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RESEARCH ARTICLE

Ethanol Extracts of Selected *Cyathea* Species Decreased Cell Viability and Inhibited Growth in MCF 7 Cell Line Cultures

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Abstract
Cancer is the cause of more than 6 million deaths worldwide every year. For centuries, medicinal plants have been used in the treatment of cancer. Chemotherapy, radiotherapy, surgery and acupuncture point stimulation are also used to treat cancer. The present study was intended to reveal the cytotoxic and anticancer potential of selected *Cyathea* species and to highlight their importance in the pharmaceutical industry for the development of cost-effective drugs. Cytotoxic studies using brine shrimp lethality bioassays and MCF 7 cell line cultures were carried out. Compared to petroleum ether, chloroform and acetone extracts, the ethanol extracts of selected *Cyathea* species were found to be more effective against brine shrimps. The ethanol extracts were further subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assays. A decrease in cell viability and an increase in growth inhibition were observed for the MCF 7 cell line. The maximum percentage of cell inhibition was observed in *Cyathea crinit*, followed by *Cyathea nilgirensis* and *Cyathea gigantea*. The results of the present study suggest that *Cyathea* species are an effective source of cytotoxic compounds.

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1. Introduction

Plant extracts are evaluated by various methods to determine their pharmacological activity, potency and toxicity. Qualitative and quantitative chemical examination is designed to detect and isolate the active ingredients [1]. There is a shift from synthetic to natural substances with medicinal properties all around the world. The major classes of bioactive compounds from plants include phenolics, terpenoids, alkaloids, essential oils, lectins, polypeptides and polyacetylenes [2]. Phytotherapy has been considered as an alternative to alleviate side effects associated with synthetic drugs [3]. Bioactive compounds in plants can be developed into natural products that can be used as substitutes for synthetic drugs. Furthermore, they provide the foundation for the development of new drugs [4]. Cytotoxic studies using brine shrimp lethality bioassay consists of exposing *Artemia salina* (*A. salina*) to test extracts in saline solution and the lethality is evaluated after 24 hours. It is a useful method due to its inexpensiveness and the ease of performing the assay [5]. It has been mainly used for the isolation of cytotoxic and antitumor compounds from plant extracts [6]. Numerous studies have illustrated the use of the brine shrimp assay to screen plant extracts [7,8].

Cancer is the cause of more than 6 million deaths worldwide every year [9]. For centuries, medicinal plants have been used in the treatment of cancer [10]. Nearly 50% of breast cancer and 37% of prostate cancer patients use herbal products [11]. More than 60% of currently used anticancer agents are derived from natural sources. Biologically active components from plants are significant and important sources of new drugs that are likely to lead to new and better treatments for breast cancer [12,13]. Cancer treatments such as chemotherapy, radiotherapy and surgery may cause treatment-induced cancer pain. These treatments destroy the normal cells along with cancer cells; cancer cells can develop resistance to treatment through mutations [14]. Acupuncture point stimulation is also used to treat cancer. Even though acupuncture is used among oncology patients to control cancer pain, the role of acupuncture in controlling cancer pain has not been clearly established through clear clinical trials. Many clinical trials have problems such as poor study design, small sample size and lack of statistical analysis [15]. Numerous randomized controlled trials (RCTs) have been conducted to treat cancer using acupuncture [16—19]. This may be used as a complementary therapy to treat cancer after successful clinical trials.

Natural products derived from medicinal plants have gained significant recognition in the potential management of cancer. Numerous experiments have evaluated plant extracts as prophylactic agents, which offers great potential to inhibit the carcinogenic process [20—22]. India is a vast country enriched in pteridophytic flora. However, there is no report on the cytotoxic properties of tree fern *Cyathea*. To fill this gap, the present study was intended to observe the cytotoxic and anticancer properties of *Cyathea nilgirensis* Holttum, *Cyathea gigantea* (Wall. ex. Hook.) Holttum and *Cyathea crinita* (Hook.) Copel.

