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Phytochemical and In Vitro Cytotoxic Screening of Chloroform Extract of *Ehretia microphylla* Lamk

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Abstract: *Ehretia microphylla* of the Boraginaceae family has been extensively used as a folklore remedy for the treatment of a wide range of ailments such as cough, cancer, allergies, and gastrointestinal and venereal disorders. Extensive literature review reports have revealed these findings due to the presence of numerous phytomolecules. To validate traditional claims for cytotoxic activity of *E. microphylla*, the present study was undertaken. Dried leaves of the plant were powdered and defatted with petroleum ether followed by hot continuous extraction with chloroform. The chloroform extract was subjected to in vitro cytotoxic screening against a panel of human cancer cell lines such as HCT-116 (colon), MCF-7 (breast), PC-3 (prostate), A-549 (lung), HL-60 (leukemia) and MiaPaCa-2 (pancreatic) at 50 µM using SRB assay. The extract exhibited noteworthy cytotoxicity activity against breast and lung cancer. It exhibited 85.55% and 77.93% inhibition against MCF-7 and A-549 cancer cell lines, respectively. The mechanism behind cell death was determined using the DAPI staining method, which induces alteration in nuclear morphology in MCF-7 cell lines evidenced through DAPI staining. Phytochemical screening of *E. microphylla* extract showed the presence of saponins, steroids, lipids, tannins and triterpenoids. The chemoprofile of the chloroform extract of *E. microphylla* leaves was established using an n-hexane:ethyl acetate solvent system in a ratio of 6:4. The developed chromatogram showed five spots both in visible and UV light at 254 nm. The information provided in the present study will enable further studies on the isolation and characterization of bioactive compounds/fractions by following bioactivity-guided fractionation, and thus, the plant has the potential to reduce proliferation and may induce cell death via apoptosis in breast cancer cells.

Keywords: cancer; cytotoxicity; scorpion bush; natural products; phenolic acids; major chemical substances; triterpenoids

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1. Introduction

Cancer is a critical non-communicable public health menace. Cancer is a general term for a large assemblage of diseases that can affect any portion of the body and is characterized by uncontrolled proliferation of abnormal cells. It is the second leading cause of mortality after cardiovascular disorders around the world, with an incidence rate of more than 9.6 million cases per year [1]. All over the world, people suffer from different types of cancers; among these, lung cancer is reported as the most prevalent in males followed by breast cancer in females [2–5]. Generally, normal body cells grow, proliferate and die in a programmed channel, whereas cancer cells do not die, but they continue to grow and proliferate in a disorderly manner. Research indicates that cancers are caused by a dysfunction of many genes coding for proteins such as anti-apoptotic proteins, transcription factors, growth factor receptors, tumor suppressor genes and cancer-promoting enzymes [6,7]. Cancer management has largely benefited from the discovery of medicinal plants. Phytomolecules and their derivatives are being explored for the prevention or cure of cancer via diverse mechanisms [8,9]. Researchers are not only tirelessly trying to isolate phytochemicals from medicinal plants to conquer this growing threat of

cancer but are also exploring the mechanism behind cell death [10,11]. A thorough survey of literature reveals that a large number of phytomolecules have been evaluated for in vitro anticancer activity in various cancer cell lines [12].

Ehretia microphylla Lamk (Boraginaceae), also known as Scorpion bush, is a medium-sized dicot shrub with a height around 1–4 m [13]. The plant is locally known as Wild Tea and Tsaang Gubat by tribal communities of Asia [14,15]. *E. microphylla* has been documented in the Siddha Materia Medica [16]. It is widely distributed in the Guangdong, Hainan, and Taiwan provinces of South China, as well as in subtropical areas of southern and southeastern Asia. The plant is widely used for its ample range of activities such as antibacterial, anti-viral, and anti-allergic as well as its wound-healing actions. [17–19]. Numerous reports documented on *E. microphylla* reveals this plant as a potential candidate for the treatment of eczema, scabies and pruritus [20], tooth decay [21] cachexia, and food poisoning [22]. It is used in the management of diarrhea, and it is antispasmodic and a disinfectant. [23]. Traditionally, the decoction of the dried leaves has been used for stomach and cough problems; moreover, the fresh leaves are used as a substitute for tea [24]. The plant is also used in the treatment of diabetes, allergies and hyperacidity [25].

