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Sapodilla Plum (*Achras sapota*) Induces Apoptosis in Cancer Cell Lines and Inhibits Tumor Progression in Mice

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Intake of fruits rich in antioxidants in daily diet is suggested to be cancer preventive. Sapota is a tropical fruit grown and consumed extensively in several countries including India and Mexico. Here we show that methanolic extracts of Sapota fruit (MESF) induces cytotoxicity in a dose-dependent manner in cancer cell lines. Cell cycle analysis suggested activation of apoptosis, without arresting cell cycle progression. Annexin V-propidium iodide double-staining demonstrated that Sapota fruit extracts potentiate apoptosis rather than necrosis in cancer cells. Loss of mitochondrial membrane potential, upregulation of proapoptotic proteins, activation of MCL-1, PARP-1, and Caspase 9 suggest that MESF treatment leads to activation of mitochondrial pathway of apoptosis. More importantly, we show that MESF treatment leads to significant inhibition of tumor growth and a 3-fold increase in the life span of tumor bearing animals compared to untreated tumor mice.

Cancer is a major disease with millions of patients diagnosed each year with high mortality around the world. Phytochemicals have been considered as conducive for cancer prevention. Preclinical and clinical studies have established plant derived dietary substances as suitable candidates for treating various types of cancers due to their broad chemical diversity. Such phytochemicals can block the action of carcinogens on target tissues thereby suppressing cancer development. Hence, the risk of cancer can be repressed by eating more fruits, vegetables and other plant products[1–3]. Meta-analyses of cohort and case control studies show significant evidence for cancer preventive effects with fruit consumption as they are good sources of vitamins, minerals and fibers[4].

Studies have explored the anticancer properties of different fruits, both in terms of their extracts and bioactive ingredients. It has been shown that *Carica papaya* extracts can induce cytotoxicity in various types of cancer cell lines[5]. Juices of pomegranate and citrus fruits have been specifically found to be effective in preventing colon cancer[6]. Besides, administration of concentrated extracts of *Morinda citrifolia* in tumor containing animals increased the immune response of the animals with a concomitant reduction in tumor burden[7]. In an interesting study, it was suggested that intake of tomato, tomato based products or lycopene can be associated with lower risk of cancer[8]. Bioactive phenolic components of cherries (*Prunus* spp.) have also been shown to possess anticancer properties[9]. Recent studies have shown that strawberry fruits possess both cancer preventive and therapeutic values[10,11]. However, little is known about the anticancer potential of Sapota fruits.

Sapodilla plum (*Achras sapota* or *Manilkara zapota*) is a tropical evergreen fruit tree belonging to the family of sapotaceae used in traditional system of Indian medicine. Ripe sapodilla fruits are eaten, which are rich in calories and contain sugars, acids, protein, phenolics, carotenoids and ascorbic acid[12–14] and possess high antioxidant properties[15]. It is also a good source of dietary fiber, minerals (potassium, copper, and iron) and vitamins (A, C, folate, niacin and pantothenic acid). Hence, Sapota is considered to be one of the healthiest fruits to alleviate micronutrient malnutrition[16].

Different components of the Sapota plant such as saponins and triterpenoids have been used in folk medicine and are known to exhibit anti-inflammatory, antioxidant, antimicrobial, analgesic and spermicidal activities[17,18]. Importantly, chemical constituents such as flavonoids, polyphenols, dihydromyrecetin, quercitin, myricitrin, catechin, epicatechin, gallocatechin and gallic acid have been isolated from fruits[14,19,20]. The decoction of young fruits along with flowers is used to treat diarrhea, dysentery and pulmonary diseases[21,22]. In a preliminary study it

has been shown that phenolic antioxidants such as methyl 4-*O*-galloylchlorogenate and 4-*O*-galloylchlorogenic acid derived from Sapota fruits can induce cytotoxicity in colon cancer cells[23].

Here, we report for the first time, anticancer properties of Sapota fruit using *in vitro, ex vivo* and *in vivo* studies. We show MESF is capable of inducing cytotoxicity in cancer cells by activating intrinsic pathway of apoptosis. Further, we demonstrate that MESF treatment on adenocarcinoma mice model can inhibit tumor progression, resulting in an increased life span in about 50% of the mice. Thus, our results indicate that inclusion of Sapota fruit in our daily diet may protect from genesis and progression of cancer.

Results

MESF induces cytotoxicity in cancer cells. In the present study, we investigate the effect of Sapota fruit on the proliferation and survival of cancer cells. In order to evaluate the cytotoxic effects of methanolic extracts of Sapota fruit (MESF), cell viability was determined in cancer cell lines of different origins. NALM6 (pre-B cell leukemia) and K562 (Chronic myelogenous leukemia) cells were treated with MESF and cytotoxicity was evaluated using trypan blue assay (Fig. 1a). Results showed that cell viability was significantly affected in NALM6 cell line, both after 48 and 72 h of MESF treatment (Fig. 1a). However, the effect was limited in K562 cells and restricted to the higher concentration of MESF (5 mg/ml) tested.

In order to confirm the cytotoxic effect of MESF on proliferation of NALM6 cells, live-dead cell assay was performed. Cells treated with different concentrations of MESF were harvested after 48 h and subjected to FACS analysis after staining with ethidium bromide staining. Consistent with above results, cell viability was affected at concentrations of 1 mg/ml onwards. Importantly, >80% cell death was observed at 2 mg/ml (Fig. 1b). Taken together, our results suggest that MESF is capable of inducing cytotoxicity in NALM6 and K562 cells with an IC_{50} of 0.9 mg/ml and 2.5 mg/ml, respectively, after 72 h of MESF treatment.

