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Research Article

IN VITRO CYTOTOXIC AND APOPTOSIS STUDY OF CHEMICAL CONSTITUENTS FROM *CLERODENDRUM PHLOMIDIS* LEAF ON MCF-7 AND A549 CANCER CELL LINES

HABEELA JAINAB N[1,2]*, MOHAN MARUGA RAJA MK[1]

[1]Department of Pharmacy, Centre for Research and Development, PRIST University, Vallam, Thanjavur - 613 403, Tamil Nadu, India. [2]Department of Pharmaceutical Chemistry, Hillside College of Pharmacy and Research Centre, Raghuvanahalli, Bengaluru - 560 062, Karnataka, India. Email: habi.asheesh@gmail.com

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ABSTRACT

Objective: Cancer is a molecularly heterogeneous diseaseand the leading cause of death worldwide. The methanol extract of *Clerodendrum phlomidis* leaf has been reported for cytotoxicity. Hence, the current investigation was planned to evaluate the cytotoxic activity of the chemical constituents isolated from the methanol extract of the *C. phlomidis* leaf against the Michigan cancer foundation-7 (MCF-7) breast cancer and adenocarcinomic human alveolar basal epithelial cells (A549) lung cancer cell lines by the apoptotic study.

Methods: Cytotoxic activity of the chemical constituents of the methanol extract of the *C. phlomidis* leaf was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT) assay against breast cancer (MCF-7) and lung cancer (A549) cell lines. The MCF-7 and A549 cell lines were tested at different concentrations to determine 50% of growth inhibition (inhibitory concentration [IC_{50}]) by MTT assay. Apoptosis of nuclei was detected by 4',6-diamidino-2-phenylindole staining assay.

Results: In MCF-7 breast cancer cell line study, Compounds 6 and 9 exhibited good cytotoxic activity with an IC_{50} value of 83.80 and 75.16 µg/ml, respectively. In A549 lung cancer cell line, again Compounds 6 and 9 exhibited good cytotoxic activity with an IC_{50} value of 84.46 and 78.60 µg/ml, respectively. Percentage of apoptosis induced by the Compounds 6 and 9 in the MCF-7 cancer cells was found to be 74.50 and 85.48, respectively.

Conclusion: The results of the current study prove that the Compounds 6 and 9 are potential agents for breast and lung cancer. In future research, these potential agents can further be evaluated by animal studies and their mechanism of action apart from the induction of apoptosis of the cancer cells can be determined.

Keywords: Methanol extract, 3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide assay, 4',6-diamidino-2-phenylindole staining, DNA fragmentation, Anticancer.

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INTRODUCTION

Cancer is a molecularly heterogeneous disease [1] and the leading cause of death worldwide. National Institute of Cancer Preventive and Research reported that the estimated number of people living with cancer in India is around 2.5 million, with over 7 lakh new cases being registered every year and 5,56,400 deaths. Cancers of oral cavity and lungs in males and cervix and breast in females account for over 50% of all cancer deaths in India [2]. Due to the existence of various types of tumors with different histopathology, genetic, epigenetic variations, and clinical outcomes [3], it has become difficult to understand this disease, the mechanism of action of chemotherapeutics and the creation of novel therapies. Studies of cancer rely on the use of primary tumors [1,4], paraffin-embedded samples [1], cancer cell lines [1,4,5], xenografts [2,6], tumor primary cell cultures [4,5], and/or genetically engineered mice [3]. These diverse models are used for different studies, mainly because certain types of manipulations for the genetic and DNA methylation analysis and drug testing are ethically, and in practice, difficult to perform in animals.

Cell lines emerge as a feasible alternative to overcome these issues, being at the same time easy to manipulate and molecularly characterize (e.g., genetic and/or epigenetically) [4]. Cancer cell lines have been widely used for research purposes and proved to be a useful tool in the genetic approach, and its characterization shows that they are, in fact, an excellent model for the study of the biological mechanisms involved in cancer [1]. The results of the research in cancer cell lines are usually extrapolated to *in vivo* human tumors [4], and its importance as models for drug testing and translational study has been recognized by biomedical and pharmaceutical industries [7]. In comparative studies made between cancer cell lines derived from earlier stage tumors and the original tumor tissues showed good concordance in several parameters, including the state of tumor protein - TP53 (100%) and epidermal growth factor - ERBB2/EGFR2 (93%). This shows that this type of cells is more representative of an original tumor [5], reflecting more accurately the events that occur in cancer cells *in vivo* [6].

