



Anticancer property of *Alstonia scholaris* linn leaf extract in MCF -7 and MDAMB -231 Breast Cancer Cell Lines

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Abstract

Breast cancer is a disease in which abnormal cells in the breast grow uncontrollably and form a tumor. It is one of the most common cancers worldwide, especially among women, but it can also occur in men. Plant materials have played an important role in cancer treatment—but usually after scientists isolate and refine specific compounds into safe, standardized medicines. The current work aims to assess the intracellular ROS production and apoptosis induction by *Alstonia scholaris* Linn (*A. scholaris*) leaf extract in breast cancer cell lines. Column chromatography was used to purify bioactive components from an *A. scholaris* Linn methanolic extract. Fraction with a significant concentration of phytochemicals was utilized for further investigation. The results of DCFH-DA staining revealed a substantial rise in intracellular ROS levels in *A. scholaris* Linn-treated cell lines. The dual staining technique (AO/EtBr) was used to examine apoptotic changes. Treatment with *A. scholaris* Linn substantially enhanced the rate of apoptosis in breast cancer cell lines.

Keywords: *Alstonia scholaris*, intracellular ros, apoptosis, breast cancer

Introduction

Breast cancer is now the leading cause of cancer related deaths in women worldwide. Breast cancer affects around 2.1 million women each year, according to the WHO. Breast cancer is detected in an Indian woman every 4 - 5 minutes, according to reports. It accounts for 14% of malignancies in Indian women, while more than 50% of Indian women have stage 3 or 4 breast cancer (Mathur P *et al.*, 2020) [18]. Breast cancer is a complicated disease that need multimodal therapy. It comprises breast removal surgery, cosmetic repair, chemotherapy, radiation, hormone therapy, and other treatments. Women undergoing therapy must deal with a wide range of physiological and psychological issues (Waks AG and Winer EP, 2019) [33]. Despite the availability of numerous modern treatment options, the survival rate is quite low. This might be due to a lack of knowledge, a delay in screening and diagnosis, or the adverse effects of hazardous drugs.

Chemotherapeutic chemicals used in cancer treatment are cytotoxic, meaning they destroy both cancer cells and healthy cells, resulting in a variety of adverse effects during and after treatment. Furthermore, the reaction to some chemotherapeutic drugs may differ between patients, and a lack of a comprehensive treatment plan may raise toxicity even further. To circumvent medication toxicity, seek for alternatives that are less toxic to normal cells or exhibit selective toxicity to cancer cells (Shareef M *et al.*, 2016) [27]. As a result, the current emphasis of research is on herbal treatments that preferentially target cancer cells. Approximately 69% of anticancer medicines authorized are natural goods or were created based on knowledge obtained from natural items (Sak K, 2014). Because of their safety, effectiveness, and cost, the World Health Organization supports medicinal plants for the treatment of different malignancies. Plants produce a diverse range of secondary metabolites known as phytochemicals, which play a role in plant defence mechanisms in a variety of ways. These

phytochemicals have been identified as novel anticancer active compounds (Newman DJ and Cragg GM, 2007) [21]. Most malignancies are accelerated by oxidative stress and inflammation, which promotes all phases of carcinogenesis. Phytochemicals have been shown to exhibit antioxidant properties, to prevent oxidative damage to different macromolecules, and to scavenge free radicals produced by biochemical processes (Kampa M and Castanas E, 2008). The majority of these antioxidant phytochemicals have also been shown to have anti-inflammatory properties. The particular method includes cell growth inhibition, carcinogen inactivation, cell cycle arrest promotion, apoptosis, and immune system modulation, among other things. Apigenin, Curcumin, Gamma Linoleic Acid, Carnosic Acid, Kaempferol, and other phytochemicals from herbal sources were shown to modulate nitric oxide (NO), TNF- α , IL-1, and thromboxane B2 (TXB2), as well as inhibit COX-2 gene expression (Ghasemian M Owlia S and Owlia MB, 2016) [9]. Many plant-derived chemicals are undergoing preclinical testing for anticancer medication development in order to assess their effectiveness, toxicity, pharmacokinetics, and safety. Many of these are in clinical studies, and some are already being used in cancer treatment (Choudhari AS *et al.*, 2020) [5].

