Int. J. Pharm. Investigation, 2020;10(4):542-547 Original Article

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

VK Muhammed Ashraf[1,*], VK Kalaichelvan[1], R Ragunathan[2], V V Venkatachalam[1]
[1]Department of Pharmacy, Annamalai University, Annamalai Nagar, Tamil Nadu, INDIA.
[2]Centre for Bioscience and Nano Science Research, Coimbatore, Tamil Nadu, INDIA.

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 IC_{50} 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.

Correspondence

Mr. Muhammed Ashraf VK
Department of Pharmacy, Annamalai University, Annamalai Nagar - 608002, Tamil Nadu, INDIA.
Phone no: +91 9747313918
Email: ashrafvkclt@gmil.com

DOI: 10.5330/ijpi.2020.4.94

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.[3,4] The management and investigation of the innovative capable molecules for the effective controlling of malignancy are still technically challenging.[5] Research in herbal products has extensively grownup in the past few decades exclusively to achieve cancer-related treatment goals.[6] 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).

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.[13] 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 ^{1}H NMR, ^{13}C 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 CO_2 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 (IC_{50}) 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-dimethylthiozol-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 IC_{50} 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 10^5 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 CO_2 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.[14,15]

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% CO_2 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 % CO_2). 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.[16]

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 hr. 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.[17]

EtBr staining

In short, 5 x 10^5 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 % CO_2 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.[18]

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 ^{1}H 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, methane and OH protons respectively (Table 1). The ^{1}H 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 ^{13}C 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 ^{13}C 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 an 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 300 (μg/mL) was evaluated and the IC_{50} value was noted as 12. 58 μg/mL. Morphological profile of the MCF-7 cells after treated with 2, 4-bis (1-phenylethyl)-phenol at different concentrations such as 1.0, 10.0, 50.0, 100 and 300 μg/mL compared to control for 24 h are given in the following Figure 5.

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

S. No	δ values (ppm)	Type of protons	No. of protons
1	1. 68-1. 69	CH_3	6
2	2. 51-2. 52	CH	2
3	6. 73-7. 42	Aromatic protons	13
4	9. 742	OH	1

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

S. No.	δ values (ppm)	Type of carbon
1	20. 68-21. 47	Methyl carbons
2	27. 63-27. 65	Methine carbons
4	112. 97-149. 48	Aromatic carbons

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

S. No.	Type of vibration	Wavenumber (in cm^{-1})
1	O-H stretch	3066. 64
2	Methyl C-H stretch	2985. 66
3	Aromatic C=C stretch	1638. 67
4	C-O stretch	1072. 22
5	Out-of-plane aromatic CH bend	724. 53

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 mucosa's integrity is lost and stain the nucleus red (Figure 8). In our study the following events are observed. *Early apoptotic cells* – cells which appeared yellow-green fluorescence; *Late apoptotic cells* - cells which appeared orange nuclear fluorescence; *Necrotic cells*- cells which appeared orange-red color fluorescence; *Live cells*-cells which

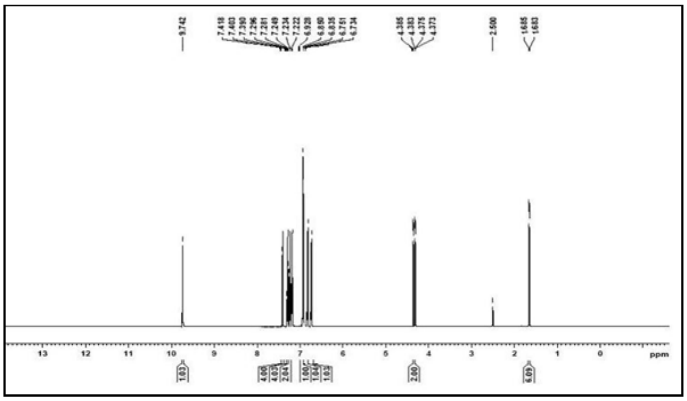

Figure 1: ^{1}H NMR spectra of 2,4-bis (1-phenylethyl) -phenol separated from aerial parts of *Clerodendrum thomsoniae* Balf.f.