Further, the effect of MESF was evaluated in human and mouse breast cancer cell lines, EAC, MCF7 and T47D. Results showed a decrease in the viability of all three cell lines, particularly at high doses of MESF (Fig. 2). In contrast, cervical cancer cell line, HeLa, was less sensitive compared to breast cancer cell lines (Fig. 2). A549, a lung adenocarcinoma cell line, showed a decrease in cell viability at concentrations between 1 and 10 mg/ml. Interestingly, MESF induced only limited cytotoxicity in normal cell lines, STO (MEF) and 293T, compared to most of the other cancer cell lines studied (Fig. 2).

We have also compared the effect of Paclitaxel, a plant derived anticancer compound on NALM6, K562 and MCF7 cells. Results showed a decrease in cell viability, with NALM6 being the most sensitive among all the cell lines tested (Suppl. Fig. 1). As Paclitaxel is a purified compound compared to methanolic extracts of Sapota, a direct comparison of effective dosage between the two cannot be evaluated. Taken together, our data suggests that MESF can induce cytotoxicity in different cancer cell lines of varying origin.

Figure 1 | Evaluation of cytotoxic effects of MESF on leukemic cell lines. (a). NALM6 and K562 cells (0.3 × 10^5 cells/ml) were seeded and treated with MESF (0.1, 0.5, 0.75, 1 and 5 mg/ml). Cytotoxicity was evaluated by trypan blue exclusion assay after 48 and 72 h of treatment. (b). Live and dead cell population following treatment with MESF on NALM6 cells. For the evaluation, MESF treated cells (0.75, 1, 2 and 5 mg/ml, 48 h) were ethidium bromide stained and subjected to flow cytometry analysis. In each panel, M1 represents live cell and M2 denotes dead cell populations. Bar graph shows the % of live and dead cells following MESF treatment. Each experiment was repeated a minimum of three independent times and the error bars are indicated.

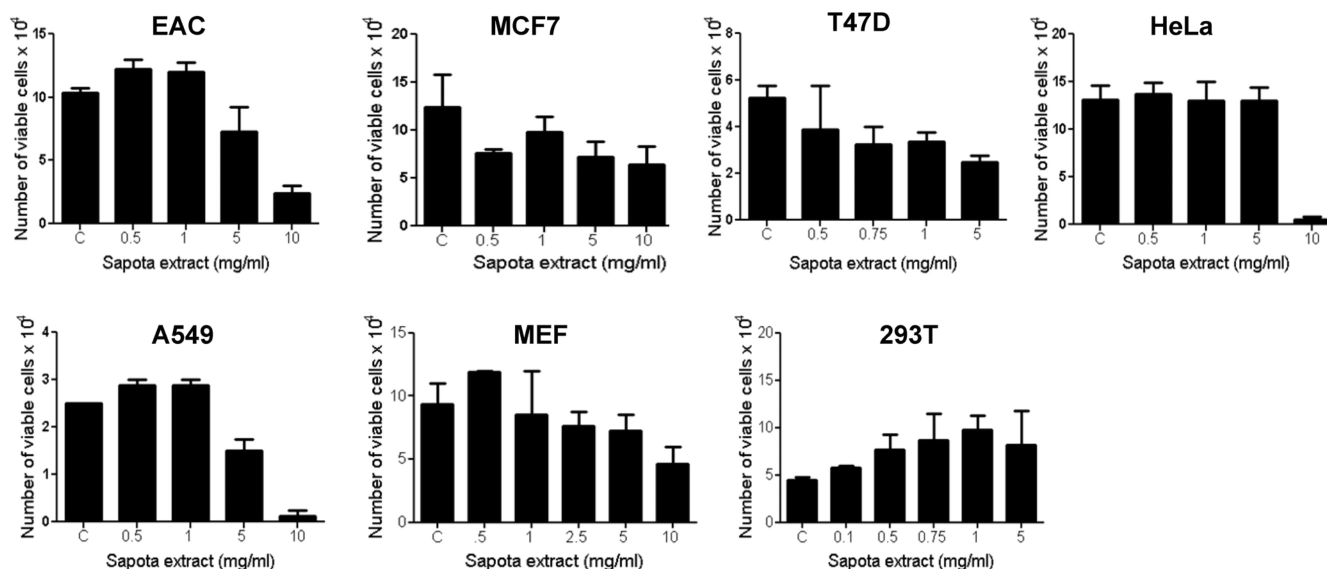

Figure 2 | Effects of MESF treatment on viability of breast, cervical and lung cancer cell lines. Breast cancer cell lines, EAC, MCF7 and T47D, cervical cancer cell line, HeLa, lung cancer cell line, A549 and normal cell lines, MEF (STO) and 293T (0.2×10^5 cells/ml) were seeded and treated with indicated concentrations of MESF. Trypan blue exclusion assay was performed after 48 h of MESF treatment and data is represented as bar diagram. Data presented is of three independent experiments and error bars are indicated.

MESF treatment does not induce cell cycle arrest. Cell cycle analysis following MESF treatment (0.5, 0.75, 1.0, 2.0 and 5.0 mg/ml) in NALM6 cells showed a dose-dependent increase in the subG1 population (hypodiploid DNA content) of cells, which is a hallmark of apoptosis (Fig. 3). However, MESF treatment did not lead to any cell cycle arrest (Fig. 3). Thus, both cell viability and flow cytometric assays suggest that MESF can indeed result in cytotoxicity.

ROS generation is considered as an intermediate step during activation of apoptosis. We examined the levels of ROS based on the fluorescence of DCFDA. Flow cytometric evaluation showed no detectable levels of ROS production upon MESF treatment (Fig. 4a). This is understandable as fruits possess high levels of antioxidants.

