Apoptosis Induction and Anticancer Activity of 2, 4-bis (1-phenylethyl) Phenol from Clerodendrum thomsoniae Balf.f. in vitro

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ABSTRACT
Objectives: Plant phenolic have extended significance as a promising therapeutic candidate for several disorders including cancer. Apoptosis is a well-organized cell death that has importance in cancer research. The present study was aimed to isolate 2, 4-bis (1-phenylethyl) -phenol from Clerodendrum thomsoniae Balf.f. and evaluation of cytotoxicity on human breast cancer cells. Methods: MTT assay was used to estimate cell viability. Apoptosis was analyzed by Annexin V/PI staining, EtBr staining, cell cycle analysis and MMP assay. Results: In this study 2,4-bis (1-phenylethyl) -phenol was separated from aerial parts of C. thomsoniae and its structure was confirmed by different spectroscopic methods. The IC50 value was determined based on cell viability rates and the value was calculated as 12.58 (μg/mL). Apoptosis analysis by annexin V/PI staining showed that died cells were stained as red fluorescence. The loss of mitochondrial potential as a result of the induction of apoptosis was noted. The EtBr assay showed early apoptotic cells, late apoptotic cells, necrotic cells and, dead cells with characteristic fluorescence. The apoptosis induction of 2, 4-bis (1-phenylethyl) –phenol was also noticed in Cell cycle analysis using flow cytometry. Conclusion: The outcome of this research work, we can conclude that 2, 4-bis (1-phenylethyl)-phenol may be a successful candidate for breast cancer therapy via apoptosis activation and farther in vivo studies are required to evaluate this compound for its safety and efficacy as a potential anticancer candidate. Key words: Anticancer activity, Apoptosis induction, Clerodendrum thomsoniae, Cytotoxicity on MCF-7, Isolation of 2, 4-bis (1-phenylethyl) -phenol.

INTRODUCTION
Cancer is one of the world's major life-threatening disorders.1 Cancer on breast, blood, cervix, lung, prostate and bone is the major cancer type found in all ages globally and the majority of them are lethal to mankind.2 As the world's largest cancer among women, breast tumor is presently the leading cause of cancer-associated death in the world.3–5 The management and investigation of the innovative capable molecules for the effective controlling of malignancy are still technically challenging.6 Research in herbal products has extensively grownup in the past few decades exclusively to achieve cancer-related treatment goals.5 Several studies have shown that natural products can superbly control in vitro and in vivo breast cancer cells' differentiation, expression and proliferation. Secondary metabolites from plants that are extracted and purified are much broadly used clinically for the treatment of various illnesses including cancer diseases.7 Different therapy using Hormonal medicine, radiation, chemo medicine and even surgery or combinations of all of these are the supreme familiar management approach for the treatment of most of the malignancy. Unfortunately, these conventional management modules have major undesirable effects on the body.8 In recent years, the researchers mostly targeted herbal source with the hope of developing some novel promising anticancer agents to achieve the therapeutic goal and most importantly to save mankind from such a terrific illness. It has been noted that around 60% of the recently used medicine for cancer treatment is from the herbal origin which may be motivational to new researchers to focus the research on the same area.9 C. thomsoniae is a twining, rambling, vine-like shrub local to tropical West Africa and the collective name is Bleeding-heart or Bag-flower.10–11 They have beautiful white flowers and are available in a different part of the world because of ornamental demand. These species are not extensively studied for pharmacological studies, even though many plants listed under the genus Clerodendrum are well studied for various pharmacological studies including cancer. According to the data available, the species C. thomsoniae have phytoconstituents with excellent free radical scavenging properties and are useful in multiple conditions such as sores, cuts, bruises and skin rashes.12 This study’s objective was to assess the apoptosis induction and anticancer activity of 2, 4-Bis (1-phenylethyl) phenol, isolated from C. thomsoniae.

MATERIALS AND METHODS
Chemicals and plant materials
DMEM medium, Penicillin/Streptomycin, Trypsin and EDTA was bought from Gibco (USA); the Alexa Fluor® 488 annexin apoptosis kit was purchased from Thermo logical (USA). MCF-7 cell was obtained from National Centre for Cell Sciences (Pune, India). EtBr and Acridine orange were obtained from Sigma Aldrich (USA), Fetal Cow-like Serum (FBS), Mitochondrial layer potential test pack was from Sigma (USA). Aerial parts of C. thomsoniae have been locally acquired from Calicut and was identified by the University of Calicut (Botany Department).

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In the same herbarium, the specimens were deposited as Reference no. 148249.

