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# Evaluation of in vitro cytotoxic activity of different solvent extracts of *Clerodendrum thomsoniae* Balf.f and its active fractions on different cancer cell lines

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## Abstract

**Background:** *Clerodendrum* is a genus of about 500 species belongs to the family Lamiaceae. Many species of this genus have been proved for the treatment of various diseases. This study was aimed to evaluate the cytotoxic effect of different solvents and their most active fractions of *Clerodendrum thomsoniae* Balf.f. in different human cancer cell lines. Aerial parts of the plant were subjected to Soxhlet extraction. Phytochemical analysis was done by using standard tests. In vitro anti-cancer activity on MCF-7, Hep-G2, A549, HT-29, MOLT-4, Hela, and Vero cell lines were evaluated by MTT assay.

**Results:** Phytochemical analysis confirmed the presence of most of the phytoconstituents in ethyl acetate extracts and the same extracts were found to be more cytotoxic activity to cancer cell lines MCF-7, Hep-G2, A549, HT-29, MOLT-4, and Hela with IC<sub>50</sub> values 29.43 ± 1.44 µg/ml, 43.22 ± 1.02 µg/ml, 56.93 ± 1.41 µg/ml, 60.68 ± 1.05 µg/ml, 69.83 ± 1.33 µg/ml, and 40.02 ± 1.14 µg/ml respectively, while it had no cytotoxic effect on normal Vero cells IC<sub>50</sub> = 367.5 ± 1.03 µg/ml. Ethyl acetate extracts were selected for the fractionation and MCF-7 cell line was used repeat MTT assay and found that fraction F5 was the most active fraction with IC<sub>50</sub> 17.33 ± 0.54 µg/ml.

**Conclusion:** These findings have proved that *Clerodendrum thomsoniae* Balf.f. have significant cytotoxicity especially for breast cancer cell lines. Further studies are required for the isolation of constituents and to explore the mechanism of action.

**Keywords:** *Clerodendrum thomsoniae* Balf.f, Anti-cancer, Cell lines, Fractions, MTT assay

## Background

Cancer is considered as one of the life-threatening ailments, which include abnormal cell growth with the capacity to continuously multiply and invade from one tissue to the other in the body [1]. The cancer had continuously been the most serious disease in humans around the world due to its high morbidity and mortality [2]. Breast cancer is a significant global health issue and one of the foremost causes of death in females [3].

The World Health Organization (WHO) expects about 15 million new cases of cancer by 2020 [4]. Chemotherapy, radiation therapy, hormonal therapy, and surgery are the common treatments for all kinds of cancer, and due to resistance and adverse or toxic side effects of these treatments, it has become necessary to hunt for an alternative anti-cancer treatment [5]. Natural products preserve vast pharmacological significance and have been considered as a key source of potential chemotherapeutic treatments [6]. Natural products obtained from plants are well-accepted sources and also many drugs are presently used from plant basis for the treatment of numerous human ailments including cancer.

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Over the past few decades, there has been a certain interest in the role of medicinal plant extracts in cancer prevention. Plants are rich sources of chemically diverse compounds, several with beneficial properties to human health. Consequently, about 50% of the anti-cancer therapeutic agents identified are derived from plants [7]. Several plant-based molecules that consist of vinblastine, vincristine, taxol, and camptothecin derivatives are used clinically to treat various types of cancers [8]. Various drugs were derived from natural sources such as plants and microorganisms is evidence for their ability to cure several types of diseases such as prostate, breast, lung, and colon cancers. In addition, the anti-cancer activities of numerous natural products are presently being studied to identify potential anti-cancer agents which could improve the efficacy of specific targeted remedies against cancer [9–13]. Most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied toward battling cancer [14, 15].

The National Cancer Institute collected about 35,000 plant samples from 20 countries and has studied around 114,000 extracts for anti-cancer activity [16]. Over 3000 species of plants with anti-tumor properties have been reported [17]. The genus *Clerodendrum* (Verbenaceae, more recently placed in the Lamiaceae) is a diverse genus with 580 species [18] of small trees, shrubs, or occasionally perennial herbs, typically in the tropical and subtropical region around the world [19]. A number of species from this genus have been used in traditional systems of medicine by several tribes in many countries like China, Japan, India, Korea, and Thailand [20]. This genus has been found to contain terpenoids as the major secondary metabolites [21, 22]. The genus also contains neo clerodane diterpenes [23], triterpenes [24], and iridoids [25]. Phenolic compounds have been frequently reported with phenyl propanoids and flavonoids as a principal class [26, 27] and few of species have been reported to have macrocyclic alkaloids and cyanogenic glycosides [28, 29]. Some of these compounds have been assessed for a number of activities mostly anti-inflammatory [30, 31], anti-asthmatic [32, 33], hepatoprotective [34], antioxidant [35], cytotoxicity [36], anti-tumor [37], and for the effects on central nervous system [38].

