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Original Article

Anticancer Activity of *Elytraria acaulis* L. Extracts on Triple Negative Breast Cancer Cell Line

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ABSTRACT

Objectives: In the present study, the anticancer potential of the plant extracts of *Elytraria acaulis* L. (*E. acaulis* L.) were investigated on human breast cancer cells (MDAMB-231). **Methods:** Different organic solvent extracts of *E. acaulis* L. were prepared using methanol, ethanol, ethyl acetate and hexane. The cytotoxicity of plant extracts were evaluated by MTT assay (-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) against MDA MB-231cells. The apoptosis associated morphological changes were also observed with the help of propidium iodide staining method. **Results:** Of all the solvent extracts, methanol extract has shown the highest activity and its IC_{50} concentration was found to be 80.8±2.08 µg/ml. The anticancer drug Cis-diammineplatinum (II) dichloride was considered as a standard. **Conclusion:** In further the bioactive compound should be isolated from the methanol crude extract, which is responsible for the cytotoxic effects.

Key words: *E. acaulis* L., Anticancer activity, Cytotoxic assay, Propidium iodide, Apoptosis.

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INTRODUCTION

The breast cancer is caused due to development of malignant cells in the lining of the mammary ductal or glandular epithelium in the breast. In Breast cancer, at first DNA damage takes place and later alterations occurs in the signaling pathways.[1] In women, it is the most common type of cancer and men can also be affected. When it is detected in an early stage, can be treated and cured according to the best practice. MDAMB-231 breast cancer cells are highly aggressive and are TNBC (triple negative breast cancer) cells.[2]

The anticancer drugs, which are being used now a day, are very expensive and are highly toxic.[3] Hence new drug combinations with less toxicity and with affordable price should be produced. From the past times, the plants and the plant extracts were used in curing different diseases. Phytochemicals are playing a crucial role in inhibiting the growth of cancer cells.[4] Commonly, plants produce different secondary metabolites to protect them from the adverse environment. These secondary metabolites are being focused more by researchers as they are the lead candidates in preventing various ailments. *E. acaulis* L. is such a plant which can survive in different stress conditions like drought, accumulation of heavy metals and salinity.[5] *E. acaulis* L. also known as yeddadugu, patharchatta is a medicinal plant belongs to the family Acanthaceae. It is a deciduous associate, distributed in Indian subcontinent, Madagascar islands and tropical Africa. *E. acaulis* L. is a perennial herb grows to 30 cm in height. Leaves are obtuse, long and hairy. Flowers are spike and white in color. Fruit is capsule. Seeds are many, small and reticulate.

In the present study, the screening for anti-cancer activities of *E. acaulis* L. has been done. The potentiality of this plant as an anticancer drug has been studied.

MATERIALS AND METHODS

Collection of Plant Material

E. acaulis L. was collected from the forest area of Seshachalam hills, Tirupati, Andhra Pradesh. Plant was cleaned under tap water and dried under shady conditions to avoid the thermal degradation. Then the dried plant was grounded into fine powder and stored in dark airtight containers at a cool and dry place to avoid the oxidation.

Extraction

Solvents such as methanol, ethanol, hexane, ethyl acetate were used to extract the bioactive compounds present in the plant with the help of orbital shaker.[6] The plant powder was weighed and dissolved with solvent to sample of 10:1(v/w) ratio in each solvent and left for 24hr at room temperature. Then each solvent with plant mixture were kept in the orbital shaker for 48 hr. Within this time, the bioactive compounds get extracted into the solvent. Then each solvent is filtered and concentrated in rota evaporator at 40°C, under reduced pressure conditions. The obtained concentrated solvent extracts were dried and stored at room temperature and were screened for further analysis.