Preparation of extracts and isolation

The aerial parts of C. thomsoniae collected were dried in the shade for three weeks, separated, pulverized. About 3 kg of air-dried plant material was removed in a set of Soxhlets with ethyl acetate. The extract was concentrated using an evaporator with a rotary vacuum. Fractionation of ethyl acetate was performed by using column chromatography as mentioned in our earlier studies. The fractions obtained were tested for cytotoxicity in MCF-7 and Fraction 5 (F5) and Fraction 6 (F6) showed better cytotoxicity activity than with other fractions.

For the isolation of 2,4-bis (1-phenylethyl) phenol, fractions F5 and F6 were subjected to chromatography techniques. For the fractionation, the borosilicate material used a vertical column of glass with a width of 40 mm and a length of 60 mm. The column was well rinsed and before packaging and wholly dried with acetone. A ball of glass wool was placed with the help of a glass rod at the base of the column. At the top of the glass wool at the height of 1 cm, seabed (grain size 50–70) was applied. The solvent was used to clean the sand particles. At level 3/4, Hexane was thrown off the tap in the column. The packaging material was used with 200 g silica gel. The hexane was used to make the silica suspension and about 2/3 of the column was spent on top of the column and simultaneously dried out the solvent to enable the column to be properly packaged. Sea sand was added at a temperature of 1 cm to the top of the suspension and the solvent was rinsed with the sand particle. 20 g F5 and F6 were mixed with a minimal quantity of hexane and rinsed with solvent from the above column along the sides. In order to avoid drying the column, a solvent level of 6 cm above the extract was maintained. The gradient elution method was used to separate the F5 and F6 fractions using high polarity low polarity solvents (hexane from hexane to methanol) in varying proportions. Different concentrations of hexane (30%, 50% and 70%) and methanol (2%, 5% and 10%) were mixed in an orthogonal design. Then, the samples were extracted with hexane-methanol (70%: 2%, V/V) in a different ratio (1:10, 1:15, 1:20, 1:25 and 1:30). The flow speed was restricted to 5 ml/min and a solution of 40 ml was obtained for each fraction. Fractions were TLC purified and spectroscopic methods such as ¹H NMR, ¹³C NMR, FTIR and MASS was used to identify the pure compound.

Cell Culture

The cell lines MCF-7 (human breast cancer cells) produced in an aqueous medium (DMEM), supplied by 10 percent bovine fetal serum (FBS), 100 μg penicillin/ml and 100 μg / ml Streptomycin and maintained at a CO₂ environment of 5 percent at 37°C. The cell line has been acquired by the National Center for cell science (Pune, India).

Cell Viability Test

By performing a standard MTT method test, the inhibitory effect of 2,4-bis (1-phenylethyl) phenol (IC₅₀) was assessed. After 48 h incubation, MCF-7 cancer cell lines were cultivated by a microtitre plate to reach a confluence of approximately 90%. The medium of use was then replaced at various concentrations by a fresh medium that contains anti-cancer compounds and incubated for approximately 48 hr. The culture medium was subsequently removed and 3-(4, 5-dimethylthiazol-2-yl)-3 was load-packed and kept for 4 hr at 37°C, with 5-diphenyl tetrazolium bromide (100 mL). DMSO (50 mL) was then carefully added to the wells and the formazan crystals were solubilized. The absorbance for every well was estimated at 570 nm and the percentage cell viability with IC₅₀ value was determined.

Apoptosis analysis by Annexin V/PI staining

The “Alexa Fluor 488 Annexin V / Dead Cell Apoptosis Kit” was used to perform the test. In short, in the DMEM medium, 5 x 10⁴ cells/ml of MCF-7 cells were placed into 24-well tissue culture plates that contain 10% FBS and 1% antibiotic solution at 37°C for 24-48 h. The wells were then washed with sterile PBS and treated in a serum-free DMEM medium with 12.58 μg / ml of 2,4-bis (1-phenylethyl) phenol. The plate was then incubated (24 h) at 37°C in a 5 percent CO₂ incubator. 10 ml of Alexa Fluor and 5 ml of PI were then added to the wells after incubation and mixed gently and incubated for a duration of 15 min. Finally, the plate was centrifuged and evaluated immediately within an hour and the cells were examined using a fluorescent filter under a fluorescent microscope.