Plant-derived natural products have long been and will continue to be extremely important as sources of medicinal agents and models for the design, synthesis, and semi-synthesis of novel substances for treating humankind diseases. Plant-derived compounds which have recently undergone development include the anticancer agents, taxol and camptothecin, the Chinese antimalarial drug, artemisinin, and the ayurvedic drug, forskolin. These and many other examples serve to illustrate the continuing value of plant-derived secondary metabolites as viable compounds for modern drug development. However, despite these many important past contributions from the plant kingdom, a great many plant species have never been described and remain unknown to science, and relatively few have been surveyed systematically to an extent for biologically active chemical constituents [8].

Clerodendrum phlomidis is a small herb common in India and Sri Lanka. The leaves and roots are used in *Ayurveda*, *Siddha*, Chinese and *Unani* medicines

[9]. *C. phlomidis* leaf has been reported for various biological activities such as antifungal [10], antioxidant [11], antibacterial [11], analgesic [12], antiasthmatic [13], antidiarrheal [14], anti-inflammatory [15], antiplasmodial [16], and hypoglycemic [17]. The methanol extract of *C. phlomidis* leaf has been reported for cytotoxicity [18]. Thus, in the current investigation, we planned to evaluate the cytotoxic activity of the chemical constituents isolated from the methanol extract of the *C. phlomidis* leaf against breast cancer (Michigan cancer foundation-7 [MCF-7]) and lung cancer (A549) cell lines with the help of apoptosis study. The findings of the current study will be useful in identifying the potential cytotoxic agent/s against the breast and lung cancer cell line with fewer side effects.

MATERIALS AND METHODS

Collection of plant material

Leaves of *C. phlomidis* were collected from the outskirts of Tirupati, Andhra Pradesh, India. Plant material was authenticated by Dr. K. Madhava Chetty, Taxonomist, Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh. A voucher specimen (CP/L/2015/1160) was also submitted.

Extraction

The leaves were dried in shade and ground to coarse powder and stored in a glass jar until use. Powdered drug was packed into Soxhlet apparatus, and extraction was carried out with methanol till exhausted. The extract was then concentrated using rotary vacuum evaporator at 50°C under vacuum. The dried residue was then stored in an opaque glass bottle for further studies.

Isolation of compounds by column chromatography

The methanol extract of *C. phlomidis* leaf was subjected to column chromatography for the isolation of chemical constituent/s with cytotoxic potential. The methanol extract was chromatographed on a silica gel column, and gradient elution technique was followed using chloroform: Methanol as the mobile phase in different ratios.

Spectral characterization studies of the isolated compounds

The isolated compounds were characterized by infrared spectra, proton nuclear magnetic resonance spectra (^{1}H NMR), carbon NMR (^{13}C NMR), and mass spectra.

3-(4,5-dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT) assay chemicals

MTT, fetal bovine serum (FBS), phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), and trypsin were obtained from Sigma-Aldrich Co, St Louis, USA. Ethylene diamine tetraacetic acid (EDTA), glucose and antibiotics were obtained from HiMedia Laboratories Ltd., Mumbai, India. 4',6-diamidino-2-phenylindole (DAPI), dimethyl sulfoxide (DMSO), ethidium bromide, glycerin, and agarose gel were procured from E Merck Ltd., Mumbai, India. MCF-7 (human breast cancer cell line) and A549 (lung cancer cell line) cell cultures were procured from National Centre for Cell Sciences, Pune, India.

Cell treatment procedure

Stock cells were cultured in DMEM supplemented with 10% inactivated FBS, penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (5 µg/ml) in a humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02 % EDTA, and 0.05% glucose in PBS). The stock cultures were grown in 25 cm^2 culture flasks, and all experiments were carried out in 96 µ titer plates.

Preparation of test solutions

To carry out the cytotoxicity studies, each of the Compounds (1, 2, 4, 6, 9, and 11) were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to get a stock solution concentration of 1 mg/ml and sterilized by filtration. Serially two-fold dilutions of 10, 50, 100, 150, and 200 µg/ml were prepared from the stock for carrying out cytotoxic studies.

