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A novel antioxidant rich compound 2-hydoxy 4-methylbenzaldehyde from *Decalepis arayalpathra* induces apoptosis in breast cancer cells

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ABSTRACT

Decalepis arayalpathra is an endangered and endemic medicinal plant, mainly used for its aromatic and ethnomedical properties. In this study, we reported the biomedical potentials of 2-hydoxy 4-methylbenzaldehyde (2H4MB) from *D. arayalpathra*. The compound 2H4MB was isolated from fresh root barks of the plants using steam-hydro distillation extraction method and quantified using HPLC analysis, which yielded 6.5 ± 0.8 μg g^{-1} of purified compound. Further, the antioxidant and anticancer effects *in vitro* were studied in detail using MDA-MB-231 cells by cell-based assays. 2H4MB was observed to display the features such as generation of reactive oxygen species (ROS), improved cytotoxicity, loss of mitochondrial membrane ($\Delta\psi_m$) potential, and nuclear DNA fragmentations, cellular apoptosis and cell cycle arrest. Further, the bioavailability of 2H4MB may facilitate its use as a potential cancer therapeutic agent.

1. Introduction

Decalepis arayalpathra is a monospecific genus of the family Periplacaceae, a leafy, perennial under a shrub, found in small groups. They are endemic to a few forest segments of Tirunelveli, Kanyakumari and Thiruvananthapuram districts of Tamil Nadu and Kerala (Gangaprasad et al., 2005). Plant parts are mainly used as an effective remedy to cure peptic ulcers, cancer-pathologies and as a tonic to regain the lost strength and stamina; the plant roots have wide-ranging therapeutic properties (Gangaprasad et al., 2005; Nair et al., 2014). As the plant has been enlisted as an endangered species, there is an urgent need to conserve and propagate this under a shrub on a large scale due to its ethnomedicinal potentials and bioavailability of plant constituents. In recent years, the natural products and food bioactive molecules derived from medicinal plants are widely used for treating a variety of symptoms and diseases (Lin et al., 2008).

Cancer, a disease which is the third major leading cause of death worldwide, accounting for about 6 million deaths with 16 million new cases every year (Ferlay et al., 2015; Miller et al., 2016). Globally, breast cancer is the most common type of cancer that occurred in women, and it is reported to be the second most cause of death. The steroid hormone estrogen, primarily estradiol plays a vital role in carcinogenesis of breast cancer (Ferlay et al., 2015; Miller et al., 2016). Triple-negative breast cancer (TNBC) is the heterogeneous group of cancer, which remains a considerable therapeutic challenge in the absence of defined targeted therapies. Conventional treatments such as chemotherapy, radiation, and surgery have well documented limitations, complications and adverse effects necessitating researchers to evolve promising anticancer agents either through synthetic or natural routes with distinct anticancer mechanisms. Chemotherapeutic options to treat TNBC suffer demerits due to non-specificity, drug resistance and less bioavailability. A promising anticancer drug candidate is characterized to induce oxidative stress in tumor or cell environment resulting in cellular damage through substantial DNA damage (McLean et al., 2008). It is also reported that the intake of bioactive molecules through consumption of phyto-based food substances or uptake of natural or synthetic

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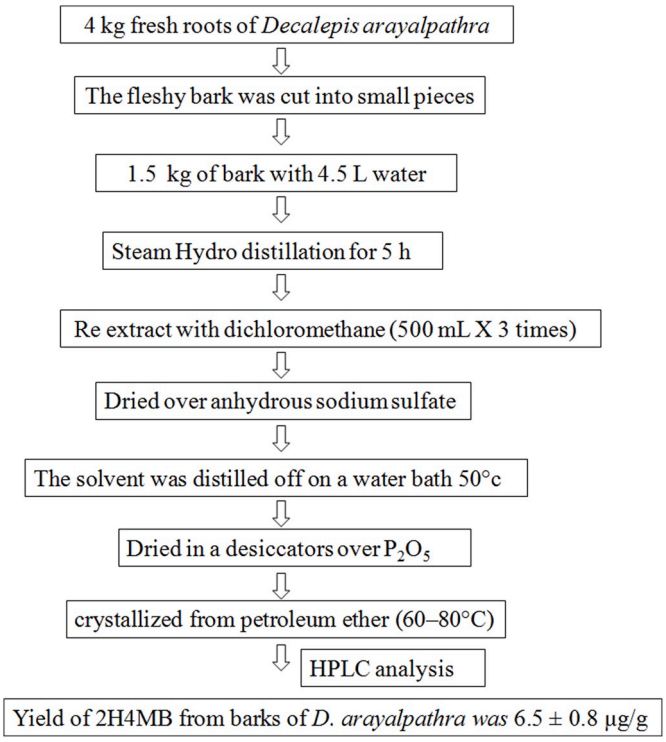

Fig. 1. Steps in the isolation of aroma rich compound 2-hydroxy 4-methyl-benzaldehyde (2H4MB) from roots of *Decalepis arayalpathra.*

derivatives of such bioactive molecules may generate reactive oxygen species (ROS) in tumor cells resulting in apoptosis (Trachootham et al., 2009; Liou and Storz, 2010).

Therefore, in this study, we attempt to study ROS accumulation and subsequent DNA damage as well as anti-proliferative, apoptotic and cytotoxic effects in MDA-MB-231 breast cancer cells treated with the compound 2H4MB derived from *D. arayalpathra* root barks. Besides, the safety of the compound is established in this study as it does not cause any pathological changes in the normal healthy cells (controls), suggesting the nutraceutical potential of the study compound.

2. Experimental section

2.1. Plant material

Approximately 4 kg of fresh roots of *D. arayalpathra* were harvested from plants planted in near simulated natural conditions of Ethno-Medicinal Garden (EMG), FRLHT, Bangalore, India. The prepared herbarium of plant species was deposited at FRLH Herbarium, Bangalore for future references.

2.2. Extraction of 2H4MB from D. arayalpathra

Extraction of 2H4MB was carried out by steam hydro-distillation process as described previously (Nagarajan and Jagan Mohan Rao, 2003). Briefly, the fresh roots of *D. arayalpathra* (~4 kg) were collected from Ethno-Medicinal Garden and subjected to manual cleaning with flowing tap water. The fleshy root barks were separated from the hard-inner core, and further cut into small pieces. The small fleshy pieces of bark were soaked immediately in water to prevent the loss of volatile aroma substances. Then, ~1.5 kg of barks in 4.5 L water was subjected to steam hydro-distillation process for about 5 h. The generated volatiles along with steam condensate were collected and extracted using dichloromethane (500 mL x 3). The combined extract was further dried over anhydrous sodium sulfate. The solvent was distilled off on a water bath <50 °C and the residual was dried in a desiccator using P_2O_5 and paraffin wax to obtain the volatile oil. This was crystallized from petroleum ether (60–80 °C) and quantified using HPLC (Fig. 1).