MMP Assay (mitochondrial membrane potential assay)

MCF-7 cells (5,000-20,000 cells/well) have been placed on a 24-well plate which is then incubated in DMEM medium for 24 h. The plate was washed with PBS after incubation and treated in a serum-free DMEM medium with 12.58 μg/ml of 2,4-bis (1-phenylethyl) phenol sample. Again, a 5% CO₂ humidified incubator for 24 h. The potential measurement of the mitochondrial membrane for treated and control cells was done according to the instructions suggested by the manufacturer. In short, the cells were incubated with the help of a JC-10 dye loading solution for 100 μl/well and the plate was protected from light. The dish was incubated for 30-60 min (at 37°C in 5 % CO₂). After incubation, 100 μl/well assay buffer B was added to each sample/well. Finally, the plate was centrifuged for 2 min at 800 rpm and fluorescence with a ratio of 490/525 and 540/590 was observed.

Cell cycle analysis by flow cytometry

Briefly, trypsinization was used to harvest the cultured MCF-7 cells, pooled in a 15 ml tube. The cells were then plated in a 6-well tissue culture plate in DMEM medium with 10 percent FBS and antibiotic solution 1% (for 24-48 hr at 37°C). The wells were treated with 12. 58 μg / mL of 2 4-bis (1-phenylethyl) phenol samples in a serum-free DMEM medium and incubated at 37°C for 24 h. The cells were harvested by trypsinization after incubation and washed in PBS for 5 min by centrifuging at 1500 rpm. In addition, by adding drop wise to the cell pellet, the cells were fixed at 4°C for 30 min using cold 70 percent ethanol. Following incubation, the cells were rinsed twice by centrifuging for 5 min at 1500 rpm with sterile PBS and the supernatant was removed. Lastly, the cells were examined by flow cytometry.

EtBr staining

In short, 5 x 10⁴ cells/ml of MCF-7 cells have been planted in the 24-well plating of tissue culture and treatment in the serum-free medium of DMEM with 12.58 μg/ ml 2,4-bis (1-phenyl ethyl) phenol sample. The plate was incubated for 24 h (at 37°C in a 5 % CO₂ incubator). 50 μl of 1 mg/ml acridine orange and ethidium bromide was added and mixed slowly in the wells after incubation. Finally, the plate was centrifuged at 800 rpm for 2 min and evaluated immediately within an hour and examined at least 100 cells by a fluorescence microscope using a fluorescent filter.

RESULTS

Isolation of 2, 4-bis (1-phenylethyl) -phenol

In our study 2, 4-bis (1-phenylethyl) -phenol were isolated from C. thomsonia and the structure was identified by NMR, IR and MASS spectroscopic method.
The types of protons and the number of protons present in the compound were confirmed with the help of ¹H NMR spectrum which showed an NMR signal at 1.68–1.69 δ ppm, 2.51–2.52 ppm, 6.73–7.42 δ ppm and 9.742 ppm which corresponds to methyl, methine and OH protons respectively (Table 1). The ¹H NMR spectra of 2,4-bis (1-phenylethyl) –phenol is given at Figure 1. The types of carbon present in the compound were confirmed by ¹³C NMR spectrum, which showed an NMR signal at 20.68–21.47 δ ppm, 27.63–27.65 δ ppm and 112.97–149.48 δ ppm which corresponds to methyl, methine and aromatic carbons respectively (Table 2). The ¹³C NMR spectra of 2,4-bis (1-phenylethyl) –phenol is given at Figure 2. The functional groups present in the compound were confirmed with the help of IR spectrum which gave IR absorption bands at 3066.64, 2985.66, 1638.67 and 1072.22 cm⁻¹ which corresponds to OH, CH, Aromatic C=C and C-O stretch of the molecule. Aromatic CH bend was observed as an IR absorption band at wavenumber of 724.53 cm⁻¹ (Table 3). The IR spectra of 2,4-bis (1-phenylethyl) –phenol is given at Figure 3. The compound’s molecular weight was confirmed with the help of a mass spectrum, which produced a molecular ion peak at m/z ratio of 302.11, that corresponds to the compound’s molecular weight (Figure 4).

### Cell viability assay (MTT assay)

The isolated 2, 4-bis (1-phenylethyl)-phenol was measured for cytotoxicity on MCF-7 cell line using cell viability assay (MTT assay). The cell viability test by using diverse concentrations ranges from 1 to 100 and 300 μg/mL compared to control for 24 h are given in the following Table 5.

### Annexin V/PI staining

The "Alexa Fluor® 488 annexin V/Dead Cell Apoptosis Kit" offers a quick and suitable assay for apoptosis. The propidium iodide (PI) is impermeable to living cells and apoptotic cells as well, but spots dead cells by red fluorescence, tightly adhered to nucleic acids in the cell. If the PI enters the cell membrane, it is supposed to be damaged. In our study it is very clear that cells have died and which are stained as red fluorescence (Figure 6).

