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Molecular Mechanism of Induction of Apoptosis by Garcinol, A Poly-Isoprenylated Benzophenone in HEPG2 Cells

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Abstract

Aim: The analysis aims to identify and collate the molecular mechanism underlying apoptosis for concealing and development of novel molecule. **Methodology:** HEPG2 and 3T3-L1 cell lines were treated with SAHA and Garcinol at different concentrations were concealed for cytotoxicity by MTT assay, Morphological features of apoptosis induced on cells were observed by Acridine orange-Ethidium bromide dual staining and Hoechst staining, Cell cycle analysis and Annexin V expression for apoptosis by Flow cytometry. **Result:** The study revealed strong data for the significant cytotoxicity induced by Garcinol, further explored as potential compound for reducing proliferation of HEPG2 cells, sparing the normal 3T3L1 cells. The morphological and internal changes induced by the Garcinol on HEPG2 confirm that the cytotoxicity induced by the compound ultimately promote apoptotic signals eventually leading to cell death. This supports the quantitative data obtained through the Annexin V expression studies by flow cytometry. From the study it was observed that the anti-cancer effects of the compound Garcinol is significantly comparable with SAHA. **Conclusion:** The results conclude the anticancer activity of Garcinol with negligible cytotoxicity to normal cells and effective to HEPG2. Garcinol reduced cell proliferation by causing significant S phase arrest in the cell cycle and induce apoptosis in HEPG2 cells as evidenced by the microscopic observations. Garcinol has significant positive result for Annexin V expression in cancer cells which proved apoptosis as the mechanism of cell death. So further studies will be very promising on the compound, Garcinol or its derivatives which can be further exploited as specific anticancer agents.

Keywords

HDAC (Histone deacetylase); Annexin V expression; Cytotoxicity; Apoptosis; SAHA (Suberoylanilide hydroxamic acid); Garcinol

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1. INTRODUCTION

Cancer can be explicated as a condition in which a set of abnormal cells uncontrollably growing by disregarding the standard rules of cell division by developing a degree of autonomy signals [1]. The manifestations are mainly based upon the category of cancer that depends on the environmental conditions (90-95 %) as well as genetic factors (5-10 %)[2][3]. Genetic factors encircle mutations of transposons, viral infections as well as the mutagens

[4]. One of the main impact is the disruption of the cell cycle machinery that regulate the progression of the cell through G1, S, G2 and M by phosphorylation and dephosphorylation [5].The suppression of apoptosis and anti-apoptotic operations lead to anomalous activation of proliferation, make it pertinent to search for a better molecule which can arrest of cancer, by blocking the generators of anti-apoptotic stimuli. The chemotherapeutics in current use may lead to the mutilation of normal cells, thus limiting the potency of the drug. Hence selective attack on apoptotic abnormalities in cancerous cells can generate a potent, pro-apoptotic stimuli leading to critical destruction of tumor cells [6] [7].

The overexpression of Histone deacetylases (HDAC) leads to histone deacetylation at the promoter site of tumor suppressor genes by causing histone 3 lysine 9 methylation, causing heterochromatinization that leads to abnormal changes in chromatin pattern followed by tumorigenesis. Altered expression of epigenetic factors is also involved in regulating cancer-relevant processes, including epithelial-to-mesenchymal transition (EMT), senescence, genome stability, and metastasis [8] [9]. Lysine acetylation, the epigenetic post translational modifications among cellular proteins regulates a variety of physiological processes including enzyme activity, protein- protein interactions, gene expression, and subcellular localization [10]. HDAC inhibitors synergize with certain anti-cancer agents, like cytotoxic agents as they functions through multitude of pathways, thus inhibiting angiogenesis through a shift in the balance of pro- and anti-apoptotic genes. Several studies indicated that HDAC inhibitors are a new class of cytostatic agents that inhibit the proliferation of tumour cells in culture and *in vivo* by inducing cell cycle arrest, differentiation and/or apoptosis.

In recent years, medicinal plants have attracted a lot of attention globally. Since long time evidence has accumulated to demonstrate promising potential of medicinal plants used in various traditional, complementary, and alternative systems especially for cancer treatment. To understand the mechanism of action, the researchers have worked at molecular level and several significant phytochemicals have been isolated based on the activity analysis of the medicinal plant extracts with different solvents. Presently, almost fifty percent of all prescribed drugs are derived or synthesized from natural products, the only available sources for which are animals, marine, plants, and micro-organisms. It is considered that because of the structural and biological diversity of their constituents, terrestrial plants offer a unique and renewable resource for the discovering of potential new drugs and biological entities. SAHA or vorinostat and Garcinol are the compounds used in the present study. Pharmacological manipulation of acetylation has been possible with the identification of inhibitors of histone deacetylases (HDAC), the enzymes responsible for deacetylating lysine residues. SAHA has been shown to bind to the active site of histone deacetylases and act as a chelator for Zinc ions. SAHA's inhibition of histone deacetylases results in the accumulation of acetylated histones and acetylated proteins, including transcription factors crucial for the expression of genes needed to induce cell differentiation [11]. Garcinol (molecular mass: 602.39) is a polyisoprenylated benzophenone derivative from *Garcinia indica*. The structural analysis confirmed the structure of Garcinol as an isoprenylated benzophenone with an enolizable 1, 3-diketone group conjugated to a 3, 4-dihydroxybenzoyl moiety and two αβ-unsaturated carbonyl groups [12]. It also inhibits topoisomerases I and II at concentrations comparable to Ectoposide, a chemotherapy medication in use for various cancers. The structure of Garcinol and SAHA is given as **(Figure 1a and 1b)** respectively.