*Clerodendrum thomsoniae* Balf.f. is a twining, rambling, vine-like shrub native to tropical West Africa. Bleeding-heart vine or Bag-flower is the collective name of *Clerodendrum thomsoniae* Balf.f. [19, 39]. The leaves and flowers of *Clerodendrum thomsoniae* Balf.f. are the main bases of several medicinally significant phytochemicals. These phytochemicals protect the human body from oxidative stress by their own capable defense mechanism and curing diseases like bruises, cuts, skin rashes, and sores [40]. The objective of the study was to evaluate in vitro cytotoxic activity of *Clerodendrum*

*thomsoniae* Balf.f. and its active fractions on different cancer and normal cell lines.

## Methods

### Plant material

Aerial parts of *Clerodendrum thomsoniae* Balf.f. were obtained locally from Calicut District (Kerala, India). The plant materials were identified and authenticated by the Botany Department, Calicut University (Calicut, India). Voucher specimens were deposited in the same department herbarium as specimen no. 148249.

### Preparation of extracts

The aerial parts of *Clerodendrum thomsoniae* Balf.f. were dried properly in shade for 3 weeks, segregated, pulverized by a mechanical grinder, and passed through a 40-mesh sieve. About 1 kg of air-dried plant material was extracted in soxhlet assembly successively with petroleum ether, chloroform, ethyl acetate, ethanol and water (order of increasing polarity). Each time before extracting with the next solvent, the powdered material was dried at room temperature. Each extract was concentrated by using a rotary vacuum evaporator. The extract obtained with each solvent was weighed and the percentage yield was calculated in terms of the dried weight of the plant material. The color and consistency of the extract were also noted. All the solvents used for this entire work were of analytical grade (Merck, Mumbai).

### Phytochemical analysis

Phytochemical tests were carried out using standard procedures to identify constituents, as described by Harborne [41], Trease and Evans [42], and Kokate [43].

### Tests for alkaloids

One milliliter of extracts and Dragendorff's reagents were mixed in a test tube. The precipitation of orange-red color indicates the presence of alkaloids in the extract. The addition of Wagner's reagent to the extract produces reddish-brown residue also represents alkaloids. Similarly, the formation of white precipitate on Mayer's reagent addition to the extract indicates alkaloid presence. The appearance of yellow precipitate on Hager's reagent addition also helps to know alkaloids' presence in the extract.

### Test for carbohydrates

One milliliter of  $\alpha$ -naphthol and 2 ml of extracts were mixed in a test tube. The presence of purple or reddish-violet color at the junction of the two liquids on the addition of concentrated sulphuric acid through the test tube's side slowly indicates the presence of carbohydrate (Molisch Test). Another test was with Fehling's reagent. In this test, equal quantities of Fehling's solution A and

B were added to 1 ml of the extract. The presence of carbohydrates was indicated by the formation of a red brick precipitate upon heating. According to Benedict's test, 1 ml of extract solution was added to 5 ml of Benedict's reagent and boiled for 2 min and cooled. The formation of a red precipitate can confirm the presence of carbohydrates.

#### Tests for proteins and amino acids

According to Biuret test, add 1 ml of 40% sodium hydroxide solution to 1 ml of the extract, followed by two drops of 1% copper sulfate solution. The formation of a violet color will be an indication of the presence of proteins. The presence of amino acids was assessed by Xanthoprotein test. According to this test, 1 ml of concentrated nitric acid was added to 1 ml of the extract that results in the formation of a white precipitate. The obtained white residue is then boiled and cooled. Subsequently, 20% of sodium hydroxide or ammonia was added. The appearance of orange color is the indication of aromatic amino acids.

#### Test for steroid

One milliliter of plant extract, an equal volume of chloroform was added, and the presence of steroids was indicated by the presence brown ring upon the addition of few drops of concentrated sulphuric acid.

#### Tests of glycosides

The extract was dissolved in pyridine, and to make it alkaline, sodium nitroprusside solution was added. The presence of glycosides was shown by the formation of the pink-red to red color (Legal test). According to Keller Killiani test, the extract was dissolved into acetic acid with traces of ferric chloride and then transferred a test tube having sulphuric acid. At the junction, glycosides were found to develop a reddish-brown color, gradually become blue.

#### Test for saponins

In this test, 2 ml of distilled water was added to 2 ml of plant extract and shaken in a graduated cylinder for 15 min. One centimeter foam layer formation indicates the presence of saponins.

