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Anticancer activity of *Biophytum sensitivum* ethanolic extract against cervical carcinoma cell lines

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Abstract

Cancer is a debilitating disease resulting from uncontrolled proliferation. One major treatment strategy for cancer is the application of chemotherapeutic drugs which kill cancer cells. In this study the anticancer potentials of plants was investigated against HeLa and HT-3 cell line. Cytotoxicity of leaves extracts was determined by MTT assay. The results showed that the ethanolic extract of *Biophytum sensitivum* possessed a moderate amount of anticancer activity and the IC_{50} value was recorded. The most potent anticancer activity was observed with the ethanolic extract of *Biophytum sensitivum* with IC_{50} values of 61.25µg/ml and 56.25µg/ml on HeLa and HT-3 cells respectively. Phytochemical analyses revealed the presence of large amount of phenols and flavonoids in the potent plant extracts which may be suggested to play an important role in their anticancer activities. The ethanolic extract would be studied further for isolation and characterization of active components for lead optimization studies.

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Introduction

Cancer is defined as an abnormal growth of tissue resulting from uncontrolled, progressive multiplication of cells and serving no physiological function, a neoplasm. Tumour can be divided into two types: Benign tumour and malignant tumour. Cancer is currently one of the foremost health challenges and a leading cause of death worldwide. According to the World Health Organization (WHO), cancer was responsible for approximately 9.6 million deaths in 2018. Environment (physical carcinogen), certain viral infections (biological carcinogen) and diets (chemical carcinogen) are the main etiologies in the occurrence of cancer. Globally, cervical cancer is one of the most common cancers in women. Cervical cancer is caused by cofactors, including oral contraceptive use, smoking, multiparity, and HIV infection. One of the major and considerable causes is the persistent infection of oncogenic human papilloma virus.

Cancer has been a constant battle globally with a lot of development in cures and preventative therapies. The disease is characterised by cells in the human body continually multiplying with the inability to controlled or stopped. Consequently, forming tumours of malignant cells with the potential to metastatic (Ochwang *et al.*, 2014). Current treatments include chemotherapy, radiotherapy and chemically derived drugs. Treatments such as chemotherapy can put patients under a lot of strain and further damage their health. Therefore, there is a focus on using alternative treatments and therapies against cancer.

For many years herbal medicines have been used and are still used in developing countries as the primary source of medical treatment. Plants have been used in medicine for their natural antiseptic properties. Thus, research has developed into investigating the potential properties and uses of terrestrial plants extracts for the preparation of potential nanomaterial based drugs for diseases including cancer (Sivaraj *et al.*, 2014). Many plant species are already being used to treat or prevent development of cancer. Multiple researchers have identified species of plants that have demonstrated anticancer properties with a lot of focus on those that have been used in herbal medicine in developing countries.

Medicinal plants have been used for thousands of years in folk medicines in Asian and African populations and many plants are consumed for their health benefits in developed nations. According to the World Health Organisation (WHO) some nations still reply of plant-based treatment as their main source of medicine and developing nations are utilising the benefits of naturally sourced compounds for therapeutic purposes (Rajeswara Rao *et al.*, 2007). Compounds which have been identified and extracted from terrestrial plants for their anticancer properties include polyphenols, brassinosteroids and taxols.

Cervical cancer

Cervical cancer belongs to a group of gynecological cancers, including vulvar and endometrial cancer that share common features, such as differentially expressed proteins, pathways, and transcription factors (Pappa *et al.*, 2015). Cervical cancer is the fourth most common cancer in women across the world (Ferlay *et al.*, 2015). The majority of cervical cancer incidents are attributed to 13 high-risk oncogenic HPV types, represented mainly by HPV16 and HPV18. HPV infection of the cervical epithelium results in the eventual expression of E6 and E7 oncogenes, leading to sequential steps of tumor progression, corresponding to discrete histological lesions such as CIN1, CIN2, and CIN3 (Schiffman and Castle, 2003).

Infection of cervical epithelium with high-risk HPV types represents the initiating event towards cervical cancer. Proteomic studies are a valuable tool in order to explore the mechanisms involved in viral infection and protein dysfunction interplay that lead to cervical carcinogenesis (Di Domenico *et al.*, 2013). Furthermore, proteomic approaches have been widely utilized for the discovery of novel putative biomarkers and also for understanding the mechanism of action of drugs in cervical cancer treatment (Kontostathi *et al.*, 2016).

Cell lines

HT-3 is a human cervical carcinoma cell line that grows in adherent culture. Although this cell line was initially classified as human papillomavirus (HPV) DNA negative, subsequent studies revealed that the cells harbor HPV30 DNA in their genome. The HT-3 cells have a homozygous mutation in the *TP53* gene, resulting in the expression of the transactivation-defective, dominant negative form of the protein. These cells form tumors when injected subcutaneously into immunocompromised mice.