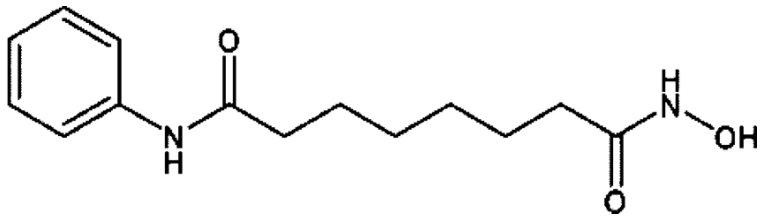

Fig. 1a Chemical Structure of SAHA

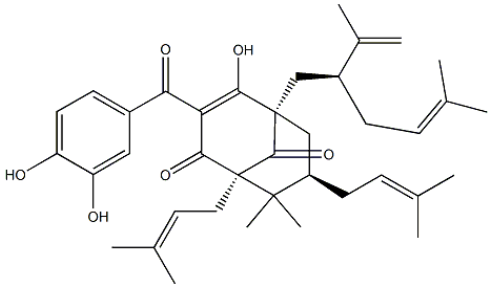

Fig. 1b Chemical Structure of Garcinol

The resulting compounds were found capable of inducing apoptosis in human leukemia HL-60 cells and inhibit NO radical generation as well as LPS-induced iNOS gene expression, respectively [13]. In addition to HL-60 cells, the chemotherapeutic potential of Garcinol has been examined on other cell lines as well such as murine macrophage RAW 264.7 cells and cyclin D1-positive cells showing similar results. Additionally, Garcinol also inhibits histone acetyltransferases and p300/CPB-associated factor (PCAF, IC50 = 5 μM, both of which are known to modulate gene expression [14]

Clinical trials using are now underway with Vorinostat or SAHA, having been approved by the FDA for treating cutaneous T-cell lymphoma (CTCL) in patients with progressive, persistent, or recurrent disease. Among the current targets for cancer chemotherapy, natural product based small molecule epigenetic disruptors are emerging as novel anti-cancer therapeutics.

Garcinol is reported to potently inhibit tumor growth and angiogenesis by targeting HATs p300 and PCAF, thereby suppressing cell proliferation, which leads to *cancer* [15]. Garcinol has shown very promising antiangiogenic and antimetastatic activities *along with* downregulation of MMP-9, IL-8, PGE-2, and VEGF, markers of angiogenesis and metastasis in pancreatic cancer cell lines, Panc 1 and BxPC3. The effects of Garcinol were even more pronounced in the Panc1 cell line, carrying the *k-ras* mutation, implicated in the majority of human pancreatic cancer patients [16].

In the present study, we aimed to study the possible potential of Garcinol in special impetus to the induction of apoptosis and the anticancer activity by inhibiting/ arresting of cell cycle in in HepG2 cells

2. MATERIALS AND METHODS

2.1 Materials

Dulbecco's Minimal Essential medium (DMEM), Bovine Ribonuclease A, Propidium Iodide, Acridine Orange, Hoechst were purchased from Sigma Aldrich, USA. Garcinol and Suberoylanilide hydroxamic acid (SAHA) were purchased from Enzo life sciences. Fetal Bovine Serum (FBS), Antibiotic-Antimycotics and Trypsin-EDTA were obtained from GIBCO, MTT from Merck. Proteinase K was purchased from MP Biomedicals. HDAC Cell - Based Activity Assay Kit was purchased from Cayman chemical. Annexin-V FITC Apoptosis Detection Kit II was bought from BD Pharmingen. Preadipocyte cell line (3T3L1) were isolated from normal adipocytes was used as normal control cell line. Liver carcinoma HepG2 cells were obtained from National centre for cell sciences (NCCS), Pune. Both the cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) as per standard culture conditions [17]. The cultures were maintained at 37^0C in 5% carbondioxide incubator (New Brunswick).

2.2 Cell Culture

2.2.1 Cell Viability Assay using MTT

100µL of the cells were seeded at a density of ($5x10^3$ cells) in each well in 96 well plates and incubated for 24 hours at 5% CO_2 incubator. On the second day, media was replaced by 100 µL of the drugs dissolved in DMSO and added at different concentrations of Garcinol (400, 200, 100, 50, 25, 12.5, and 6.25 µM) at different time incubations (48 & 72 hours). Followed by overnight incubation, the cells were incubated in MTT solution for 2 hours, followed by addition of 100 µL of lysis buffer (100% DMSO) to dissolve the formazan crystals formed. The absorbances of the samples were measured at 570 nm using a Multiskan Microplate Spectrophotometer (Thermo scientific) [18]. The percentage of cytotoxicity obtained with each drug was calculated as follows

$$\% \text{ Cytotoxicity} = 100 - \{\frac{Absorbance\ of\ Test}{Absorbance\ of\ control}\} * 100$$

IC_{50} values were calculated from the plotted graph using easy plot software with concentration of the compound plotted along X axis and percentage of cytotoxicity along Y axis. The concentrations which exerted maximum cytotoxicity on cancer cells and the most appropriate time incubation for the activity was selected and chosen for further apoptotic and HDAC inhibition studies.

2.2.2 Fluorescence Microscopy

The morphological features of the cells treated with different compounds undergoing apoptosis were observed using under the microscope and images

were captured using a fluorescent microscope [19] (LEICA).

2.2.3 Acridine Orange – Ethidium Bromide Dual Staining

The treated cells were incubated with 100 µL of Acridine Orange-Ethidium Bromide (1:1) solution for 3 minutes followed by sterile PBS wash and observed under Fluorescent microscope at 450-490 nm [20].

2.2.4 Hoechst Staining

The treated cells were washed with PBS and incubated with 100 µL of Hoechst solution for 10 minutes, again washed with PBS and observed under Fluorescent microscope at 350-460 nm [21].

2.2.5Assessment of Apoptosis

To identify the different stages of apoptosis, cells were treated with different concentrations of Garcinol and harvested after 48 hours of treatment. The cells were washed twice with cold PBS and prepared for acquisition using Annexin V- FITC apoptosis detection kit (BD Biosciences, cat no: 556547). The cells were acquired using flow cytometry (BD FACS Calibur™) and the results were interpreted using Cell quest Pro software analysis.

2.2.6 Cell cycle analysis

The phase of the cell cycle at which Garcinol treated cancer cells got arrested was determined using flow cytometry. Briefly, the cells were grown in tissue culture flasks and treated with different concentrations of Garcinol. After 48 hours of treatment, the cells were harvested and centrifuged at 3500 rpm for 7 minutes. The cells were then fixed in 70% ethanol for 30 minutes. After centrifugation, the pellet was dissolved in PBS and 5 µL of RNase A (10mg/ml) was added and incubated for 30 minutes at 37°C. 10 µg/ml of propidium iodide (PI) was added and after 15 minutes of incubation in dark, the cells were filtered through 0.75 µm filter and analysed using cell quest Pro software analysis.

2.3 Statistical Analysis

All the statistical analysis was done using the GraphPad Insta Software. Data were expressed as mean of three independent experiments in *in-vitro* cytotoxicity experiments. The statistical significance of difference was tested using one-way ANOVA. A value of $p < 0.05$ is considered significant.