#### Test for flavonoids

One milliliter of the extract, magnesium ribbon was added, followed by 1–2 drops of concentrated hydrochloric acid. Formation of the red color showed the presence of flavonoids.

#### Test for tannins and phenolic compounds

The ferric chloride was added to 1 ml of the extract. The presence of tannins can be confirmed by the

formation of a dark blue or greenish-black color. In distilled water, a little extract was dissolved. A few drops of lead acetate solution have been added to this. White precipitate formation indicates the presence of phenolic compounds.

#### Test for triterpenoids

Chloroform was added to 5 mL of the extract together with a few drops of concentrated sulphuric acid. The mixture was shaken and kept for a while. The red color appears in the lower layer indicates the presence of steroids and the yellow color formation in the lower layer indicates the presence of triterpenoids.

#### Test for quinones

One milliliter of sulphuric acid was added to 1 ml of extract. Red color formation indicates the presence of quinone in the extract.

#### Test for anthraquinones

A few drops of 10% ammonia solution were added to 1 ml of plant extract, the formation of pink color precipitate indicates the presence of anthraquinones

#### Fractionation of ethyl acetate extract

The ethyl acetate extracts were subjected to column chromatography using silica gel (mesh size 60–120). Fifty grams of the extract was submitted to flash chromatography using silica gel (mesh size 60–120) as the stationary phase. The silica column was prepared using ethyl acetate by wet packing method and the column was washed using 100 ml of ethyl acetate. Then, the ethyl acetate extract was mixed with silica gel and made fine powder for easy distribution of the sample. The powdered sample mass was placed on the top of the pre-packed silica column. The elution was done using increasing solvent polarity made of hexane: ethyl acetate and methanol mixtures. Every 10 ml of fractions were collected in vials and further analyzed by thin layer chromatography. Similar fractions were pooled together to produce seven fractions (F1–F7), evaporated to dryness and kept in the dark for subsequent analysis.

**Table 1** Percentage yield and color of various solvent extracts from *Clerodendrum thomsoniae* Balf

S. no.	Extracts	Color	Yield % (W/W)
1.	Petroleum ether	Greenish Yellow	3.17
2.	Chloroform	Green	2.86
3.	Ethyl acetate	Brownish Green	12.58
4.	Ethanol	Brown	7.24
5.	Water	Chocolate Brown	9.62

**Table 2** Phytochemical analysis of *Clerodendrum thomsoniae* Balf

S. no.	Phytochemical constituents	Petroleum ether	Chloroform	Ethyl acetate	Ethanol	Water
1.	Alkaloids	–	–	+	+	–
2.	Carbohydrates	–	+	–	+	+
3.	Glycosides	–	–	+	+	+
4.	Terpenoids	–	–	+	–	–
5.	Proteins	–	–	–	–	+
6.	Amino acids	–	–	+	+	+
7.	Steroids	–	–	+	+	+
8.	Flavonoids	–	+	+	+	–
9.	Phenols	–	+	+	–	–
10.	Tannins	–	–	–	–	–
11.	Quinones	–	–	–	–	–
12.	Anthraquinones	–	–	–	–	–
13.	Saponins	–	–	–	–	+

Key: + presence, – absence

**Cell culture**

MCF-7 (human breast cancer cells), Hep-G2 (human liver cancer cells), A549 (human lung cancer cells), HT-29 (human colon cancer cells), MOLT-4 (human acute T lymphoblastic leukemia cells), Hela cells (human cervical cancer cells), and Vero (African green monkey kidney cells) cell line were cultured separately in liquid medium (DMEM) supplemented 10% fetal bovine serum (FBS), 100 µg/ml penicillin and 100 µg/ml streptomycin, and maintained under an atmosphere of 5% CO<sub>2</sub> at 37 °C. All cell line was purchased from the National Centre for Cell Sciences (Pune, India).

**In vitro anti-cancer activity assay by MTT method**

The test sample was evaluated for in vitro cytotoxicity on selected cell lines by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Briefly, the cultured cells were harvested by trypsinization, pooled in a 15 ml tube. Then, the cells were plated at a density of  $1 \times 10^5$  cells/ml cells/well (200 µL) into 96-well tissue culture plate in DMEM medium containing 10% FBS and 1% antibiotic solution for 24–48 h at 37 °C. The wells were washed

with sterile PBS and treated with different concentrations of the test sample in a serum-free DMEM medium. Each sample was replicated three times and the cells were incubated at 37 °C in a humidified 5% CO<sub>2</sub> incubator for 24 h. After the incubation period, MTT (20 µL of 5 mg/ml) was added into each well and the cells incubated for another 2–4 h until purple precipitates were clearly visible under an inverted microscope. Finally, the medium together with MTT (220 µL) were aspirated off the wells and washed with 1× PBS (200 µl). Furthermore, to dissolve formazan crystals, DMSO (100 µL) was added and the plate was shaken for 5 min. The absorbance for each well was measured at 570 nm using a micro plate reader (Thermo Fisher Scientific, USA) and the percentage cell viability and IC<sub>50</sub> value was calculated using GraphPad Prism 6.0 software (USA).

