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Antiproliferative effect of methanolic extract of *Allophylus cobbe* in C127I cell lines

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ABSTRACT

Scientific world is in search of newer and effective therapies against cancer and nature form a good source of drugs. The present study was undertaken to assess the antiproliferative potential of methanolic extract of *A. cobbe* in C127I cell lines. The leaves of *A. cobbe* were shade dried and was extracted using methanol and qualitative phytochemical analysis was performed. The extract was assessed for its cytotoxicity by MTT dye reduction assay in C127 I cells maintained using DMEM and 10 per cent foetal bovine serum at concentrations of 320, 160, 80, 40, 20, 20 and 5 µg/mL and the percent cell inhibition and IC_{50} were calculated. Acridine Orange/Ethidium bromide staining was used to detect the possible mechanism of cytotoxicity. From the results of MTT assay, it could be seen that there was a dose dependent inhibition of cell proliferation of C127I which was maximum at a concentration of 320 µg/mL. The IC_{50} value of the methanolic extracts was found to be 64.63 µg/mL respectively. The effect was comparable to doxorubicin. The extract and positive control treated cells showed orange to red fluorescence when stained with Acrdine Orange/ Ethidium bromide compared to greenish fluorescence in the control cells indicating apoptosis in the treated cells. The study concluded that methanolic extract of *A. cobbe* induced cytotoxicity by apoptosis of cancer cells.

Keywords: A. cobbe, C127I, cytotoxicity, Apoptosis.

INTRODUCTION

Cancer is the uncontrolled proliferation of cells that can affect any organ system in the body. One characteristic of cancer is the quick development of aberrant cells that expand outside of their normal borders, infiltrate other body components, and eventually move to other organs. The main reason of death in cancer patients is widespread metastases. After skin cancer, breast cancer is the second most frequent malignancy among women. Key risk factors for breast cancer include age, gender, inheritance, mutations, menopause, obesity, diet, smoking, and exposure to radiation. Radiation, chemotherapy, and surgery are regularly used treatment modalities for breast cancers. Resistance developing to commonly used medications and their significant risk of organ toxicity are two of the major challenges with anticancer therapy. For the past 30 years, natural products have been the mainstay of cancer chemotherapy, and it is expected that they will provide many of the lead structures that can be employed as templates to create new compounds with improved biological features. Some bioactive compounds show no cytotoxicity in normal cells but hold significant antioxidant and anticancer activities.

Allophylus cobbe a small shrub belonging to the family sapindaceae,is widely seen in Western Ghats of India. It is traditionally used for treating fractures and also relieves rashes [1, 2]. It also possesses antibacterial, analgesic, anti-inflammatory, antioxidant and wound healing activities [3]. The present study used C127I cell lines which is non transformed murine mammary tumor for assessing the antiproliferative activity of *A. cobbe*.The presence of high amount of antioxidants in these plants may have the potency in inhibiting cancer cells of this nature. Hence the present study was undertaken to explore the cytotoxic potential of the methanolic extract of leaves of *A. cobbe* in C127I cells and to identify the possible mechanism of cytotoxicity.

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MATERIALS AND METHODS

Plant material

The leaves of *A. cobbe* was collected from Wayanad District of Kerala. The leaves were then shade dried, pulverized and powder were extracted using methanol in a Soxhlet apparatus and further concentrated in a rotary vacuum evaporator under reduced pressure and temperature (40 °C). After the complete evaporation of solvent, the extract was kept in an air tight container in a refrigerator [4].

Phytochemical Analysis

The qualitative phytochemical analysis of the methanolic extract was done as per Harborne [5].

Cell line

C127I, mouse mammary tumor cell line, was obtained from the National centre of cell science in Pune. It was then cultured in tissue culture flasks with Dulbecco's Modified Eagle Medium (DMEM) combined with 10% foetal bovine serum and 1% of gentamicin (50mg/mL). The cells were cultured in an incubator with 5% CO_2 at a temperature of 37°C.

Cytotoxicity studies: 3-(4,5- dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay

According to Riss and Moravec [6], the *in-vitro* cytotoxic capability of the methanolic extract of *A. cobbe* was evaluated in C127I using MTT reduction assay. The cells were uniformly cultured at a concentration of 5×10^4 cells/mL in microtiter plate (96 wells) and then incubated for 24 hours for maximum active cell growth. The diluted extract was added to the cells at concentrations of 320,160,80,40,20 and 10µg/mL. Doxorubicin was used as positive control. After 24 hours of incubation, 10µL of MTT at a concentration of 5mg/mL was added to each well and incubated along with 100µL of serum free medium for 4 hours. 100µL of DMSO was added to stop the reaction and the absorbance is measured using enzyme-linked immunosorbent assay, also called ELISA at a wavelength of 594nm. Percent cell viability, percent cell inhibition was calculated as follows.