2.3. Quantification of 2H4MB

Quantification of compound 2H4MB from a crystallized extract of

Table 1
Isolated compound 2H4MB in free radical generation assessed using antioxidant activity.

Antioxidant activity	IC_{50} (mg mL^{-1})
DPPH	0.5 ± 0.04
Hydroxyl	1.9 ± 0.09
Superoxide	2.1 ± 0.1
Nitric oxide	0.53 ± 0.03

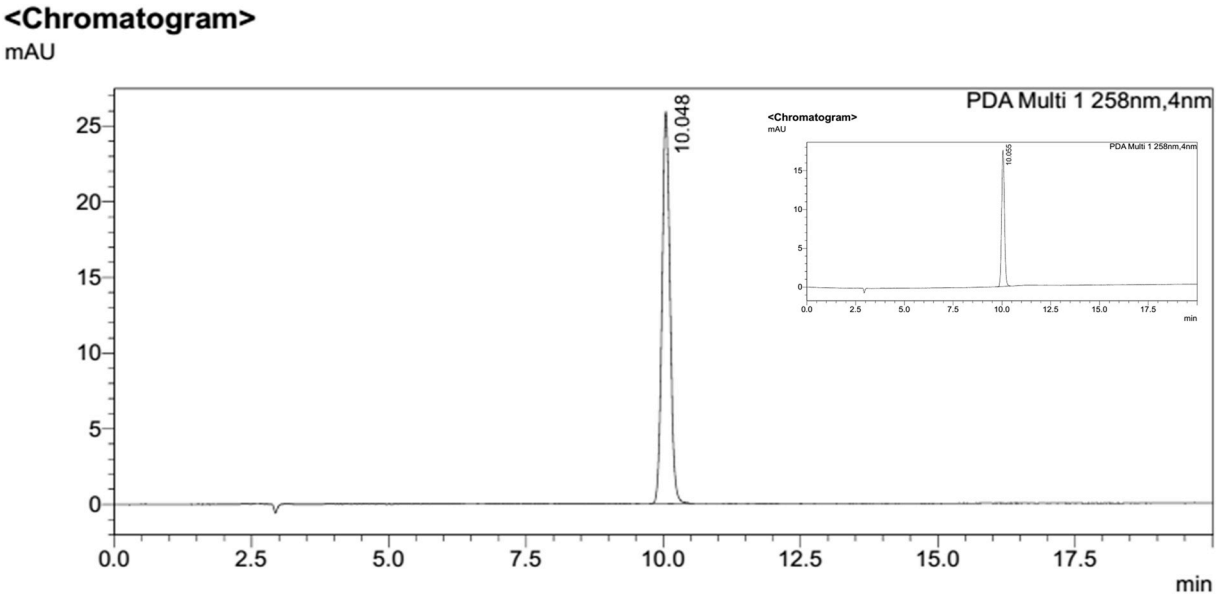

Fig. 2. Reverse phase HPLC chromatograms showing the resolution of 2H4MB, purified from roots of *D. arayalpathra,* with peak identity (detected at 220 nm) as compared with standard (inset figure).