Plant secondary metabolites and their derivatives such as vinca alkaloids, taxanes, podophyllotoxins and camptothecin have been clinically employed in combating cancer [3]. The outcome of these studies not only signifies the use of phytomolecules but has also attracted the attention of scientists/researchers to explore the anticancer potential of other traditionally used and medicinally promising plants [26,27].

Keeping in view its efficacy and relative safety, it seems to be a viable approach to investigate this traditional plant to search for new anticancer molecules. Several researchers have established that *E. microphylla* contains numerous bioactive metabolites such as triterpenes, alkaloids, glycosides, tannins, α-amyrin and β-amyrin, saponins, flavonoids and rosmarinic acid [28,29].

2. Results

2.1. Plant Extract

The yield of *E. microphylla* extract as well as color and physical appearance are presented in Table 1.

Table 1. Yield of extracts of *E. microphylla*.

Sr. No.	Extract (g/Kg)	Percentage Yield	Color	Physical Appearance
1	Chloroform (54.347)	5.43	Green	Sticky mass

2.2. Phytochemical Screening of Chloroform Extract of E. microphylla

Crude extract of chloroform of *E. microphylla* was subjected to phytochemical screening to reveal the presence of phytomolecules, viz., carbohydrates, proteins, amino acids, lipids, alkaloids, glycosides, flavonoids, saponins, tannins, steroids, triterpenoids and phenolic compounds, which are depicted in Table 2.

2.3. Thin-Layer Chromatography (TLC) Finger Printing

Thin-layer chromatography is a technique for examining mixtures and determining the number of components present in them. It is also used for identification and to check the purity of a compound. The chemoprofile of chloroform extract of *E. microphylla* was developed using a solvent system (n-hexane:ethyl acetate: 6:4), and the chromatogram showed five spots. The developed chromatogram depicted in Figure 1 and retention factor (Rf) for each spot was calculated and presented in Table 3.

Table 2. Results of phytochemical screening of chloroform extract of *E. microphylla*.

Sr. No.	Class of Phytoconstituents	Tests	Chloroform
1	Carbohydrates	Molisch's test	−
2	Proteins	Biuret test	−
3	Amino acid	Ninhydrin test	−
4	Lipids	Solubility test	+
5	Alkaloids	Mayer's test	−
6	Glycosides	Legal test	−
7	Flavonoids	Shinoda test	−
8	Saponins	Froth test	+
9	Tannins and phenolic compounds	Ferric chloride test	+
10	Steroids and triterpenoids	Liebermann–Burchard test	+

(+) present; (−) absent.

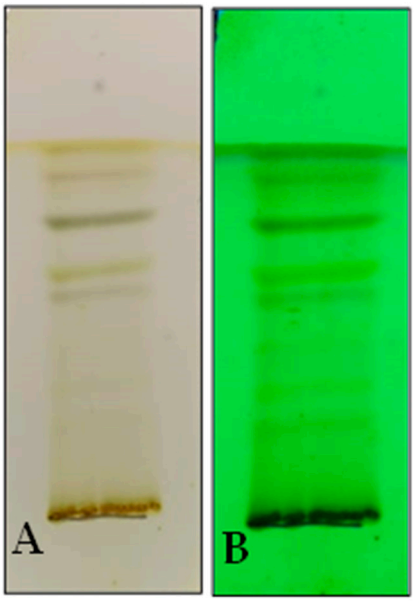

Figure 1. Thin-layer chromatogram of chloroform extract of *Ehretia microphylla* leaves. (**A**) TLC chromatogram observed in visible light; (**B**) TLC chromatogram observed in UV light at 254 nm in UV chamber.

Table 3. Results of TLC fingerprinting of chloroform extract of *E. microphylla* leaves.