In vitro cytotoxicity MTT assay

MTT assay is based on the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water-soluble substrate MTT into an insoluble, colored formazan product which was measured spectrophotometrically. Since only the metabolically active cells are capable of reducing MTT, the level of the activity is a measure of the cells. The MTT cell proliferation assay was used to evaluate the cytotoxicity of the compounds against human cancer cell lines, namely, MCF-7 (breast cancer) and A549 (lung cancer) at the concentration ranging from 10 to 200 µg/ml.

Determination of cell viability by MTT assay

For screening experiment, the cells were seeded into 96 well plates in 100 µl of DMEM containing 5% FBS, at a plating density of 10,000 cells/well and incubated at 37°C, 5% CO_2, 95% air, and 100 % relative humidity for 24 h before the addition of samples. 100 µl of the sample solution was added to the respective wells. The plate was then incubated for 48 h maintaining the following conditions of 37°C, 5% CO_2, 95% air, and 100% relative humidity. The medium without the sample served as control. After 48 h, 15 µl of MTT (5 mg/ml) in PBS was added to each well and incubated at 37°C for 4 h. The medium with MTT was then flicked off, and the formed formazan crystals were solubilized in 100 µl of DMSO, and then the absorbance was measured at 545 nm using microplate reader. The concentration of the samples required to inhibit the cell growth by 50% (inhibitory concentration [IC_{50}]) value was calculated from the dose-response curve. The percentage growth inhibition was calculated using the following formula [19,20].

$$\%\,\text{Growth Inhibition} = \frac{\text{OD control} - \text{OD sample}}{\text{OD control}} \times 100$$

Apoptosis induction assay - nuclear staining with DAPI fluorescent dye

Apoptosis of nuclei was detected by a DAPI staining assay. DAPI dye is a fluorescent dye that stains the nuclear DNA of a cell and is, therefore, used to determine the effect of samples on inducing morphological changes in the nuclei of cancer cells undergoing apoptosis. MCF-7 cell line at 500 µl (1×10^6 cells/well) was seeded on 24 well plates and incubated until cell growth at log phase for 24 h. The cells were then treated at $2\times IC_{50}$ of test samples (at the highest concentration of 75.03 and 83.80 µg/ml, 0.1% DMSO) for 24 h. After treatment, the cells were washed with PBS (1.0 ml) and then fixed with 50 µl of methanol and water (1:1) under −20°C for 10 min. The fixed cells were washed and stained with 100 µl (1 µg/ml) of DAPI dye and then incubated at 37°C in a dark room for 30 min. The excess dye was then removed and 20 µl of PBS: Glycerin (1:1) was added to the mixed cells. The cells undergoing apoptosis, represented by the morphological changes of apoptotic nuclei, were observed and imaged at 40× magnifications under an inverted fluorescence microscope [21,22]. Percentage of apoptotic cells was calculated as follows:

$$\%\text{ of apoptotic cells} = \frac{\text{Amount of apoptotic nuclei}}{\text{Amount of all cells}} \times 100$$

DNA fragmentation detection assay

DNA fragmentation was used to determine the induction of apoptosis by observing the biochemical change. After the cancer cells were treated with $2\times IC_{50}$ of samples for 24 h, the cells were collected and washed with media. Then, cell suspensions were transferred to microcentrifuge tubes (1.5 ml) and centrifuged at 300 rpm for 5 min to collect the cell pellets. The DNA in the cell pellet was extracted with DNA extraction Kit, 2 µg of DNA was electrophoresed on 2% agarose gel containing 0.1 mg/ml ethidium bromide staining. After electrophoresis, DNA fragments were analyzed with an ultraviolet illuminated camera [23].

RESULTS AND DISCUSSION

Need for the novel anticancer agents arises due to the development of multidrug resistance in patients, severe side effects associated with the long-term treatment of cancer drugs, potential of the cytotoxic drugs to be very harmful to the body unless they are specific to cancer cells.

