

Research Article



Induction of Apoptosis by Ethanolic Extracts of Two Species of *Biophytum* DC. (Oxalidaceae) in Four Human Cancer Cell Lines

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ABSTRACT

The study of apoptosis is of profound importance in cancer biology and cancer genetics. Apoptosis can be triggered by a diverse array of stimuli. Apoptotic cells are monitored by a number of assays such as loss of cell viability, DNA fragmentation, DNA ladder assay and DNA condensation. In the present study, ethanolic extracts of two species of *Biophytum*, *B. veldkampii* Shanavas and *B. reinwardtii* (Zucc.) Klotzsch. were screened for their apoptotic effect by nuclear condensation assay in four human cancer cell lines, MCF-7, MDA-MB-231 (breast cancer cell lines) He La (cervical cancer cell line), and NCI/ADR- Res (ovarian cancer cell line). Two concentrations (5, 10µg/ml) of the extracts were used for the assays. The study revealed that, ethanolic extracts of *B. reinwardtii* and *B. veldkampii* showed potent activity against all the cancer cell lines tested. The apoptotic activity exhibited against MCF-7 was highest [*B. reinwardtii* (96%), *B. veldkampii* (90%)] followed by He La, MDA-MB-231 and ADR Res in that order. The DNA in condensed chromatin of apoptotic cells stains strongly with fluorescent dyes which allows for differentiation of apoptotic from non-apoptotic cells. The percentage of apoptotic cells in the presence of plant extracts increased in a concentration-dependent manner and is characterized by morphological changes in the cells or its contents. In the control group (cell lines not treated with extracts), very few apoptotic cells could be (2-3%) observed. Thus the nuclear condensation assay proved useful for the scientific validation of the anticancer activity of the two species of *Biophytum*.

Keywords: *Biophytum veldkampii*, *B. reinwardtii*, apoptosis, human cancerous cell lines, nuclear condensation assay, Hoechst-33342 staining

INTRODUCTION

Cancer is caused by a complex, poorly understood interplay of genetic, molecular and environmental factors. Cancer progression results in the production of malignant cells that continue to proliferate but do not die due to the absence of apoptosis. It is the second leading cause of death in the world and there is as yet no completely effective medicine to treat most cancers. The development of effective treatment regimens remains one of the greatest challenges in the area of cancer chemotherapy. Furthermore, many cancer treatments are very expensive.

Over the past few decades a significant progress has been made in cancer prevention and treatment. Research on plant or plant parts have great importance in drug discovery as there is high scope for exhaustive studies to identify new medicinal plants for such life-threatening diseases. The identification of new and effective anticancer drugs has always been a focal point in cancer research. During the 1960s, the National Cancer Institute (USA) began to screen plant extracts with antitumor activity¹. Natural compounds isolated from medicinal plants have received increasing interest ever since they have been identified to be rich sources of novel anticancer drugs².

For a long time, plants have played a very important role in human life. Herbal formulations consisting of single and multiple herbs are commonly prescribed as an alternative way to treat cancer. Recently, intensive studies have been

conducted to examine the mechanisms responsible for the anticancer effects of medicinal plants and plant-based drugs^{3,4}. Many chemo-preventive agents have been associated with anti-proliferative and apoptotic effects on cancer cells because of their high antioxidant activity, targeting signalling molecules, and preventing or protecting cells from further damage or transformation into cancer cells⁵. Chemotherapeutic agents have been associated with side effects and deleterious health diseases. Therefore scientists are trying to isolate new potentially valuable anticancerous drugs from medicinal plants with lesser side effects.

The development of human cancers is often mainly a consequence of deregulated cell cycle control and/or suppressed apoptosis⁶. Therefore apoptotic assays have become an important tool for researchers studying cancer biology and cancer genetics. Apoptosis is an important homeostatic mechanism that balances cell division and cell death, thus maintaining the appropriate cell number in the body. Apoptosis can be triggered by a diverse array of stimuli, and is characterized by a number of morphological changes, which are utilized for the identification of apoptosis. These include cell shrinkage, chromatin condensation, DNA fragmentation, membrane blebbing and formation of apoptotic bodies.