Cell culture

The human breast cancer cells (MDAMB-231) were brought from NCCS, Pune. The MDAMB-231 cells were cultured in the laboratory under sterile conditions in the tissue culture flasks. The RPMI-1640 medium provided to the cells which is supplemented with 10% FBS (fetal bovine serum), streptomycin sulphate (100g/ml) and penicillin (100IU/ml). Cells were incubated at 37°C, 5% CO_2 and 100% humidity.

Trypan blue dye exclusion assay

Trypan blue dye exclusion assay was done, and the cell density and cell viability were estimated.[7] The intactness of membrane of the cell plays a major role in determining the viability of cells. The cells will exclude the dye if they have intact cell membrane. The viability count can be known accurately by this test. MDA MB 231 cells were analyzed for the cell density and cell viability was analyzed separately. Trypan blue dye solution was made in 0.2% PBS. 0.1ml (each cell suspension) and 0.9ml (trypan blue solution) are mixed. 10µl of mixture was loaded into haemocytometer (neubar chamber) and immediately examined under the microscope at low magnification. The total number of cells present was counted and the % cell viability was calculated.

Formula for calculating the % cell viability –

$$\% \text{ Cell Viability} = \frac{(\text{Average number of live cells})}{(\text{Total number of cells})} \times 100$$

The Formula for estimating the cell density –

$$\text{Cells/ml} = \text{Average number of cells} \times \text{Dilution factor} \times 10^4$$

Cell viability assay

The cytotoxicity of *E. acaulis* L. extracts on MDA MB 231 was evaluated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay according to the method described by Kedari *et al.* 2016[8] with slight modifications.[9] The cells were seeded into treated 6 well plates having density 2.5 X 10^6 to 3.5 X 10^6. After 24 hr of incubation, the breast cancer cells (MDA MB 231) were treated with increasing concentrations of cisplatin and *E. acaulis* L. plant extracts (methanol, ethyl acetate, ethanol, hexane). These cancer cells were incubated for 24 hr. Then, washed with PBS and 1 ml of MTT (0.1% w/v in PBS) solution was added to the cells and followed by incubation for 4 hr at 37°C in dark. The blue colored crystals will be formed which indicates the formation of formazan crystals. For dissolving these crystals, the MTT was removed from the wells and 1ml of DMSO (Dimethyl Sulphoxide) was added. After 1hour, with the help of microplate reader (Wallac 1420 Multi label counter, PerkinElmer) the purple color intensity was measured using at a wave-length of 560 nm. The data is presented as percent post treatment recovery (% live cells), whereas the absorbance from untreated control cells is defined as 100% live cells. The general formula used for estimating the percentage viable cells was

$$\% \text{ Cell Viability} = \text{O.D of treated cells/ untreated cells} \times 100$$

The IC_{50} concentrations were interpolated from the graph by plotting % cell viability on Y axis against concentration on X axis.

Microscopic Study

On treatment with different extracts of *E. acaulis* L. and cisplatin the MDA MB 231 cells undergone morphological changes and was observed using phase contrast inverted microscope. The MDA MB 231 cells were cultured in 6 wells plate and treated with IC_{50} concentrations of *E. acaulis* L. (methanol, ethyl acetate, ethanol and hexane) and cisplatin for 24 hours at 37°C in a CO_2 incubator. After the incubation period, using a phase contrast microscope, photomicrographs were taken.

Fluorescence Imaging

For observing the morphological changes occurred in the cells Propidium iodide (PI) staining method was used. The MDA MB 231 cells were cultured in 6 wells plate and treated with IC_{50} concentrations of *E. acaulis* L. (methanol, ethyl acetate, ethanol, hexane) and cisplatin for 24 hr at 37°C in a CO_2 incubator. After the incubation, with PBS the cells were washed. After washing, the cells were fixed for half an hour in absolute alcohol at 40°C, rehydrated with PBS. 100 µl of propidium iodide (25µm) was added and incubated at 37°C for 5 min. After the incubation period, using a fluorescent microscope, photomicrographs were taken.