HeLa cell, a cancerous cell belonging to a strain continuously cultured since its isolation in 1951 from a patient suffering from cervical carcinoma. The designation HeLa is derived from the name of the patient, Henrietta Lacks. HeLa cells were the first human cell line to be established and have been widely used in laboratory studies, especially in research on viruses, cancer and human genetics. HeLa cells are a common source of cross-contamination of other cell lines and a suspected cause of numerous instances of cell line misidentification. The HeLa cell genome has also been shown to be highly unstable, housing numerous genomic rearrangements (e.g., abnormal numbers of chromosomes) in a phenomenon known as chromothripsis.

Biophytum sensitivum

Biophytum sensitivum (L: Linnaeus) DC belonging to Division: Magnoliophyta, Class: Magnoliopsida, Order: Oxalidales, Family: Oxalidaceae, found in wet lands of tropical India, South Asia and Africa. Normally, it is present in the shades of trees and shrubs, in grass lands at low and medium altitudes. It is commonly known as Life plant (English). In India, it is also known by various vernacular names, Jhalai (Bengali), Laajjaalu, Lakshmana (Hindi), Hara muni, Jalapushpa (Kannada), Mukkutti (Malayalam), Lajwanti (Marathi), Vipareetalajjaalu, Jhulapushpa (Sanskrit), Nilaccurunki, Tintaanaalee (Tamil), Attapatti, chumi, Jalapuspa, (Telugu). It has been used in traditional medicine for various ailments, especially in Indian medicine (Jirovetz *et al.*, 2004). The flower of this plant is considered as one of the ten sacred plants which are called as Dasapushpam in tradition and culture of Kerala state in India (Varghese *et al.*, 2010). Due to seeds remaining dormant, the cultivation of this plant has become very difficult. Shivanna *et al.* (2008) established a protocol to regenerate *B. sensitivum* through indirect and direct and somatic embryogenesis from its various explants.

Various crude extract of this plant have shown multifarious activities which includes antioxidants, anti-inflammatory and antitumor activity. Medicinal plants become the main source for cancer drug development (Tian *et al.*, 2007). Pharmacological screening from leaves of the *B. sensitivum* showed significant antitumor activity in Dalton's Lymphoma Ascites (DLA)-bearing mice (Bhaskar and Rajalakshmi, 2010) and this represents *B. sensitivum* as a valuable medicinal plant with therapeutic effects. Continued research to elucidate the molecular mechanisms behind the role of antitumor activity is worthwhile. The present article aims to provide a comprehensive review on its state of knowledge about morphology, phytoconstituents and its various biological activity of *B. sensitivum* as a revolutionary therapeutic agent to combat life-threatening diseases flourished during the last decade.

Apoptosis, or programmed cell death, is one of the most finely coordinated regulatory functions for maintenance of the homeostasis in the living organism. It involves the continuous checking of the cellular integrity and cascade-like events of self-destruction when the integrity of the organism is endangered. Morphological hallmarks of apoptosis are nuclear condensation, cell shrinkage, membrane blebbing and the formation of apoptotic bodies.

These changes are accompanied by biochemical features, including DNA fragmentation and the proteolytic cleavage of a variety of intracellular substrates. The present investigation was taken up for evaluating the antiproliferative potential possessed by

the ethanolic leaves extract of *Avicennia marina* against different cancer cell lines.

Materials and methods

Collection and identification of plant material

For the study, the whole plant of *Biophytum sensitivum* belongs to Oxalidaceae family was collected from Kerala, South India. The whole plant were identified taxonomically and authenticated according to various literatures, Flora of Madras Presidency and Wealth of India including other pertinent taxonomic literature.

Preparation of plant materials and extract

The leaves were carefully cleaned, shade dried and powdered. The powdered material was stored in a closed air-tight plastic container at low temperature. The powdered plant material (50 g) was extracted with 300 mL of each solvent ethanol by maceration (3×24 h) at room temperature. The collected solvents were concentrated by rotary vacuum evaporator at 45°C and then dried using a freeze dryer. All extracts and acyclovir (extracted from commercial tablet) were dissolved in dimethyl sulphoxide (DMSO). The final concentration of DMSO was 0.1% v/v in cell culture environment.

Phytochemical analysis

The preliminary phytochemical evaluation of leaves was carried on extract prepared by successive extraction method in Soxhlet. The resultant extracts were evaporated to dryness under vacuum. These extract were subjected to chemical test for different phytoconstituents viz. alkaloids, carbohydrates, phenolics, flavonoids, proteins, amino acids, saponins, mucilage and resins etc. Chemical tests were identifying the phytochemicals as described (Trease and Evans, 1983; Harborne, 1973). Alkaloids, carbohydrates, tannins and phenols, flavonoides, gums and mucilage, fixed oils and fats and saponins were qualitatively analyzed.