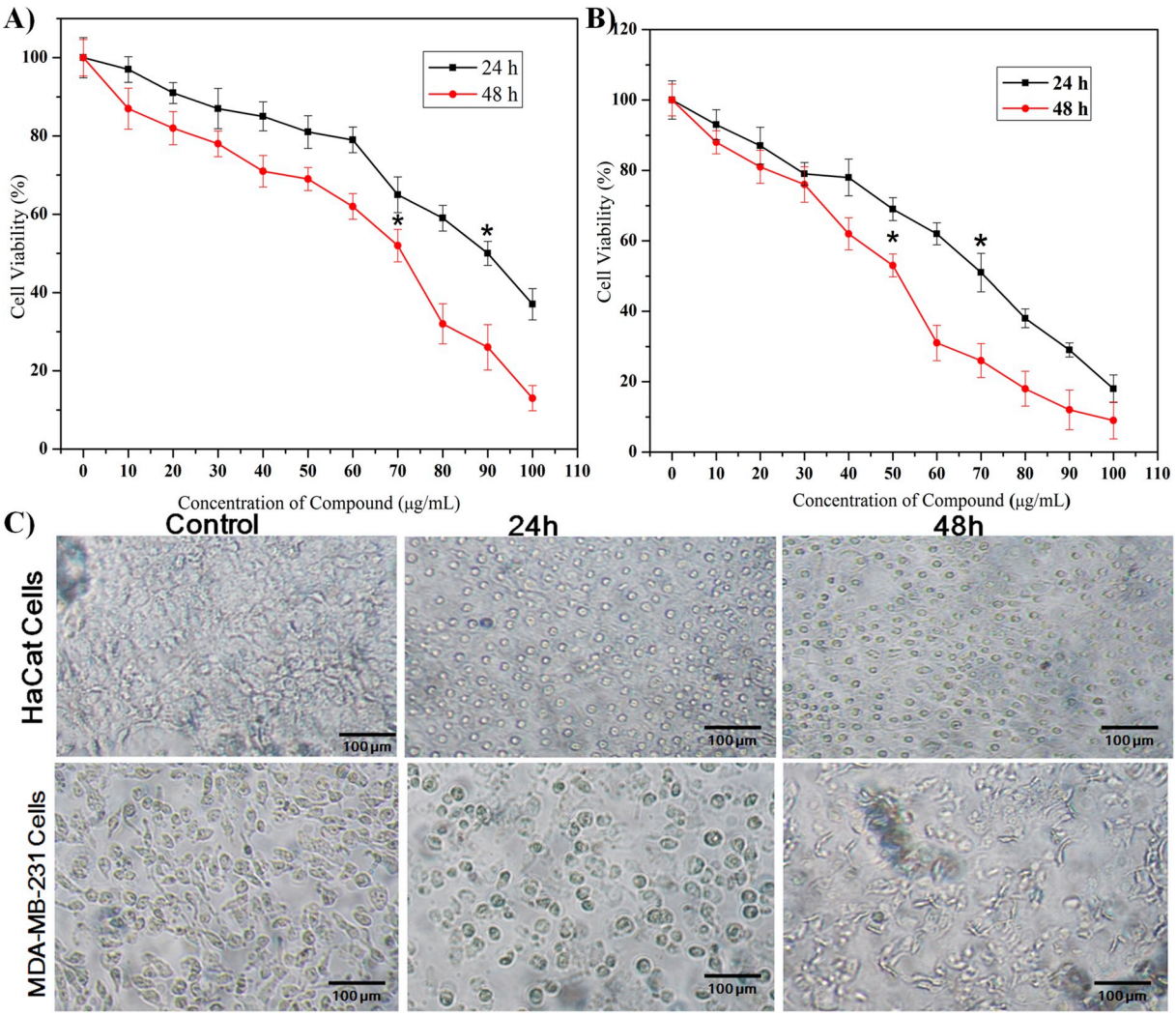

Fig. 3. (A, B) Cytotoxicity effects of 2H4MB in human normal (HaCat) and metastatic breast cancer (MDA-MB-231) cells during 24 h and 48 h of incubation (A and B). These cells are treated with series of dilutions (10–100 μg mL^{-1}) of isolated compound 2H4MB to study the dose dependent cytotoxicity in 24 and 48 h time intervals by MTT assay. The isolated compound effectively inhibits cell viability of MDA-MB-231 cells at the IC_{50} concentration of 70 μg mL^{-1} and 50 μg mL^{-1} in 24 and 48 h respectively; it shows less cytotoxicity in HaCat cells (90 μg $mL^{-1}$and 70 μg mL^{-1}) during 24 and 48 h incubation duration. This assay was performed in triplicates, mean ± SD. *$p \leq 0.05$ significant, as compared with cell control. **(C)** The microscopic images show the typical morphological apoptotic features in MDA-MB-231 cells at their IC_{50} concentrations. The image analysis of 2H4MB treated HaCat and MDA-MB-231 cells displays normal cell morphology in control group (untreated) whereas treated cancer cells shows shrinkage in cell morphology, membrane blebbing and echinoid shaped cell structures at different concentrations.

D. arayalpathra was estimated using Reverse Phase HPLC method as described previously (Sircar et al., 2007). The concentration of 2H4MB was determined by high-performance liquid chromatography (HPLC) after appropriate dilution with the mobile phase, an isocratic mixture of aqueous 1 mM TFA and methanol, in the ratio of 70:30 (v/v). The HPLC system was equipped with Waters HPLC 525 Pump, Waters 2998 Photodiode Array detector and Rheodyne 7725i manual injector. The chromatographic separation were performed on Symmetry C-18 analytical Column (250 mm × 4.6 mm, 5 μm) with an appropriate standard molecule, at each concentration level, and the isolated compound was compared with a standard to confirm the purity.

2.4. Antioxidant assays

2.4.1. Free radical-scavenging assay

The antioxidant activity of purified 2H4MB was assessed using DPPH, as described by Lim et al. (2007). The purified compound was added with PBS and 180 μL of DPPH solution. After 30 min. of incubation, the absorbance was measured at 492 nm using multiwell plate reader (Thermo Multiskan, Waltham, MA). The ability of free radical scavenging was calculated using the below formula:

$$\text{Scavenging } (\%) = \frac{1 - Abs.sample - Abs.blank}{Abs.control} \times 100$$

L-ascorbic acid was used as positive control.

2.4.2. Nitric oxide radical-scavenging assay

The nitric oxide (NO) scavenging ability of the purified compound (2H4MB) was determined according to the previous report (Tsai et al., 2007). Briefly, 60 μL of different concentrations of the serially diluted compound was pipetted into a 96-well plate. Following this, 60 μL of 10 mM sodium nitroprusside is dissolved in PBS, and the plate was incubated under light at room temperature for 150 min. After incubation, an equal volume of Griess reagent was added in order to quantify the nitrite contents. The NO scavenging ability of 2H4MB was expressed as IC_{50}, which denoted the concentration of the compound required for quenching 50% of NO radicals released by sodium nitroprusside. Here, L-ascorbic acid is used as a control.

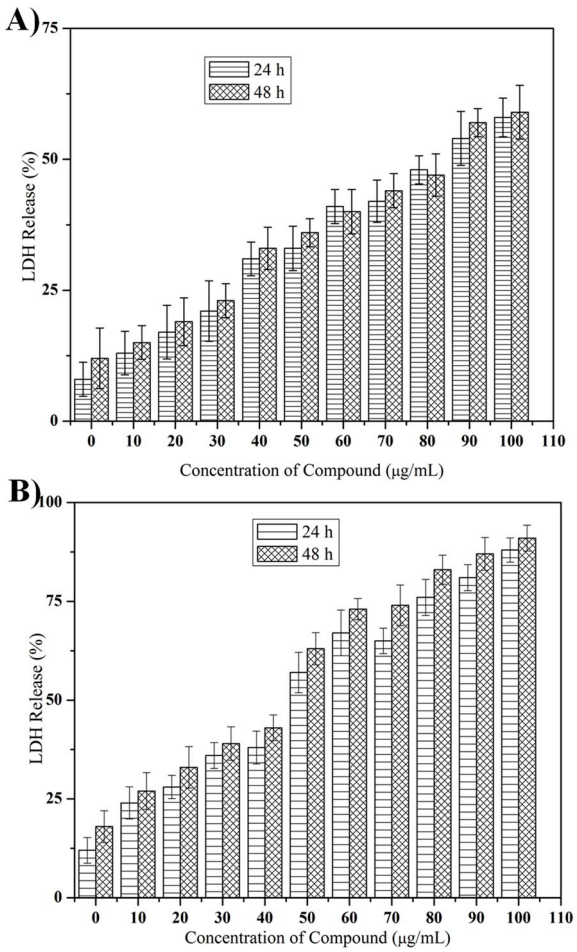

Fig. 4. Assessment of 2H4MB on cell membrane damage by measuring the release of LDH 24 and 48 h at different concentrations. **(A)** Positive Control group cell line (HaCat) shows less degree of membrane damage as evidenced by lower LDH release whereas the level of LDH is significantly higher in cancer cell (MDA-MB-231) group **(B).** The study indicates minimal extent of membrane damage in normal cells when compared against breast cancer cells. The results are expressed in Mean ± SD of triplicate values.

$$\text{Scavenging } (\%) = \frac{1 - Abs.sample - Abs.blank}{Abs.control} \times 100$$

2.4.3. Hydroxyl radical-scavenging activity assay

The hydroxyl radical scavenging ability of purified compound (2H4MB) was investigated as per the method reported by Costa et al. (2010). Hydroxyl radicals were generated using 3 mL sodium phosphate buffer (150 mM, pH 7.4) containing 10 mM $FeSO4{\cdot}7H_2O$, 10 mM EDTA, 2 mM sodium salicylate, 30% H_2O_2 (200 mL) and varying compound concentrations. In control, sodium phosphate buffer was replaced with H_2O_2. The solutions were then incubated at 37 °C for 1 h, and the presence of the hydroxyl radical was detected by measuring the OD at 510 nm. Catechin was used as positive control.

$$\text{Scavenging } (\%) = \frac{1 - Abs.sample - Abs.blank}{Abs.control} \times 100$$

2.4.4. Superoxide radical scavenging assay

This assay was conducted to assess the capability 2H4MB to inhibit photochemical reduction of nitroblue tetrazolium (NBT) in riboflavin-light-NBT system, as followed by an earlier report of Costa et al. (2010). Each 3 mL of reaction mixtures (13 mM methionine, 2 mM riboflavin, 100 mM EDTA, 75 mM NBT) was prepared which contained 50 mM PBS (pH 7.8), and 1 mL sample solution. Due to formation of formazan crystals, there was an increase in absorbance at 560 nm. L-ascorbic acid was used as positive control.