MESF induces depolarization and loss of mitochondrial transmembrane potential ($\Delta\psi_m$). Loss of mitochondrial transmembrane potential ($\Delta\psi_m$) is well known to be an early event during apoptosis. We measured the loss of mitochondrial membrane potential in MESF treated NALM6 cells (48 h) using flow cytometry following staining with JC-1 dye (Fig. 4b). $\Delta\psi_m$ was measured from the shift in the ratio of red to green fluorescence emitting cells following MESF treatment. Results showed an increase in green fluorescence in presence of MESF in a concentration dependent manner, indicating a loss of mitochondrial transmembrane potential in treated cells (Fig. 4b,c). Thus, our data suggests that MESF induces depolarization and mitochondrial transmembrane potential collapse in cells leading to activation of apoptosis.

MESF induces apoptosis in cancer cells. Since MESF treatment resulted in significant decrease in cell viability, we wondered whether it induces apoptosis. To test this, annexin V-FITC/PI double-staining followed by flow cytometry was performed in NALM6 and MCF7 cells following treatment with MESF (48 h) (Fig. 5). Results showed four sets of populations; unstained viable cells, early apoptotic cells (annexin V positive), necrotic cells (PI positive cells) and late apoptotic cells (annexin V and PI positive cells) in both NALM6 and MCF7 cells (Fig. 5a,c,d). MESF treatment led to significant increase in the population of cells undergoing late apoptosis after 48 h of treatment (Fig. 5). Our results suggest that MESF induces translocation of phosphatidyl serine from inner to outer leaflet of the cell membrane, which is a hallmark of apoptosis. Further, the annexin V-FITC/PI double-stained cells indicate occurrence of extensive cell membrane damage, resulting in the nuclear staining in those cells.

We further verified the annexin V-FITC stained cells by confocal microscopy. Results showed that, while MESF treated cells were stained by annexin V-FITC (green), untreated cells showed limited or no staining suggesting disruption of cell membrane of NALM6 upon treatment with MESF (Fig. 5b).

MESF activates intrinsic apoptotic pathway in NALM6. In order to understand the mechanism by which MESF induces apoptosis, we checked for changes in the expression levels of apoptotic proteins following exposure of MESF to NALM6 cells. Whole cell extracts were prepared after 48 h of treatment with MESF (0, 0.5, 1.0 and 2 mg/ml) and then subjected to immunoblotting. Results showed that MESF treatment led to significant upregulation of proapoptotic proteins like BAD and t-BID (Fig. 6). Besides, activation of apoptotic marker, MCL-1 resulting in its increased cleavage (pro-apoptotic form) was also observed in a dose-dependent manner, compared to the control (Fig. 6). PARP-1 cleavage triggered by caspases is considered as a hallmark of apoptosis. By immunoblotting analysis, we found that MESF treatment led to significant increase in cleavage of PARP-1 and Caspase 9 (Fig. 6), suggesting the activation of intrinsic pathway of apoptosis. Thus, our results suggest that MESF activates the mitochondrial pathway of apoptosis to induce cytotoxicity in cancer cells.

MESF treatment inhibits the tumor progression in mice. A mouse tumor model was used for investigating the *in vivo* anticancer property of Sapota fruit extracts. Tumor was induced by injecting Ehrlich ascites carcinoma cells, a breast adenocarcinoma of mouse origin in Swiss albino mice. Pilot studies showed that a dose of 10 g/kg body weight (b.wt.) of MESF exhibited good antitumour activity without noticeable side effects (data not shown). In order to evaluate the anticancer properties of Sapota extracts, mice bearing tumor (5 days post tumor injection) were treated daily with MESF (500 mg/kg b.wt). Results showed a significant decrease in the tumor load when mice were treated with MESF, in comparison to untreated tumor

Figure 3 | Effect of MESF on cell cycle progression. NALM6 cells were treated with different concentrations of MESF (0.5, 0.75, 1, 2 and 5 mg/ml). After 48 h of treatment, distribution of cell cycle phases and apoptotic population was quantitated based on flow cytometric analysis. Bar diagram shows the % of cells present in different phases of cell cycle.

animals, wherein progressive increase in tumor size was observed (Fig. 7a, c). Gross appearance of 16th day thigh tissue of animals treated with Sapota extracts showed significant reduction in the tumor size (Fig. 7c). Importantly, ~3-fold increase in lifespan was also observed at least in 50% of the mice (6/12 mice) when tumor bearing mice were treated with MESF (10 g/kg b.wt.) (Fig. 7b). Therefore, our results suggest that MESF treatment inhibits tumor progression and significantly increases the life span in mice. These results indicate that Sapota fruit can be used as a potent chemotherapeutic agent. However, further studies are warranted for evaluating anticancer activity of Sapota extracts in other animal models.

MESF treatment reduces tumor burden by inducing apoptosis. Gross appearances of tissues from normal, untreated and treated animals show restoration of morphological alterations in the liver and spleen of tumor bearing animals following MESF treatment (Suppl. Fig. 2). To understand the underlying mechanism of tumor regression in mice models, histological and immunohistochemistry (IHC) studies were carried out. Haematoxylin-eosine (HE) staining showed reduction in the infiltrated tumor cells in thigh tissue of MESF treated animals, when compared to untreated control (Fig. 8a). Besides, IHC studies showed that Ki67 positive cells, indicative of proliferation, were significantly less in MESF treated thigh tissues (Fig. 8b).