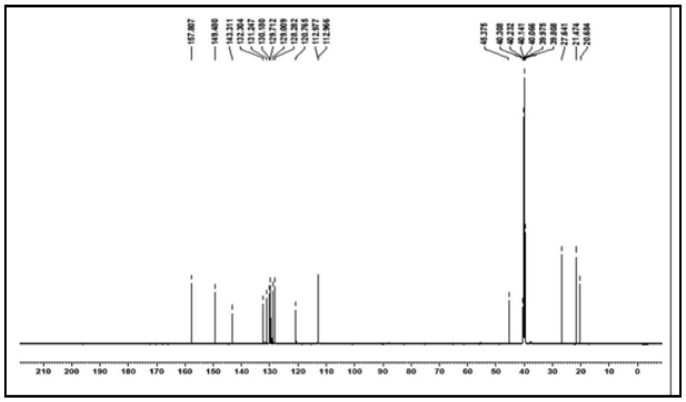

Figure 2: ^{13}C NMR spectra of 2,4-bis (1-phenylethyl) -phenol separated from aerial parts of *Clerodendrum thomsoniae* Balf.f.

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.[19] 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.[21] 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.[23] Pterostilbene is a natural resveratrol analog that has been shown to induce apoptosis in different cell lines.[24] Pterostilbene also can reduce mitochondrial membrane potential, which has significant importance in apoptosis.[25] The Hesperetin is a naturally occurring flavanon-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

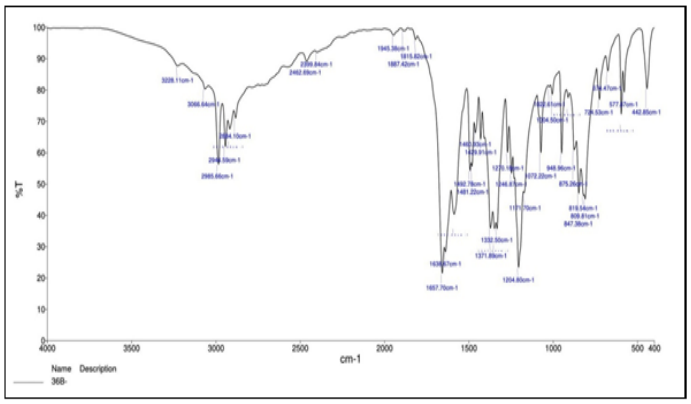

Figure 3: FT-IR spectra of 2,4-bis (1-phenylethyl) -phenol separated from aerial parts of *Clerodendrum thomsoniae* Balf.f.

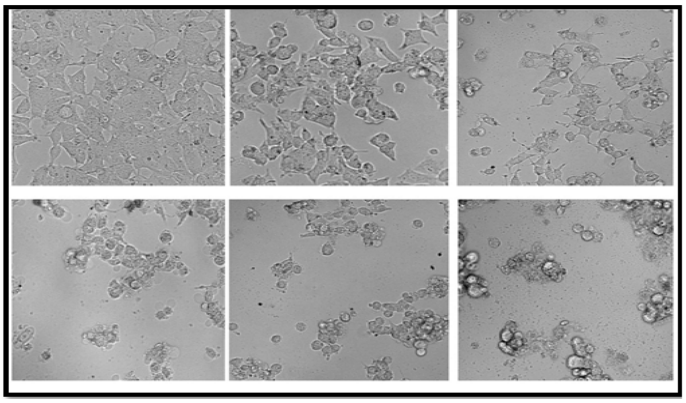

Figure 5: Morphological profile of the MCF-7 cells after treated with 2,4-bis (1-phenylethyl)- phenol 1. 0 (b)10 (c) 50 (d) 100 (e) and 300 µg/mL (f) compared to control (a) for 24 h.(100 x enlargement).