Per cent cell viability = (Average absorbance of treated cells /Average absorbance of untreated cells) ×100

Per cent cell inhibition = 100 - percent cell viability.

The IC_{50} values of extracts were calculated by plotting the concentration against percent cell inhibition using graph pad prism v.5.0.

Acridine orange / Ethidium bromide (AO/EB) staining

The cells are seeded to a 6 well plate at a concentration of 1×10^5 cells/mL. The cells were exposed to the IC_{50} concentration of extract of *A. cobbe* for 24 hours. The acridine orange / ethidium bromide (AO/EB) staining procedure was followed to examine the active, apoptotic and necrotic cells. Twenty-five microlitres of the extract treated, positive control or untreated cells were stained with 5µL of acridine orange (10 µg/mL) and ethidium bromide (10 µg/mL) and analysed under trinocular research fluorescence microscope, DM 2000 LED, Leica with blue excitation (488nm) and emission (550nm) filters at 10X magnification.

RESULTS

Qualitative Phytochemical Analysis

The phytochemical analysis of the leaves of the extract showed the presence of flavonoids, saponins, terpenes and tannins and absence of alkaloids and steroids. The results of the qualitative phytochemical analysis of the extract is summarized in table 1.

Cytotoxic evaluation of methanol extract of *A. cobbe* in C127I cell line:

The per cent cell viability after 24 hours of treatment with methanolic extract is depicted in table 2. There was a dose dependent decrease in the per cent cell viability and the cells treated with 10µg/mL showed maximum viability. The per cent inhibition of cell proliferation as studied by MTT assay 24 hours post treatment with methanolic extract in C127I cell line is presented in table 3. The IC_{50} value of *A. cobbe* as obtained from MTT assay was 64.63µg/mL. The graph showing the IC_{50} analysis is depicted in fig.1 Maximum inhibition was shown when cells were exposed to 320µg/mL and the inhibition of the cells was 100. 8$\pm$ 0.38 per cent.

Acridine Orange / Ethidium Bromide (AO/EB) staining:

The results of Acridine orange / Ethidium bromide (AO/EB) staining revealed the presence of necrotic, early and late apoptotic cells that were treated with the extract. The extract treated cells showed orange to red fluorescence as shown in fig.2 whereas the untreated cells showed green fluorescence (fig. 3) indicating the live cells. Hence it could be concluded that the extract induced apoptosis in the cell lines.

DISCUSSION

Breast cancer is a highly invasive cancer commonly seen among women. The present study investigated the cytotoxic potential of *A. cobbe*in murine mammary tumor cell line, C127I. *A. cobbe* is known for their antioxidant, cytotoxic, and anti-cancer activities [7]. Qualitative phytochemical analysis revealed the presence of phenolic compounds, flavonoids, terpenes and tannins. The presence of phenolic compounds in the extract may be the reason for the potential cytotoxic activity of the plant extract [8]. In the MTT assay, viable cells can use NADH to transform the compound MTT into a purple formazan product and the dead cells lose the ability to do so. This color formation is read in a spectrophotometer and there is a correlation between the developed color and viability [9]. In the present study the extract of *A. cobbe* reduced the cell viability in a dose dependent manner with the least viability was measured at a dose rate of 320µg/mL. The MTT assay is unable to differentiate between necrosis and apoptosis as the reason of reduction in cell growth. One of the main mechanisms by which cytotoxic agents act to kill cancer cells is through apoptosis. The morphological and apoptotic mechanisms of the cell were evaluated using the AO/EB staining, which made it easy to distinguish between live, early, and late apoptotic cells [10]. The result of present study demonstrated that extract of *A. cobbe*is promoting apoptosis in a dose-dependent manner. The nuclei of normal cells get pigmented by AO penetration, which turn green by adhering to DNA. In contrast, EB hues

the nuclei of late apoptotic and necrotic cells red. The current research demonstrated a dose-dependent activation of apoptosis of cancer cells.