$$\text{Scavenging } (\%) = \frac{1 - Abs.sample - Abs.blank}{Abs.control} \times 100$$

2.5. Anticancer assays

2.5.1. Cell viability assay

The MDA-MB-231 (metastatic triple negative breast cancer) and HaCat (keratinocyte) cells were cultured in DMEM and supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 µg mL^{-1} streptomycin, and maintained at 37 °C with 5% CO_2. The cell viability of the compound 2H4MB was studied using MTT cell viability assay (Mosmann, 1983). Briefly, the MDA-MB-231 and HaCat cells were cultured and seeded at the density of 1×10^4 cells/mL in 96-well tissue culture plates for 48 h to reach 70–80% confluence. After treating with 2H4MB (2-hyroxy 5-methylbenzaldehye) (dilutions of 10–100 µg mL^{-1}), the plates were incubated for 24 and 48 h in triplicates. An untreated group was used as a negative control, and the normal cell line was used

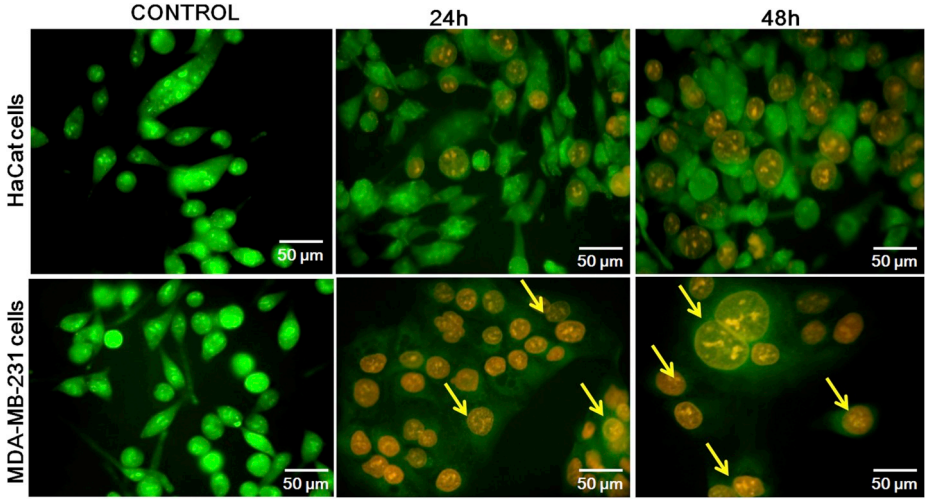

Fig. 5. Fluorescent microscopy images of acridine orange/ethidium bromide staining of 2H4MB treated MDA-MB-231 cells reveal the induction of apoptosis at IC_{50} concentrations. Active viable cells (green), dead cells (red), and apoptotic cells (orange; as indicated by arrow) are shown. Experiments were performed in triplicates keeping untreated cells as control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

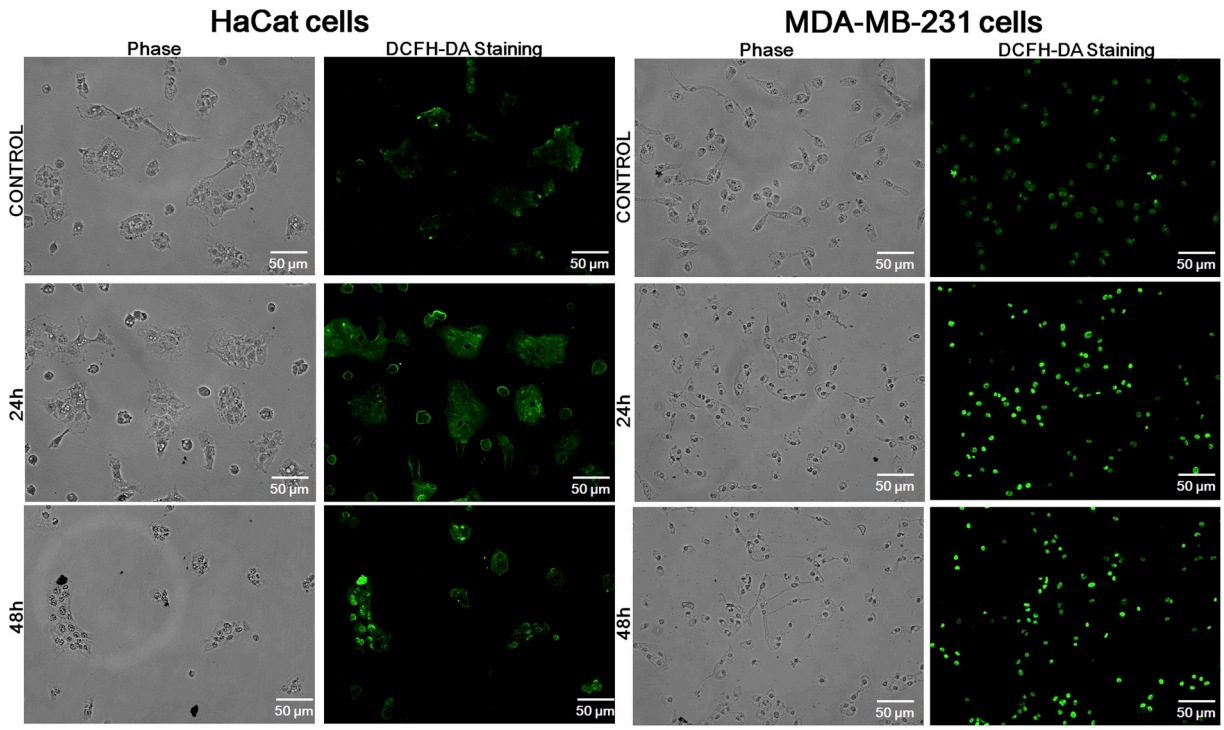

Fig. 6. 2H4MB facilitates generation of intracellular ROS and mitochondrial dysfunction in MDA-MB-231 cells during treatment with dose dependent concentrations for 24 and 48 h. (A) 2H4MB does not have any significant effect on normal cells (HaCat) as evidenced by absence of ROS production upon treatment of cells. (B) The MDA-MB-231 cells incubated with compound for 24 and 48 h reveal ROS generation when compared to normal and compound untreated cells as visualized by fluorescence images of DCFH DA stained cells.