The genus *Biophytum* (Oxalidaceae) contains herbaceous annuals or perennials and about 70 species are reported to be distributed in the tropical and subtropical regions of the world⁷. The genus is reported to be represented by 17



species in India and nine species in Kerala⁸. The flowers of this plant are considered as one among the ten sacred flowering plants, called as the 'Dasapushpas'. This group of ten flowers is given due to importance in the traditional and cultural folklore in the state of Kerala, India. *Biophytum* has been used variously to treat numerous diseases. The plant is a good medicinal herb used to clean the uterus after delivery. It is also used for treating heavy bleeding seen in women. Therefore it is also called "Teendanaazhi".

In the present study, the two commonly available species of *Biophytum*, *B. veldkampii* and *B. reinwardtii* are being considered. *B. veldkampii* looks like a miniature palm and is native to India. The plants are usually found in wet lands (mostly plains) of tropical Africa, Asia and India, normally in the shades of trees and shrubs, in grasslands, open thickets, at low and medium altitudes. *B. reinwardtii* (Reinwardt's Tree Plant) is found in India, Ceylon, S. China, Malaysia, and also in the Himalayas, from Garhwal to Nepal, at altitudes up to 1000 m. It is also an annual herb which also looks like a miniature tree. The present study was carried out to determine the apoptotic efficiency of ethanolic extracts of two species of *Biophytum* against four human cancer cell lines.

MATERIALS AND METHODS

In the present study, two commonly available species of *Biophytum*, *B. veldkampii* Shanavas and *B. reinwardtii* (Zucc.) Klotzsch were used. The two species of *Biophytum*, were collected from Thiruvananthapuram, Kerala.

Preparation of Extract

The two species of *Biophytum* were collected, washed and shade dried. The shade dried plants were ground to fine powder and subjected to Soxhlet extraction using 70% ethyl alcohol. The extract was concentrated under reduced pressure and preserved in the refrigerator until further use.

Cell lines used for the Experiment

He La, MCF-7, NCI/ADR-RES, MDA MB-231

He La cell is an immortal cell line used in scientific research.

MCF-7, a breast cancer cell line, is the acronym of Michigan Cancer Foundation-7, referring to the institute in Detroit where the cell line was established⁹.

NCI/ADR-RES cells are derived from OVCAR-8 ovarian adenocarcinoma cells¹⁰.

MDA MB-231 is an estrogen negative human breast cancer cell line.

All these cell lines were purchased from National Centre for Cell Science (NCCS), Pune, India.

Hoechst 33342 staining assay was carried out in the above-mentioned human cancer cell lines, to distinguish

the morphological variations in the apoptotic cells.

Determination of apoptosis by Hoechst-33342 staining

Chromatin condensation is a late apoptotic event. Hoechst staining determines the chromatin condensation. Hoechst dyes are non-intercalating, benzimidazole, a derivative that bind preferentially to AT base and emit fluorescence when excited by UV radiation at about 350 nm. Transport of Hoechst stain across the plasma membrane is altered in apoptotic cells. Dye is diluted in the ratio 1:1000. The four cell lines were cultured in RPMI 1640 (Himedia) supplemented with 20% FBS (Gibco, NY, USA), penicillin (100 units/ml) and streptomycin (50 units/ml) in a humidified incubator with 5% CO₂ atmosphere at 37°C. In this step, the cells were taken out from -80°C or liquid nitrogen and made active again. The cells were revived with RPMI containing 20% FBS and used for the apoptosis assay.

Apoptosis Assay

The cells (1x10⁶) were grown in 96 wells plate and apoptosis was induced in them using the drugs at different concentrations (5 and 10µg/ml). About 60µl of the medium was removed from the wells and the same amount of the diluted Hoechst dye was added. The cells were then incubated at 37°C for 10 min in a 5% CO₂ incubator. About 60µl of the media was removed from the wells and observed under fluorescent microscope (Eclipse E-600, Nikon, NY, USA) and the percentage of apoptosis was determined¹¹.

RESULTS

The proportion of dead cells and type of cell death after treatment of different cell lines with different concentrations (5 and 10µg/ml) of ethanol extracts of both the species of *Biophytum* was estimated by Nuclear condensation assay. The study was carried out in four different human cancer cell lines using the chromatin-specific dye Hoechst 33342. The ethanolic extracts of both species showed potent activity against all the cancer cell lines studied. Among the four cell lines studied, MCF-7 showed the highest (96%) rate of apoptosis when treated with *B. reinwardtii* extract followed by *B. veldkampii* (90%). This was followed by the effect in He La cells (Table 1).