**Statistical analysis**

All the data expressed as mean ± SEM were analyzed by one-way analysis of variance (ANOVA), using GraphPad Prism version 6 software.

**Table 3** IC<sub>50</sub> of different solvent extracts of *Clerodendrum thomsoniae* Balf on different cell lines

Extracts	IC <sub>50</sub> value in µg/ml						
	Hep-G2	A549	MCF-7	HT-29	Hela	MOLT-4	Vero
Petroleum ether	213.12 ± 2.14	312.43 ± 1.42	174.43 ± 3.45	287.21 ± 3.56	259.22 ± 2.44	317.11 ± 1.67	522.45 ± 2.21
Chloroform	112.43 ± 2.48	213.54 ± 2.34	256.23 ± 1.72	157.44 ± 2.84	312.32 ± 2.65	247.33 ± 2.34	425.45 ± 2.14
Ethyl acetate	43.22 ± 1.02	56.93 ± 1.41	29.43 ± 1.44	60.68 ± 1.05	40.02 ± 1.14	69.83 ± 1.33	367.5 ± 1.03
Ethanol	178.23 ± 1.09	183.87 ± 2.45	119.22 ± 3.47	213.11 ± 2.34	229.12 ± 2.67	311.24 ± 3.47	574.29 ± 2.27
Water	389.22 ± 2.46	299.11 ± 2.89	234.39 ± 2.94	253.39 ± 3.56	211.23 ± 3.78	159.34 ± 3.61	>600

All values are in mean ± SEM (n = 3)



## Results

### Extraction yield

The percentage yield and color of all five extracts are listed in the following (Table 1). The highest percentage was observed in ethyl acetate extract (12.58% w/w), and the lowest was noted in chloroform extract (2.86% w/w).

### Phytochemical analysis

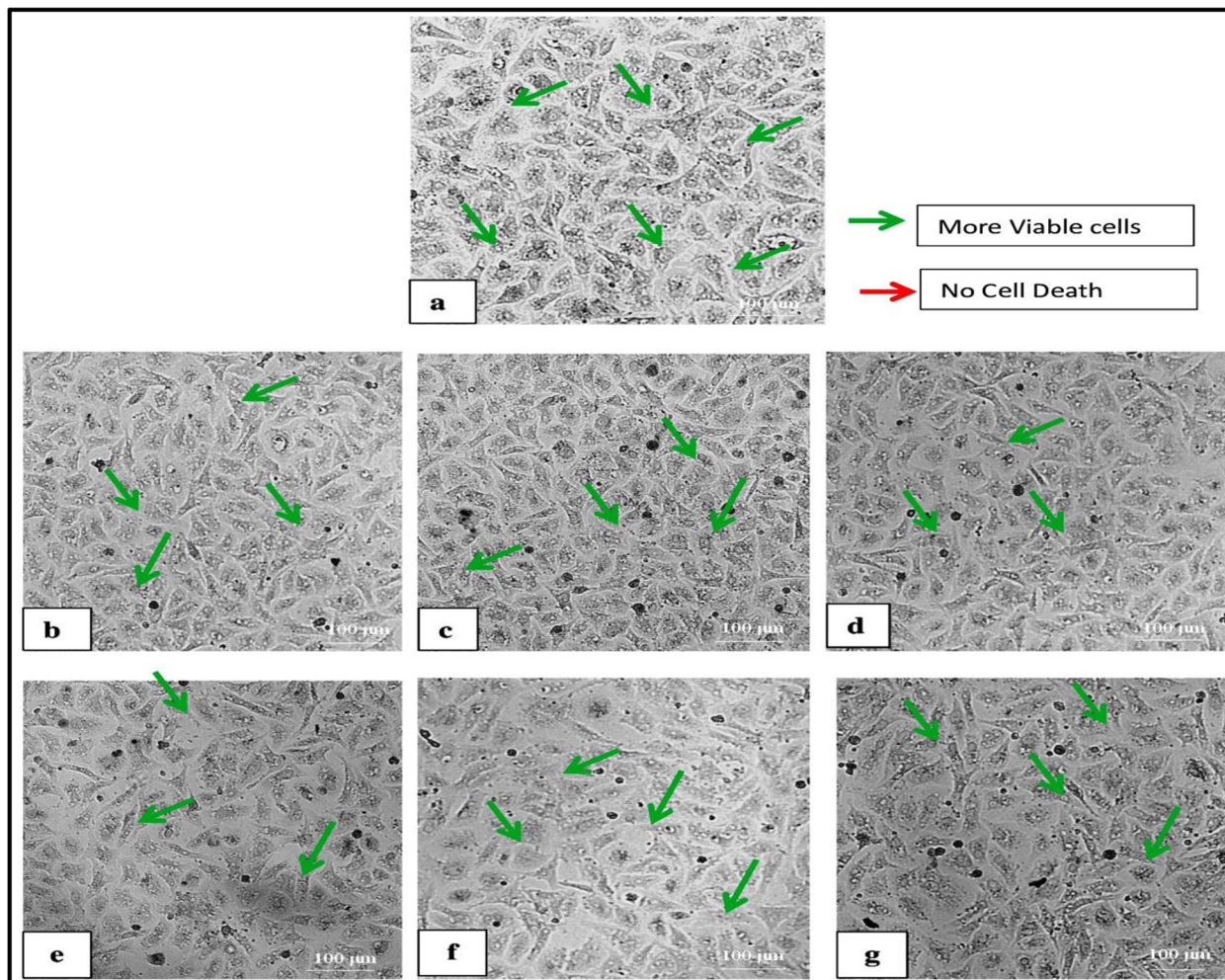
The phytochemical analysis showed that most of the phytoconstituents are present in ethyl acetate extracts. Detailed reports are listed in the following table (Table 2). The chloroform extract contains flavonoids and phenols to a minor extent. Ethyl acetate extracts showed a high presence of steroids, flavonoids, terpenoids, and phenols.