2. Materials and methods

2.1. Collection of plant materials

Samples for the present study were collected from different parts of Tamil Nadu, South India. *C. nilgirensis* Holttum were harvested in and around Kakkachi stream (1,725 m), Tirunelveli hills (8° 44′ N, 77° 44′ E), *C. gigantea* (Wall. ex. Hook.) Holttum from the road sides near Nadugani (2,637 m), Nilgiris hills (11° 24′ N, 76° 44′ E) and *C. crinita* (Hook.) Copel. from the Anglade Institute of Natural History, Shenbaganur, Kodaikanal (2,195 m), Palni hills (10° 13′ N, 77° 32′ E), Western Ghats, South India. The specimens were identified based on the "Pteridophyte Flora of the Western Ghats, South India" by Manickam and Irudayaraj [23]. Herbarium specimens were deposited in the St. Xavier's College Herbarium (XCH), Palayamkottai for further reference (*C. nilgirensis*, XCH 25423; *C. gigantea*, XCH 25422; and *C. crinita*, XCH 25424).

2.2. Preparation of extracts

The collected species of *Cyathea* were thoroughly washed with tap water and then by distilled water. They were blotted on the blotting paper and spread out at room temperature in shade to remove excess water content. The shade dried plant samples were ground to fine powder using a mixer grinder. The powdered materials were stored in a refrigerator for further use. Thirty-gram powdered samples were extracted successively with 180 mL petroleum ether, chloroform, acetone and ethanol using the Soxhlet extractor for 8—12 hours at a temperature not exceeding the boiling point of the solvent. The extracts were concentrated in a vacuum at 40°C using a rotary evaporator.

2.3. Cytotoxic activity — brine shrimp lethality bioassay

Cytotoxic activity of different extracts of selected *Cyathea* species were evaluated using the brine shrimp lethality bioassay method [24]. Approximately 1 g of *A. salina* cysts was aerated in a 1-L capacity glass jar containing filtered seawater. The air stone was placed in the bottom of the jar to ensure complete hydration of the cysts. After 24 hours incubation at room temperature (25—29°C), newly hatched free-swimming nauplii were harvested from the bottom outlet. As the cyst capsules floated on the surface, this collection method ensured pure harvest of nauplii. The freshly hatched free-swimming nauplii were used for the bioassay. Thirty clean test tubes were taken and separated by 10 mL in each test tube. Twenty-five tubes were used for the samples in five different concentrations ranging from 100 mg/mL to 500 mg/mL and five tubes for controls. With the help of a Pasteur pipette, 20 nauplii were transferred to each test tube containing various concentrations (100 mg/mL, 200 mg/mL, 300 mg/mL, 400 mg/mL, and 500 mg/mL) of petroleum ether, chloroform, acetone, and ethanolic extracts of *C. nilgirensis*, *C. gigantea*, and *C. crinita*. Five replicates were made for each concentration and a control dimethyl sulfoxide was also maintained. Standard

plumbagin was used as a positive control (Fig. 1). The setup was allowed to remain for 24 hours under constant illumination. After 24 hours, the dead nauplii were counted with a hand lens. Using the recorded observations, 50% lethal concentration (LC_{50}), 95% confidence interval, 90% lethal concentration (LC_{90}), and χ^2 values were calculated.

2.4. Cytotoxic activity — MTT cell proliferation assay

2.4.1. Cell line and culture

The MCF 7 cell line (human breast carcinoma) was obtained from the National Centre for Cell Science, Pune, India. The cells were cultured in Dulbecco modified Eagle medium (pH 7.4), supplemented with 10% fetal bovine serum and antibiotics, penicillin (100 U/mL) and streptomycin sulfate (100 μg/mL).

2.4.2. MTT assay

The cytotoxicity of *C. nilgirensis*, *C. gigantea*, and *C. crinita* ethanolic extracts against MCF 7 cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [25]. The cells were seeded in 96-well microtiter plates at 3000 cells per well with 100 μL Dulbecco modified Eagle medium. The plates were incubated for 24 hours at 37°C under 5% CO_2 in a humidified atmosphere. Later, the medium was removed and fresh growth medium containing different test doses of *C. nilgirensis*, *C. gigantea*, and *C. crinita* (12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, and 200 μg/mL) ethanolic extracts were added. Five wells were included in each concentration. After 3 days incubation at 37°C under 5% CO_2, the medium was removed. Twenty microliters of 5 mg/mL MTT (pH 4.7) was added per well and cultivated for a further 4 hours, and the supernatant fluid was removed. One hundred microliters of dimethyl sulfoxide was added per well and shaken for 15 minutes. The absorbance at 570 nm was measured with a UV spectrophotometer, using wells without cells as blanks. All the experiments were performed in triplicate. The absorbance of untreated cells was considered as 100%. The 50% inhibitory concentration (IC_{50}) was determined graphically. The conventional anticancer drug adriamycin was used as a positive control. The