In conclusion, our results show that extracts prepared from Sapota induce cytotoxicity by inducing apoptosis in different cancer cell lines and inhibit tumor progression in mice leading to an increase in lifespan.

Discussion

Dietary intake of fruits has been a wholesome approach for the treatment of various cancers due to their high content of antioxidants and polyphenolics[24,25]. In the present study, we show by various experimental approaches that Sapota fruits possess anticancer property. Firstly, it induced cytotoxicity in cancer cell lines by activating the intrinsic pathway of apoptosis. Secondly, it inhibited tumor cell proliferation in a breast adenocarcinoma model. Thirdly, treatment of MESF could significantly improve lifespan in treated tumor bearing mice. Other than the present study, to date, there is only a single report on Sapota fruits to evaluate their anticancer efficacy, in which methyl 4-*O*-galloylchlorogenate and 4-*O*-galloylchlorogenic acid obtained from Sapota fruits were analyzed for their cytotoxic effects on colon cancer cell lines[23].

Sapota fruits induce cytotoxicity in cancer cells by activating intrinsic pathway of apoptosis. Cytotoxicity studies in different cancer cell lines showed that MESF affected cell viability in a dose-dependent manner, although at varying levels. MESF treatment led to

Figure 4 | Effect of MESF on production of reactive oxygen species and mitochondrial integrity. (a). Histogram representing the effect of MESF on ROS production in NALM6 cells. Cells were treated with MESF (2 mg/ml) and harvested after 5, 15, 30, 60 and 120 min of treatment. Cells were stained with DCFDA and subjected to flow cytometric analysis. (b). Effect of MESF on the mitochondrial membrane potential as assayed by JC-1 dye staining. Histogram shows spectral shift from red to green upon treatment with methanolic extracts of Sapota (0.75, 1.0, 2.0, 5.0 mg/ml). 2,4 DNP was used as positive control. Quantitation is also shown as bar diagram.

the production of low amounts of intracellular ROS levels. Cell cycle analysis suggested dose-dependent activation of apoptosis which was evidenced by accumulation of subG1 peak (Fig. 3). Such hypodiploidy is a known to be a sign of DNA degradation due to activation of endogenous nucleases during apoptosis[26]. MESF treatment led to a collapsed mitochondrial transmembrane potential which in turn led to the release of Cytochrome C from the intermembrane space of mitochondria into the cytoplasm resulting in the activation of the Caspase cascade eventually leading to activation of mitochondrial (intrinsic) pathway of apoptosis[27].

Induction of the translocation of phosphotidylserine from internal layer to outer surface of plasma membrane is also an indicator of apoptosis[28]. Majority of cancer cells after treatment with MESF showed cell death by apoptosis. An increase in both the early and late apoptotic populations of NALM6 and MCF7 cells was observed when treated with MESF. Translocation of phosphatidyl serine by flow cytometry and confocal microscopy of double-stained cells indicate extensive damage to cell membrane. Hence, our results demonstrate that Sapota fruit extracts potentiate the apoptotic effects on cancer cells rather than necrosis.

Apoptosis involves a complex network of protein-protein interactions that essentially rely on the balance between the antiapoptotic (MCL-1) and proapoptotic (BAX, BAD, BID) proteins[29]. It is also regulated by ratio of antiapoptotic and proapoptotic proteins which belong to BCL2 family[30]. The observed increase in the proapototic proteins indicates activation of mitochondria mediated apoptosis. PARP-1 cleavage is considered as another marker for apoptosis and is one of the major targets for caspases[31]. The observed PARP-1 cleavage and Caspase 9 activation suggest that MESF triggers activation of mitochondrial pathway of apoptosis[32].

Breast adenocarcinoma derived EAC cells are predominantly used for inducing tumors in mice as well as for assessing the anticancer activity of compounds *in vivo*[10,33,34]. Our results showed that MESF treatment in mice bearing tumor resulted in significant reduction in tumor volume. More importantly, we observed ~3-fold increase in the survival of tumor bearing mice following MESF treatment. This indicates that MESF treatment affected the viability of cancerous cells. This could possibly be due to the different phenolic antioxidants present in Sapota fruit, which would have exerted anticancer effects by their action on cellular events such as tumor initiation and progression. Further, histopathological and IHC studies suggest reduction in the proliferating tumor cells in treated tumor animal tissues. Importantly, a previous study with Sapota stem bark extracts was also consistent with our observations[35,36]. Hence, the observed inhibition in tumor progression upon treatment with MESF emphasizes its therapeutic potential.

As methanolic fractions of crude extracts of Sapota has been used in the study, determining dosage of effective compounds responsible for the anticancer effects is difficult. However, identification of active ingredients exhibiting anticancer effect will be of great therapeutic value. Nevertheless the present study provides proof of principle evidence that Sapota fruit, which is commonly ingested world wide, possess effective anticancer properties and opens avenues for future research.