### Evaluation of $IC_{50}$ using MTT assay

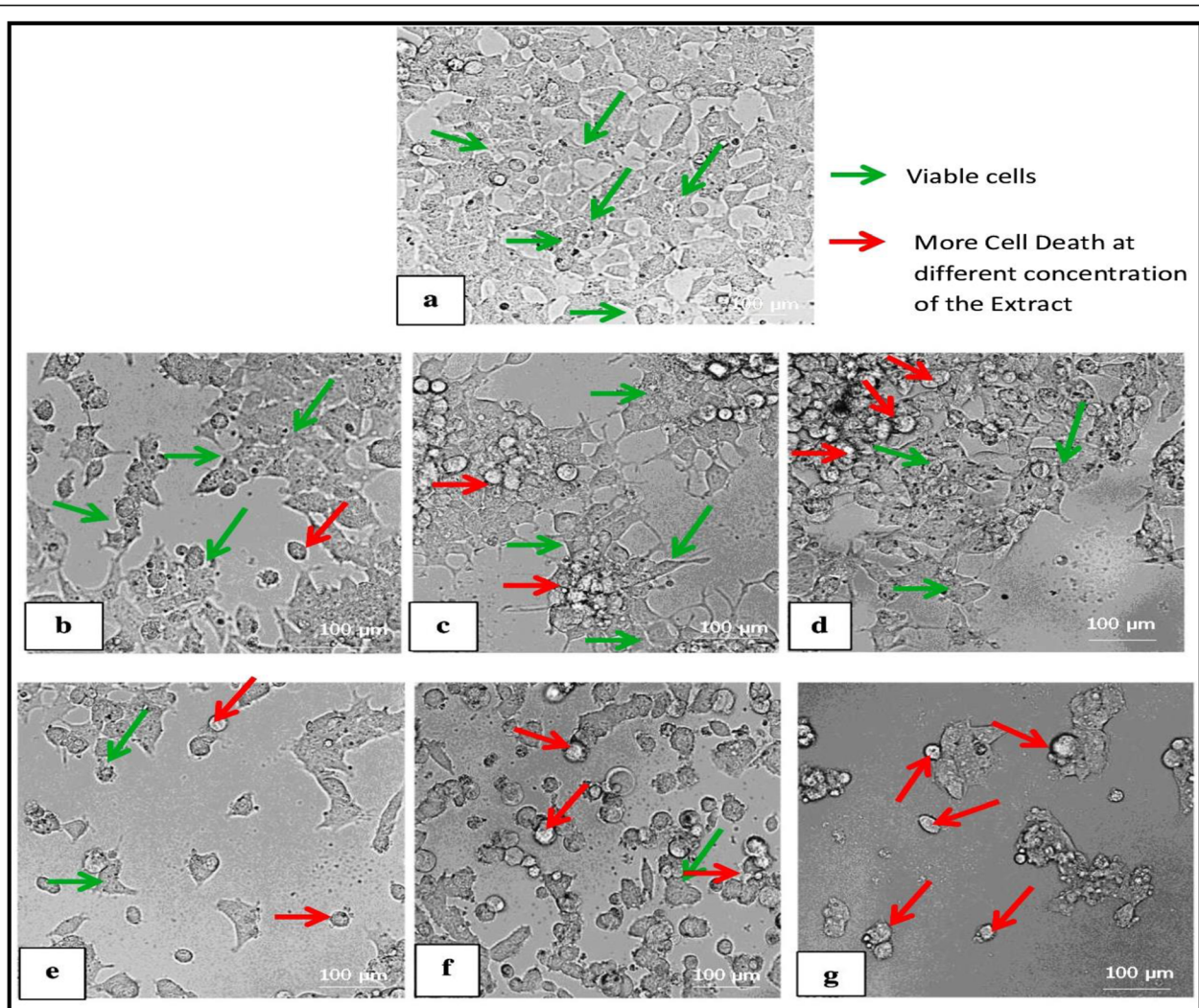
The  $IC_{50}$  value was determined based on cell viability rates. The results are listed in the following table (Table 3). The