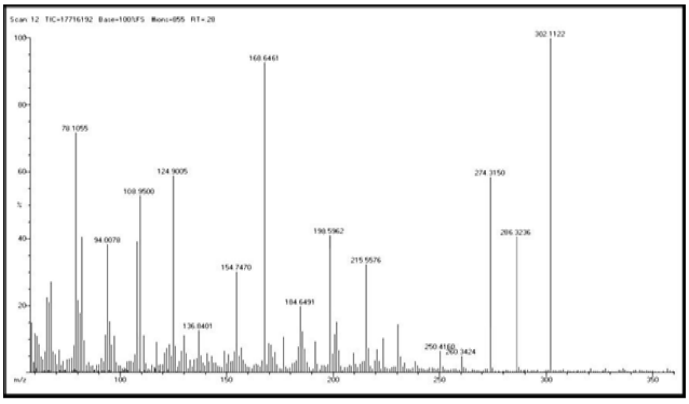

Figure 4: MASS spectra of 2,4-bis (1-phenylethyl) -phenol separated from aerial parts of *Clerodendrum thomsoniae* Balf.f.

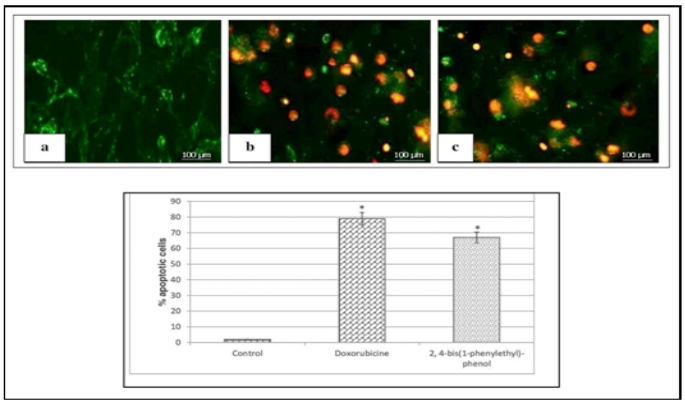

Figure 6: Apoptotic effect of 2, 4-bis (1-phenylethyl) -phenol on MCF-7 cells analyzed by Annexin V/PI staining method; (a) Control MCF-7 cell (b) Doxorubicin and (c) treated with 2,4-bis (1 phenylethyl) -phenol . All values are in mean ± SEM; *$p < 0.05$ compared to control; $n = 3$.

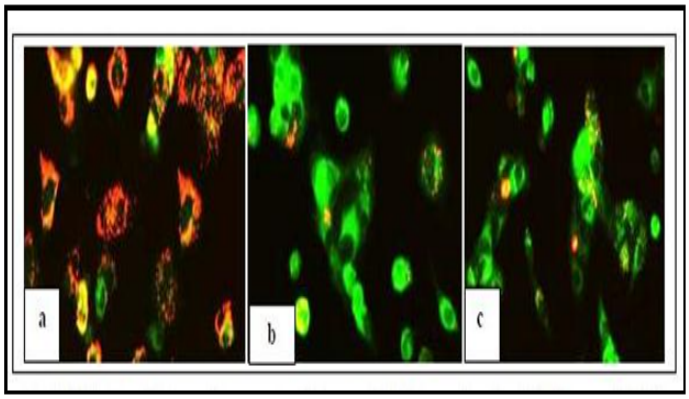

Figure 7: Mitochondrial membrane potential assay (MMP assay) using JC-10 assay kit, (a) Control cells (MCF-7) (b) Doxorubicin and, (c) 2, 4-bis (1-phenylethyl)–phenol.

