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Empirical Study of *Simarouba glauca* Induced Cell Death Through Parafacial Anticancer in Breast Cancer (T47d)

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Abstract

Cancer treatment, particularly conventional chemotherapy, poses significant challenges due to long-term impacts and severe adverse reactions. This has led to a growing interest in natural compounds, with an emphasis on plant-derived remedies to enhance the effectiveness of anticancer treatments. The study primarily focuses on the determination of anticancer activity of *Simarouba glauca* against T47-D breast cancer cells by MTT assay. The results demonstrate that this extract effectively inhibits the growth of T47D cells in a time- and dose-dependent manner, with an IC50 value of 125µg. Moreover, the study investigates the gene expression levels of p53 and Bcl-2 in response to the plant extract by RT-PCR. Notably, Bcl-2 gene expression is significantly down regulated, while p53 gene expression is unregulated. In conclusion, this study underscores the anticancer potential of *Simarouba glauca*, particularly in inhibiting T47D breast cancer cells and inducing apoptosis. Further exploration of this plant's compounds for more effective and less toxic alternatives in breast cancer treatment is warranted. The study contributes to the understanding of the molecular mechanisms underlying the anticancer effects of *Simarouba glauca* and opens avenues for future research.

***Key words*:** Anticancer activity, T47D breast cancer cells, *Simarouba glauca,* p53, Bcl-2

Cancer is one of the most powerful challenges in modern medicine, and millions of lives have been affected by its relentless advancement. In recent years, significant improvements have emerged in cancer treatment, giving patients and physicians the same confidence. These developments include targeted treatments, immune treatments, and accurate clinical approaches, which have revolutionized cancer treatment. However, despite these significant developments, a strong obstacle has emerged. Breast cancer is one of the most common cancers among women worldwide and is the second major cause of cancer-related deaths in women following lung cancer [1]. Breast cancer development involves the planned implementation and dysfunction of various genes to promote cell proliferation. In this context, the wild-type p53 protein plays an important role in regulating apoptosis in response to DNA damage induced by anti-cancer drugs. This tumor suppressant protein can stop the growth of potential cancer cells in the G1 phase or induce apoptosis [2]. In contrast, Bcl-2, a founding member of the genetic family that manages apoptosis, acts as an anti- apoptosis gene by preventing early stages of apoptosis and preventing projected cell death [3].

Cancer treatment can now be said to be a critical battle because current cancer drugs have lost their ability to cure cancer, and this can be called resistance. Various therapies have been proposed to treat cancer, many of which use plant-derived products. To address these challenges and combat their illness, cancer patients often turn to alternative therapies. Plants still have enormous potential to deliver new drugs, and they are a stagnation of natural chemicals that can provide chemical protection energy against cancer. Recently, Greenwell and Rahman [4] have recommended several compounds from medicinal plants with anti-cancer activities.

Simarouba glauca DC is an evergreen edible oil tree belonging to the family Simaroubaceae. This family includes 32 genera and more than 170 species of trees and bushes of pantropical distribution. The Simaroubaceae family possesses a wide variety of chemicals and can be characterized as having potential bioactive molecules with remarkable research potential. More than 200 currently known chemicals are isolated and identified in the Simaroubaceae family. The properties of *Simarouba glauca* have been extensively studied, including its potential as an antimalarial, antidysenteric, antipyretic, anticancer, and antibacterial agent. In 2018, Prajapati *et al*. [5] and colleagues reported on the anticancer activity of leaf extracts against leukemic cancer cell lines. Furthermore, Mahesh *et al*. [6] documented the anticancer potential of *Simarouba glauca* against HELA and MCF-7 cancer cells. However, no one determined the anticancer activity of *Simarouba glauca* against T47D and the expression of p53 and Bcl-2 genes. Therefore, present studies evaluated the anticancer activity of *Simarouba glauca* and determined the gene expression levels of p53 and Bcl-2 to gain insights into the molecular mechanisms underlying the anticancer effects of *Simarouba glauca* on T47D cells.

MATERIALS AND METHODS

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Preparation of plant materials

The leaf of *Simarouba glauca* was collected from the Erode area, Tamil Nadu, India. The collected plant leaf was dried for 15 days and powdered with a blender. One hundred grams of the dried material were exhaustively extracted with ethanol by Soxhlet extraction. The ethanolic extracts were filtered and evaporated to dryness under reduced pressure by a rotatory evaporator. The resulting residues of each part of the plants were stored at -20 °C.