RESULTS

Trypan Blue Dye Exclusion Assay

The cancer cells MDA MB 231 and human blood lymphocytes were treated with different dose concentrations (5µg-150µg) of *E. acaulis* L. methanol plant extracts and cisplatin. After 24 hr of incubation period, the cell viability was analyzed by trypan blue dye exclusion method using heamocytometer. In the study, preliminary viability assay trypan blue dye exclusion test was performed to estimate the viability. As shown in (Figure 1), plant extract inhibited cancer cell growth effectively in parallel with chemotherapeutic drug. The anti-proliferative activity was observed in a dose dependent manner. The cell viability was assessed by considering the untreated cells.

Microscopic study

The methanol and ethyl acetate extracts of *E. acaulis* L. have effectively shown cytotoxic activity against breast cancer cells MDA MB 231. It was found that, methanol extract have more cyototoxic activity than compared to the ethyl acetate, ethanol and hexane extracts. The present study is correlating with the earlier cytotoxic studies on crude extracts as well metabolites extracted from acanthaceae plants against cancer cell lines *in vitro*. In the present study, the IC_{50} concentrations of these *E. acaulis* L. solvent extracts against were found to *E. acaulis* L. against MDA MB 231 after 24hr of treatment were found to be 80.8µg/ml, 85.3 µg/ml, 90.4 µg/ml and 120µg/ml respectively.

Cell viability assay

Anticancer activity was done by using cell viability assay (MTT Assay). MTT [3 - (4, 5-dimethyl thiazol-2-yl) -2, 5-diphenyl tetrazolium bromide] is a tetrazolium salt. The anti proliferative effect of aqueous, methanol, ethanol, ethyl acetate, chloroform and hexane extract of *E. acaulis* L. on the growth and morphology of human breast cancer metastatic cell line (MDA MB 231) was studied. Cells were exposed to concentrations ranging from 10 to 500 µg/ml of aqueous, methanol, ethyl acetate, chloroform ethanol, ethyl acetate, chloroform and hexane extract of *E. acaulis* L. A chemotherapeutic drug, cisplatin was considered as a standard and cells were treated (1-50 µg/ml) simultaneously along with extracts. The

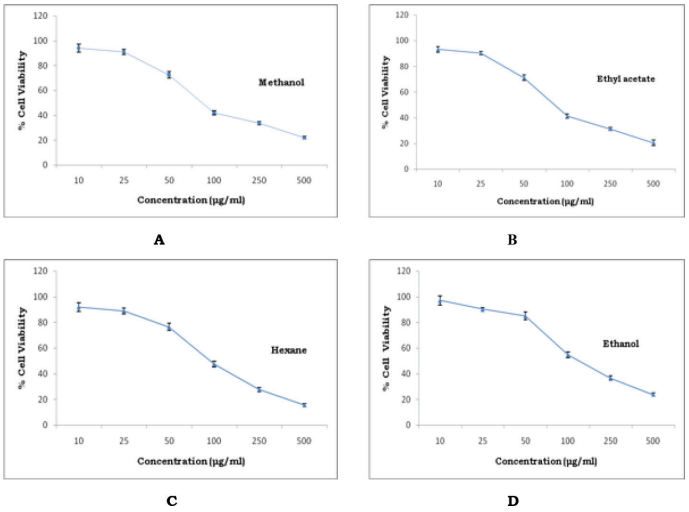

Figure 1: Graphical illustration showing cytotoxic effect of different concentrations of plant extracts on MDA MB 231 after 24 hrs treatment. % cell viability measure by MTT assay.