S. No.	Extract	Solvent System	No. of Spots	R_f Value
1	Chloroform extract	Hexane: ethyl acetate	5	0.57, 0.65, 0.78, 0.87, 0.92

2.4. Biological Evaluation

2.4.1. In Vitro Cytotoxicity

The dried chloroform extract of *E. microphylla* (PSEM) was tested for in vitro cytotoxicity against human cancer cell lines HCT-116 (colon), MiaPaCa-2 (pancreatic), PC-3 (prostate) A-549 (lung), and HL-60 (promyelocytic leukemia) malignant cell lines using sulforhodamine B. The results of the preliminary screening of SRB assay are presented in Table 4. Careful observation of the results reveals that the cell lines HCT-116, PC-3, A-549 and MCF-7 were the most sensitive, while HL-60 and MiaPaCa-2 cell lines were almost resistant to the chloroform extract at 50 µg/mL. The chloroform extract produced significant effects against PC-3, HL-60, HCT-116 and MCF-7 cell lines. The chloroform extract elicited 75.45%, 74.50%, 77.93% and 85.55% inhibition against HCT-116, PC-3, A-549 and MCF-7, respectively, which are presented in Table 4.

Table 4. Percentage inhibition of cancer cells by different extracts of *E. microphylla* at 50 µg/mL.

Entry	HCT-116 (Colon Cancer)	MiaPaCa-2 (Pancreatic Cancer)	PC-3 (Prostate Cancer)	A-549 (Lung Cancer)	HL-60 (Leukemia Cancer)	MCF-7 (Breast Cancer)
PSEM	75.45 ± 0.0399	19.36 ± 0.0422	74.50 ± 0.0473	77.93 ± 0.0451	34.67 ± 0.0410	85.55 ± 0.0389

Results are represented as mean ± SD (n = 3).

The chloroform extract displaying more than 70% inhibition was only evaluated against those cell lines at different concentrations (10, 20, 30, 40 and 50 µg/mL), and the IC_{50} values (Table 5) were calculated. The chloroform extract of *E. microphylla* was also assessed for selective killing against "FR2" normal human breast cells. The extract did not attain IC_{50} values until >97 µM, as depicted in Table 5.

Table 5. IC_{50} values of chloroform extract against different human cancer and normal cells.

Entry	HCT-116 (Colon Cancer)	MiaPaCa-2 (Pancreatic Cancer)	PC-3 (Prostate Cancer)	A-549 (Lung Cancer)	HL-60 (Leukemia Cancer)	MCF-7 (Breast Cancer)	FR2 (Normal Human Breast Epithelial)
PSEM	26.40 ± 1.2445	ND	21.56 ± 1.5473	29.47 ± 1.6532	ND	16.13 ± 0.9765	>97
Adriamycin	——	1.75 ± 0.143	——	——	——	1.10 ± 0.437	ND

Results are represented as mean ± SD (n = 3); ND, not determined.

Careful observations of Table 5 reveal that the IC_{50} values of PSEM were found to be 16.13 ± 0.9765 and 26.40 ± 1.2445 µg/mL against MCF-7 and HCT-116 human cancer cell lines, respectively.

2.4.2. Effect of Chloroform Extract on Nuclear Morphology

The apoptotic effect of chloroform extract of *Ehretia microphylla* was observed using 4',6-Diamidino-2-phenylindole (DAPI) staining. Noticeable changes were observed in the nuclear morphology of the MCF cell lines. The formation of apoptotic bodies and condensed nuclei was observed in a concentration-dependent manner, which is indicated in white colored arrows in Figure 2, whereas the nuclei of untreated MCF- 7 cells exhibited normal intact morphology.