Cell culture

The human breast cancer cell line T47D was purchased from the National Centre for Cell Science (Pune, Maharashtra, India). The cells were cultured in RPMI-1640 (Himedia, India) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). The cells were allowed to adhere to the culture dish and form a monolayer. The cells were subculture once they reached confluence.

MTT assay

The cytotoxicity of *Simarouba glauca* extract on T47D cells was assessed using the MTT assay, which relies on the reduction of MTT by mitochondrial dehydrogenase to form purple formazan crystals, indicating cell viability. T47D cells were plated in 96-well plates and exposed to various concentrations of the plant extract for 48 hours. A negative control using 0.2% DMSO and a positive control using Cyclophosphamide were included. Following incubation, MTT was introduced, and cells were incubated for an additional 4 hours. Cell viability was determined by measuring the optical density (OD) at 570 nm, and the IC50, representing the concentration inhibiting 50% of cell growth, was calculated. The resulting data were expressed as a percentage of relative viability compared to the control conditions. Cell viability and cytotoxicity were computed using the following formulas, as described by Roshene *et al*. [7]:

$$\text{Cell viability \%} = (\text{test OD}/\text{ control OD}) \times 100 \text{ and}$$
$$\text{cytotoxicity \%} = 100 - \text{viability \%}.$$

Extraction of RNA and cDNA synthesis

To extract RNA from both treated and untreated cancer cells, an Aura RNA extraction kit (India) was employed. Subsequently, for cDNA synthesis, the Aura cDNA synthesis kit (Cat. No: MPC -1) was utilized. The following materials were combined in a sterile tube on ice: 5X reaction buffer (4 µl), 10X Random Primers (2 µl), Reverse Transcriptase (1 µl), 25X dNTPs (0.8 µl), Rnase Inhibitor (0.3 µl), and water (1.9 µl), resulting in a total volume of 10 µl. Then, 10 µl of RNA (ranging from 20 ng to 2 µg) was added, thoroughly mixed, and subjected to a cDNA synthesis program, which involved incubation at 25 °C for 10 minutes, followed by 55 °C for 60 minutes, and inactivation at 85 °C for 5 minutes.

RT-PCR analysis

The semi quantitative PCR of the Bcl2 and P53 genes was performed based on the previous study of Roudkenar *et al*. [8]. The following primers were used for this study: p53 Reverse: 5′-CTCATTCAGCTCTCGGAACATCTCGAAGCG-3′ and Bcl-2 Forward: 5′-GTTCGGTGGGGTCATGTGTGTGGAGA-3′; Bcl-2 Reverse: 5′-GCTGATTCGACGTTTTGCCTGAAGAC-3′). As an internal control, the housekeeping gene β-Actin (β-Actin Forward: 5′-ACGGGGTCACCACACTGTGC-3′; β-Actin Reverse: 5′-CTAGAAGCATTTGCGGTGGACGATG-3′) was co-amplified in each reaction. The PCR reaction was carried out in a final volume of 20 µl containing master mix 10µl (1x PCR buffer, 5 U/µl Taq polymerase, 1.5 mM MgCl2, and 0.2mM of each dNTP) (Aura, India), 0.4 µM of each 1 µl primer and make up to 20 µl with molecular grade water. The template was denatured for 5min at 94°C, followed by amplification cycles at 94°C for 1min, 69°C (for p53, 64°C for Bcl-2 and 57°C for β-actin) for 1 min and 72°C for 1.20 min, and terminated with an additional extension step for 8 min at 72°C. The PCR products were visualized using 1.2% agarose gel electrophoresis with ethidium bromide staining.

RESULTS AND DISCUSSION

Chemotherapy is currently used as an effective treatment option for various cancer types, either as a complete treatment or in combination with radiotherapy [9]. Based on the long-term impacts and severe adverse reactions associated with conventional chemotherapy, contemporary research efforts focus on finding natural compounds that can correct these unwanted side effects. In recent times, the importance of plants is evident in improving the effectiveness of many clinically valuable anticancer drugs, with more than 60% of the currently used antiproliferative agents having their appearance, either directly or indirectly, in natural sources. Significant emphasis is placed on traditional pharmaceutical herbal remedies [10].