morphological profile of the Vero cells after treated with ethyl acetate extract of *C. thomsoniae* is given at Fig. 1. The  $IC_{50}$  values showed that ethyl acetate extracts showing more toxicity on all cancer cell lines used, especially more toxicity selectively to MCF-7 cells ( $IC_{50} = 29.43 \pm 1.44 \mu\text{g/ml}$ ). The  $IC_{50}$  observed for ethyl acetate extract on MCF-7 was  $43.22 \pm 1.02$ ,  $56.93 \pm 1.41$ ,  $60.68 \pm 1.05$ ,  $40.02 \pm 1.14$ , and  $69.83 \pm 1.33$  for Hep-G2, A549, HT-29, Hela, and MOLT-4, respectively (all values are in  $\mu\text{g/ml}$ ). The selective cytotoxic activity of ethyl acetate extract may be due to the presence of different phytoconstituents present in this plant and most of them are soluble in ethyl acetate. The morphological profile of the MCF-7 cells after treated with ethyl acetate extract of *C. thomsoniae* is given at Fig. 2.

The results showed Vero cells were not showing toxicity any of the extracts treated which reveals the safety of this plant. Ethyl acetate extracts was selected for fractionation because of its specific toxicity on MCF-7 when compared to other cell lines.  $IC_{50}$  value of ethyl acetate



**Fig. 1** Morphological profile of the Vero cells after treated with ethyl acetate extract of *Clerodendrum thomsoniae* Balf 1.0  $\mu\text{g/mL}$  (b), 10  $\mu\text{g/mL}$  (c) 50  $\mu\text{g/mL}$  (d) 100  $\mu\text{g/mL}$  (e), 300  $\mu\text{g/mL}$  (f), and 500  $\mu\text{g/mL}$  (g) compared to control (a) for 24 h ( $\times 100$  enlargement)



**Fig. 2** Morphological profile of the MCF-7 cells after treated with ethyl acetate extract of *Clerodendrum thomsoniae* Balf.f. at 5.0 µg/mL (b),10 µg/mL (c), 50 µg/mL (d), 100 µg/mL (e), 300 µg/mL (f), and 500 µg/mL (g) compared to control (a) for 24 h (× 100 enlargement)

extract on MCF-7 was  $29.43 \pm 1.44$  µg/ml. IC<sub>50</sub> value of ethyl acetate extract fractions showed that Fraction F5 was more cytotoxic to MCF-7, then F6, F7, F4, F3, F2, and F1, respectively (Table 4). The morphological profile of the MCF-7 after treated with fraction 5(F5) of ethyl acetate extract of *C. thomsoniae* is given at Fig. 3.

Discussion

Cancer is the foremost cause of death worldwide, and as reported by WHO, it caused more deaths than AIDS, tuberculosis, and malaria in 2012. Among the