3. RESULTS

3.1 Cell viability by MTT assay

Garcinol induced low cytotoxicity of 22% and 25% at 1600 µM, 21% and 24% at 800 µM, 18 % and 25 % cytotoxicity at 400µM, 17 % and 12% at 200µM and 18 % and 17 % at 100µM at both 24- and 48-hour incubation, respectively. The percent cell cytotoxicity values of Garcinol in preadipocyte cells were negligible and insignificant and hence can be proceed for further studies **(Fig. 2a).**

The percent cytotoxic values of HEPG2 cells treated with Garcinol showed maximum cytotoxicity of 82% at 72-hour incubation at 100 µM concentration **(Fig. 2b)**. IC_{50} values of Garcinol on HepG2 cells were calculated graphically using Easy plot software. The concentration of the Garcinol required to cause 50 % cytotoxicity of the HEPG2 cells at different time incubations are given in the **Table 1**.

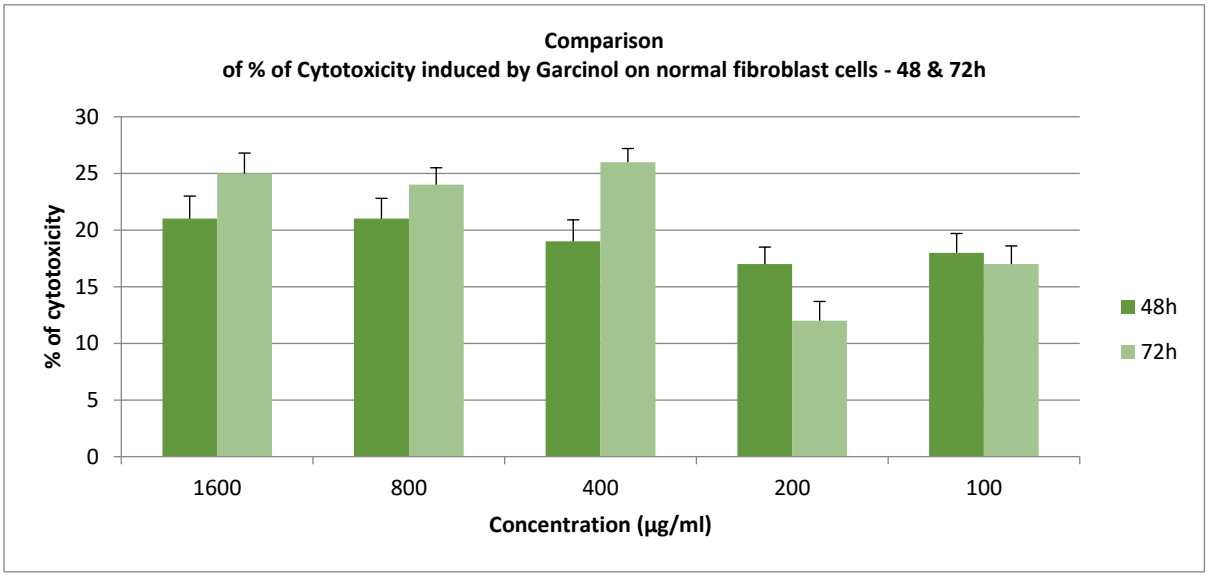

Fig. 2a Confluent 3T3L1cells on 96 well plates were treated with different concentrations of Garcinol; (1600, 800, 400,200,100µM) were used. Experiments were done in triplicates. After 48 and 72 h, the plates were read at 570 nm using a Multiskan Microplate Spectrophotometer (Thermo Scientific) and % of cytotoxicity was calculated from the obtained OD and was represented in graphical forms ($p < 0.05$).

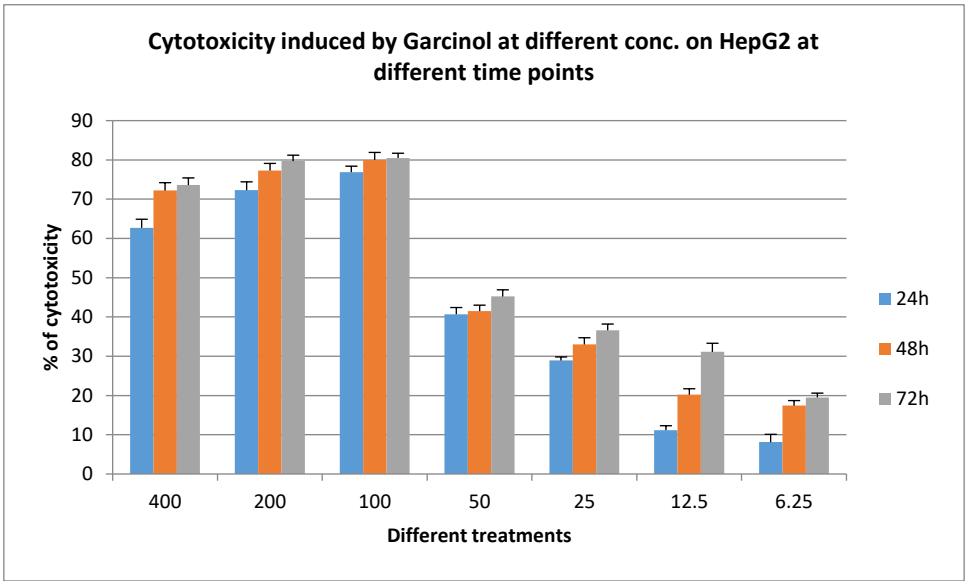

Fig. 2b Confluent HEPG2 cells on 96-well plates were treated with different concentrations of Garcinol; (400, 200, 100, 50, 25, 12.5 and 6.25µM) were used. Experiments were done in triplicates. After 24, 48 and 72 h, the plates were read at 570 nm using a Multiskan Microplate. Spectrophotometer (Thermo Scientific) and % of cytotoxicity was calculated from the obtained OD and was represented in graphical forms ($p < 0.05$).

Time Incubation Hours	IC 50 Value
24	62.82
48	61.26
72	58.53

Table 1. MTT Cytotoxicity Assay: IC50 value calculated using Easy plot software represents the concentration of the Garcinol required to cause 50 % cytotoxicity of the HEPG2 cells at 24, 48 and 72 Time Incubation Hours.

3.2 Effect of Garcinol and SAHA on morphological features of HepG2 Cell lines

Comparing with negative control all the treated cells showed reduction in cell number whose effects were most pronounced in SAHA. Cells were toxic when treated with 200µM concentration and almost all cells lost their normal morphology and were round. The characteristics like rounding, shrinking, and clumping of cells were also observed. Higher concentration of Garcinol 200µM showed more cytotoxic morphology than that of cells treated with Garcinol 100µM **(Fig. 3a)**.