Several medicinal herbs and their secondary metabolites have been shown to have cytotoxic properties against breast cancer cells (Kooti W *et al.*, 2017) [6]. *Alstonia scholaris* Linn (*A. scholaris*) belongs to the Apocynaceae family and is a popular ornamental tree in India. This plant, known as saphthaparna, has been used for millennia in Ayurvedic medicine to cure a variety of ailments. It has a diverse set of pharmacological characteristics, including antioxidant, anti-inflammatory, antimutagenic, and immunomodulatory effects (Meena AK *et al.*, 2011) [19]. Various investigations (Sharma V *et al.*, 2010) [28]; Nanditha R *et al.*, 2021) have demonstrated its cytotoxic effects on lung cancer cells, oral

cancer cells, neuroblastoma cells, colon cancer cells, hepatic cells, mammary cells, and other cancer cells. The aqueous and ethanolic extracts of *A. scholaris* bark were found to have a strong cytotoxic impact on human cervical cancer cell lines (Chellappa, LR and Prabakar J, 2019) [4]. A mixture of alkaloids and terpenoids isolated from *A. scholaris* leaves was evaluated for apoptosis and immunomodulatory activity in human lung cancer cell line A549 and Lewis tumor-bearing C57BL/6 mice. They demonstrated strong anti-proliferative activity in A549 cells and substantial suppression of tumour development in C57BL/6 mice with tumours. In addition, immunological indices were shown to be elevated in treated mice (Feng L *et al.*, 2013) [7]. Its anti-inflammatory efficacy, decreased tumour multiplicity, and neuroprotective potential have all been demonstrated in studies. Of experimental animals, the alkaloids and flavonoids in *A. scholaris* decrease the generation of inflammatory cytokines and neuropathic pain (Singh H *et al.*, 2017) [29]. Though numerous investigations have identified multiple pharmacological characteristics of *A. scholaris*, the molecular processes and genetic engrossment of its activity have yet to be well illustrated. In-vitro breast cancer analysis with cell lines is a highly challenging procedure since the clonal population comprising of any single cell line could not well capture the heterogeneity of breast tumors at the intra-tumoral level. Success in long-term propagation has been a limiting factor, needed to cover the inter-tumoral cancer heterogeneity. Very few breast cancer cell lines (MCF7, T-47D, MDAMB231), regardless of the total number of established ones, have been frequently used for research purpose for the sake of cultivation easiness, rendering the transportability of results etc (Dai X *et al.*, 2017) [6]. So an attempt was made in this work to analyse the cytotoxic impact of *A. scholaris* at the genetic level in MCF 7 and MDAMB 231 breast cancer cell lines.

Methodology

Methanol, Hexane, Chloroform, Minimum essential medium (MEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), 0.25% trypsin EDTA, penicillin, and streptomycin mixture were obtained from Lonza Biosciences India. 2,7-diacetyl dichlorofluorescein (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Ethidium Bromide (EtBr), Rhodamine-123, Acridine Orange (AO) were purchased from Sigma, St. Louis, USA. Monoclonal primary antibodies and secondary conjugated anti-mouse were purchased from Santacruz.

Preparation of Plant Extract: The young leaves of *A. scholaris* were gathered from Erode (DT), Nasiyanoor (village) at month of October, cleaned under running tap water, and shade-dried for 20 days at room temperature. Using a grinder, the dried plant material was crushed into a fine powder (mixer). In a Soxhlet extraction device with 150 ml of methanol, 9 g of powdered material was extracted (Vogel, 1988). The solvents were evaporated (at 40°C) using the heating mantle. The resulting greenish compounds were evaporated to dryness, and percentage yields were computed (Beyer *et al.*, 1997). The crude extract was then fractionated using column chromatography, and the main fraction with a high concentration of phytochemicals was chosen for further study (Shanmugapriya D and Jayanthi G, 2019) [26].

Cell Culture: MCF-7 breast cancer cell lines and MDA-MB 231 triple-negative breast cancer cell lines were obtained from NCCS in Pune, India. Cells were cultured in DMEM media and kept at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

Cytotoxicity Assay: MTT test was used to determine the effect of *A. scholaris* on the cytotoxicity of MCF-7 and MDA-MB 231 (Marks DC *et al.*, 1992) [17]. The cells were seeded in microtiter plates at a density of 5000-10000 cells per well in a final volume of 100µl of MEM medium and incubated for 24 hours. Cells were exposed to a different concentration of *A. scholaris* after 24 hours of incubation. It was then incubated for another 24 and 48 hours. The MTT solution 10 µl, 5 mg/ml in PSB (phosphate buffered saline) was then added to each well and left for four hours at 37°C. The MTT reagent was withdrawn after incubation, and 100µl 100% DMS was added to dissolve the purple formazan crystals. The plate was read at 570nm in an ELISA plate reader (Robonic, India).