Conclusion. Our results suggest the potential of Sapota fruits in inhibition of tumor development and progression leading to

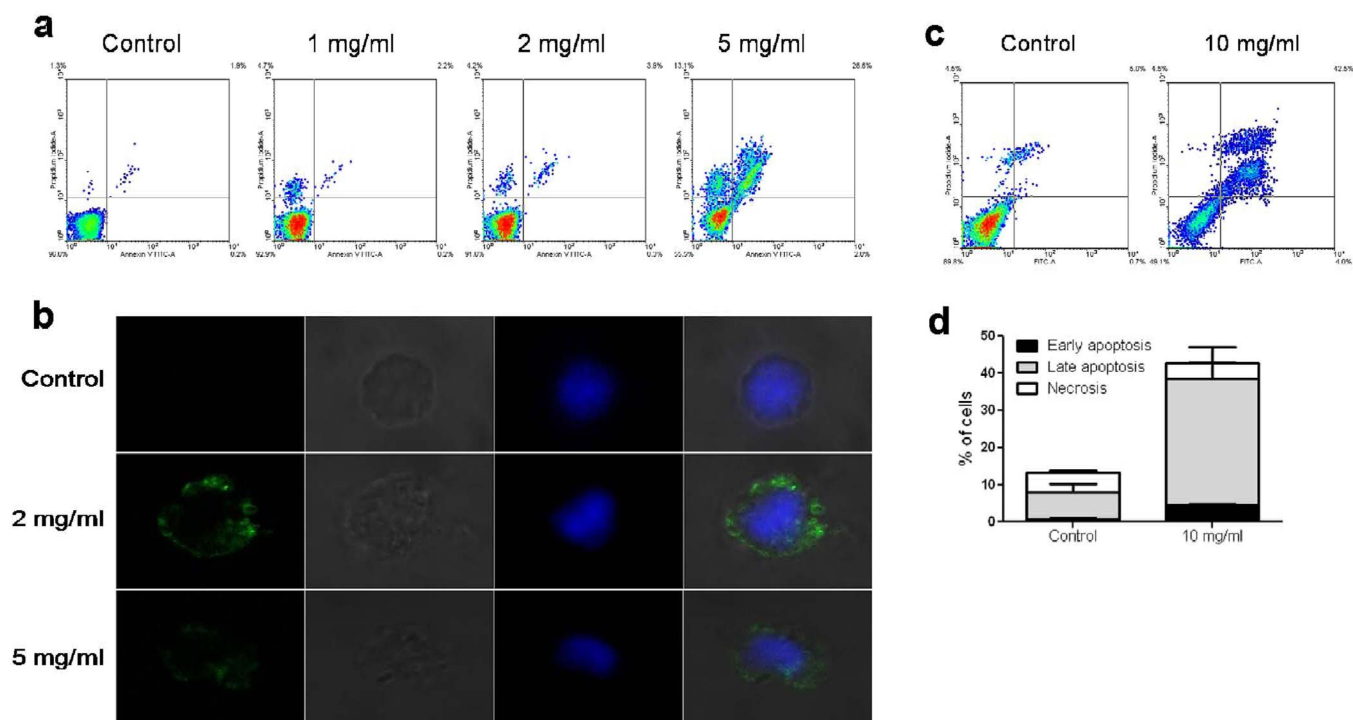

Figure 5 | Detection of apoptosis induced by MESF in leukemic and breast cancer cells. (a). NALM6 cells were stained with annexin V-FITC and PI, following treatment with MESF and analyzed by flow cytometry. In each panel, lower left quadrant shows cells which are negative for both annexin V-FITC and PI, lower right shows annexin V positive cells which are in the early stage of apoptosis, upper left shows PI positive cells which are dead, and upper right shows both annexin V and PI positive, which are in the late stage of apoptosis. (b). Visualization of apoptotic cells stained with annexin V-FITC using confocal microscopy after treatment with MESF (0, 2 and 5 mg/ml). DAPI staining was used as nuclear marker. (c). Density plot showing annexin V-FITC and PI stained untreated and MESF treated (10 mg/ml) MCF7 cells. (d). Bar diagram showing the distribution of early, late and necrotic cell populations.

cancer prevention. The observation needs to be verified in other model organisms including humans, but this study suggests the health beneficial aspects of Sapota.

Methods

Cell culture. Human Chronic mylegenous leukemia cell line, K562; human breast adenocarcinoma cell line, MCF7; human ductal breast epithelial tumor cell line, T47D; mouse Ehrlich ascites carcinoma, EAC; human cervix adenocarcinoma cell line, HeLa; human lung carcinoma cell line, A549; mouse embryonic fibroblast cell line, STO and human embryonic kidney epithelial cell line, 293T were purchased from National Center for Cell Science, Pune, India. Human B cell leukemia, NALM6 cell line was a kind gift from Dr. M. Lieber, USA. Cells were grown in RPMI 1640/DMEM/MEM or Ham's F12 medium (SERA LAB, USA) containing 10% FBS (GIBCO BRL, USA) and antibiotics in appropriate conditions.

Chemicals and reagents. All the chemicals and reagents used in the present study were obtained from Sigma Chemical Co. (St. Louis, MO) and SRL (India). Annexin V-FITC and antibodies were purchased from Santa Cruz Biotechnology, USA.

Preparation of methanolic extracts of Sapota fruit (MESF). Ripened Sapota fruits were sliced, shade dried and pulverized using grinder. The powdered material was extracted with methanol twice a day. The extracts were filtered through Whatman filter paper No.1 and concentrated under vacuum to get crude extract. After filtration, the solvent was evaporated under reduced pressure as described earlier[10]. The solvent free methanolic extracts of Sapota fruit was used for the present study, which is abbreviated as MESF throughout the study.