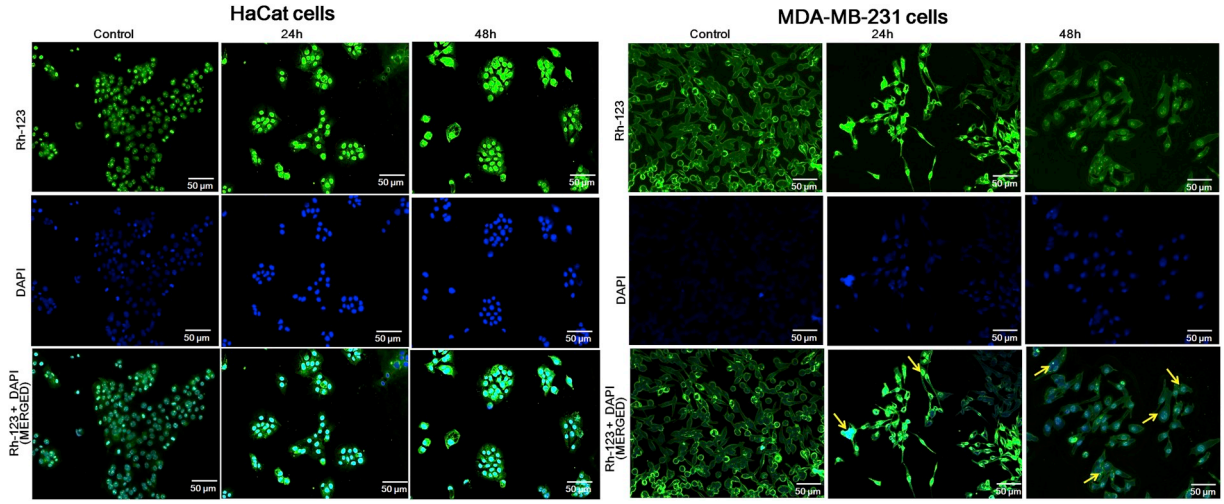

Fig. 7. Analysis of mitochondrial membrane potential loss and nuclear fragmentation by fluorescence microscopy through treating HaCat cells **(A)** and MDA-MB-231 cells **(B)** with the compound 2H4MB at dose dependent IC_{50} concentrations in 24 and 48 h of incubation. The panels of images indicate shows loss of mitochondrial membrane potentials through Rhodamine 123 (green) staining (indicated by arrow) and nuclear fragmentation by DAPI staining. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

as positive control. Further, the treated cells in each well were added with 100 µL of MTT solution (5 mg mL^{-1}), and incubated for another 4 h at 37 °C in 5% CO_2 incubator to allow MTT reduction. The formazan crystals formed by cells were dissolved using 100 µL of DMSO, and the optical density (OD) was measured using micro-titer plate reader at 570 nm. The percentage of viable cells was calculated as follows:

$$Cell\ Viability\ (\%) = \frac{OD\ of\ treated\ cells}{OD\ of\ control\ cells} \times 100$$

2.5.2. LDH assay

Lactate Dehydrogenase (LDH) assay was used to analyze the conversion of lactate to pyruvate in the presence of LDH with a parallel reduction of NAD^+ during the release of LDH from the cell membrane of cancer cells. The cultured cell suspensions of 1×10^5 cells/mL were seeded in six well plates and grown for 48 h under 5% CO_2 at 37 °C. After 48 h, the cells were treated with series of 10–100 µg mL^{-1} compound 2H4MB and incubated for 24 and 48 h to observe cytotoxicity. Then, the medium was collected, and the dead cells in the medium were transferred to the fresh centrifuge tubes. To supernatant and cell suspensions

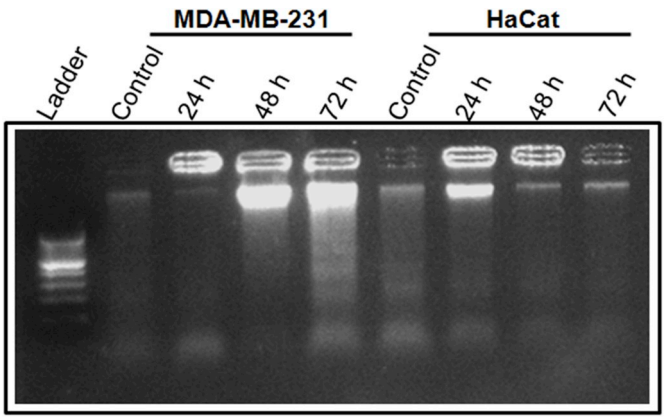

Fig. 8. The nuclear DNA fragmentation analysis of 2H4MB treated HaCat and MDA-MB-231 cells at IC_{50} concentration for 24, 48 and 72 h of incubation shows DNA laddering pattern of cells indicating the occurrence of apoptotic event.

of each group), the LDH reduction chemicals were added and incubated at 37 °C for 15 min. The cell membrane damage was analyzed by LDH leakage measurements from dead cells, which also indicated the percentage of cytotoxicity in compound treated cells. The OD of formazan solution was measured on a microplate reader at 570 nm after dissolving with DMSO. The cytotoxic effects of compound treated wells were studied in terms of the release of LDH along with untreated control.

2.5.3. Fluorescence microscopy analysis for apoptosis induction

To assess the extent of apoptosis induction in MDA-MB-231 and HaCat cells, the cells were seeded on 13 mm coverslips, placed in 6-well plates (Coster, USA) with a density of 1.5×10^5 cells/well, and grown for 48 h. The cells were further treated with its dose dependent IC_{50} concentrations. The cells were washed with PBS and stained using equal concentration of 20–50 µL of acridine orange and ethidium bromide (AO/EB) combinations. The stained cells were immediately visualized under fluorescence microscopy with an excitation filter at 480 nm to detect the live (green), apoptotic induced (orange) and necrotic (red) cells upon treatment with 2H4MB.

2.5.4. Loss of mitochondrial membrane potential (ΔΨm) analysis

The MDA-MB-231 and HaCat cells were cultured in six-well plates (2×10^5 cells/well) and allowed to reach 60–70% confluence for a day before treatment with dose dependent IC_{50} concentrations of 2H4MB. After the given time intervals (24 and 48 h), the treated cells were fixed using Acetic acid: Methanol (1:3) combination, further washed twice with PBS, and exposed to $\Delta\psi_m$ specific Rhodamine 123 (Rh-123) stain (10 µg mL^{-1}) for 20 min at ambient temperature in dark. The stained cells were washed with absolute methanol, then evaporated, and analyzed for changes in mitochondrial membranes using fluorescence microscope with an excitation and emission wavelengths of 505 nm and 534 nm, respectively.

2.5.5. Nuclear DNA damage fragmentation analysis

The nuclear DNA damage was analyzed by genomic DNA fragmentation assay, in which the cells were treated with dose dependent concentrations of 2H4MB for 24 h, 48 h and 72 h. The treated cells were added with 10 mL of a solution containing 10 mM Tris HCl, 10 mM EDTA (pH 8.0) and 20 mg mL^{-1} proteinase K for isolation of fragmented DNA. Mixtures were incubated at 37 °C for 3 h, followed by DNA extraction with phenol: chloroform: isoamyl alcohol solution (25:24:1). Then the extracted DNA was treated with DNase free RNase at a concentration of 20 mg mL^{-1} at 4 °C for 45 min and precipitated with 100 mL of 2.5 M sodium acetate and 3 volumes of ethanol. Subsequently, DNA fragmentation analysis was carried out using an electrophoresis for 45 min at 100 V on a 2% agarose gel and visualized under Gel-Doc System (Alpha Innotech Image viewer, version 6.0.0, Japan).