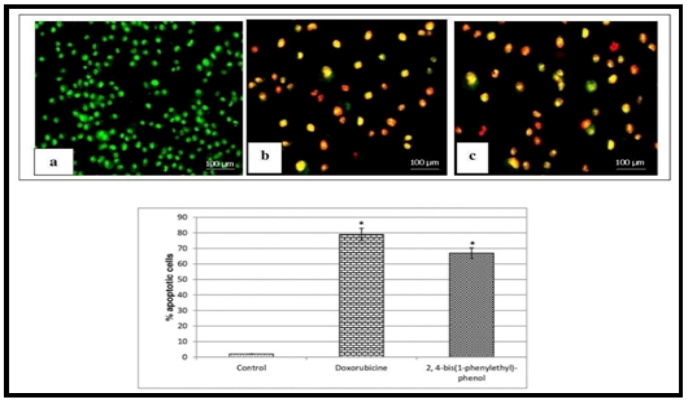

Figure 8: Apoptotic impact on MCF-7 cells assessed by ETBr/AO dual staining: (a) control MCF-7 cells, (b) Doxorubicin and, (c) 2,4-bis (1-phenylethyl) -phenol Values as mean ± SEM; *$p < 0.05$ compared to control; $n = 3$.

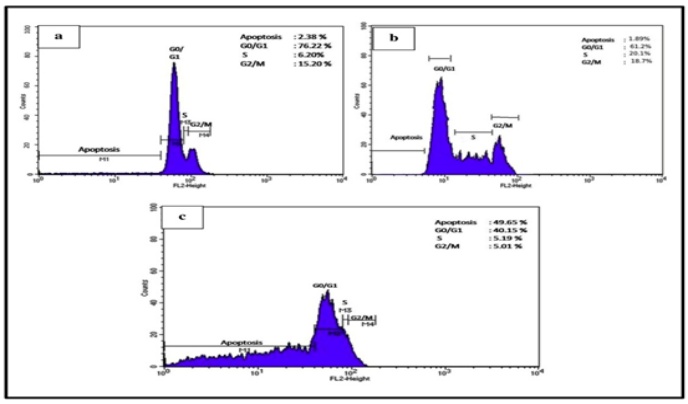

Figure 9: Effect of 2 ,4-bis (1-phenylethyl) -phenol on MCF-7 cells assessed by cell cycle analysis.(a) Control cells (MCF-7), (b) Doxorubicin and (c) treated with 2,4-bis (1-phenylethyl) -phenol.

cells is deliberated as one of the major therapeutic goals for any cancer healing.[36]

Doxorubicin was used as the standard drug in our study. Doxorubicin is a strong anticancer and is useful for the treatment of breast cancer. They produce anti-cancer effects through several mechanisms. The predominant way is by induction of apoptosis by enhancing caspases activity and also influencing proteolytic processing of Bcl-2 family. Doxorubicin also can decrease the oxidative stress by influencing ROS damage in human breast tissues.[37] 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.[38,39] For the research of the mechanisms of cell cycle development and cell death, the quantitative investigation of the cell cycle is essential.[40,41] The cell cycle investigation by flow cytometric procedure estimates the occurrence of apoptotic cells by staining them with DNA dyes.[42] 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*.[43] 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.[44]

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

We would like to thank all colleagues for their help. We specially thanks Dr. P. Manoj Kumar, Principal, "The Dale View College of Pharmacy and Research Centre" Trivandrum, for helping spectroscopic interpretation of compound isolated.

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.

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Article History: Submission Date : 19-08-2020; Revised Date : 20-09-2020; Acceptance Date : 31-10-2020.
Cite this article: Ashraf MVK, Kalaichelvan VK, Ragunathan R, Venkatachalam VV. Apoptosis Induction and Anticancer Activity of 2, 4-bis (1-phenylethyl) Phenol from *Clerodendrum thomsoniae* Balf.f. *in vitro*. Int. J. Pharm. Investigation, 2020;10(4):542-7.