Previous studies related to the preliminary phytochemical screening of methanol leaf extract of *C. phlomidis* indicated the presence of alkaloids, steroids, coumarins, and flavonoids. The methanol leaf extract of *C. phlomidis* has been reported for significant cytotoxic and antioxidant potential [18]. Compounds (1, 2, 4, 6, 9, and 11) were isolated from the methanol extract of the *C. phlomidis* leaf [24]. From the spectral studies the isolated compounds were found to be 3-hexen-1-yl benzoate (Compound 1), 2,3-dihydroxypropanal (Compound 2), 1- (2,4,5-trihydroxyphenyl)-1-butanone (Compound 4), 3,6,7-trihydroxy-2-(3-methoxyphenyl)-4H-chromen-4-one (Compound 6), isopropyl linoleate (Compound 9), and oleic acid eicosyl ester (oleic acid ester) (Compound 11).

MTT cytotoxicity assay

In vitro cytotoxicity test is mainly done to screen potentially toxic compounds that affect basic cellular functions [25]. The molecular cancer cell lines profiling is essential for the development of new anticancer drugs, for understanding the mechanism of action and the patterns involved in cell resistance to chemotherapeutics already used in the treatment of cancer. Moreover, cancer cell lines can be a powerful tool for the identification of gene alterations or pathways related to cancer and for the discovery of putative drug targets [26].

Previous studies have evaluated the cytotoxic activity on mouse embryonic fibroblasts cell line (NIH 3T3) and HeLa cell lines using MTT assay on the crude extracts of petroleum ether, ethyl acetate, chloroform, and ethanol obtained from the root of the *C. phlomidis* plant. Ethanol extract had no cytotoxic activity, and the other extracts had moderate to weak cytotoxic activity on both the cell lines [27]. Evaluation of the *in vitro* antioxidant and cytotoxic activity of isolated compounds on roots of *C. phlomidis* has revealed that phenylacetic acid and ethyl-2-hydroxy-4-methyl benzoate from the ethanolic extract represent, a new group of cytotoxic and antioxidant agents [28]. Lakshmi and Bai have reported that *C. phlomidis* (L) leaves extract (CPLE) and its silver nanoparticles, plays a dual role by blocking carcinogen metabolic activation and enhancing carcinogen detoxification. They have also reported that the protective properties of the CPLE may be due to the presence of phytochemicals such as flavonoids, terpenoids, and alkaloids [29]. Although cell line studies have been reported for various solvent extracts of *C. phlomidis*, there was no much systematic study performed leading to an active cytotoxic chemical constituent against breast cancer (MCF-7) and lung cancer (A549) cell lines with the help of apoptosis study.

In MCF-7 breast cancer cell line study, Compounds 6 (3,6,7-trihydroxy-2-(3-methoxyphenyl)-4H-chromen-4-one) and 9 (isopropyl linoleate) exhibited good cytotoxic activity with an IC_{50} value of 83.80 and 75.16 µg/ml, Compounds 1 (3-hexen-1-yl benzoate) and 4 (1-(2,4,5 trihydroxyphenyl)-1-butanone) exhibited moderate cytotoxic activity with an IC_{50} value of 120.08 and 152.30 µg/ml, and Compounds 2 (2,3-dihydroxypropanal) and 11 (oleic acid eicosyl ester) exhibited less cytotoxic activity with an IC_{50} value of 187.50 and 208.22 µg/ml (Table 1). In A549 lung cancer cell line, Compounds 6 and 9 exhibited good cytotoxic activity with an IC_{50} value of 84.46 and 78.60 µg/ml, Compounds 1 and 4 exhibited moderate cytotoxic activity with the IC_{50} value of 107.31 µg/ml and 139.01 µg/ml, and Compounds 2 and 11 exhibited less cytotoxic activity with the IC_{50} value of 168.48 and 190.50 µg/ml against A549 lung cancer cell lines (Table 2). The effect of isolated compounds on percentage growth of MCF-7 and A549 cell line is illustrated in Figs. 1 and 2. The results clearly indicate that the Compounds 6 and 9 decreases cell viability and induces the cytotoxic effect in MCF-7 cells and A549 cells more efficiently compared to the other isolated compounds.

Apoptotic study

Apoptosis is a process of programmed cell death. Biochemical events lead to characteristic cell changes (morphology) and death. Morphological changes in the cell include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and mRNA decay.