2.5.6. Analysis of nuclear condensation using DAPI staining

The MDA-MB-231 and HaCat cells ($\sim 2 \times 10^5$ cells/well) were cultured in six well plates and treated with their dose dependent IC_{50} concentrations of 2H4MB for 24 and 48 h, including the appropriate control group (without compound treatment). Further, the cells were washed thrice with PBS and subsequently with acetic acid: methanol combination (25:75) for 20 min at room temperature. Fixed cells were stained with 2.5 µg mL^{-1} of DAPI under dark condition. After staining, the cells were washed with methanol and dried. The stained plates were visualized under fluorescence microscopy.

2.5.7. Detection of reactive oxygen species and cell apoptosis

The generation of intercellular ROS from cells during oxidation at intracellular level was analyzed with the ROS-sensitive 2′,7′-dichlorodihydrofluorescein di-acetate using fluorescence microscopy. One day before the treatment of cells, the selected cancer and normal cells were seeded in 6-well plates at a density of 2×10^5 cells/well. Prior to treatment, the adherent cells were washed with PBS, and stained with 50 µM dye diluted in methanol. Subsequently, the cells were incubated at 37 °C for 30 min to allow incorporation and activation of DCF-DA, followed by removal of free dye by washing with methanol. These stained plates were analyzed for fluorescence intensity (485/530 nm) using Floid Cell Imaging Station, Life Technologies.

2.5.8. Flow cytometry analysis

The measurement of the cellular DNA content in various cell cycle phases was carried out using flow cytometry. In brief, the MDA-MB-231 cells (1×10^6) were seeded in culture dish and allowed to attach for 24 h. Then the cells were treated with IC_{50} concentrations of 2H4MB for 16, 24, and 48 h of incubation. Further, the treated cells were harvested by trypsinization and pelleted out 2500 rpm for 5 min at room temperature. The cells were re-suspended in 300 µL of PBS–EDTA to which 700 µL of chilled 70% ethanol was added drop-wise with slow mixing. The solution was added gently to ensure complete mixing of ethanol, and the samples were stored at 0 °C overnight. Subsequently, 1:100 volumes of 20 mg mL^{-1} RNase were added to the cell mixture, and the mixture was incubated at 37 °C for 1 h. The freshly prepared propidium iodide (50 µg ml^{-1}) was added to a final concentration and it was incubated for 10–20 min. The stained cells were analyzed for DNA histograms and the cell cycle phase distribution analysis was performed using flow cytometry (Becton Dickinson Immuno cytometry System) (Thangam et al., 2012).

2.6. Statistical analysis

All the data are expressed as mean ± S.D. of three independent experiments. The IC_{50} values were calculated with the help of Graph-Pad Prism software Version 5.0 (Graph-Pad Software, Inc., USA). *$p \leq 0.05$ values were considered to be statistically significant.

3. Results and discussion

3.1. Quantitative and qualitative analysis

The study compound 2H4MB, extracted and purified from the roots of *Decalepis arayalpathra,* is an aromatic aldehyde and is structurally similar to vanillin, which is an important flavoring agent in food industry. This study derives importance as 2H4MB may be used as a potential substitute for vanillin. Thus, the study focuses on the purification of 2H4MB by reverse phase HPLC (Fig. 2) analysis as well as evaluation of the compound for anticancer features through specific induction of apoptosis in cancer cells. Our phytochemical analytical results showed that the fleshy root bark contained 6.5 ± 0.8 µg g^{-1} of 2H4MB and the

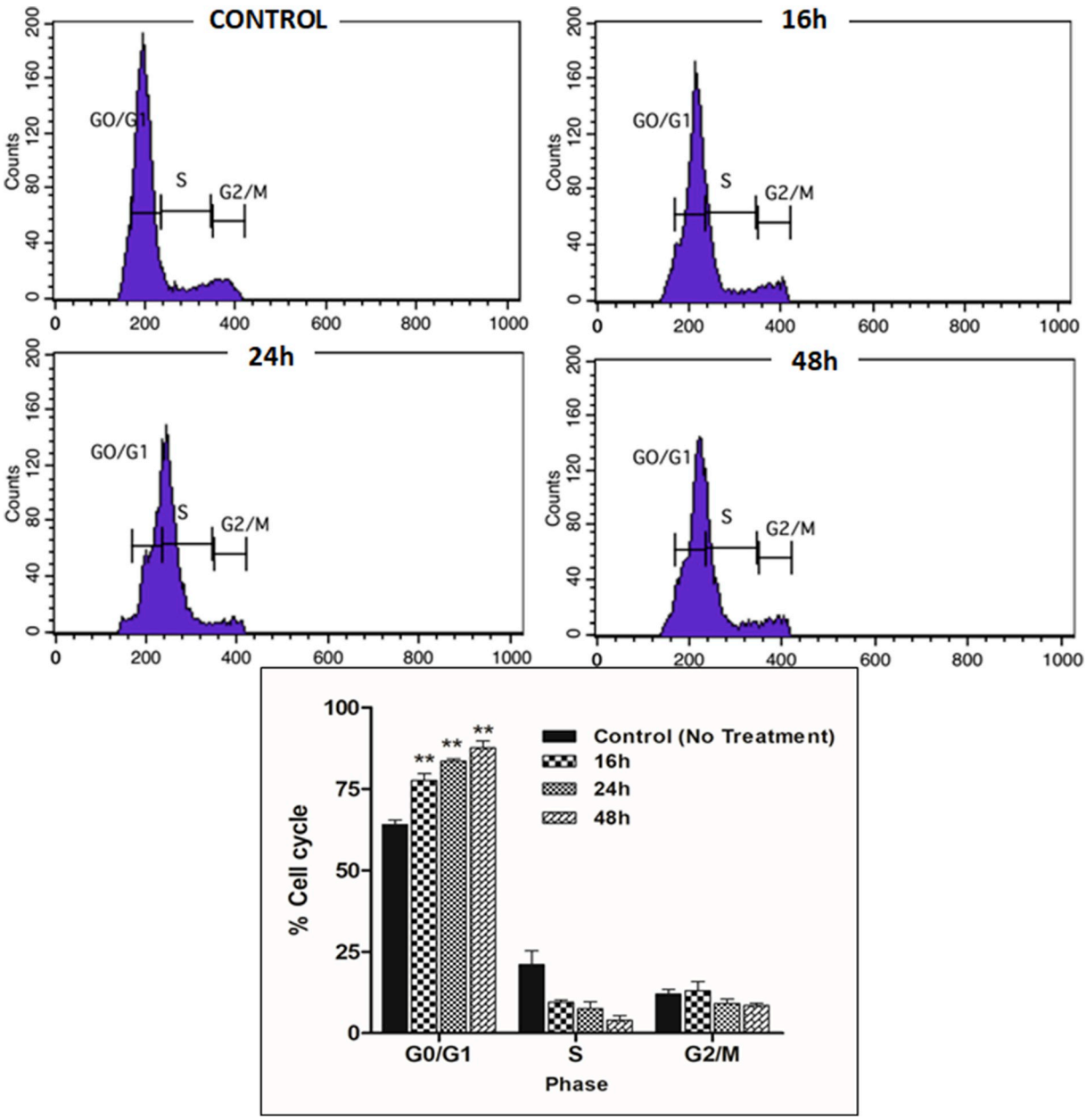

Fig. 9. Cell cycle analysis by flow cytometry. Cell cycle population (%) of cancer cells treated against 2H4MB at IC_{50} concentrations in different time intervals (16, 24, 48 h) as studied by flow cytometry with respective control group. The histogram shows cell population in different phases of cell cycle as compared to cell control group (**$p \leq 0.05$). Cell cycle arrest in the G0/G1 phase is associated with an increased abundance of cell distributions due to apoptotic induction as compared to untreated group.