The nuclei of the apoptotic cells stained blue. Very few apoptotic cells (2-3%) were observed in the control culture, while the percentage of apoptotic cells increased in a concentration-dependent manner in the presence of plant extracts in both species. In the control group, the nuclei were less bright and more homogeneous. Hoechst 33342 staining showed that there were significant morphological changes of the nuclear chromatin in the extract treated cells. Shrinkage, condensation of chromatin and cytoplasm, detachment of the cells from the neighboring cells, fragmentation of the nucleus and membrane blebbing were observed in the apoptotic cells (Figure 1-4).



Table 1: The percentage of cell death induced by two species of *Biophytum* in four human cancerous cell lines

Plant samples	Concentrations of extract applied (µg/ml)	Cell Lines (% of apoptosis)			
		MCF-7	He La	MDA-MB-231	NCI/ADR RES
Control	-	2±0	2±0	3±0	3±0
<i>B. veldkampii</i>	5	88±0.080	83±0.070	80±0.20	73±0.40
	10	90±0	88±0.010	86±0.090	76±0.30
<i>B. reinwardtii</i>	5	92±0	90±0.020	88±0.070	75±0.040
	10	96±0	93±0.010	90±0.080	77±0.050

Values are mean ± S.D of four separate determinations

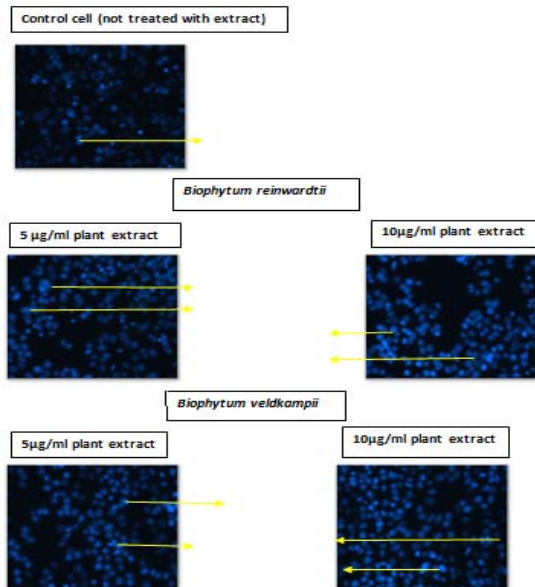


Figure 1: Apoptosis induced by ethanolic extracts of two species of *Biophytum* in MCF-7 cell line

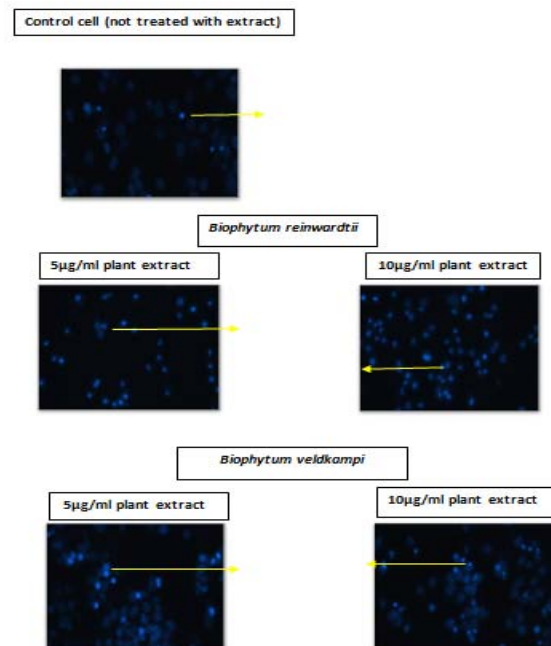


Figure 3: Apoptosis induced by ethanolic extracts of two species of *Biophytum* in NCI/ADR RES