Table 1: Phytochemical constituents present in the methanolic extract of *A. cobbe*

Constituent	**Phenolics**	**Alkaloids**	**Steroids**	**Glycosides**	**Tannins**	**Terpenes**	**saponins**	**Flavonoids**
Methanolic	+	-	-	+	+	+	+	+

Table 2: The per cent cell viability of C127I cells after 24 hours treatment with methanolic extract of *A. cobbe*

Concentrations (µg/mL)	**% Cell Viability (Mean±SEM)**
320	-0.8 ±0.038
160	9.2±10.7
80	34.9±8.9
40	71.3±9.8
20	82.1±3.7
10	106.2±7.4

Table 3: The per cent cell inhibition of C127I cells after 24 hours treatment with methanolic extract of *A. cobbe*

Concentrations(µg/mL)	**% Cell Inhibition (Mean±SEM)**
320	100. 8± 0.38
160	90.8±0.07
80	65.1± 0.89
40	28.7± 0.98
20	17.9± 0.37
10	-06.2±0.074
IC_{50}	**64.63 µg/mL**

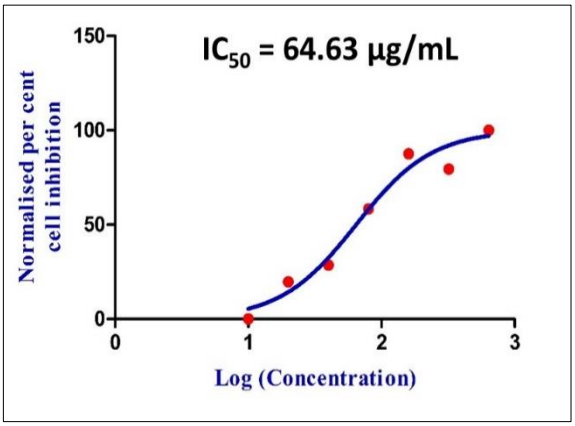

Figure 1: Calculation of IC_{50}

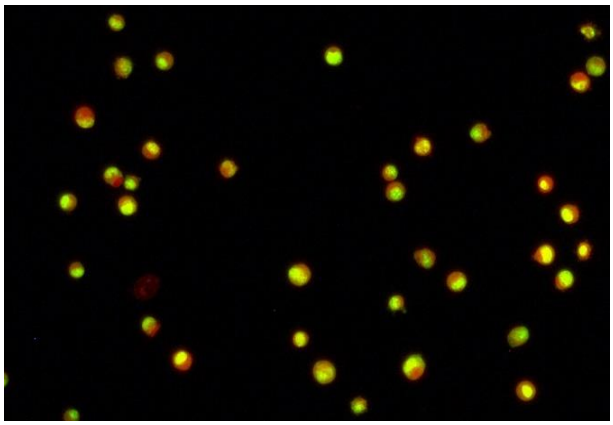

Figure 2: AO/EB staining of C127I cells after treatment with *A. cobbe*

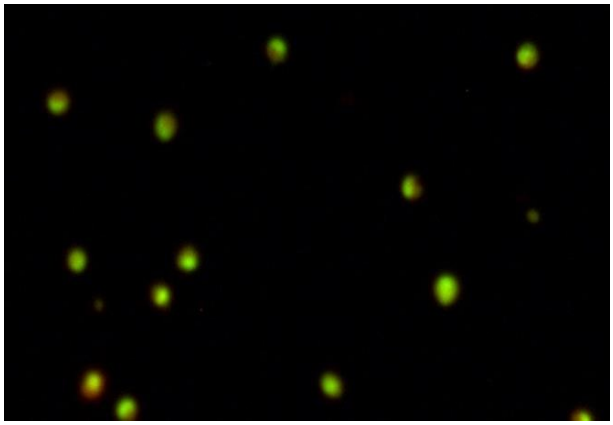

Figure 3: AO/EB staining of C127I control cells without treatment with *A. cobbe*

CONCLUSION

The current study showed that *A. cobbe* possesses significant cytotoxic potential against cancer cell lines via MTT Assay. AO/EB staining showed marked apoptosis of the cell lines through intrinsic pathway. The presence of polyphenolic compounds and terpenes may be reason for the cytotoxic activity of *A. cobbe*. From the result of the present study it could be concluded that the methanolic extract of *A. cobbe* contains significant antiproliferative effect on C127I and hence can be developed as a lead molecule for anticancer therapy with appropriate modelling.

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Conflict of Interest

The authors declare no conflict of interest.

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