obtained purity is 84.6 ± 6.6 in crystallized extracts as assessed by RP-HPLC, and these observations were in line with previous reports (Verma et al., 2014).

3.2. Antioxidant activities

Display of antioxidant feature is one of the important therapeutic strategies for various diseases. The bioactive plant products are being used as a potent source of developing antioxidant therapeutic strategies due to their minimal pathological and toxic effects in contrast to synthetic drugs (Singh and Bhat, 2003; Ling et al., 2013). Besides, the emerging therapeutic approaches combine the antioxidant feature along with other specific functions such as modulations in different cellular and metabolic pathways including signaling cascades, regulation of apoptosis through altered gene expressions distinguishing the normal and pathological conditions including various types of cancers (Srivastava and Shivanandappa, 2009). In this present study, we demonstrated both antioxidant and regulation of apoptosis events that distinguish normal and cancer cells through detailed studies on free radical scavenging assays, cytotoxicity, apoptotic induction, and cell cycle analysis using the at isolated compound 2H4MB.

The DPPH radical scavenging property of the compound from *D. arayalpathra* is presented in Table 1 and it is shown that the compound 2H4MB exhibits strong free radical scavenging effects with an IC_{50} of 0.5 ± 0.04 mg mL^{-1}. It was reported that the hydrogen-donating ability or of the antioxidant molecules might contribute to the free radical scavenging or antioxidant feature (Chen and Ho, 1995).

The superoxide radicals, usually generated during normal physiological processes mainly in mitochondria, initiate lipid peroxidation and cell membrane damage (Valko et al., 2007; Sundaresan et al., 1995). It is

shown in the study that 2H4MB inhibits superoxide radical significantly at the IC_{50} of 2.1 ± 0.1 mg mL^{-1} (Table 1). In biological systems, the nitric oxide (NO) is generated by the catalytic action of nitric oxide synthase (NOS) on L-arginine and NO is an important endothelium-derived relaxation factor (Tsuchiya et al., 1999). However, the role of NO is indicated in various pathological conditions as it is known to injure cells and tissues at high concentrations (Sueishi et al., 2011). The study compound 2H4MB has a potent scavenger of nitric oxide and the NO scavenging effect is detected at IC_{50} of 0.53 ± 0.03 mg mL^{-1}. Besides, the compound displays significant hydroxyl radical scavenging ability (Table 1) and thereby it protects the cells from DNA damage due to hydroxyl radicals (Aruoma et al., 1998; Moorhouse et al., 1985).

3.3. Assessment of cytotoxicity

2H4MB was analyzed for its inhibitory effects on triple negative breast cancer cells (MDA-MB-231) and normal (HaCat) cells by MTT assay. The MDA-MB-231 and HaCat cells were treated with 2H4MB for about 24 h and 48 h. It was observed that the molecule effectively inhibited cell proliferation with its potent cytotoxicity having IC_{50} values of about 70 μg mL^{-1} (24 h) and 50 μg mL^{-1} (48 h) against breast carcinoma cells; however, it was noted to be a less toxic against normal cells as evidenced by its IC_{50} values of 90 μg mL^{-1} (24 h) and 70 μg mL^{-1} (48 h) (Fig. 3). Importantly, the compound showed weaker anti-proliferative effects in normal cells as compared to cancer cells as observed by the results of MTT assay (Fig. 3C). The changes in cell morphology were also analyzed by microscopic studies after the compound treatment and it was shown that the cancer cells were exhibiting the characteristic apoptotic morphological changes such as cell shrinkage, membrane blebs etc. (Fig. 3C). It was also observed that the changes in morphological features between compound 2H4MB treated cells (MDA-MB-231), normal cells (HaCat) and untreated (control) cells (Fig. 3C) as these were prominent in cancer cells when compared to normal and untreated control cells. This *in vitro* result supports the use of this compound 2H4MB as a potent anticancer lead.

The LDH assay to evaluate the dose-dependent inhibitory effects of the study compound was performed by treating the cells for 24 h and 48 h (Fig. 4). The results showed higher release in LDH in the cancer cell group as these cells displayed extensive cell membrane damage (Fig. 4b). The mechanism of anticancer activity of the compound is not studied and the study results demonstrate the potential anticancer property of 2H4MB through both MTT and LDH assays (Fig. 3 and Fig. 4), and the current study unveils the systemic anticancer functions of 2H4MB as a potent bioactive molecule to inhibit breast cancer cell proliferation (Fig. 4b).

3.4. Induction of apoptosis

The double staining with ethidium bromide and acridine orange was used to assess the presence of viable and apoptotic cells upon treatment of cells by 2H4MB. The viable cells appeared in green, whereas apoptotic induced cells and dead cells appeared in orange and red (Fig. 5). It was found that the compound decreased the viability of MDA-MB-231 cells by damaging nuclear DNA. The presence of apoptotic MDA-MB-231 cells increased during treatment, while there was a reduction in the proportion of apoptotic HaCat and control (untreated) cells indicating the specific apoptotic effects of compound against the cancer cells (Fig. 5). At the dose-dependent IC_{50} concentrations, the compound displayed apoptosis induction features in MDA-MB-231 cells, as compared to normal (HaCat) and untreated control cells. The treated cancer cells were shown to have features such as membrane blebs, apoptotic bodies and high degree of nuclear condensation (Fig. 5), and these observations were in agreement with previous reports using natural and synthetic compounds having apoptosis induction and cell growth inhibition features in cancer cells (Surh, 2003). However, our study compound 2H4MB exhibits the above features in addition to induction of ROS associated apoptosis and free radical scavenging antioxidant activities specifically in normal cells (Table 1 and Fig. 6). These results suggest that 2H4MB has apoptotic induction effects against cancer cells but possesses distinct cell protective effects on normal cells through free radical scavenging effects.