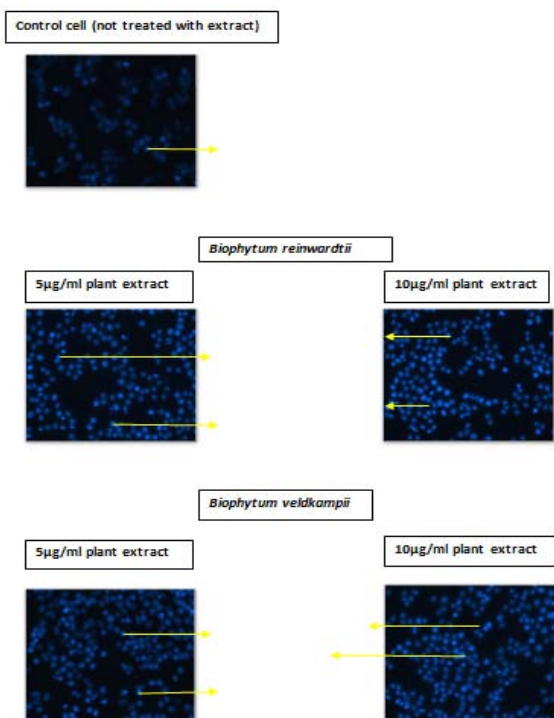


Figure 2: Apoptosis induced by ethanolic extracts of two species of *Biophytum* in He La cell line

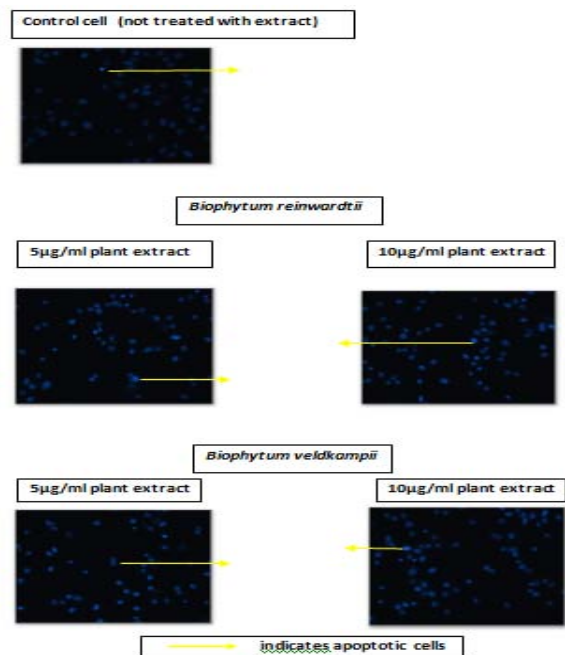


Figure 4: Apoptosis induced by ethanolic extracts of two species of *Biophytum* in MDA-MB-231

DISCUSSION

Determination of cytotoxicity, commonly used to evaluate the biological activity of natural products, is helpful to confirm whether plant extracts have potential antineoplastic properties¹² and induction of DNA damage in cancer cells is a well recognized therapeutic strategy for killing cancer cells¹³.

In the present study, the ability of the ethanolic extracts of two species of *Biophytum* to induce cell death was estimated by analyzing its effect on cell morphology.

The proportion of dead cells and type of cell death after treatment of different cell lines with different concentrations of the extract was estimated by Hoechst-33342 DNA staining.

Fluorescent microscopic image of different cells after Hoechst-33342 staining showed characteristic apoptotic morphology emitting bright fluorescence after 24 h extract treatment (Fig 1-4).

In the present study, both the species of *Biophytum* were found to show potent anticancerous activity against the various human cell lines studied.

The nuclear morphological changes by Hoechst staining were assessed using four cell lines (He La, MCF-7, NCI/ADR-RES and MDA MB-231) and it was found that, more than 70% of cells underwent apoptosis when treated with extracts at a concentration of 5µg/ml after 24 h and in 80%–90% of the cells, fragmented nuclei were detected by fluorescence microscopy.

The viable cells were uniformly blue, whereas the apoptotic cells were blue and contained bright blue dots in their nuclei, representing the nuclear fragmentation.

MCF-7 cell lines were sensitive to both the species and showed the highest percentage of apoptosis. However of the two extracts, the *B. reinwardtii* extract was found to be more effective in causing death of cancer cells.

The ethanol extracts of both species exhibited significant cytotoxic activity on MCF-7 cell lines followed by He La (Table 1). In the present study, both the species of *Biophytum* were found to show potent anticancerous activity against the various human cell lines studied.

CONCLUSION

The results of the nuclear condensation study showed that the ethanol extracts of two species of *Biophytum* exhibited strong anticancer activity against all the cell lines, especially against the MCF-7 cells.

Thus cytotoxic effect exhibited against cancer cell increases the prospect that these plants contain compound(s) which could serve as leads for the development of novel anticancer drugs.

However, further work should be carried out to isolate, purify, and characterize the active constituents responsible for the specific activity of these plants.

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Conflict of Interest: None.