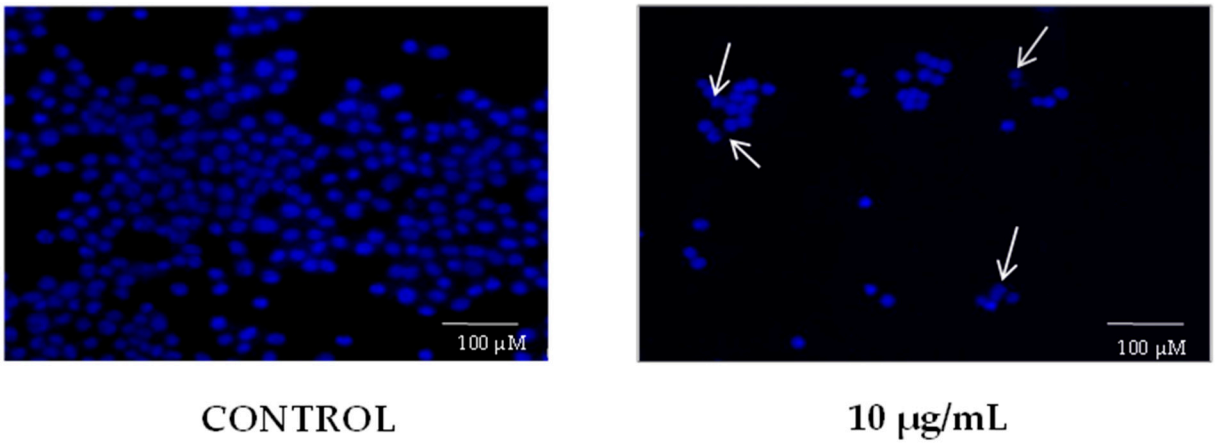

Figure 2. *Cont.*

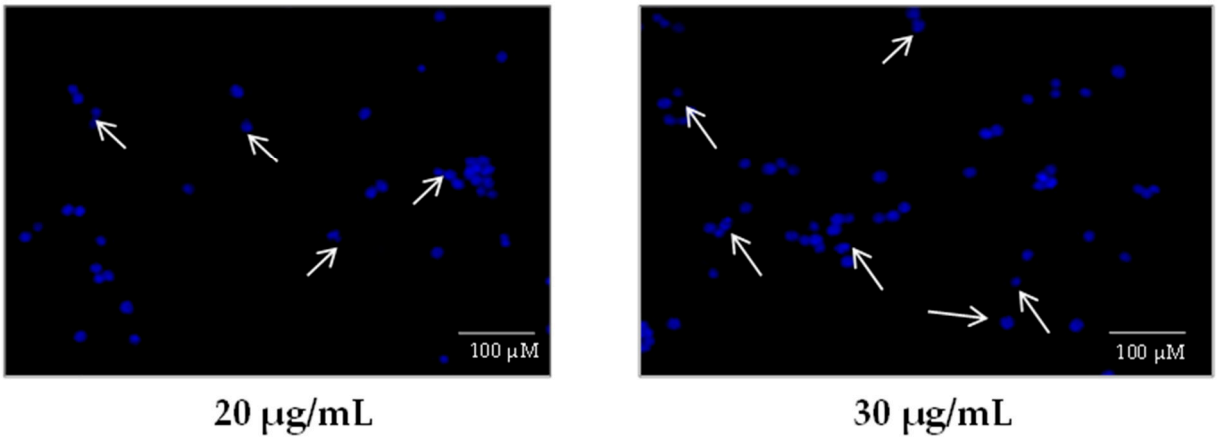

Figure 2. DAPI staining in MCF-7 cell lines. Variation in nuclear morphology induced with PSEM in a concentration-dependent manner, observed under fluorescence microscope. PSEM induced the formation of apoptotic bodies, as shown by arrows. The photographs depicted here are magnified at ×20, and scale bars, 100 µm.

3. Discussions

Cancer is one of the foremost threats to mankind worldwide after cardiovascular disorders. Numerous chemotherapeutic agents are clinically available and are approved by the FDA for the mitigation of cancer. Cancer therapy in the form of surgery or radiotherapy is effective when the disease is detected early, but many cancers are still diagnosed after the cells from a primary tumor have metastasized to other parts of the body, and chemotherapy is the main form of treatment at this point [30]. Chemotherapy involves delivering drugs systemically so that they can reach and kill tumor cells, but most of these drugs have severe side effects in patients and must therefore be used at low doses. Researchers claim that developing new drugs that are more selective for cancer cells and have fewer side effects is a major challenge [31].

Integrative medicine, which combines traditional western medicine with alternative or complementary treatments such as herbal medicine, acupuncture, massages, biofeedback, yoga, and stress management techniques, is being used to supplement orthodox medicines and treatment approaches in the management of cancer patients. Despite the availability of chemotherapeutic agents, the scientific community is still facing challenges to seek out novel entities for the treatment and management of different types of cancer because of the agents' harmful side effects.