### Mitochondrial membrane potential assay

JC-10 was used to determine the possible depletion of the mitochondrial membrane (MMP) cells. JC-10 will directly penetrate mitochondria and alter its color. In our study loss of mitochondrial potential as a result of induction of the apoptosis was noted (Figure 7).

### EtBr staining

The EtBr intercalate inside the double helix of DNA. The acridine orange (AO) can form complexes with either dual or single-stranded DNA and RNA. One AO molecule can also interact with one single-stranded DNA or RNA phosphate group to form an aggregated or stacked structure emitting red fluorescence at a maximum wavelength of 650 nm. Ethidium bromide is taken up only by cells when the cytoplasmic membrane (MMP) cells. JC-10 was used to determine the possible depletion of the mitochondrial membrane (MMP) cells. JC-10 will directly penetrate mitochondria and alter its color. In our study loss of mitochondrial potential as a result of induction of the apoptosis was noted (Figure 7).

### Table 1: ¹H NMR spectrum details of 2,4-bis (1-phenylethyl) -phenol isolated from aerial parts of Clerodendrum thomsoniae Balf.f.

<table>
<thead>
<tr>
<th>S. No</th>
<th>δ values (ppm)</th>
<th>Type of protons</th>
<th>No. of protons</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.68-1.69</td>
<td>CH₃</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>2.51-2.52</td>
<td>CH</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>6.73-7.42</td>
<td>Aromatic protons</td>
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<tr>
<td>4</td>
<td>9.742</td>
<td>OH</td>
<td>1</td>
</tr>
</tbody>
</table>

### Table 2: ¹³C NMR spectrum details of 2,4-bis (1-phenylethyl) -phenol isolated from aerial parts of Clerodendrum thomsoniae Balf.f.

<table>
<thead>
<tr>
<th>S. No</th>
<th>δ values (ppm)</th>
<th>Type of carbon</th>
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<tbody>
<tr>
<td>1</td>
<td>20.68-21.47</td>
<td>Methyl carbons</td>
</tr>
<tr>
<td>2</td>
<td>27.63-27.65</td>
<td>Methine carbons</td>
</tr>
<tr>
<td>3</td>
<td>112.97-149.48</td>
<td>Aromatic carbons</td>
</tr>
</tbody>
</table>

### Table 3: FT-IR spectra details of 2,4-bis (1-phenylethyl) -phenol isolated from aerial parts of Clerodendrum thomsoniae Balf.f.

<table>
<thead>
<tr>
<th>S. No</th>
<th>Type of vibration</th>
<th>Wavenumber (in cm⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>O-H stretch</td>
<td>3066.64</td>
</tr>
<tr>
<td>2</td>
<td>Methyl C-H stretch</td>
<td>2985.66</td>
</tr>
<tr>
<td>3</td>
<td>Aromatic C=C stretch</td>
<td>1638.67</td>
</tr>
<tr>
<td>4</td>
<td>C-O stretch</td>
<td>1072.22</td>
</tr>
<tr>
<td>5</td>
<td>Out-of-plane aromatic CH bend</td>
<td>724.53</td>
</tr>
</tbody>
</table>
appeared green fluorescence; Dead cells - cells which appeared red color fluorescence.

**Cell cycle analysis by flow cytometry**

The impact of 2,4-bis (1-phenylethyl) -phenol on cell cycle was estimated with the aid of flow cytometry in MCF-7 cells. The proportion of cells were observed in phases G0/G1, S and G2/M of the cell cycle. The outcome of our study showed that apoptosis induction in 2, 4-bis (1-phenylethyl) -phenol treated cells. Phase S of the cell cycle in MCF-7 cells was arrested by doxorubicin (Figure 9).