Cytotoxicity assays. Cytotoxic effect of MESF on NALM6, K562, MCF7, T47D, HeLa, A549, EAC, 293T and STO cell proliferation was determined by trypan blue exclusion assay by harvesting cells after either 48 and/or 72 h. Briefly, the cells were seeded (0.3×10^5/ml for NALM6 and K562; 0.2×10^5/ml for adherent cells) in six-well plates and different concentrations of MESF (0.1, 0.5, 0.75, 1.0, 5.0, 10 or 20 mg/ml) were added to the cells. Cells were counted under a microscope after trypan blue staining and plotted as described earlier[37–39]. Alternatively, we also

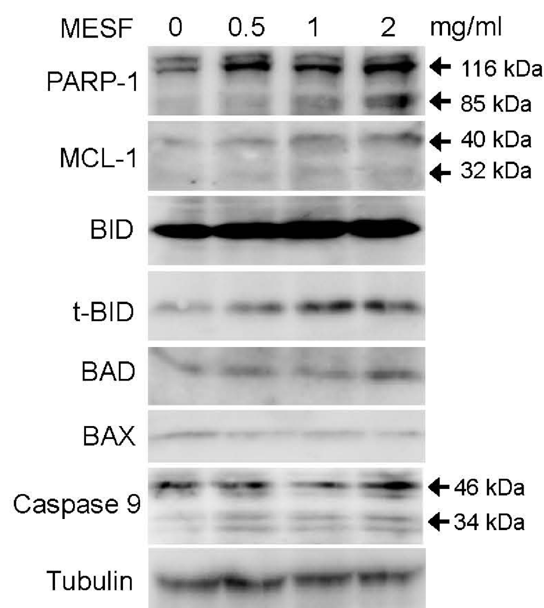

Figure 6 | Effect of MESF on expression of different apoptotic proteins. Cell lysate was prepared after 48 h of addition of MESF (0, 0.5, 1 and 2 mg/ml) in NALM6 cells. Untreated cells grown for 48 h were used as control. Cell extract (30–40 μg) was resolved on SDS-PAGE and western blot analysis was performed using apoptotic markers. Antibodies against PARP-1, MCL-1, BID, t-BID, BAD, BAX and Caspase 9 were used. Tubulin was used as the loading control. The blots shown are derived from multiple gels. Membrane was cut based on the molecular weight, probed with antibody of interest and band of interest is indicated with an arrow.

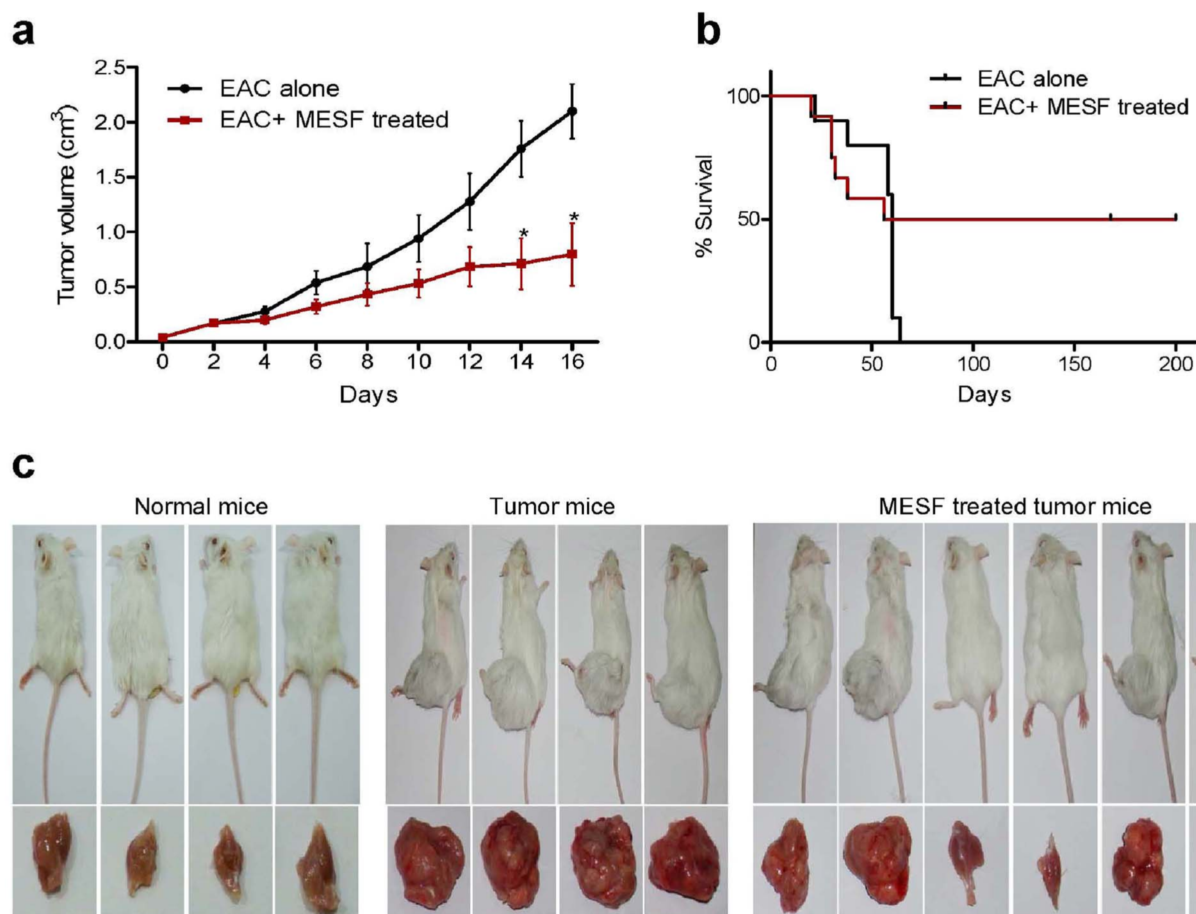

Figure 7 | Effect of MESF on tumor progression and survival in mice. Solid tumor was induced by injecting 1×10^6 EAC cells/animal. After five days of EAC injection, Sapota fruit extracts (0.5 g/kg b. wt, every day) were orally administered throughout the experimental period. (a). Tumor volume following MESF treatment in mice. (b). Kaplan–Meier survival curves of MESF treated and untreated mice bearing tumor. (c). The gross appearance of normal, control tumor and treated tumor animals and their tumor tissues on 16th day of MESF treatment.

monitored the ratio of live to dead cells following addition of MESF (0, 0.75, 1.0, 2.0 and 5 mg/ml) in total cell population by Live-Dead cell assay using flow cytometry as described[33]. Experiments were repeated a minimum of three times and data is presented as bar diagram with error bars.