Compound 6 (3,6,7-trihydroxy-2-(3-methoxyphenyl)-4H-chromen-4-one), a flavonoid and Compound 9 (isopropyl linoleate) showed good cytotoxic activity against the breast and lung cancer cell lines. Flavonoids are part of the polyphenol class of phytonutrients. Research on flavonoids had shown major developments in anticancer drug discoveries with the potential to destroy cancer cells through apoptotic induction [30]. Flavonoids exert protective effects against various types of tumors including oral and pharyngeal, gastric, pancreatic, colorectal, hepatic, prostate, ovarian, endometrial, breast, and lung cancers [31,32].

Table 1: Effect of the isolated compounds from methanol extract of *C. phlomidis* leaf on MCF-7 cell lines

Concentration (µg/ml)	% Growth inhibition					
	CPD-1	CPD-2	CPD-4	CPD-6	CPD-9	CPD-11
10	19.55±0.60	24.61±0.39	15.88±0.26	33.86±0.60	34.90±0.66	17.80±0.88
50	34.21±0.25	28.97±0.70	27.23±0.49	42.58±0.50	42.76±0.87	27.57±0.61
100	47.47±0.14	37.00±0.18	39.97±0.73	51.31±0.59	55.67±0.86	30.54±0.39
150	53.23±0.31	43.63±1.14	48.87±0.87	60.21±0.27	67.54±0.60	43.11±0.63
200	72.60±0.19	52.88±0.86	60.56±0.68	84.82±0.64	83.42±1.11	47.99±1.08
IC_{50}	120.08	187.50	152.30	83.80	75.16	208.22

All the values are expressed as mean±standard deviation, n=3. CPD-1, CPD-2, CPD-4, CPD-6, CPD-9, CPD-11 represents the isolated Compounds 1, 2, 4, 6, 9, and 11.
C. phlomidis: Clerodendrum phlomidis, MCF-7: Michigan cancer foundation-7

Table 2: Effect of the isolated compounds from methanol extract of *C. phlomidis* leaf on A549 cell lines

Concentration (µg/ml)	% Growth inhibition					
	CPD-1	CPD-2	CPD-4	CPD-6	CPD-9	CPD-11
10	20.37±0.80	18.72±0.45	16.87±0.64	27.78±0.41	23.46±0.27	22.63±0.88
50	38.68±0.54	24.69±0.63	29.84±0.56	35.80±0.37	47.94±0.75	22.51±0.70
100	46.50±0.75	37.86±0.08	41.77±0.60	54.53±0.25	59.05±0.41	36.01±0.87
150	64.81±0.73	42.59±0.71	52.67±0.40	76.54±0.67	74.07±0.79	41.56±0.85
200	72.84±0.47	58.85±0.78	63.99±0.52	83.13±0.40	78.40±0.35	53.09±0.70
IC_{50}	107.31	168.48	139.01	84.46	78.60	190.50

All the values are expressed as mean±standard deviation, n=3. CPD-1, CPD-2, CPD-4, CPD-6, CPD-9, CPD-11 represents the isolated Compounds 1, 2, 4, 6, 9, and 11.
C. phlomidis: Clerodendrum phlomidis