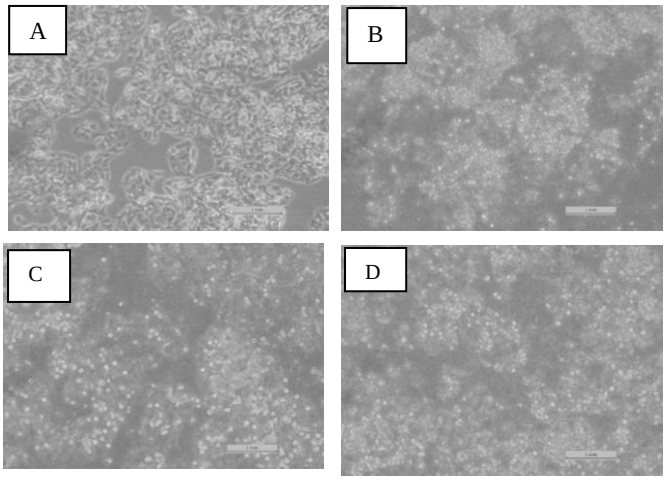

Fig. 3a Confluent HEPG2 cells in 96-well plates were treated with different concentrations of compounds; A) Negative Control, B) SAHA 12.5µM, C) Garcinol 100 µM & D) 200 µM. Experiments were done in triplicates.

After 48 h, the plates were observed for morphological changes using a fluorescent microscope (LEICA) and photographed (10X).

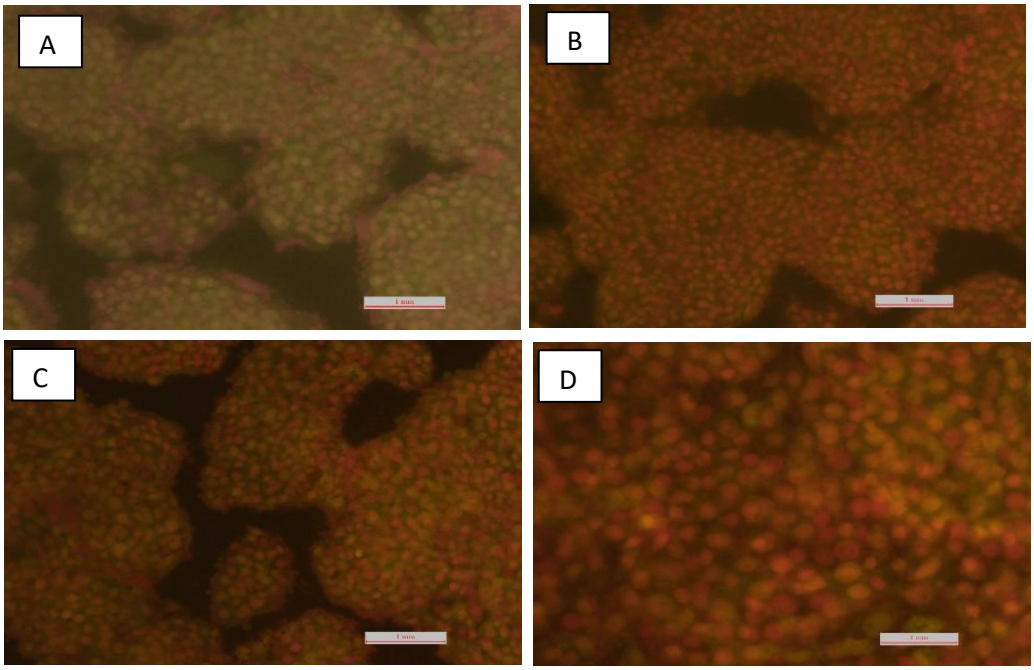

Fig. 3b Confluent HEPG2 cells in 96-well plates were treated with different concentrations of compounds; **A) Negative Control, B) SAHA 12.5µM, C) Garcinol 100 µM & D) 200 µM**. After 48 h, the cells in each well were stained using 100 µL of AO-EtBr (1:1) and incubated for 1-2 minutes and were observed and photographed (20X) using fluorescent microscope (LEICA) . Viable and dead cells were visible in green and reddish-orange respectively.

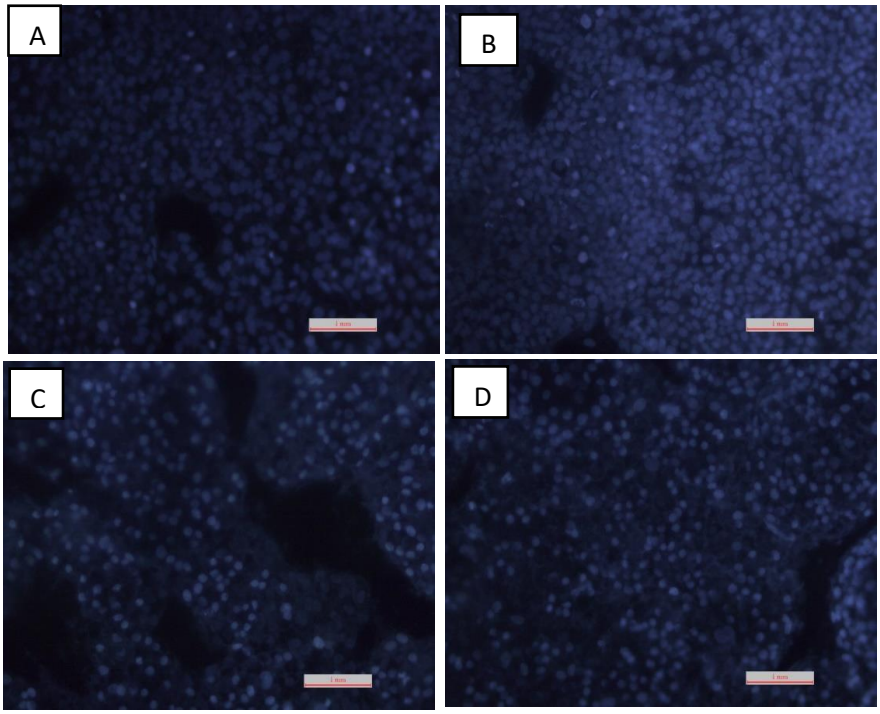

Fig. 3c Confluent HEPG2 cells in 96-well plates were treated with different concentrations of compounds; A) Negative Control, B) SAHA 12.5µM, C) Garcinol 100 µM & D) 200 µM. After 48 h, the cells in each well were stained using 100 µL of Hoechst solution (6µg in 1mL PBS) and incubated for 10 minutes and were observed and photographed (20X) using fluorescent microscope (LEICA). Cells without condensed chromatin were observed in blue color and with chromatin condensation in bright blue color.