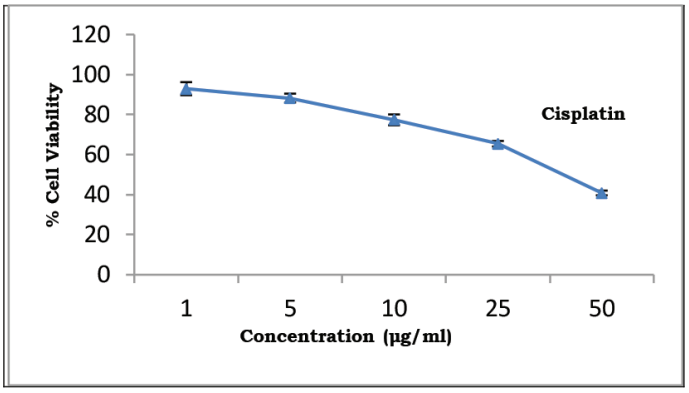

Figure 2: Graphical illustration showing cytotoxic effect of different concentrations of cisplatin on MDA MB 231 after 24 hr treatment.

cytotoxicity was determined by the MTT assay. The photomicrographs of the untreated cells, cells treated with IC_{50} concentrations of methanol, ethanol, ethyl acetate and hexane extracts of *E. acaulis* L. and cisplatin are shown in Figure 2.

Cellular morphology analysis using phase contrast inverted microscope

Cellular morphological changes were observed under phase contrast inverted microscope. After trypsinization, the cells were seeded in 12 well plate at a cell density of $1x10^5$ cells per well. Following day, cells were treated with ethanol, methanol, acetone and diethyl ether extracts at their respective IC_{50} concentrations. As hexane extract has not shown much activity in the tested concentration, cells were treated with a concentration of 700 µg/mL hexane extract. After 18 hr, cells were washed with phosphate buffered saline and cell morphology was captured using phase contrast microscope at 100X magnification (Figure 3).

Evaluation of Genotoxicity and Fluorescence imaging

To estimate the cumulative number of apoptotic cells in culture and the morphological alterations of treated cells, a rapid and precise propidium iodide staining was performed. This method yields quantitative measure of apoptotic cell population. Propidium Iodide (PI) is a fluorochrome that intercalates with DNA or RNA at a specific ratio. It has fluorescence excitation/emission maxima at 535nm/617nm. The level of PI fluorescence in a cell is directly proportional to the DNA content of that cell as RNase treatment was used. Fluorescence imaging studies revealed the existence of apoptotic bodies in all the cell populations treated with kaempferol, drugs and combinations which prove the induction of apoptosis (Figure 4) in both the cell lines. Untreated cancer cells served as control.

DISCUSSION

Previously, Das *et al.*, 2011[10] performed experiments on different flavonoids to investigate the molecular mechanism underlying apoptosis caused by them in human malignant neuroblastoma (SH-SY5Y). For example, methanol extract of *Andrographis paniculata*, methanol root extracts of *Rhina canthusnasutus*, compounds like Elenoside from *Justicia hyssopifolia*, justiflorinol, justicinol from *J. patentiflora*, showed cytotoxic activity against NCI – H187, A 549, K-526, HeLa, MCF7, KB, HT-29, HL-60 etc.[11,12,13] Programmed cell death (apoptosis) or necrosis may be the possible mechanisms involved in cell death. In contrast to apoptosis, necrosis is associated with rapid cytoplasmic swelling and leads to local inflammation due to plasma membrane rupture and organelle break-