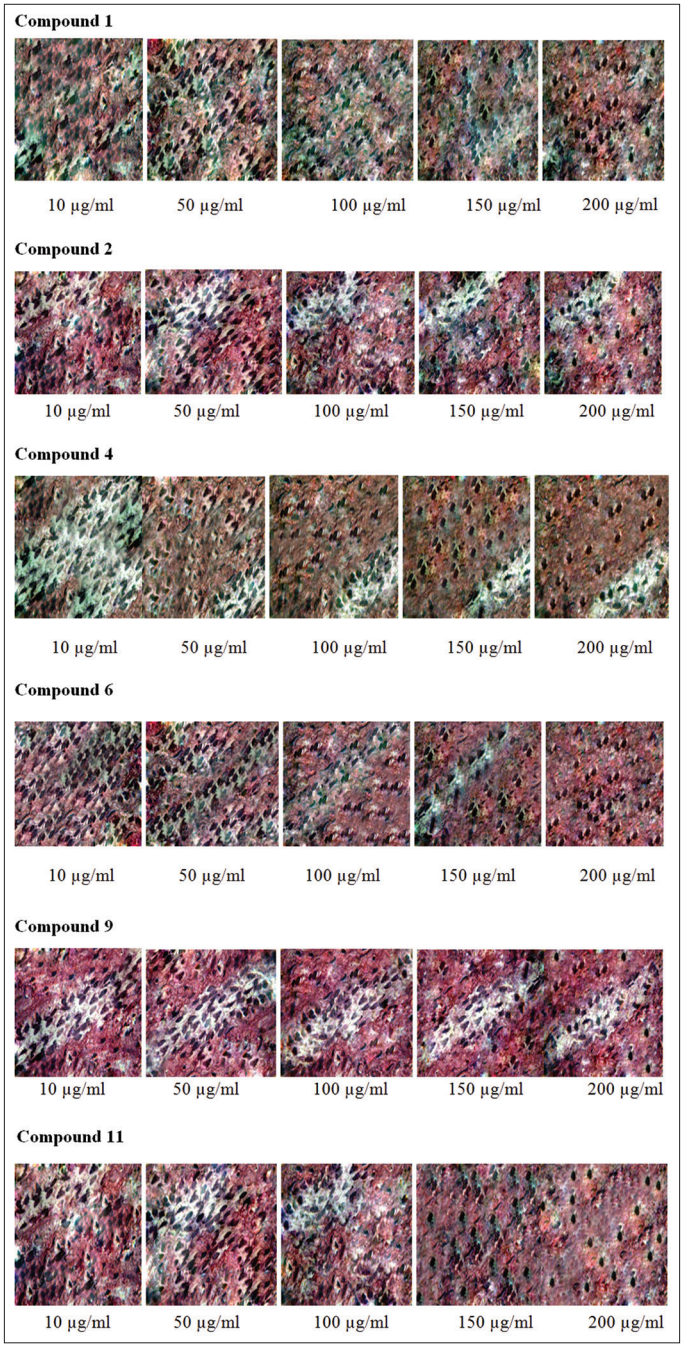

Fig. 1: Cytotoxic activity of the isolated compounds on Michigan cancer foundation-7 breast cancer cell lines at different concentrations

The molecular mechanism of flavonoids in cancer prevention remains unclear; some studies have proposed that flavonoids interact with different types of genes and enzymes. Some mechanisms of action of flavonoids have been identified, such as inactivation of carcinogens, antiproliferation, cell cycle inhibition, apoptotic induction, inhibition of angiogenesis, antioxidants, or the combination of such mechanisms [33].

To find out whether the cytotoxic activity of the isolated chemical constituents was due to apoptosis, MCF-7 cells were treated with the Compounds 6 and 9 at 2× IC_{50} for 24 h. There were no prominent morphological or nuclear changes in the control or untreated MCF-7 cells; the stained nuclei were rounded and evenly stained with DAPI. The cancer cells treated with the sample Compounds 6 and 9 showed the presence of condensed chromatin and apoptotic bodies that are the characteristic of the early and late stages of apoptosis. Compounds 6 and 9 induced 74.50 and 85.48% apoptosis in MCF-7 cells (Table 3 and Fig. 3). Determination of apoptosis was further carried out by evaluating the DNA laddering as a result of DNA fragmentation, which was typical of the late stage of apoptosis. DNA laddering was observed in the MCF-7 cells treated with the Compounds 6 and 9.

CONCLUSION

Among the chemical constituents isolated from the methanol extract of *C. phlomidis* leaf, Compounds 6 and 9 exhibited good cytotoxic activity against the breast cancer and lung cancer cell lines. Moreover, Compounds 6 and 9 induced apoptosis in MCF-7 cell lines. The results of the current study prove that the Compounds 6 and 9 are potential agents for the breast and the lung cancer disease. In future research, these potential agents can further be evaluated by animal studies and their mechanism of action apart from the induction of apoptosis of the cancer cells can be determined.