3.3 Acridine Orange – Ethidium Bromide Dual Staining

Comparing with negative control, dead cells were observed as orange due to the uptake of EtBr. Damaged cells were more or less rounded and cell shrinkage was also seen. Nuclear invagination and fragmentation were also observed. Clumping of cells due to membrane blebbing and fusion of cells were seen. Formation of apoptotic bodies was visible. Significant effect was shown by the cells treated with 200 µM Garcinol compared to the positive control SAHA **(Fig.3b)**.

3.4 Hoechst Staining

Cells took up Hoechst stain and nuclei of the untreated cells were observed in blue color. In compound treated cells, the nuclei were observed as bright blue color compared to negative control which indicated chromatin condensation following apoptosis. Fragmented DNA was also observed. Pronounced effect was shown by the cells treated with 200 µM Garcinol compared to the positive control SAHA **(Fig. 3c)**.

3.5 Annexin V Expression For Apoptosis

From the flow cytometric analysis of Annexin V expression study on HepG2 cells, it was confirmed that Garcinol induces apoptosis similar to SAHA as given in **(Fig.4a)**. Quantitative analysis using the Annexin V/PI assay showed the proportion of apoptotic cells (Annexin V+/PI+) increased significantly from 29.1% in control to 43.5 %, 87.3%, 39.1% and3.3% in cells treated with 25µM SAHA, 400 µM Garcinol, 200µM Garcinol and 100 µM Garcinol respectively after 24 hours treatment. Garcinol treated cells induced apoptotic cells approximately two times than that of SAHA (***p, 0.001-highly significant)

The flow cytometric analysis of Annexin V expression study after 48 hours in HepG2 cells revealed induction of apoptosis from 2.9 % in untreated control to 2.8 %, 76.7%, 17.5 and 5.5% in cells treated with 25µM SAHA, 400 µM Garcinol, 200µM Garcinol and 100 µM Garcinol respectively **(Fig. 4b)**. The apoptotic effect was highly significant (***p, 0.001) at 400 µM Garcinol and moderately significant (**p, 0.01) at 200 µM Garcinol compared to SAHA (**Fig. 4c**).

3.6 Cell Cycle Analysis of HepG2 Cells

Cancer cells have the inherent property of abnormal proliferation. Most of the antitumor agents are known to arrest cell cycle progression at S, G2 or M phase. Control of cell cycle progression in cancer cells is considered to be a potentially effective strategy for the control of tumor growth. The number of cells arrested in the G1 phase of cell cycle increased from 2% in control cells to 28% in SAHA treated cells and 48, 52, 72% in Garcinol treated cells at concentrations 400, 200, 100µM respectively **(Fig. 5a).**

The results indicated that there was a G1-S Phase arrest in the compound treated cells. The percentage of treated cells accumulated in the S phase significantly decreased than that of the control and positive cells which indicated that one of the mechanisms that may act to inhibit the proliferation of cancer cells is the inhibition of cell cycle progression. The 400, 200 and 100 µM of Garcinol have high significance (***p, 0.001) in arresting the cell cycle of the hepato carcinoma cells compared to that of SAHA **(Fig. 5b)**.

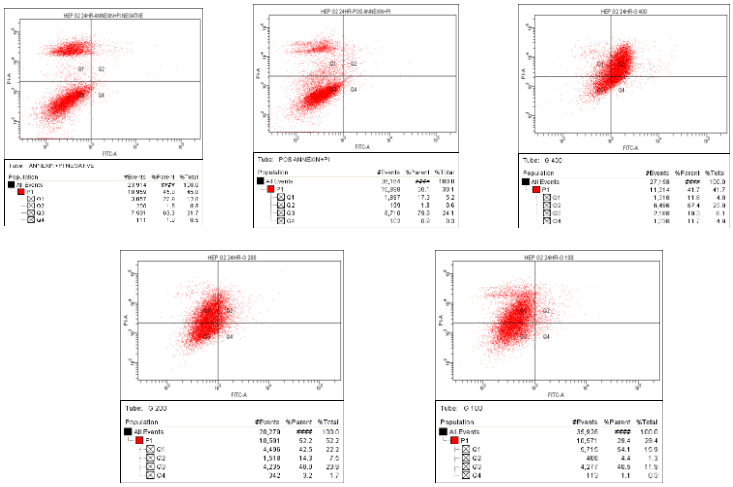

Fig. 4a Assessment of apoptosis by Annexin V/PI on human liver cancer cells (HEPG2). The cells were treated with media only A) Control, B) SAHA C) 400µM Garcinol, D) 200µM Garcinol & E) 100µM Garcinol, for 24 h and apoptosis was examined by flow cytometry (BD Biosciences Pharmingen) after Annexin V-PI double staining. Necrotic cells lose membrane integrity, permitting PI entry. Viable cells exhibit Annexin V (-)/PI (-); early apoptotic cells exhibit Annexin (+)/PI (-); late apoptotic cells or necrotic cells exhibit Annexin V (+)/PI (+).