The present study aimed to assess the effect of the ethanol extract of *Simarouba glauca* on the apoptosis of T47-D breast cancer cells. Numerous studies have shown that *Simarouba glauca* exert influential effects on malignancies such as breast [11], HCT 116 [12], lung [13], skin, and colon cancers [14]. However, the precise molecular mechanism of *Simarouba glauca* in the induction of apoptosis in cancer cells has to be clarified. Therefore, the present study focuses on the molecular mechanisms of the p53 and bcl2 genes.

As seen in (Fig 1-2), *Simarouba glauca* inhibits the growth of the cells. This effect is increased with increasing the concentration of *Simarouba glauca* so that the cells were dead completely at a concentration of nearly 1mg and the IC_{50} value was 125µg. In other words, it seems that the effect of *Simarouba glauca* on the T47D breast cancer cell line is time- and dose-dependent. It should be noted that the specific changes in cell shape observed during the MTT assay varied widely depending on the concentration of the plant extract. This could include cell shrinkage, membrane blebbing, or other morphological changes associated with cell stress and damage. These morphological alterations offer vital insights into the possible cytotoxic or therapeutic qualities of plant extracts and are frequently a key signal of their effects on cell health and viability [15].

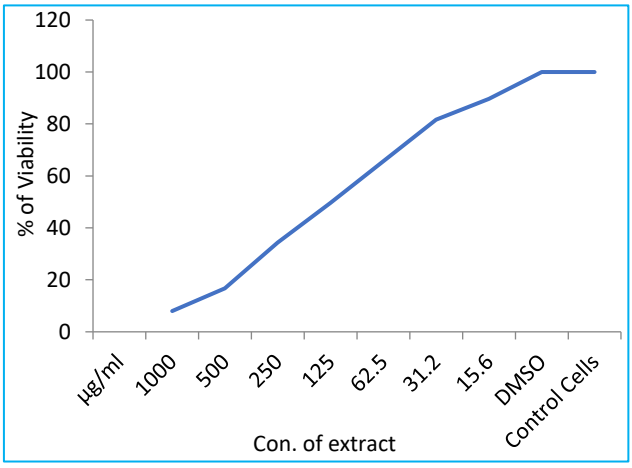
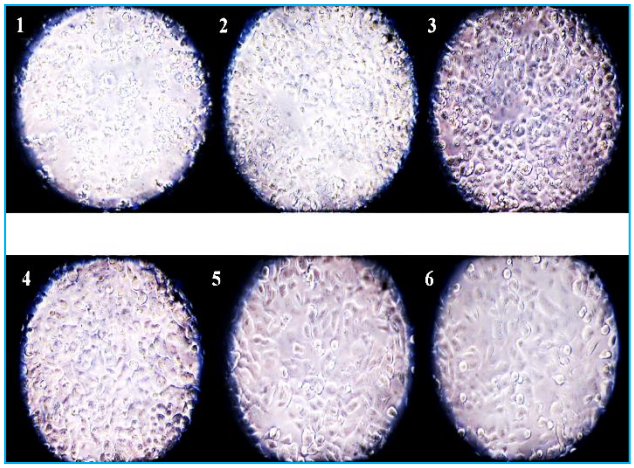


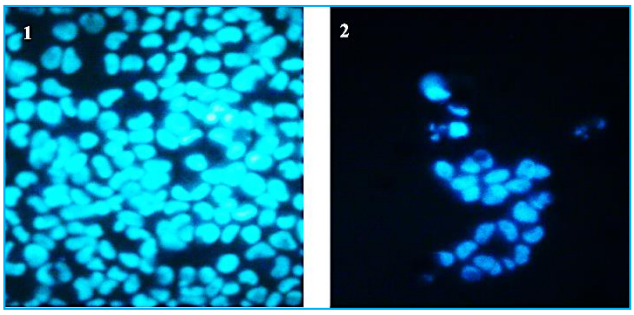
Fig 1 Determination of anticancer activity of *Simarouba glauca* against the T47D breast cancer cell line



(1) 1000µg/ml, (2) 250µg/ml, (3) 62.5µg/ml, (4) 15.6µg/ml, (5) Control cell, (6) Treated with DMSO

Fig 2 Detection of cytotoxicity activity of *Simarouba glauca* by MTT assay

Furthermore, the cytotoxicity of the plant extract was confirmed with the DAPI staining method. It was clearly visible in treated cells after DAPI staining that (Fig 3) the *Simarouba glauca* ethanol extract-treated cells displayed maximum condensation of the nucleus; the 125µg concentration of the extract was highly efficient in inducing apoptosis in cancer cells. In DAPI staining, a bright blue condensed or blebbing nucleus is an indication of apoptosis [16]. Extensive research has been conducted on the leaf extract of *Simarouba glauca* for its potential in cancer treatment, and it has been found that the main constituents responsible for its medicinal properties are Quassinoid. This is because quassinoid and alkaloids extracted from the plant exhibit cytotoxic and antiproliferative properties. A search of the literature revealed that no studies had previously documented *Simarouba glauca's* ability to suppress T47D breast cancer. However, one review paper did contain data suggesting that glaucarubinone, a substance present in *Simarouba glauca*, had action against T47D breast cancer.