**DISCUSSION**

The need for novel anticancer agents has prime importance due to several reasons mainly because of the development of multidrug resistance and severe side effects associated with long-term treatment of cancer drugs. The potential of the harmful effects of cytotoxic drugs will be high unless they are specific to cancer cells. According to available research data, several secondary metabolites from plants, especially phenolic compounds, are reported for anticancer activity due to their capability of inducing apoptosis. Phenolic compounds isolated from herbal bases consist of simple phenols, tannins, flavonoids, lignans, lignin’s, xanthone and coumarins. Phenolic compounds can produce anticancer activity by several approaches such as induction of cell arrests, modulation of ROS levels, activation of tumor suppressor genes, angiogenesis, blocking of the signals in oncogenic cascades, controls the cell proliferation and apoptosis induction.20 The phenolic compound curcumin, yakuchinone B and resveratrol are capable of inducing apoptosis in different human cancer cells.24 The 8-prenylnaringenin is a flavonoid from Humulus lupulus was reported for anticancer activity by inducing apoptosis on human breast cancer cell.22 Protocatechuic acid is phenolic acid reported for anticancer activity due to apoptosis induction on several types of cancer cells such as prostate, cervix, breast, lung, liver and cancer.25 Pterostilbene is a natural resveratrol analog that has been shown to induce apoptosis in different cell lines.26 Pterostilbene also can reduce mitochondrial membrane potential, which has significant importance in apoptosis.25 The Hesperetin is a naturally occurring flavon-glycoside reported for anticancer activity by inducing apoptosis of esophageal cancer cells.26 Triticuside A is a flavonoid that has been shown to induce apoptosis on human breast cancer cells.27 Genistein is an isoflavone reported for anticancer activity due to apoptosis induction on breast cancer cells.28 Artocarpesin and cycloartocarpesin are flavonoids from Morus have been shown to induce apoptosis in CCRF-CEM leukemia cells.29 Loss of apoptosis is frequently found in the majority of the drug-resistant cancers.30,31 The apoptotic disturbance is deemed to be a major hallmark of cancer.32,33 Many research data have shown that numerous anticancer agents produce activity by inducing programmed cell death called apoptosis.34,35 Along these lines, the initiation of apoptosis in the tumor
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ROS damage in human breast tissues. Our results show that 2-4-bis (1-phenylethyl)-phenol has exercised significant cytotoxic effects on MCF-7 breast cancer cells through significant morphological changes related to the induction of apoptosis. Medicines that induce selective apoptosis of cancer cells with the least side-effects on normal cells are very favorable for cancer treatment. For the research of the mechanisms of cell cycle development and cell death, the quantitative investigation of the cell cycle is essential. The cell cycle investigation by flow cytometric procedure estimates the occurrence of apoptotic cells by staining them with DNA dyes. During the process of apoptosis, the destruction of the mitochondrial membrane potential (MMP) occurs due to the collapse of the mitochondrial transition pores. This will enable cytochrome c to discharge into the cytosol that initiates other downstream incidents in the apoptotic cascade. JC-10 was used to decide the damage of the mitochondrial membrane potential (MMP) in MCF-7 cells. JC-10 can penetrate mitochondria correctly and reversibly transforms the color from green to orange with increased membrane potential. The resulting loss of membrane potential was observed in our study after treatment with 2, 4-bis (1-phenylethyl)-phenol that allows us to assume apoptosis induction on MCF-7 cells. Acridine orange (AO) is a vibrant dye with the ability to pigment dead and live cells that can pigment necrotic cells orange. Cells only take up ethidium bromide (EtBr) when the cytoplasmic membrane's integrity is damaged and the nucleus stains red. The viable cells thus have a normal green colored nucleus. The early apoptotic cells have a green nucleus and have a chromatin that is consolidated and split, whereas the late apoptotic cells have dense orange chromatin. Our results showed that the cells treated with 2, 4-bis (1-phenylethyl) -phenol exhibited cell death, which was induced by apoptosis.

2,4-bis(1-phenylethyl) phenol also was isolated from Zanthoxylum integrifoliolum. The available literature review shows that 2,4-bis(1-phenylethyl) phenol is not tested for cytotoxicity studies in any normal or cancer cell lines. This is the first research data that report isolation of 2,4-bis(1-phenylethyl) phenol from Clerodendrum thomsoniae Balf.f. and its ability to induce apoptosis in human breast cancer cells. Similarly substituted compound 4-Isopropyl-2,6-bis(1-phenylethyl) phenol which was isolated from Cordyceps bassiana has been reported for anti-proliferative and apoptosis-inducing activities on cancer cells.

CONCLUSION
The present study successfully isolated 2, 4-bis (1-phenylethyl)-phenol from Clerodendrum thomsoniae Balf.f. According to this research, we can conclude that 2, 4-bis (1-phenylethyl) -phenol may be a successful candidate for breast cancer therapy via apoptosis activation. Even though the in vitro trials confirm the anti-cancer property, further in vivo studies are required to analyze its potential usage as an anti-cancer drug candidate.

ACKNOWLEDGEMENT
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CONFLICT OF INTEREST
The authors declare no Conflict of interest.

ABBREVIATIONS
MTT: 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide; MMP: mitochondrial membrane potential; EtBr: Ethidium bromide;
HCl: Hydrochloric acid; WSI: Water solubility index; FBS: Fetal bovine serum; NCCS: National Centre for Cell Science; DMEM: Dulbecco’s Modified Eagle Medium; DMSO: Dimethyl sulfoxide; AO: Acridine orange; PI: Propidium iodide.

REFERENCES


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