Cell cycle analysis. NALM6 cells were cultured, treated with different concentrations of MESF (0.5, 0.75, 1.0, 2.0 and 5.0 mg/ml) and harvested after 48 h. The cells were then stained with propidium iodide, subjected to flow cytometry[40,41] (FACS Canto II, BD Biosciences, USA). A minimum of 10,000 cells

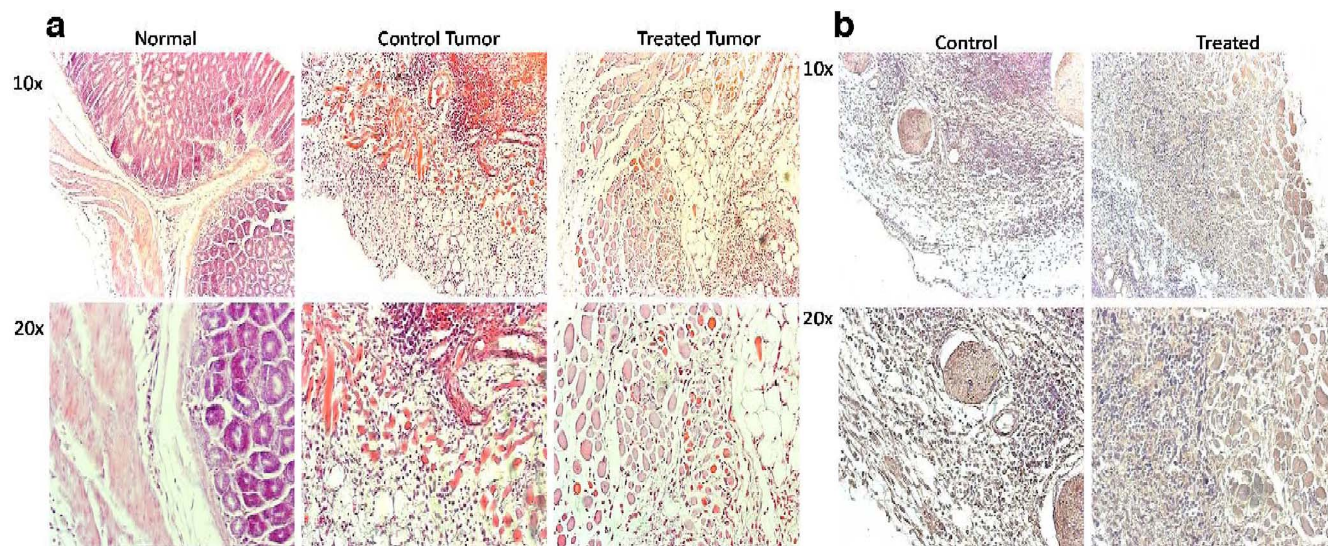

Figure 8 | Histological and immunohistochemical studies on MESF treated tumors. Sections prepared from tumor tissues of control and MESF treated EAC tumor mice were used for the study. (a). Images representing HE stained sections of normal, control tumor and MESF treated tumor tissues. Both 10× and 20× magnifications are shown. (b). Ki76 antibody stained paraffin sections of control and treated tumors, respectively.

were acquired per sample and histogram was analyzed using WinMDI 2.8 software.

Detection of generation of intracellular reactive oxygen species (ROS). Levels of net intracellular ROS production upon treatment with MESF (2 mg/ml for 5 min–2 h) was measured by using 2,7 dichlorodihydrofluorescein diacetate (H_2DCFDA) as described[39]. Following the MESF treatment, cells were washed and the flourescence intensity was analyzed by flow cytometry.

Detection of mitochondrial transmembrane potential ($\Delta\psi_m$). Alterations in mitochondrial membrane potential were analyzed by flow cytometry using JC-1 dye (5,5′,6,6′ tetrachloro 1,1′3,3′tetraethylbenzimidazol-carbocyanine iodide; Calbiochem, USA) as described earlier[42]. Briefly, NALM6 cells treated with MESF (0.75, 1.0, 2.0 and 5.0 mg/ml) for 48 h, were incubated with JC-1 (0.5 μM) and subjected to flow cytometric analysis. The ratio of red to green fluorescence was measured for each treatment and plotted. 2, 4-Dinitrophenol-treated cells (2, 4-DNP) served as the positive control.

Detection of apoptosis by Annexin V/PI double staining. The early and late apoptotic cells were identified and quantitated using annexin V-FITC/PI staining as described[43]. After treating NALM6 (1.0, 2.0 and 5.0 mg/ml) and MCF7 (10 mg/ml) cells for 48 h with MESF, cells were stained with annexin V- FITC (0.2 μg/μl) and propidium iodide (PI) (0.05 μg/μl) and subjected to FACS analysis as described[43].

Confocal microscopy. NALM6 cells treated with MESF (2.0 and 5.0 mg/ml) were harvested after 48 h and used for annexin V-FITC staining. The cells were then observed under inverted confocal laser scanning microscope (Ziess LSM 510 MK4, Germany) and images were captured. DAPI was used as a nuclear marker.