Measurement of Intracellular ROS Generation: ROS generation in MCF-7 and MDA-MB was mediated by *A. scholaris*. Dichloro-dihydrofluorescein diacetate (DCFH-DA) staining revealed 231 cells (Carter WO *et al.*, 1994) [2]. Cells were sown (1 × 10⁶ cells/well) on a 6-well plate; following harvesting, cells were treated with *A. scholaris* at different incubation times and maintained in a CO₂ incubator for 24 and 48 hours, respectively. After appropriate incubation time, 5 µg DCFH-DA was added to the wells, and it was incubated for 30-45 mins in a dark environment. Fluorescent intensity for DCFH-DA was measured with excitation and emission filters set at 485 ±10 and 530 ±12.5 nm, respectively (Multimode reader, Tecan).

Acridine Orange/Ethidium Bromide Staining Assay: Using two staining techniques (acridine orange/ethidium bromide staining), *A. scholaris* mediated apoptotic morphological alterations in MCF-7 and MDA-MB 231 cell lines were identified (Kasibhatla S *et al.*, 2006) [10]. In a 6-well plate, 1×10⁵ cells were plated; cultivated cells were treated with *A. scholaris* and incubated. After incubation, the treated cells were rinsed with ice-cold Phosphate Buffered Saline (PBS) and stained for 30 minutes at 37°C with acridine orange/ethidium bromide (10 L/mg AO and 10 L/mg EtBr) solution. The labelled apoptotic and live cells were examined under a fluorescence microscope.

Results

***A. scholaris* (AS) has an inhibitory effect on breast cancer cell viability:** MTT assay was used to determine the cytotoxicity of *A. scholaris* on MCF-7 and triple-negative breast cancer MDA-MB 231 cell lines. The IC₅₀ value was 22 g/ml for 24 hours incubation and 34 g/ml for 48 hours incubation. Doxorubicin was utilised as a control medication, and its IC₅₀ value was 1.8 µg/mL for 24 hours incubation and 2.5 µg/mL for 48 hours incubation.

***A. scholaris* induces overproduction of intracellular ROS levels in MCF-7 and MDA-MB 231 cell lines:** Using DCFH-DA staining, the effect of AS on intracellular ROS production in MCF-7 and MDAMB 231 cell lines was investigated. When compared to AS-treated MCF 7 and MDAMB 231 cell lines, the intensity of DCF fluorescence,

which indicates intracellular ROS, was insignificant. Furthermore, the intensity of fluorescence was observed to rise considerably with time in AS-treated cell lines.

A. scholaris induces apoptosis in MCF-7 and MDA-MB 231 cell lines: Dual labelling (AO/EtBr) was used to examine the apoptotic morphological alterations in the MCF-7 and MDA-MB 231 cell lines (Figures 5 and 6). At 24 and 48 hours, AS administration dramatically enhanced the rate of apoptosis in MCF-7 and MDA-MB 231 cell lines. The proportion of apoptotic cells was much greater after 48 hours of AS therapy, according to the bar diagram.

A. scholaris mediated mRNA expression of p53, Bcl-2, and BRAC1 in MCF-7 and MDA-MB 231 cell lines: The RT-PCR assay was used to examine the AS-mediated mRNA expression levels of the p53, Bcl-2, and BRAC1 genes, with GAPDH serving as a control. Densitometry was used to quantify the mRNAs of interest. According to the figure, the expression level of the p53 gene is raised in both MCF-7 and MDA-MB 231 cell lines after 24 and 48 hours of AS treatment. In contrast, Bcl-2 and BRAC1 gene expression was reduced following 24 and 48 hours of AS therapy.

A. scholaris mediated protein expression of TNF- α and IFN- λ in MCF-7 and MDA-MB 231 cell lines: Western blotting was used to examine the AS mediated protein expression of TNF- α and IFN-1 in MCF-7 and MDA-MB 231 cell lines. Densitometry was used to quantify the proteins, which were then normalized to their respective β -actin loading controls (Figures 9 and 10). TNF- α and IFN-1 concentrations were lower in AS-treated MCF-7 and MDA-MB 231 cell lines at 24 and 48 hours compared to controls.