Researchers are currently inclined toward the search for natural products because of the presence of abundant therapeutically accepted phytomolecules such as flavonoids, tannins, amino acids, alkaloids, sugars, tannins and polyphenolic compounds, despite their ethno-pharmacological significance in the mitigation of jaundice, inflammation, asthma, cancer, diarrhea, wound healing and skin diseases by tribes of Africa, Australia and Asia [15,32].

Kudera et al. established in vitro antiproliferative and antibacterial actions of ethanolic extract of *E. microphylla* against intestinal human cancer cell lines such as Caco-2 and HT-29 with IC_{50} values of 37.89 ± 2.68 and 130.89 ± 13.99 µg/mL, respectively, of which this plant was used earlier as folk medicine in the mitigation of diarrhea, dysentery and abdominal pain. [33,34]. Similarly, Shukla et al. reported antioxidant, antibacterial and antiallergic effects of ethanolic leave extract of *E. microphylla* [35]. Using another approach, Simpol et al. documented that the leaves of the plant Scorpion Bush were traditionally used for numerous medicinal purposes, viz., as an antispasmodic, body cleanser and mouth wash, which are attributed due to the presence of phenolic acids, benzoquinone, fatty acids and flavonoids [36]. Henry et al. also reported some bioactive metabolites from *E. mircophylla* such as ursolic acid, baurneol, microphyllone, hydroxymicrophyllone, and

dehydromicrophyllone and recognized them for the cure of scabies, itching, stomach ache, psoriasis and skin allergies [37].

Charisse et al. documented an ample range of pharmacological actions from the different parts of *E. microphylla*, including the aerial part, leaves, root, stem and fruits. The plant is recognized to contain a variety of phytoconstituents such as rosmarinic acid, bauerenol, α-amyrin, β-amyrin, kaempferol-3-*O*-rutinoside and kaempferol-3-*O*-glucoside from methanol extract of the leaves of *E. microphylla*. Methanol extract of the plant contains ehretianone, stigmasterol, β-sitosterol and α-spinasterol. *E. microphylla* exhibited numerous activities, viz., anticancer, antioxidant, anti-inflammatory, wound healing, antimicrobial, anti-allergy and anti-angiogenesis. Methanolic extract of leaves of the plant induces apoptosis and DNA fragmentation [38,39]. With this background, several studies of this plant motivated us to explore the anticancer potential for chloroform extract of the leaves of *E. microphylla*, as this plant has not yet been explored as an anticancer agent, keeping in view that *E. microphylla* is documented to have numerous phytomolecules that are responsible for its use in the mitigation of cancer. In the present investigation, the phytochemical screening and cytotoxic evaluation of chloroform extract of *E. microphylla* have been established. The chloroform extract of *E. microphylla* exhibits significant anticancer activity and is attributed to the presence of triterpenoid, saponins and phenolic compounds. The available literature also reveals that triterpenoids and phenolic compounds are the most bioactive compounds that are responsible for anticancer potential of most traditionally used plants such as α-amyrins and β-amyrins and ursolic acid, which belong to the triterpenoid class, whereas bergenin and gallic acid are phenolic compounds that exhibit their significant anticancer activities against several human cancer cell lines [14,40–45].

Observations of the present study are in agreement with aforementioned reports, as phenolic compounds and triterpenoids have been observed as the main compounds in the phytochemical screening of chloroform extract of *E. microphylla* leaves. The thin-layer chromatographic pattern of chloroform extract of *E. microphylla* leaves reveals the presence of five spots that have established the chemoprofile of chloroform extract of *E. microphylla*. Identification of separated compounds can be achieved on the basis of their retention factor (Rf) values [46].

The cytotoxicity results reveal that HCT-116, PC-3, A-549 and MCF-7 were the most sensitive, while HL-60 and MiaPaCa-2 cell lines were almost resistant to chloroform extract at 50 µg/mL. The chloroform extract displaying more than 70% inhibition was only evaluated against those cell lines at different concentrations and to determine the IC_{50} values. The chloroform extract of *E. microphylla* exhibits selective cytotoxicity toward human breast cancer cell lines and has been found to be non-toxic against "FR2" normal human breast epithelial cells. Furthermore, the IC_{50} value of the extract was found to be high in normal human breast epithelial cells (FR2), which validates its effectiveness as a potential candidate for the mitigation of cancer. Careful observation of Table 5 reveals that the IC_{50} value of chloroform extract of *E. microphylla* is 16.25 µg/mL against MCF-7 cell lines.