Western blot anaysis. Whole cell lysate was prepared following treatment with MESF on NALM6 cells (0, 0.5, 1.0, 2.0 mg/ml, 48 h) as described[34,44]. Western blotting analysis was performed by using 30-40 μg of protein. Samples were electrophoresed on 8–12% SDS-PAGE, proteins were transferred to PVDF membrane (Millipore, USA) and probed with respective primary and secondary antibodies. The primary antibodies used were, MCL-1, BAX, t-BID, BID, BAD, BAX, PARP-1, Caspase 9 and Tubulin (loading control). The blots were developed using chemiluminescent reagents (Immobilon™ western, Millipore) and scanned by gel documentation system (LAS 3000, FUJI, JAPAN). Blots were stripped subsequently as per standard protocol and reprobed with anti-tubulin.

***In vivo* experiments.** *Ethical statement.* Mice were maintained as per the principles and guidelines of the ethical committee for animal care, Indian Institute of Science in accordance with Indian National Law on animal care and use. The experimental design of the present study was approved by Institutional Animal Ethics Committee (Ref. CAF/Ethics/125/2007/560), Indian Institute of Science, Bangalore, India.

Experimental animals. Swiss albino mice, 8–10 weeks old, weighing 18–24 g were purchased from central animal facility, Indian Institute of Science, India and used for the present study. The animals were housed in polypropylene cages and provided standard pellet diet (Agro Corporation Pvt. Ltd., Bangalore, India) and water *ad libitum*. The standard pellet diet composing 21% protein, 5% lipids, 4% crude fiber, 8% ash, 1% calcium, 0.6% phosphorus, 3.4% glucose, 2% vitamin, and 55% nitrogen-free extract (carbohydrates) was used for feeding. The mice were maintained under controlled conditions of temperature and humidity with a 12 h light/dark cycle.

Preparation of Ehrlich ascites carcinoma (EAC) cells and induction of tumor development. Ehrlich ascites carcinoma was chosen for the study, as it is of mouse origin and can be easily transplanted in immunocompetant mouse. A fixed number of viable EAC cells collected from donor mice were injected into the peritoneal cavity of each recipient mouse and were allowed to multiply. The cells were withdrawn after 8–10 days of inoculation, diluted in saline (1×10^6 cells/animal) and injected to left thigh of experimental animals for developing the solid tumor[33,34].

Determination of the anticancer effect of MESF on solid tumor regression. For life span studies a total of 18 animals were used per batch and divided into three groups of 6 animals each (2 batches). Out of 18 animals, 12 were injected with EAC cells into left thigh for developing solid tumor. Group I animals served as untreated controls and received no other treatment. EAC injected animals were sub-divided into group II and III containing 6 animals each. Group II animals served as tumor controls and group III animals received oral administration (with drinking water) of Sapota fruit extracts (10 g/kg b.wt) with a gap of two days, starting after 12 days of tumor cell injection and continued throughout the experimental period. The increase in life span of MESF treated mice was calculated and compared with untreated tumor bearing animals. The life span of experimental animals was monitored and calculated by using the formula $((T - C)/C) \times 100$, where 'T' indicates the number of days the treated animals survived and 'C' indicates that number of days that tumor animals survived[33,34].

In an independent study, a total of 12 animals were injected with EAC cells into left thigh for developing solid tumor while four animals served as normal controls. Among the 12 EAC injected mice, five were used as vehicle tumor controls, while rest were fed with MESF from 5th day of tumor development (0.5 g/kg b.wt, every day) using oral gavages, throughout the experimental period. Control mice received equal volume of water as the MESF was dissolved in it. Tumor size was measured using vernier calipers on alternative days for tumor animals and tumor volume was calculated using the formula $V = 0.5 \times a \times b^2$, where 'a' and 'b' indicates the major and minor diameter, respectively[33,34]. At the end of 16th day of experimental period, animals from each group were sacrificed and kidney, liver, thigh and spleen were collected from normal, tumor and Sapota fruit extracts treated animals and were evaluated for morphological changes. HE staining and immunohistochemistry studies were carried out using 25th day control as well as treated tumor tissues.

Histological evaluation of tumor tissues. Tumor tissues of control and MESF treated animals were collected and processed for histological evaluations as described previously[34,39]. Briefly, tissues were fixed with 4% paraformaldehyde and embedded in paraffin wax and 10 μm sections were made using Microtome (Leica Biosystems, Germany). Sections were stained with haematoxylin and eosin, analyzed using light microscope and images were captured (Zeiss, Germany).

Immunohistochemistry. Immunohistochemical analysis of tumor tissues were carried out using sections derived from control and MESF treated tumor tissues as described earlier[33,34]. Sections were deparaffinized at 65°C using xylene, rehydrated, treated with 3% H_2O_2 and antigen retrieval was carried out at 100°C in 0.01% sodium citrate buffer. Sections were blocked using 0.1% BSA and 10% FBS for 1 h at room temperature and incubated with primary antibody (Ki67, 1 : 100) overnight at 4°C. After washing, sections were treated with secondary antibody conjugated with biotin (1 : 200) for 2 h at room temperature followed by streptavidin-HRP conjugated antibody (1 : 500) for 1 h. Finally, sections were treated with DAB, H_20_2 and counterstained with haematoxylin, and images were captured (Zeiss, Germany).

Statistical analysis. Values are expressed as mean ± SEM for control and experimental samples and each experiment is repeated a minimum of 3 times, independently. Statistical analysis was performed by one-way ANOVA followed by Student 't' test using GraphPad software prism 5.1.

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Author contributions

S.C.R. and M.S. designed experiments; M.S., M.H., K.K.C., S.B., J.K. and B.C. performed experiments; S.C.R. and M.S. interpreted the data and wrote the manuscript.

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