(1) Control cells of T47D breast cancer, (2) Treated with *Simarouba glauca* extract

Fig 3 DAPI staining

Due to the fact that cancer is a heterogeneous group of diseases with numerous underlying causes, the molecular processes behind the anticancer activity of various medicines and therapies are diverse and complex. It's essential to comprehend these systems in order to create cancer treatments that work. Therefore, cancer can be prevented by understanding its molecular mechanism. In this work, after treating T47D cells with an ethanol extract of *Simarouba glauca*, we looked at the levels of mRNA expression of two cancer-related genes, p53 and Bcl-2 (Fig 4). Notably, the extract drastically down regulated the expression of the Bcl-2 gene while up regulating the expression of the p53 gene. These observed outcomes imply that secondary metabolites may be responsible for the plant's bioactivity. Mahesh *et al.* [6] recently investigated the anticancer activity of *Simarouba glauca* against T47D cancer cell lines, but they did not investigate the mechanisms of gene expression. *Simarouba glauca*, which contains tricaproin, was previously utilized to treat colorectal cancer cells, and Jose *et al.* [17] assessed the levels of caspase-3 in these cells after the treatment. In summary, gene expression studies in cancer cells play a vital role in cancer research, diagnosis, treatment, and drug development, ultimately contributing to improved patient care and outcomes.

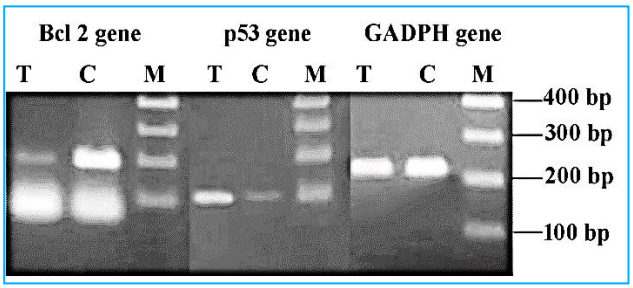


Fig 4 RT PCR analysis for determination of gene expression: T47D Breast cancer cells treated with *Simarouba glauca* extract, C: Control cell, M: 100bp DNA marker

In this era of precision medicine, understanding the molecular underpinnings of resistance to cancer treatment has become paramount. Researchers are delving deep into the intricacies of cancer biology to identify the key factors driving treatment resistance. Insights gained from such studies hold the promise of developing strategies to overcome or circumvent resistance, ultimately improving patient outcomes.

Drug resistance to cancer is a multifaceted problem with far-reaching social impacts. Beyond its medical challenges, it imposes a greater emotional and financial burden on patients and their families, which often leads to increased quality of life and health care [18]. Furthermore, therapeutic resistance prevents improvements in cancer research and health systems, limiting the effectiveness of existing treatments. To address this issue, we need joint efforts between healthcare providers, researchers, policymakers, and the community to develop innovative strategies, raises awareness, and improves access to sophisticated treatments. Ultimately, winning drug resistance is not just a scientific effort but a social imperative [19].

CONCLUSION

In conclusion, the overall results of the present study provide evidence of the anticancer activities of *S. glauca*. The selectivity of the plant proved to have promising anticancer potential by inhibiting T47D cancer cell lines. However, the search for new compounds from *S. glauca* with more effective and less toxic compounds constitutes interesting alternatives for the development of anticancer drugs for breast cancer treatment. Thus, further studies are in process to evaluate the potent fraction of the active plant. The present study revealed that an ethanol leaf extract of *Simarouba glauca* induced cell death through apoptosis. It seems that the Simaroubaceae family contains compounds that can have effective anticancer properties.

Limited scope

The study concentrates on one specific breast cancer cell line (T47D), which may not represent the heterogeneity of breast cancer as a whole. Different subtypes of breast cancer may respond differently to treatment, so the findings may not be universally applicable.

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