marked decrease in the viability of hepatic cancer cells (Fig 1). This shows the cytotoxic effects of punicalagin. Our next step was to identify the mechanism by which the growth and proliferation of these cells are hindered. The basic underlying pathological response towards any insult is generation of oxidative stress. The role of free radicles and theloss of antioxidant enzymes are the two important characteristic feature of oxidative stress. Thus in our study we intended to evaluate the oxidative stress in the HepG2 cells by measuring the antioxidant enzyme levels among the groups. The levels of antioxidant enzymes SOD and CAT in the punicalagin treated group showed a significant increase compared to untreated group. Compared to 100 µg/ml concentrations, 150 µg/ml concentration of punicalagin showed apotential increase thereby ameliorating the oxidative stress produced by the cancer cells (Fig 2 &3).

Phosphatidylinositol-3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling plays an important role in the cell growth regulation i.e., cell growth, motility, survival, metabolism, and angiogenesis. Targeting this signaling pathway helps in identification of new anti-cancer therapeutic drugs (Yang et al., 2019). PI3K is activated by a number of factors namely growth factors, cytokines, and hormones. Upon ligand receptor complex formation, PI3K is activated and initiates the progression of signaling cascade. Activated PI3k phosphorylates the PIP2, which in turn phosphorylates PIP3. PIP3 then recruits the pleckstrin-homology which then triggers the PDK1 and AKT molecules. Activation of AKT stimulates the mTOR family which bypasses the nuclear membrane and binds to specific sites at the DNA to promote cell proliferation (Yang et al., 2019). Cytochrome c is an important signaling molecule that is involved in the induction of apoptosis pathway (Shakarchi et al., 2018). This molecule is released from the mitochondria and causes cell death acting via apoptosis pathway. In this context, we conducted mRNA expression analysis to identify the fold changes in the gene expression. Our study results showed a positive indication with increased expression of PI3k, AKT and cytochrome c genes in the untreated HepG2 cells. Coherently, the 100 and 150 µg/ml treated groups showed down regulation in the expression of these genes (Fig 4-6).

Thus we conclude that punicalagin, a major compound isolated from the pomegranate peel extracts, has the potential to hinder and halt the growth and proliferation of hepatic cancer cell lines acting via the PI3K/AKT pathway and by inducing cell death by recruiting cytochrome c; thereby exhibiting its potent anti-cancer activity.

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