Tumour cell lines

Cell lines of different tissue origin such as HeLa and HT-3 were used. Cells were cultured in MEM (Minimum Essential Media) supplemented with Sodium Bicarbonate, EDTA, FCS (Foetal Calf Serum) and incubated in humidified atmosphere of 5% CO_2 and 37°C. The culture medium was changed every two days. All cell lines used were of human origin in order to more closely mimic how plant extracts would affect human cancer cells. Cells were generally cultured in 10 mL of appropriate medium in 75 cm^2 tissue culture (T-75) flasks at 37˚C in a humidified atmosphere of 5% CO_2/ 95% air. Cells were passages weekly and medium replaced fortnightly.

MTT assay (Mossman, 1983)

Antiproliferative effects were measured *in vitro* by using MTT ([3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide]) assays. After treatment, the living cells were assayed by the addition of 20 µl of 5 mg/ml MTT solution. Finally, the reduced MTT was assayed at 545 nm wells with untreated cells were utilized as controls. Antiproliferative and cytotoxic effects were distinguished by cell number and the duration of treatment (72 h, 5000 cells/w, and 24 h, 25000 cells/w, respectively). Stock solutions of the tested materials were prepared with dimethyl sulfoxide (DMSO). The highest DMSO concentration (0.3%) of the medium did not have any significant effect on the cell proliferation.

Extracts which demonstrated potent activity (growth inhibition > 50%) were selected for further in vitro testing (dose-response curve and cytotoxicity). To study the interactions between acridones and doxorubicin, a checkerboard method was applied. A series of 2-fold dilutions of the acridones was tested in combination with 2-fold dilutions of doxorubicin. The cell growth rate was determined with MTT staining drug interactions were evaluated according to the following system (fractional inhibitory index = FIX):

Synergism if FIX < 0.5
Additive effect if FIX = 0.51-1
Indifferent effect if 1 < FIX < 2
Antagonism if FIX > 2

Results and discussion

Phytochemical studies of *B. sensitivum* showed that it contains a number of phenolic and polyphenolic compounds, saponin, essential oil, polysaccharides and pectin. The main bioactive constituents found are bioflavonoid, amentoflavone with minute amount of cupressoflavone (Abinash Bharati and Alakh Sahu, 2012). All of the extracts from the *Biophytum sensitivum* contained saponin, phenols, tannins, glycosides, terpenoids, flavonoids, alkaloids, and coumarins, according to a preliminary phytochemical examination.

With the exception of the chloroform extract's lack of saponins, glycosides, and coumarins and the extract from ethyl acetate's absence of saponin. The results of the phytochemical analysis are displayed in Table 1. A higher degree of biological activity derives from the presence of a high concentration of phytochemicals in the plant.

Table 1. Qualitative phytochemical screening on extracts of *Biophytum sensitivum*

Name of test	Test applied/ Reagent used	Ethanol	Water	Chloroform	Hexane	Acetone	Ethyl acetate
Alkaloids	Mayer's	+++	++	++	++	+++	++
	Wagner's	+++	++	++	++	+++	++
	Hagner's	+++	++	++	+++	+++	++
	Dragndorff's test	++	++	++	++	++	+
Flavonoids	HCl and magnesium turnings	+++	++	+	++	+	++
Carbohydrate	Molisch's test	+	+	+	+	+	+
Tannins & phenols	10% Lead acetate	+++	+	++	++	++	++
	$Fecl_3$	+++	+	++	++	++	++
Test for steroids	Salkowski's test	++	++	++	++	++	++
	Libermann-Burchard's test	++	++	++	++	++	++
Gums & mucilages	Alcoholic precipitation	-	-	-	-	-	-
Fixed oil & fats	Spot test	+	-	+	+	-	-
Saponins	Foam test	+	+	+	+	+	+
Phytosterols	LB test	+	+	+	+	+	+
Volatile oils	Hydro distillation method	+	+	+	+	+	+
Protein & free amino acids	Biuret test	++	++	++	++	++	++
	Ninhydrin	+++	++	++	++	++	++
	Xanthoprotein	+++	++	++	++	++	++

This plants growing under natural conditions contain the spectrum of secondary metabolites such as phenols, flavanoids, quinones, coumarins, tannins and their glycosides, alkaloids, essential oils etc., the importance of these substance as microbial agents against the pathogen has been emphasized (Sofowora, 1993). In the present study, it was clearly understood that the ethanolic extracted maximum amount of the different type of metabolites present in the *Biophytum sensitivum*. Boominathan and Ramamurthy (2009) reported that the phytochemical analysis of the *H. indicum* and *C. procumbens* extracts showed the presence of tannins, alkaloids, flavonoids and phenolic compounds. Tannins have been found to form irreversible complexes with proline-rich proteins.