3.5. ROS formation and signs of apoptosis

It is reported that the reactive oxygen species (ROS) production is considerably involved in triggering apoptosis in normal as well as cancer cells by activating multiple cell death pathways (Liou and Storz, 2010). The abnormal elicitation of ROS levels in cancer cells due to oxidative stress potentially activates apoptotic signaling cascades (Lin and Beal, 2006). It was noticed from our study that 2H4MB, at its IC_{50} concentrations, was capable of generating high levels of ROS in MDA-MB-231 cells when compared to insignificant ROS levels in normal HaCat cells. During treatment, the extent of intracellular generation of ROS was determined by the DCFH-DA fluorescence of DCF in live cells. (Fig. 6). 2H4MB at concentrations of 70 μg mL^{-1} (24 h) and 50 μg mL^{-1} (48 h) generate ROS sufficient to induce apoptosis in MDA-MB-231 cells. This study results suggested that the ability of ROS formation by 2H4MB could be related to its apoptotic induction potentials as the study describes both ROS generation and apoptotic induction features. Studies report that the excessive production of ROS in cells results in induced oxidative stress, damage to DNA, lipids, and protein and these events are triggering factors of cancer and apoptotic conditions involving regulation of multiple signaling pathways, nuclear and mitochondrial functions (Irani, 2000; Trachootham et al., 2009). Medicinal chemistry research with several compounds of synthetic and natural origin such as paclitaxel, cisplatin, doxorubicin and natural compounds, quercetin, EGCG, curcumin etc. explored the potential of these chemicals for anticancer features including the activation of ROS-dependent apoptosis (Green and Kroemer, 2004). (Fig. 6). The antioxidant phenomenon is a fundamental feature of bioactive food compounds, contributing health-protecting abilities, including anti-mutagenic, anti-carcinogenic and anti-aging functions. ROS scavenging preserves the genomic stability of cells through the elimination of carcinogens and interference with DNA adduct formation (Collins et al., 2005; Gawlik-Dziki et al., 2012; Liou and Storz, 2010) in normal cells. However, ROS also involve in physiological regulation of signaling pathways that determine cell proliferation and motility (Liou and Storz, 2010; Zhang et al., 2008). The phytochemicals can directly interfere with cellular signaling cascades involved in the regulation of inflammatory processes, angiogenesis and cancer invasion and the synergistic inhibitory effects on the function of protein kinases (De Raedt et al., 2011; Zhang et al., 2008).

3.6. Disruption of mitochondrial membrane integrity in cancer cells

The main role of mitochondria is the regulation of intrinsic apoptosis (Hengartner, 2000; Kroemer and Reed, 2000; Thornberry, 1998). This organelle plays vital roles in division, proliferation, trafficking, signaling and migration of cancer cells (Brindley, 2004). Optimal electrochemical gradient is vital for maintaining mitochondrial transmembrane potential ($\Delta\psi_m$) as well as ATP synthesis (Pierroz et al., 2012) and the loss of $\Delta\psi_m$ results in mirochondrial dysfunctions and subsequent cell death. The study results demonstrated that 2H4MB could potentially induce mitochondrial dysfunctions through membrane potential ($\Delta\psi_m$) loss in treated cancer cells (Fig. 7). We notice that the 2H4MB exert mitochondrial depolarization as shown in Rh-123 stained cells. We also noticed that the DNA fragmentation and nuclear fragmentation (DAPI staining) in compound treated cells resulted in the induction of cellular apoptosis (Ellis, 2001). Besides, the fluorescent images (Rh-123-DAPI) revealed that there were features such as abnormal organization of mitochondrial network as well as nuclear disintegration and fragmentation in compound treated cancer cells and these apoptotic features

were prominent in compound treated cancer cells than positive control (HaCat) and untreated cells (Fig. 7).

The anticancer impact of the compound 2H4MB is also attributed to its potential to facilitate mitochondrial membrane permeabilization (Chen et al., 2010; Trachootham et al., 2009; Valko et al., 2006). These events in addition to the previously mentioned findings such as elevated ROS levels to cause oxidative stress (ROS) and specific cytotoxic effects against cancer cells substantiate the potential of the compound to serve as anticancer lead for development of novel chemotherapeutics.

3.7. Nuclear morphology changes through apoptosis activation in cancer cells

DAPI staining of 2H4MB treated cancer cells showed nuclear condensation and fragmentation (Fig. 7 and Fig. 8). These results on apoptotic activation are correlated with other observations on ROS generation, loss of $\Delta\psi_m$ and cytotoxicity in MDA-MB231 cells as compared with normal cells. The fluorescence images of DAPI staining confirm the nuclear condensation in compound treated cells as compared to untreated cells and the control cells (HaCat). Rh-123 and DCFH-DA staining of experimental cells reveals the morphological changes due to mitochondrial mediated apoptotic initiation (Fig. 6, Fig. 7 and Fig. 8). The genomic DNA fragmentation is a unique event indicating the state of apoptosis (Martín et al., 2009). The electrophoresis analysis showed extensive DNA fragmentation, and this was an important feature of the cellular response to our selected compound 2H4MB (Fig. 8). Hence, it is proposed that the cytotoxicity of our selected compound is exhibited by means of apoptosis induction as revealed by morphological changes (Fig. 8) (Morris and Geller, 1996; Zhang et al., 2008).

3.8. Effects of 2H4MB on cell cycle regulation

Further to analyze the effect of 2H4MB in regulation of cell cycle, flow cytometry experiments were performed after staining the cells with propidium iodide (Fig. 9). The treatment of MDA-MB-231 cells using the IC_{50} concentration of 2H4MB for 16, 24, 48 h resulted in the decrease of cells of G2/M and S phases while compared with the control group; however, it was observed that there was an increase in the population of cells in G0/G1 phase suggesting the cell cycle arrest effected in cancer cells. This experimental findings indicate that the isolated compound 2H4MB directly induced cellular apoptosis in MDA-MB-231 cells via G0/G1 phase cell cycle arrest as much of the cells could not progress to subsequent cell cycle phases (Thangam et al., 2012). Overall, our study findings substantiate the anticancer properties of 2H4MB by means of cancer cell specific effects such as cytotoxicity, induction of apoptosis due to loss of mitochondrial membrane potential as well as nuclear fragmentation and cell cycle arrest and these features of the lead plant based compound provide promising insights for developing it into a novel anticancer therapeutic agent to reduce the burden associated with cancer.

4. Conclusion

In conclusion, we state that the plant-based medications are widely used to treat various diseases including cancer. The findings of our study suggest that the isolated compound 2H4MB from *D. arayalpathra* has potential antioxidant and anticancer activities in addition to apoptosis induction through loss of mitochondrial membrane potentials and cell cycle arrest in breast cancer cells (MDA-MB-231) but not in normal cells (HaCat). The results suggest that the compound 2H4MB could potentially be used as a natural dietary antioxidant supplement. Therefore, this study provides a renewed interest on developing chemotherapeutic strategies against cancer cells through targeting mitochondrial functions with cell cycle arrest.

Conflict of interest

None.

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