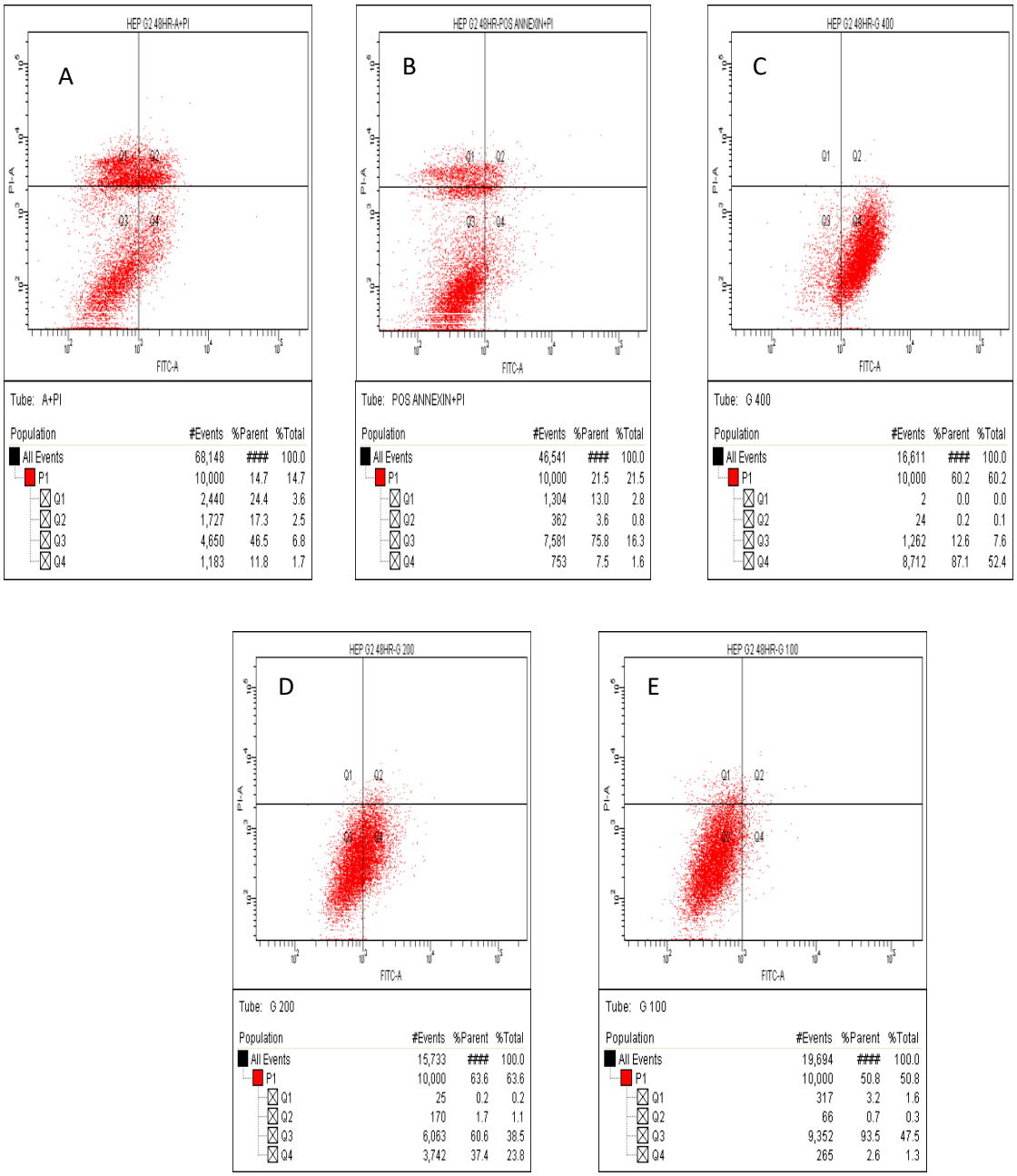

Fig. 4b Assessment of apoptosis by Annexin V/PI on human liver cancer cells (HEPG2). The cells were treated with media only A) Control, B) SAHA C) 400µM Garcinol, D) 200µM Garcinol & E) 100µM Garcinol, for 48 h and apoptosis was examined by flow cytometry (BD Biosciences Pharmingen) after Annexin V-PI double staining. Necrotic cells lose membrane integrity, permitting PI entry. Viable cells exhibit Annexin V (-)/PI (-); early apoptotic cells exhibit Annexin (+)/PI (-); late apoptotic cells or necrotic cells exhibit Annexin V (+)/PI (+).

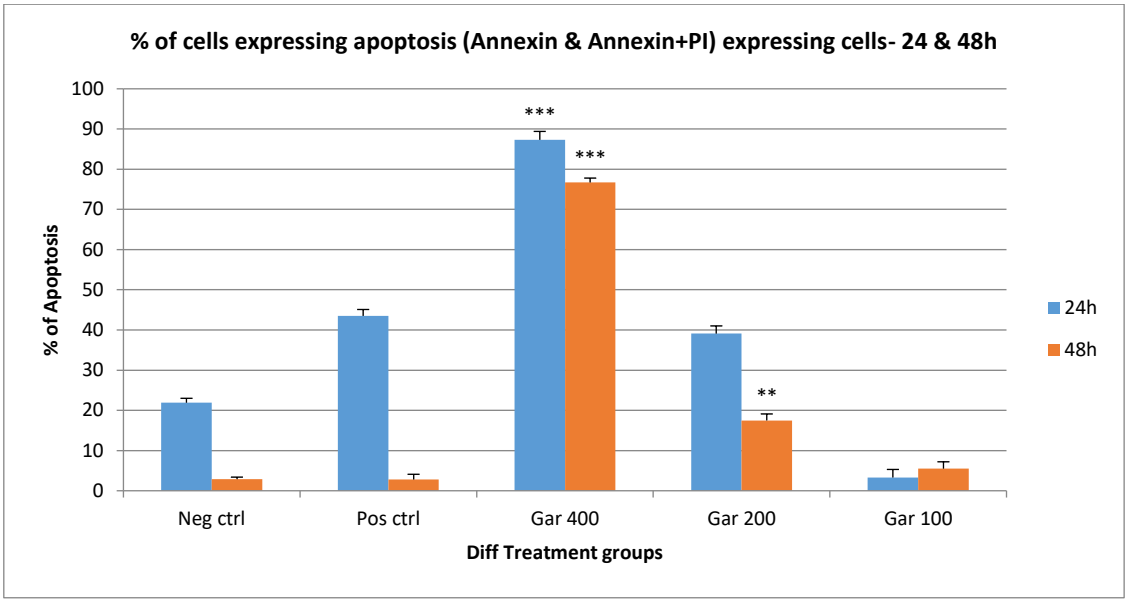

Fig. 4c **The percentage of apoptotic cells obtained from the Annexin V expression study in HepG2 cell lines: The Cells were treated with 25µM SAHA, 400µM, 200µM Garcinol and 100 µM Garcinol respectively.** Garcinol treated cells induced apoptotic cells approximately two times than that of SAHA (***p, 0.001-highly significant) in case of 24 hour time incubation. The apoptotic effect was highly significant (***p, 0.001) at 400 µM Garcinol and moderately significant (**p, 0.01) at 200 µM Garcinol compared to SAHA in case of 48 hour incubation.