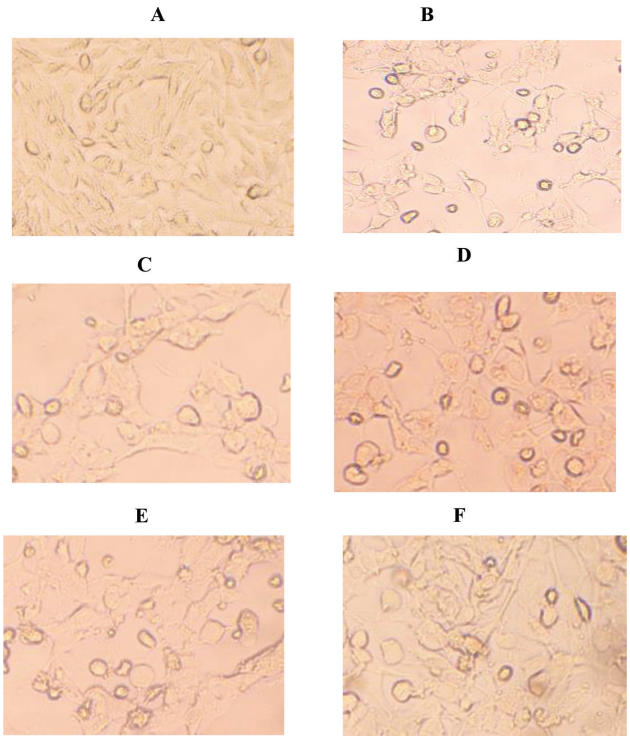

Figure 3: Effect of the plant extracts of *Elytraria acaulis* L. and cisplatin on MDA MB 321 cells. (A) Untreated control, (B) treated with methanol extract (80.8 µg/ml) (C) treated with ethyl acetate extract (85.3 µg/ml) (D) treated with ethanol extract (120 µg/ml) (E) treated with hexane extract (90.4 µg/ml) (F) treated with cisplatin (40.6µg/ml).

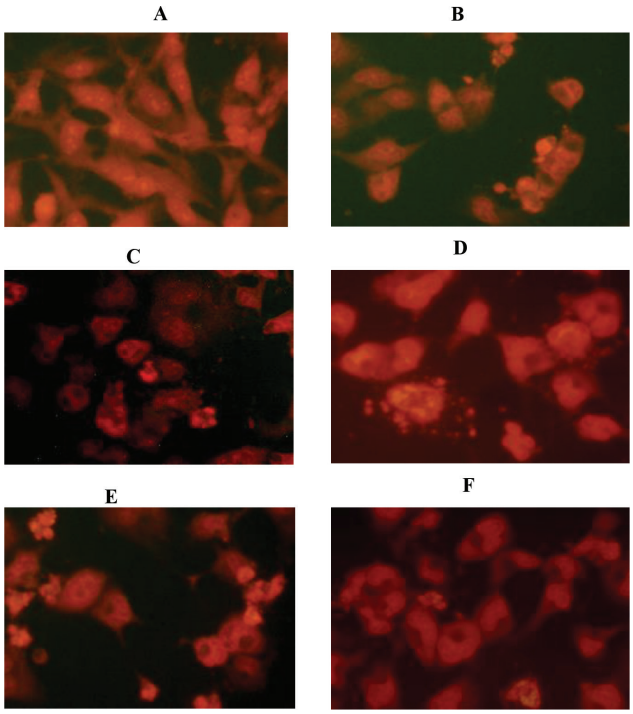

Figure 4: MDA MB 231 cells undergoing nuclear condensation, chromatin fragmentation and apoptotic bodies due to treatment. (A)Untreated control (B) treated with methanol extract (80.8µg/ml) (C) treated with ethyl acetate extract (85.3µg/ml) (D) treated with ethanol extract (90.4µg/ml) (E) treated with hexane extract (120µg/ml) and (F) treated with cisplatin (40.6µg/ml).

down.[14] Therefore, induction of apoptosis has become one of the most ideal targets for cancer therapy.[15] During the process of apoptosis, DNA cleavage progressively leads to shedding of apoptotic bodies.

CONCLUSION

The present study revealed that the extracts of *E. acaulis* L. showed dose dependent anticancer activity. MCF – 7 cells showed apoptotic related morphological changes on treatment with the extracts. Among all the extracts, diethyl ether extracts of *E. acaulis* L. showed the highest anti-cancer activity against human breast adenocarcinoma cells MDA MB-231. Further separation of the extract has to be done to isolate, purify and characterize the bioactive compound having anticancer potential.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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