Apoptosis is the hallmark of cancer and plays a critical role in the amelioration of breast cancer. The mechanism behind cell death was determined using the DAPI staining method. DAPI is a fluorescent stain that binds to DNA at furrows of the A-T region. The results revealed that the chloroform extract of *E. microphylla* induces apoptosis in a concentration-dependent manner. Thus, these classes of phytomolecules may be responsible for anticancer activity. However, further work is required to elucidate the mechanism behind cell death in MCF-7 cell lines, and thus, *E. microphylla* can be a potential lead and therapeutic candidate for next level of evaluation for the mitigation of cancer.

4. Materials and Methods

4.1. Plant Materials

The dried leaves of *E. microphylla* were procured from the Department of Botany, Sri Venkateshwara University, Tirupati. The authentication of *E. microphylla* (0850) was confirmed by Assistant Professor Dr. K. Madhava Chetty from the department of Botany of

S.V. University, Tirupati, Andhra Pradesh. Specimen voucher 0850 was submitted to the herbarium of the same department.

4.2. Chemicals and Reagents

The chemicals, solvents, and reagents such as chloroform, hexane, ethanol and ethylacetate were purchased from Loba chem., CDH, and Spectro chem India. RPMI-1640, fetal calf serum, minimum essential medium (MEM), trypan blue, trypsin, streptomycin, gentamycin, penicillin, dimethyl sulfoxide, (DMSO), mitomycin-C, sulforhodamine, and adriamycin were from Sigma Aldrich, Chemical Co., Burlington, MA, USA. Distilled water, Tris buffer, Tris-EDTA buffer, (Hi-Media) acetic acid, sodium bicarbonate, hydrochloric acid were from spectrochem. All other chemicals used in this study were of analytical grade and were purchased from local dealers.

4.3. Preparation of the Extract

The shade-dried leaves of *E. microphylla* were ground using mixer to form coarse powder. The dried coarsely powdered plant (1 Kg) was defatted by Soxhlation apparatus using petroleum ether 60–80 °C (5 L). The defatted material was then extracted using chloroform (5 L) by soxhlation process. The exhaustive extraction was carried out at a temperature of 80°C. The extraction was continued until few drops of solvent collected during siphoning did not leave any residue after evaporation on watch glass. Lastly, chloroform extract was dried under vacuum [32]. The dried extract was further subjected to phytochemical screening and cytotoxicity studies.

4.4. Phytochemical Screening of Extract of E. microphylla

The chloroform extract was subjected to phytochemical screening to detect the presence of phytomolecules such as carbohydrates, proteins, amino acids, alkaloids, glycosides, flavonoids, saponins, tannins, steroids, triterpenoids and phenolic compounds as per the standard protocol mentioned in the literature [47].

4.5. Thin-Layer Chromatography (TLC) of Extracts

The chloroform extract of *E. microphylla* leaves was subjected to thin-layer chromatography to develop a chromatographic pattern to analyze the number of constituents present in it. The chemoprofile of chloroform extract of *E. microphylla* was developed on pre-coated silica gel plates (Merck) using solvent system n-hexane:ethyl acetate: 6:4, with detection by visible light and UV light at 254 nm [46].

4.6. Cell Culture, Growth Conditions, and Treatment

HCT-116 (colon), MiaPaCa-2 (pancreatic), PC-3 (prostate) A-549 (lung), HL-60 (leukemia), MCF-7 (breast) human cancer cells and fR2 (normal human breast epithelial) lines used in this study were obtained from the National Cancer Institute (NCI), Frederick, Burlington, MA, USA. All human cancer cells were grown and maintained in RPMI1640/MEM/DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine (0.3 mg/mL), penicillin (100 units/mL), streptomycin (100 µg/mL), 0.37% $NaHCO_3$ and pyruvic acid (0.11 mg/mL). Cells were grown in CO_2 incubator (Thermocon Electron Corporation, Waltham, MA) at 37° C temperature, 90% humidity and 5% CO_2 gas environment, and subjected to treatment with chloroform extract of *E. microphylla* dissolved in DMSO, while untreated control received only DMSO (<0.2%) as a vehicle.