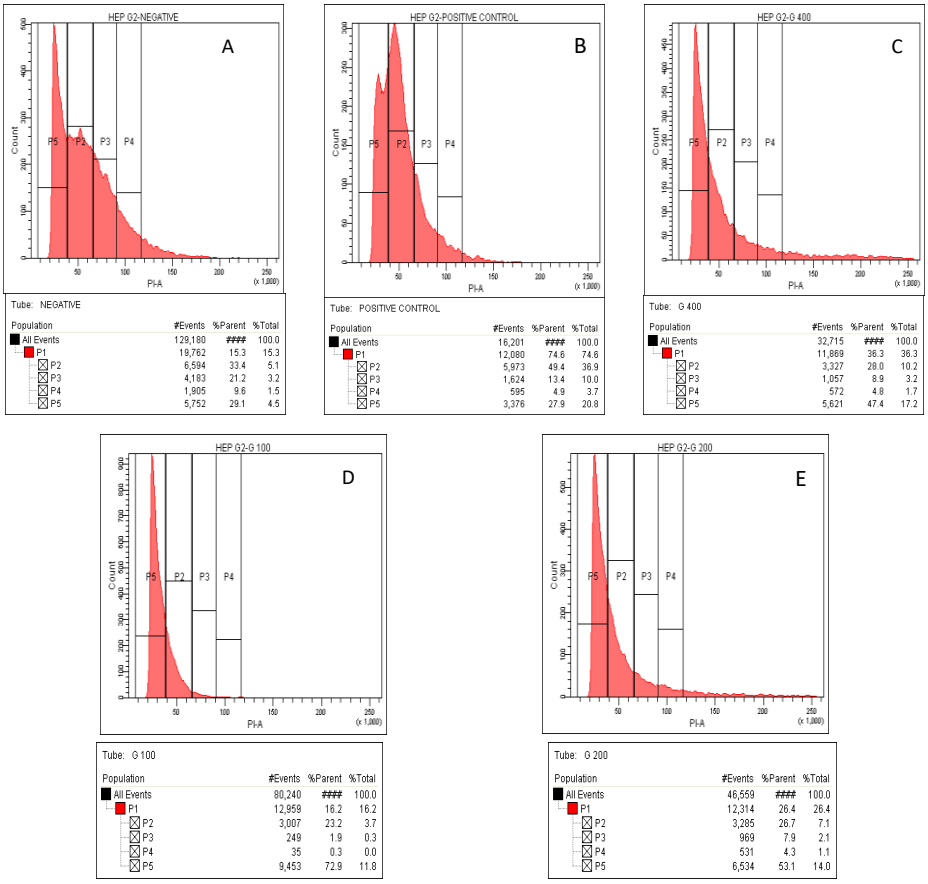

Fig. 5a Cell cycle distribution of human liver cancer cells (HEPG2): The cells were treated with media only A) Control, B) SAHA C) 400µM Garcinol, D) 200µM Garcinol & E) 100µM Garcinol,for 48 h and apoptosis was examined by flow cytometry (BD Biosciences Pharmingen)

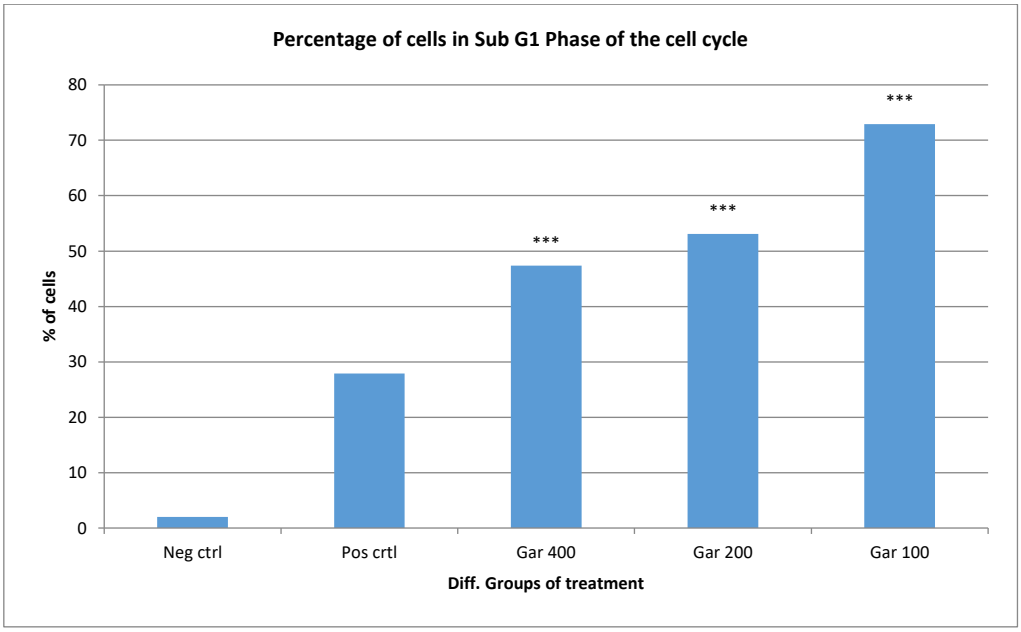

Fig. 5b Cell cycle distribution of human liver cancer cells (HEPG2): The cells were treated with media only A) Control, B) SAHA C) 400µM Garcinol, D) 200µM Garcinol & E) 100µM Garcinol,for 48 h and apoptosis was examined by flow cytometry (BD Biosciences Pharmingen). The 400, 200 and 100 µM of Garcinol have high significance (***p, 0.001) in arresting the cell cycle of the hepato carcinoma cells compared to that of SAHA.

DISCUSSION

Natural products remain an important source of new lead molecule and chemical entities. As stated by the report of World Health Organization, World's population increasingly depends on traditional medicine for health care needs. The market statistics states that approximately 60% plant-based anticancer drugs and medicinal plant products accounts for alternative strategies for cancer treatment [22], [23]. Cytotoxic screening of plants has been done to correlate the anticancer activity for their further scope in the drug development. Owing to potential benefits of plant-based drugs for cancer treatment, there is a tremendous increase in the use from 10% to 40%. Activation of apoptosis is the key molecular mechanism responsible for the anti-cancer activities. Defective apoptosis represents a major causative factor in the development and progression of cancer. Antitumour agents that can modulate apoptosis may be able to affect the steady state of cell populations that are helpful in the management and treatment of cancer. The ability of tumor cells to evade engagement of apoptosis can play a significant role in their resistance to conventional therapeutic regimens. Apoptosis induces cell shrinkage, chromatin condensation and margination at the nuclear periphery, with the eventual formation of membrane-bound apoptotic bodies containing organelles, cytosol and nuclear fragments, which are then phagocytosed without triggering inflammatory processes in the surrounding tissues in contrast with necrosis in which the cellular contents are uncontrollably released into the extracellular environment, causing damage to surrounding cells and a strong inflammatory response in the corresponding tissues[24].