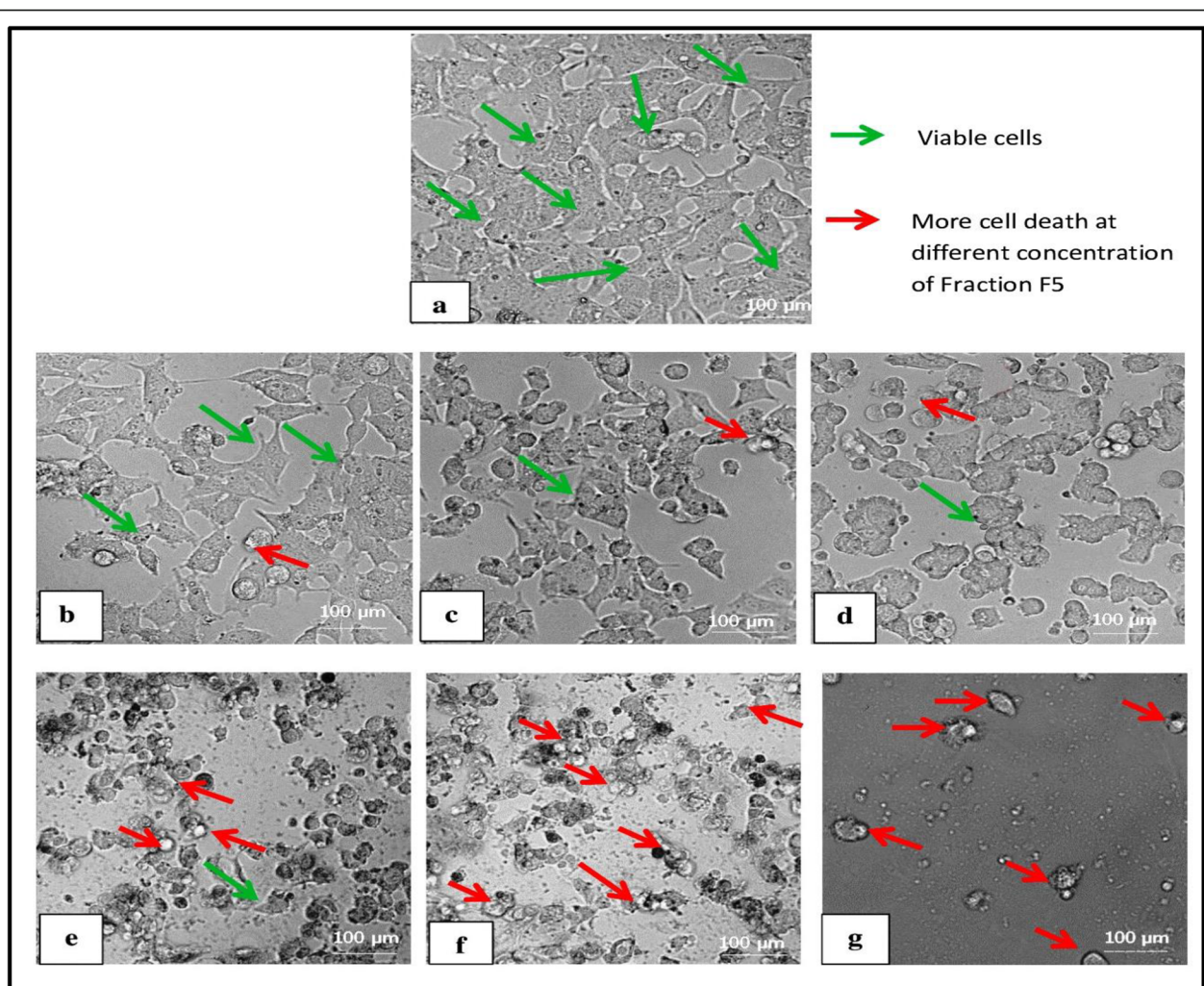
14 million cancer occurrences in 2012, commonly identified cancers were lung, breast, and colorectal, and the most common causes of cancer death were lung, liver, and stomach cancer (WHO, 2013). The management of cancer relies on surgery, radiotherapy, chemotherapy, or a combination of these methods [43]. Numerous essential anti-tumor drugs have been isolated from plants [44]. Almost one-third of prescribed drugs in the world are derived from plants, and anti-cancer properties of over 3000 plant species have been identified [45].

**Table 4** IC<sub>50</sub> of fractions of ethyl acetate extract of *Clerodendrum thomsoniae* Balf on MCF-7

Cell line	MCF-7						
Fractions	F1	F2	F3	F4	F5	F6	F7
IC <sub>50</sub>	85.50 ± 0.33	48.58 ± 1.43	44.87 ± 1.45	26.12 ± 1.43	17.33 ± 0.54	19.49 ± 1.4	26.53 ± 1.94

All values in mean ± SEM (n=3), F = fraction





**Fig. 3** Morphological profile of the MCF-7 after treated with fraction 5(F5) of ethyl acetate extract of *Clerodendrum thomsoniae* Balf.f. 1.0 µg/mL (b), 10 µg/mL (c), 50 µg/mL (d), 100 µg/mL (e) 300 µg/mL (f), and 500 µg/mL (g) compared to control (a) for 24 h (x 100 enlargement)