For instance, the presence of flavonoids suggest that the plant have been reported to exert multiple biological effects including, anti-allergic, anti-inflammatory, anti- microbial antioxidant, anti-cancer activity (Kunle and Egharevba, 2009). It also suggests that the plant might have diuretic properties (Jayvir *et al.*, 2002). The presence of tannins shows that the plant is astringent as documented and suggests that it might have antiviral and anti-bacterial activities and can relief in wound healing and burns (Haslem, 1989). Saponins and glycoside are also very important classes of secondary metabolites as some are cardio-active and used in treatment of heart conditions (Oloyode, 2005). Some researchers have also investigated that some saponins have anti-cancer and immune modulatory properties (Evans, 2002).

Volatile oils are used in the industries for various purposes, both as a pharmaceutical/ cosmetic raw material for production of emollients and active ingredient for the respiratory tract infections. Anticancer activity of *Biophytum sensitivum* was studied in different mammalian cell line.

Anticancer activity of ethanolic extract of *B. sensitivum* as well as standard was determined through MTT cytotoxicity assay. In the preliminary study, the ethanolic extract showed the good yielding capacity of phytocompounds activity. In this regards, the present investigation the ethanolic extract of *B. sensitivum* was studied in HeLa and HT-3cell lines and its result labelled in the Table 2 and also made with standard drug tamoxifen.

The minimum cell viability (18.6%) and maximum cell inhibition (81.4%) were noted in 200 µg/ml concentration of *B. sensitivum*. The IC_{50} value was calculated for anticancer activity of ethanolic extract of *A. marina* against HeLa and HT-3cell lines. The tamoxifen used as a standard for this study. In the standard, the minimum cell viability (17.5%) and maximum cell inhibition (82.5%) were observed in higher concentration. The percentage of cell inhibition was noted in the different concentrations of ethanolic extract of *B. sensitivum* ranges from 20 to 200 µg/ml. The lowest cell inhibition (20.5%) was recorded in the lowest concentration and highest cell inhibition (84.5%) was noted in the higher concentration of ethanolic extract of *B. sensitivum*.

Table 2. Survival analysis of cancer cells treated with extracts of *Biophytum sensitivum*

Concentrations (µg ml-1)	HeLa		HT-29	
	Cell viability (%)	Cell inhibition (%)	Cell viability (%)	Cell inhibition (%)
20	79.5	20.5	74.7	25.3
40	67.4	32.6	62.5	37.5
60	56.6	43.4	53.7	46.3
80	48.7	51.3	45.6	54.4
100	37.5	62.5	40.2	59.8
125	29.6	70.4	28.5	71.5
150	21.1	78.9	22.4	77.6
200	18.6	81.4	17.5	82.5
Vehicle control (DMSO)	100	0	100	0

Anticancer properties of many natural compounds isolated from different plant extracts have been reported. Research is being carried out throughout the world to find a lead compound which can block the development of cancer in humans. Nature has always been a great contributor towards this goal. Plant-derived natural products such as flavonoids, terpenoids and steroids have received considerable attention due to their diverse pharmacological properties, which include cytotoxic and chemopreventive effects (Abdullaev, 2001). They were the first agents to advance into clinical use for the treatment of cancer (Cragg and Newman, 2005).

Withania somnifera as a potential source of new molecules that can curtail cancer growth were studied by Dredge *et al.* (2003). *A. marina* has also been shown to inhibit the growth of human cancer cell lines comparable to that produced by tamoxifen. The peels extract produced antiproliferative activity on HeLa and HT-3 tumor cell lines. Jayaprakasam (2003) reported that the inhibitory concentrations obtained was 25.1±0.91 against colon cell line HCT-116, but in this study plant extracts from different cancer cell treatments of *B. sensitivum* cultivated in fly ash containing soil had shown more than 85% inhibition against human cell lines. Furthermore this study has reported growth inhibitory importance in *B. sensitivum* against various cancer cell lines, i.e., HeLa and HT-3 tumor cell lines. Hence, this study has revealed remarkable anticancer potential in the leaves extract of *B. sensitivum*.

Conclusion

Nowadays plants are extensively used for the research purpose and it possesses more than one chemical

entity so it has been widely used for the research investigations. Anticancer properties of many natural compounds isolated from different plant extracts have been reported. Research is being carried out throughout the world to find a lead compound which can block the development of cancer in humans. Nature has always been a great contributor towards this goal. Furthermore this study has to prove the cytotoxic effects of ethanolic leaves extract of *Biophytum sensitivum* may be conducted in clinical trials on patients suffering from cancer disease. To the best of our knowledge, the present study concluded that the *Biophytum sensitivum* have an anticancer activity against HT-29 and HeLa cell line. From this study, it is clear that *Biophytum sensitivum* extract have significant anti-cancer activity in cell line. The anti-cancer activity is probably due to the presence of phytocompounds.

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