SAHA is a lead Histone deacetylase inhibiting compound which is now under clinical trials. Garcinol shows anticancer activities in certain type of cancers like breast cancer, colon cancers, lung cancers and leukemias[25]. MTT assay of Garcinol was considered to check the cytoxicity of compounds. A significant decrease in cell viability was observed in HEPG2 cell lines. Comparitively, the maximum cytotoxicity was observed in HEPG2 cell line. Garcinol does not show significant cytotoxicity in normal preadipocytic 3T3L1 cells. Since the maximum cytotoxicity was seen in HEPG2 cells can be explored as potential candidate compounds in effectively reducing the proliferation and inducing cell death. So irrespective of tumor cell toxicity, tumor cell apoptosis is a more valuable tool. So, the compound toxicity in inducing apoptosis should be confirmed by different methods. The cytotoxicity of these compounds was confirmed by observing morphological features. Compounds treated with SAHA and Garcinol caused a reduction in proliferation and cell number inducing cell death. Morphological changes included shrinking of cells resulted in the loss of morphology and rounding of cells [26] .This was due to the shrinking of cytoplasm and nuclear fragmentation, as a result of cytotoxicity induced by the compounds. Clumping of cells was

also observed and this was due to the formation of membrane blebbing ultimately leading to fusion of cells. Changes in morphological features of HEPG2 cell line treated with compounds were studied. AO-EtBr staining a qualitative/ quantitative method for the detection of Apoptosis and this can be used to distinguish cells in different stages of apoptosis [27]. Cell death induced by Garcinol was confirmed by AO-EtBr dual staining. These fluorescent dyes were used to visualize morphological and internal changes of cells induced by the compounds. AO is permeable to both viable and nonviable cells while EtBr enters only damaged and dead cells, whose membrane integrity was lost [28]. Therefore, live cells appear as green and damaged cells as orange in colour. The compounds showed morphological and internal changes in HEPG2 cells with effective shrinkage, blebbing of membrane, condensed chromatin, fragmentation of nucleus and formation of apoptotic bodies. Hence, we would confirm the cytotoxicity induced the compounds ultimately induced apoptotic signals eventually leading to cell death in which SAHA as positive control with single concentration while Garcinol was used as dose dependent.

Chromatin condensation was an important feature of apoptosis [29]. Hoechst dye was a nuclear staining dyes and showed more specificity to condensed chromatin. It bound more to double stranded DNA regions with AT rich regions. Emission of Hoechst dye was at 460 nm [30]. This feature distinguished treated and untreated cells, the compounds produced more pronounced effects in causing chromatin condensation, more dye would get bound to condensed chromatin and this resulted in bright blue, fluorescent signal than untreated one. The compound showed positive effects on chromatin condensation and resulted in brighter and intense blue nucleus comparing to untreated cells. Fluorescent intensity varied with the concentration. So the effect of compound on chromatin condensation was confirmed and their role in inducing apoptosis was confirmed once again by Hoechst staining. To understand the nature of death, double labelling technique using fluorescent labelled Annexin V/Propidium Iodide was used to distinguish between apoptotic and necrotic cells. During the early stages of apoptosis, the cell membrane being intact, the cells are impermeable to Propidium iodide whereas during the later stages of apoptosis, the cells become permeable to both the dyes. On the contrary, the necrotic cells are permeable only to PI and not to Annexin for there is no Phosphadidyl serine (PS) translocated to the extracellular leaflet of the membrane to which Annexin binds. Effects of compounds to induce apoptosis was confirmed and apoptosis was quantified by Annexin V-FITC. Annexin V is a 35-36 kDa Ca2+ dependent phospholipid binding-protein and that showed high affinity for phosphatidylserine (PS) and bound to cells with exposed PS [31]. PS is a membrane phospholipid, and which translocated from inner to the outer leaflet of the plasma membrane during apoptosis. Annexin V was conjugated with PI and this form served as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis; cells that were viable were Annexin V-FITC and PI negative; Cells that were in early apoptosis were Annexin V positive and PI negative; and cells that were in late apoptosis or already dead were both Annexin V-FITC and PI positive[32]. The movement of cells through these three stages suggested apoptosis. From the data from Annexin V-FITC, the mechanism of cell death induced by the compounds was proved as through the induction of apoptosis. Percentage of Annexin expressed apoptotic cells in HEPG2 cells were graphically represented in fig: 11. The percentage of cells expressing apoptosis was found to be dosage dependant. The percentage of apoptotic cells increased from 5% to 85% at concentrations 100µM to 400µM. This data was in accordance with the experiment by Ahmad et al., in breast, prostate and pancreatic cancer cells [33]. So Garcinol can also be used in treatment of liver cancer. Cell cycle analysis is a method to understand the effect of the compounds on cell cycle phases which ultimately lead to cell cycle arrest. Cell cycle analysis was done in HEPG2 cells. Supporting with the results obtained from the above experiments, the DNA damage of the cells induced by the compounds were confirmed by the results obtained from cell cycle analysis. Cell cycle analysis showed S phase arrest and so DNA damage and apoptosis were confirmed

CONCLUSION

The results prevailed from the current study conclude that Garcinol possess anticancer activity as well as were less cytotoxic to normal cells and also, comparable with those obtained using SAHA, which is a clinically used HDAC inhibitor. The compound was found to reduce cell proliferation by causing S phase arrest in the cell cycle and induced apoptosis in liver cancer cells which was evidenced by the microscopic observations. The effective result for Annexin V expression in cancer cells which proved apoptosis as the mechanism of cell death. So further studies will be very promising on the compound, Garcinol or its derivatives which can be further exploited as specific anticancer agents.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interest.

AUTHORS CONTRIBUTIONS

"Dr. Lakshmi. S and Dr. A Jayakumaran Nair conceived this research and designed experiments; Dr. Lakshmi. S participated in the design and interpretation of the data; Miss. Athira Venugopal and Miss. Liz Thomas performed experiments and analysis; Dr. Lakshmi. S and Miss Shehna Sharaf wrote the paper and participated in the revisions of it. All authors read and approved the final manuscript."

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