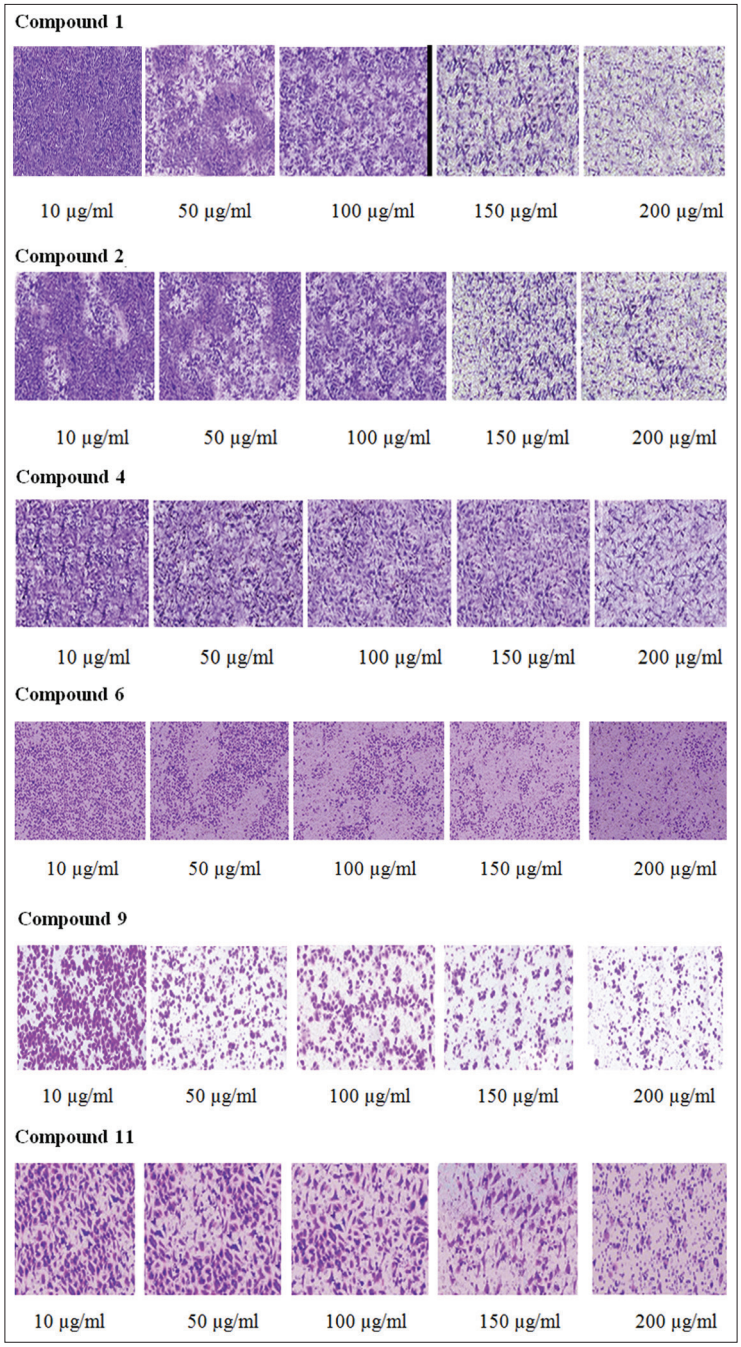

Fig. 2: Cytotoxic activity of the isolated compounds on A549 cell lines at different concentrations

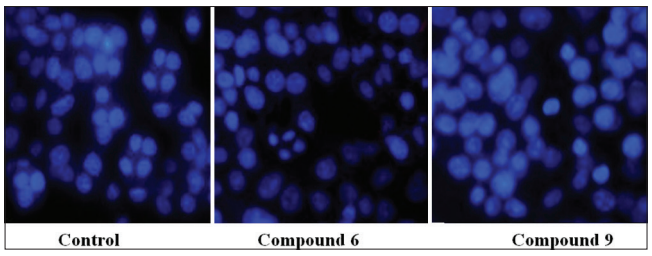

Fig. 3: Nuclear morphological changes in Michigan cancer foundation -7 cell lines after treatment with the Compound 6 and Compound 9

Table 3: Percentage of apoptotic cells of the Compounds 6 and 9 in apoptosis study of MCF-7 cell lines

S. no	Isolated compounds	Percentage of apoptotic cells
1	CPD-6	74.50±6.03
2	CPD-9	85.48±2.95

All values are expressed as a mean±standard deviation, n=3. MCF-7: Michigan cancer foundation-7

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

MKMM Raja conceived the original idea and supervised the project. NH Jainab carried out the experiment and wrote the manuscript with support from MKMM Raja.

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