The *Clerodendrum* genus has been a good source of herbal medicinal products. The pharmacological investigation has shown that these compounds and extracts from the *Clerodendrum* genus have a wide-range of activities, such as anti-cancer, anti-inflammatory, analgesic, antioxidant, anti-hypertensive, anti-microbial, anti-diarrheal, hepato-protective, hypoglycemic, hypolipidemic, memory enhancing, and neuroprotective activities [46]. Earlier studies on some *Clerodendrum* species such as *Clerodendrum quadriloculare* [47], *Clerodendrum trichotomum* [48], *Clerodendrum bungei* [49], *Clerodendrum trichotomum* [50], *Clerodendrum kiansiense* [51], and *Clerodendrum serratum* [52] have reported for cytotoxic activity against tumor cell lines.

The percentage yield of extracts will be different in plant parts as well as among plant species. It varies with solvent systems too. In the present study, ethyl acetate extract yield was better when compared with other

extracts. The plant parts possessed a varied level of secondary metabolites. The phytochemical analysis revealed that most phytoconstituents are present in ethyl acetate extracts such as phenols, flavonoids, steroids, and alkaloids. In this study, ethyl acetate extracts show more cytotoxicity on breast cancer cell line (MCF-7) when compared to the other cell lines. It has been noted that ethyl acetate extract also showing cytotoxicity to other cell lines used in the present study, such as Hep-G2 ( $IC_{50} = 43.22$ ), A549 ( $IC_{50} = 56.93$ ), HT-29 ( $IC_{50} = 60.68$ ), Hela ( $IC_{50} = 40.02$ ), and MOLT-4 ( $IC_{50} = 69.83$ ). According to the literature review, several plant extracts and fractions were used for the evaluation of in vitro cytotoxicity studies. The  $IC_{50}$  value of Doxorubicin was reported as  $9.5 \pm 1.0$  µg/ml,  $13 \pm 1.0$  µg/ml, and  $11 \pm 1.0$  µg/ml for A-549, HeLa, and HepG2 cell lines, respectively [53]. The  $IC_{50}$  value of Doxorubicin on MCF-7 cells was reported as  $2.20 \pm 0.30$  µg/ml [54]. Because

of ethyl acetate extract's selective toxicity on these cell lines, we had performed the fractionation of ethyl acetate to find the more active fraction on the MCF-7 cell line. We observed that fraction F5 ( $IC_{50} = 17.33 \mu\text{g/ml}$ ) is more active when compared to other fractions tested. According to our earlier studies, GC-MS analysis of the fraction F5 showed different compounds present and noted that the major part was identified as some phenolic compound 2,4-bis(1-phenylethyl)-phenol and 2-(1-phenylethyl)-phenol [55]. According to the data available, a similar compound 4-Methyl-2, 6-bis (1-phenylethyl) phenol was reported for anti-cancer potential [56]. Another research has reported that similar compound 4-Isopropyl-2, 6-bis(1-phenylethyl)phenol from butanol fraction of *Cordyceps bassiana* for anti-proliferative and apoptosis-inducing activities in selected cancer cell lines [57]. It has been reported that phenolic compounds possess biological properties such as anti-apoptosis, anti-aging, anti-carcinogen, anti-inflammation, and inhibition of angiogenesis and cell proliferation activities. So the presence of this compound may be responsible for the cytotoxicity observed in our study, and farther experiment is going on in our laboratory to isolate the active compound responsible for the toxicity on the cancer cell line.

## Conclusion

This study concluded that *Clerodendrum thomsoniae* Balf.f. having significant cytotoxic activity on different cancer cell lines and it is more selective to breast cancer cell lines based on MTT analysis. These findings suggest that *C. thomsoniae* is a promising source of active compounds against cancer.

## Abbreviations

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide; WHO: World Health Organization; AIDS: Acquired immunodeficiency syndrome; ANOVA: Analysis of variance; DMEM: Dulbecco's modified Eagle's medium; FBS: Fetal bovine serum

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## Authentication of plant material

The plant materials were identified and authenticated by Dr. A.K. Pradeep, Assistant Professor -Department of Botany, Calicut University (Calicut, India). Voucher specimens were deposited in the same department herbarium as specimen no. 148249.

## Authors' contributions

All authors contributed to the manuscript. MAVK performed the works, VKK and VVV supervise the works. RR has done data analysis. All the authors read and approved the final manuscript.

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## Availability of data and materials

The data that support the findings of this study are available from the corresponding author, upon request.

## Ethics approval and consent to participate

Not applicable  
Consent for publication  
Not applicable

## Competing interests

The authors declare that they have no competing interests.

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