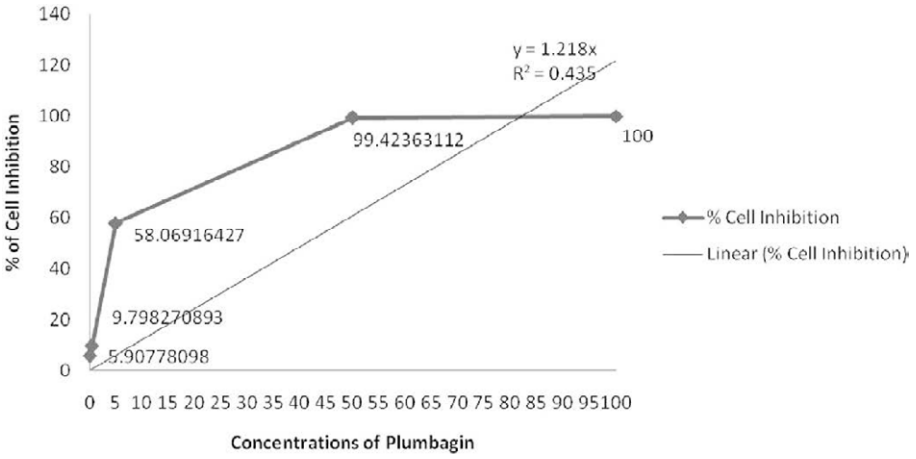

Figure 1 Effect of Plumbagin against MCF 7 cell line.

Table 1 Cytotoxic effects of studied *Cyathea* species against *Artemia salina*.

Species	Extracts	LC_{50} (mg/mL)	95% confidence interval		LC_{90} (mg/mL)	χ^2
			Lower	Upper		
Cyathea nilgirensis	Pet. ether	563.27	501.07	668.69	958.57	0.57
	Chloroform	328.08	299.80	358.40	637.71	1.54
	Acetone	567.00	461.34	910.36	968.73	4.27
	Ethanol	304.73	273.62	336.21	642.72	0.90
Cyathea gigantea	Pet. ether	543.59	449.60	821.95	901.17	4.67
	Chloroform	279.31	253.14	304.41	548.69	0.47
	Acetone	345.58	298.43	401.95	608.52	4.09
	Ethanol	277.45	204.62	340.50	973.77	0.50
Cyathea crinita	Pet. ether	421.12	312.69	891.76	728.46	15.53
	Chloroform	331.08	251.65	433.57	738.91	4.78
	Acetone	340.99	311.61	373.57	663.81	0.23
	Ethanol	287.44	254.30	319.82	512.88	2.45

Pet. ether = petroleum ether.

Table 2 Effect of ethanolic extracts of *Cyathea* species against MCF 7 cell line.

Conc. (μg/mL)	*Cyathea nilgirensis*		*Cyathea gigantea*		*Cyathea crinita*	
	% cell viability	% cell inhibition	% cell viability	% cell inhibition	% cell viability	% cell inhibition
12.5	100	0	100	0	100	0
25	99.73	0.27	100	0	100	0
50	97.77	2.23	99.24	0.76	96.38	3.62
100	95.40	4.60	94.28	5.72	84.93	15.07
200	83.95	16.05	85.42	14.58	74.74	25.26

Conc. = concentration.

inhibition of cell growth was calculated as the percent anticancer activity using the following formula:

$$\% \text{ Cell Inhibition} = 100 - \text{Sample Absorbance/Control Absorbance} \times 100$$

3. Results

3.1. Cytotoxic activity — brine shrimp lethality bioassay

The cytotoxic effects of petroleum ether, chloroform, acetone and ethanolic extracts of *C. nilgirensis*, *C. gigantea*, and *C. crinita* were found to be concentration dependent. The degree of lethality was found to be directly proportional to the concentration of the extract. The results of brine shrimp lethality bioassay are given in Table 1. The tested extracts were found to be toxic ($LC_{50} < 1{,}000$ mg/mL) in the brine shrimp bioassay. Ethanolic extracts of *C. nilgirensis*, *C. gigantea* and *C. crinita* were found to be more effective against brine shrimps with LC_{50} values of 304.73 mg/mL, 277.45 mg/mL, and 287.44 mg/mL, respectively. Chloroform and acetone extracts of *Cyathea* species showed moderate lethality level. Petroleum ether extract exhibited fewer cytotoxic effects when compared to other extracts. The standard plumbagin showed 100% mortality of brine shrimp nauplii at 0.046 mg/mL.