4.7. Biological Evaluation

4.7.1. In Vitro Cytotoxic Assay

In vitro cytotoxicity of chloroform extract of *E. microphylla* was determined using semi-automated sulforhodamine-B (SRB) assay against a panel of human cancer cell lines, viz., HCT-116 (colon), MiaPaCa-2 (pancreatic), PC-3 (prostate), A-549 (lung), HL-60 (leukemia),

MCF-7 (breast) and FR-2 (normal human breast epithelial) malignant cells (Monks et al., 1991). Cancer cells/mL of suspension were counted and seeded in 96-well plates at a density of 10,000 cells per well/100 µL. Seeded plates were incubated at 37 °C in an environment of 90% relative humidity and 5% CO_2 for 24 h. Stock solutions of chloroform extract (20 mg/mL) were prepared in DMSO, and 50 µL concentrations were prepared using serial dilution method. Then, 50 µL of test material in 100 µL of complete growth medium was added and allowed to incubate for 48 h at 37 °C temperature, 90% relative humidity and 5% CO_2 environment. After that, 50 µL of 50% chilled TCA (trichloroacetic acid) was added to each well and kept for 1 h to immobilize the cells at 4 °C. After that, plates were washed five to six times with water, air dried and stained with sulforhodamine-B dye (100 µL, 0.4% *w*/*v* in 1% acetic acid) for 30 min. Plates were again washed with 1% (*v*/*v*) acetic acid to remove the unbound dye. Plates were air dried, and absorbed dye was dissolved by adding 100 µL of 10 mM Tris buffer (pH 10.5). Thereafter, plates were placed on plate shaker for 10 min. Wells with no treatments were considered as control wells. The optical density (OD) was measured using an ELISA reader at 540 nm. Cell growth was obtained by subtracting the mean OD (optical density) values of blanks and mean OD values of the treatments. Percent growth in the presence of extracts was calculated considering the growth devoid of any test substance (chloroform extract) as 100%, and in turn, % age growth inhibition in the presence of test substance was determined. Extracts showing greater than 70% inhibition were selected to determine the dose–response curve (IC_{50}) [48–54].

4.7.2. Effect of Chloroform Extract of *E. microphylla* on Nuclear Morphology

Human breast cancer cells (MCF-7) were seeded at a density of 1×10^4 cells/well in a 12-well plate. The cells were treated with or without different concentrations of chloroform extract of *E. microphylla* for 24 h and incubated at 37 °C temperature, 95% air and 5% CO_2 atmosphere for 6 h. The treated and untreated cells were collected and washed with ice-cold PBS and fixed with methanol: acetic acid (3:1) for 30 min at room temperature. The cells were stained using DAPI (1 µg/mL) for 20 min and analyzed under fluorescent microscope. (Nuclear morphology was examined using DAPI staining as described earlier.) The presented images were magnified at ×20 and scale bars, 100 µm [53].

5. Conclusions

The results of the present investigation revealed that chloroform extract of *E. microphylla* holds great potential. The induction of apoptosis leading to cell death was determined using DAPI staining. The results of the DAPI staining revealed that chloroform extract of *E. microphylla* exhibited apoptotic body formation in a dose-dependent manner. The IC_{50} values of PSEM were determined against selected human cancer cell lines. It is inferred from the results that chloroform extract of *E. microphylla* is highly effective against MCF-7 and HCT-116 cell lines, as evident from their lower IC_{50} values against MCF-7 and HCT-116 human cancer cell lines versus other cell lines. Moreover, it was found to be non-toxic against normal human breast epithelial cells, and it could be developed as a source of a novel anticancer molecule. The anticancer activity of the plant may be attributed to alkaloids, triterpenoids and phenolic compounds, as apparent from preliminary phytochemical screening. The research work is in progress to isolate and characterize bioactive chemical constituent(s)/bioactive fraction.

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