Discussion

ROS are highly reactive chemicals that are essential for a variety of biological activities, including gene expression. One of the most significant roles of ROS is to cause programmed cell death (PCD) (Perillo B *et al.*, 2020) [22]. Increased ROS levels have been observed in virtually all malignancies, where they aid in tumour growth and progression in a variety of ways. Tumor cells also generate more antioxidant proteins in order to cleanse themselves. The antioxidant proteins are needed for cancer cell activity and must be kept in balance with the ROS level. Excessive ROS levels in cancer cells can cause oxidative stress and cell death (Tarhouni-Jabberi S *et al.*, 2017) [30]. Most chemotherapy medicines increase intracellular ROS and disrupt redox-homeostasis in cancer cells, resulting in cell damage (Liou GY and Storz P, 2010; Yang H *et al.*, 2018) [14, 35]. The intracellular ROS production in breast cancer cell lines was examined using the DCFH-DA staining technique in this study, and it was discovered that A. scholaris treated cell lines showed a substantial increase in fluorescence intensity when compared to the control. Because fluorescence intensity is directly related to ROS concentration, A. scholaris certainly enhanced intracellular ROS generation in MCF-7 and MDA-MB 231 breast cancer cells. The mechanism may also be similar to other chemotherapeutic drugs; A. scholaris treatment helps in cell injury and cell death in cancer cells.

Apoptosis is a series of processes driven by proteolytic enzymes known as caspases that cleaves certain proteins,

the nucleus, and the cytoplasm, ultimately leading to cell death (Raff and Martin, 2022). Cancer cells that undergo insufficient apoptosis eventually avoid apoptosis through a variety of ways. Cancer cells often resist apoptotic signals, upregulate anti-apoptotic signals such as BCL-2 and MCL1, and downregulate pro-apoptotic signals such as BAX, BAK, and p53, resulting in defective apoptosis (Lopez J and Tait SWG, 2015) [15]. Triggering apoptosis is one of the finest treatments and is successful in all types of cancer. It has the potential to stop the uncontrolled development of cancer cells (Lopez *et al.*, 2015) [15]. The majority of herbal substances utilized in cancer treatment are apoptosis inducers. They work through a variety of methods, including cell cycle disruption, inhibiting the signal transduction pathway that transforms normal cells to cancer cells, immunomodulation, and so on (Safarzadeh E *et al.*, 2014) [25]. MCF-7 and MDA-MB 231 breast cancer cell lines were treated with A. scholaris, and morphological alterations associated with apoptosis were examined. When A. scholaris-treated cell lines are compared to controls, the intensity of the fluorescence that shows the proportion of apoptosis increases dramatically over time. According to the findings, A. scholaris successfully causes apoptosis in cancer cells, demonstrating its anticancer properties.

Regardless of the peripheral mediators, a cell's genetic makeup eventually speaks for itself. p53 is a nuclear transcription factor with a pro-apoptotic activity that has been designated as one of the classical type tumour suppressors; it regulates cell cycle and is capable of inducing cell cycle arrest to allow for DNA repair and apoptosis (Vogelstein B and Kinzler KW, 1992) [32]. In human cancers, the p53 gene is the most often mutated gene. Its expression is inadequate under normal circumstances. When there is DNA damage, p53 is transformed into an active form by post-translational modification mechanisms, which cause cell cycle arrest and death, allowing cells to repair damaged DNA. (Lacroix, M *et al.*, 2006) [12]. Furthermore, the Bcl-2 and BRAC1 genes are required for proper cell function as well as apoptosis. Bcl-2 is an apoptosis suppressor gene that can prevent or postpone the start of apoptosis. BRAC is an acronym for the Breast Cancer gene, which aids in DNA repair. Mutations in these genes have been found in a variety of malignancies (AbdelMohsen MA *et al.*, 2016; Chan and Wood-Yee, 2000) [3]. The current study's RT-PCR results show that A. scholaris therapy dramatically enhanced the mRNA expression of p53 in both MCF-7 and MDA-MB 231 cell lines, allowing the cells to repair DNA damage. Further A. scholaris treated cancer cell lines showed downregulation of Bcl-2 and BRAC1 genes allows the cancer cells for proper apoptosis and DNA repair.

Conclusion

The results show that A. scholaris efficiently suppresses cell growth and causes apoptotic cell death in MCF-7 and MDA-MB 231 breast cancer cells. Furthermore, the observed anticancer effects of A. scholaris may be due to its ability to increase intracellular ROS generation and decrease TNF- α and IFN-1 concentrations, as evidenced by the upregulation of the p53 gene and downregulation of the Bcl-2 and BRAC1 genes in MCF-7 and MDAMB 231 breast cancer cells. Thus, the findings of this study give evidence for A. scholaris anticancer action and more significantly, the molecular basis for its impact.

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