3.2. Cytotoxic activity — MTT cell proliferation assay

The MTT assay is based on the reduction of MTT by mitochondrial dehydrogenase by purple formazan product. The ethanolic extracts of selected *Cyathea* species were subjected to MTT cell proliferation assay (Table 2). The microscopic observations explained the apoptosis of MCF 7 cells. Decreased viability and increased growth inhibition were observed in MCF 7 cells in a concentration dependent manner. Maximum percentage cell inhibition was observed in *C. crinita* followed by *C. nilgirensis* and *C. gigantea*. As the concentration increased, there was an increase in cell growth inhibition but it was found to be less with only 25.26% growth inhibition in *C. crinita* at 200 μg/mL. The IC_{50} value of *C. crinita* was greater than 400 μg/mL. Similarly, *C. nilgirensis* and *C. gigantea* showed IC_{50} values of 714.28 μg/mL and 806.45 μg/mL, respectively. The results showed that ethanolic extracts of *Cyathea* species had a moderate anticancer activity against MCF 7 cells.

4. Discussion

The evaluation of the toxicity of plant extracts is necessary for safe treatment. It enables identification of the intrinsic toxicity of the plant and the effects of acute overdose [7]. The brine shrimp lethality bioassay is used in screening the crude extracts as well as in the isolated compounds to assess the toxicity. It could also provide an indication of possible cytotoxic properties of the tested plant extracts. It is frequently used as a model system to measure cytotoxic effects of variety of toxic substances and plant extracts against brine shrimps nauplii [8]. It is also considered as a reliable indicator for the preliminary assessment of toxicity and it can be extrapolated for cell line toxicity and antitumor activity [5,26]. A number of novel antitumor and pesticidal natural products have been isolated using this bioassay [24]. Ethanolic extracts of *C. nilgirensis*, *C. gigantea* and *C. crinita* are more effective against brine shrimps with LC_{50} values of 304.73 mg/mL, 277.45 mg/mL and 287.44 mg/mL, respectively, compared to chloroform, acetone and petroleum ether extracts. The results obtained from the brine shrimp lethality bioassay can be used as a guide for the isolation of cytotoxic compounds from the ethanolic extracts.

In vitro cytotoxicity test using cell lines was performed to screen potentially toxic compounds that affect basic cellular functions. The MCF 7 cell line had a cobblestone-like phenotype with strong cell—cell adhesion. However, when the cells were exposed to cytotoxic components, two distinct modes of cell death were recognized, namely, apoptosis and necrosis. Apoptosis or programmed cell death involves a sequential cascade of cellular events, resulting from chromatin condensation, DNA fragmentation, cytoplasmic membrane blebbing and cell shrinkage [27]. Our results showed that ethanolic extracts of *Cyathea* species caused marked cell growth inhibition in the MCF 7 cell line. Most of the MCF 7 cell membranes blebbed during shrinkage and the apoptotic bodies were formed around cells treated with ethanolic extracts of *Cyathea* species. Manosroi et al [28] suggested that samples with IC_{50} values of 200—5,000 μg/mL were considered to have moderate potential to develop into a cancer therapeutic agent. Similar to previous observations, ethanolic extracts of *Cyathea* species with IC_{50} values of 400—806.45 μg/mL showed moderate cytotoxic effects. Moderate cytotoxic activity shown by *Cyathea* species crude extracts may be attributed

mainly to the presence of bioactive compounds present in the ethanolic extracts. This should be further assayed using animal models to confirm antitumor activity and may lead to new breast cancer chemotherapeutic agents with novel structures and mechanisms of action.

Disclosure statement

The author declares to have no conflicts of interest and no financial interests related to the material of this manuscript.

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