RESEARCH ARTICLE

Editorial Process: Submission:08/26/2023 Acceptance:01/19/2024

In-vitro Antioxidant Activity and Flow Cytometric Analysis of *Simarouba glauca* DC Bark Extract Induced Apoptosis in Triple Negative Breast Cancer Cells

Ramachandra Mohan Rao Pandhari, Tarikere Chandrashekharappa Taranath*

Abstract

Objective: Ethnomedicinally *Simarouba glauca* DC is an important plant containing major class of phenols and terpenoids as bioactive compounds. The present study focuses on the evaluation of the anticancer effects of *S. glauca* bark UAE-EA (Ultrasonicator Assisted Extraction – Ethyl Acetate) fraction (SG-Fraction) against MDA-MB-231 triple negative breast cancer cell lines. **Methods:** UAE-EA technique was used for the extraction of phytochemicals from *S. glauca* bark. Fractionation method was carried out to obtain Ethyl acetate fraction and PPS, TPC, and DPPH assays were performed to characterize the extract. MTT assay was then applied to analyse the viability of cells and MMP assay to confirm the initiation of drug induced apoptosis. Apoptotic morphology and quantification were assessed by DAPI and Annexin V/propidium iodide (PI) staining. **Results:** UAE yielded 53g of crude extract in methanol. 16g Ethyl acetate fraction was obtained from fractionation. Phytoconstituents such as alkaloids, phenols, flavonoids, and triterpenoids were detected. The TPC was 278.65 mg GAE/100ml. The SG-Fraction showed maximum 66.38% RSA at 200 µg/ml and IC_{50} value was 101.72 µg/ml. MMP confirmed the induction of apoptosis. DAPI showed the reduction of nuclei with bright chromatin condensation, blebbing, nuclear fragmentation and apoptotic bodies. Annexin-V FITC/PI study showed 59.48% apoptosis induction. This fraction showed a similar trend of antioxidant effect as compared to ascorbic acid but, prominently lower cell viability than Camptothecin ($P<0.005$). In line with higher TPC in the SG-fraction, free radical scavenging activity was increased ($r = 0.098^{**}$, $p=0.002$) and cell viability was reduced significantly ($r = -0.097^{***}$ $p<0.01$). **Conclusion:** These results indicate that UAE-EA fraction of *S. glauca* bark inhibits the growth of MDA-MB-231 cells and can be considered for further neo-adjuvant chemotherapy drug research.

Keywords: Anti-cancer activity- Cell viability- DAPI staining- Mitochondrial membrane potential

Asian Pac J Cancer Prev, **25 (1)**, 201-210

Introduction

Cancer remains a serious health concern worldwide, affecting physical, mental, and social status of an individual [1]. Since past few decades breast cancer has become the most common type of cancer worldwide with nearly a million cases every year [2]. It is the second most leading cause of cancer-induced death and the primary cause of death among the women at menopause globally [3]. Triple Negative Breast Cancer (TNBC) is a subtype of breast cancer that exhibits Estrogen Receptor (ER-) negative, Progesterone Receptor (PR-) negative and Human Epidermal Growth-factor Receptor 2 (HER2-) negative immunohistochemical profile. Hence it remains undetected with aggressive metastatic patterns. TNBC comprises 20% of invasive breast cancers [4]. The global susceptibility of breast cancer is 1:8 among women and based on the molecular subtype and stages of breast cancer, treatments vary from the removal of cancerous tissue to chemotherapy, radiotherapy, and hormone therapy [5].

Radiotherapy, chemotherapy, and surgery are modern treatments modalities, however accompanied with serious histological and physiological side effects [6]. Hence modern research aims to screen traditional medicine for more efficient and safe drugs to treat cancers. Phytochemicals, i.e., secondary metabolites derived from plants are being used as potential drugs, with no or very minimal side effects [7]. These drugs are emerging as safe chemotherapy agents in cancer treatment [8].

Simarouba glauca DC belongs to the family Simaroubaceae, commonly known as "The Paradise Tree" or "King Oil Seed Tree" or "Laxmitaru Taru" [9], is a multipurpose evergreen tree native to Republic of El Salvador a country in Central America, and it was introduced to India in 1960. Leaves and barks of this plant are rich sources of phytochemicals like glaucarubin, glaucarubinone, glaucarubol, glaucarubolone, triterpenoids, flavonoids and quassinoids which have

*Environmental Biology and Green Nanotechnology Laboratory, P. G. Department of Studies in Botany, Karnatak University, Dharwad, India. *For Correspondence: tctaranath@gmail.com*

prominent anticancer properties [10]. Bark is used as an important herbal medicine across the world. Prominently bark is used against dysentery hence it is also known as dysentery bark. The pharmacological studies of bark extract of Simarouba have shown its anti-cancerous, haemostatic, anti-pyretic, anti-microbial and anti-septic properties [11].

Induction of apoptosis by intrinsic or extrinsic pathways is the primary mode of action of chemotherapy drugs. Therefore, the percentage of induced apoptosis is a major parameter in evaluation of their anticancer properties.

Flow assisted cell sorting is a widely used method to investigate cellular activities including apoptosis, as it offers rapid and multi-parameter analysis of millions of cells for a single event. Several cytofluorometric dyes are available for detecting different aspects of cellular changes occurring during apoptotic processes. Fluorochrome-labelled Annexin-V stain binds to negatively charged phospholipids exposed due to loss of asymmetry of cell membrane upon induction of apoptosis, thus, detecting apoptotic cells. Propidium iodide (PI), is a phenanthrene derivative stain excluded by the living cells and trapped by dead cells. Cytofluorometric PI exclusion test is used to distinguish apoptotic cells from necrotic and living cells, in a fashion independent of the DNA fragmentation [12].

The present study focused on evaluating the total phenolic content, antioxidant activity, and flowcytometric detection of apoptosis induction capacity of Ethyl acetate fraction of *Simarouba glauca* DC bark UAE methanolic extracts in MDA-MB-231 TNBC Cells.

Materials and Methods

Source and Maintenance of Cancer Cell line

MDA-MB-231 cells were purchased from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were maintained in T25 culture flask (#12556009, Biolite-Thermo) containing 10 ml of DMEM (#AL111, HIMEDIA) high glucose media supplemented with 10% FBS (#RM10432, HIMEDIA) and 1% antibiotic-antimycotic solution, incubated in the atmosphere of 5% CO2, 18 – 20% O2 at 370 C temperature in the CO2 incubator (Heal Force, China), and sub-cultured for every two days. 200µl cell suspension was seeded in a 96-well plate (Corning, USA) at a density of 20,000 cells per well and were incubated in a 5% CO2 incubator for 24 hrs at 37° C.

Treatment groups

Negative control: cells cultured in the same medium without any drug/compound. Positive control: cell treated with Camptothecin, Test group: cells treated with desired concentrations of SG-fraction prepared in 0.1% DMSO.

Collection and preparation of sample

Fresh bark sample was collected from Mr. K. M. Hegde's Simarouba plantation, Bhairumbe, Sirsi, Uttara Kannada; 14°41'43.0"N 74°49'27.2"E; GPS co-ordinates of the plant: 14.695268, 74.824211; Plus Code: MRWF+4M4 Ashisara, Karnataka, India; Altitude: 576.00m/1,889.65 ft and authenticated by Prof. K. Kotresha, Professor and Taxonomist, Department of Botany, Karnataka Science College, Dharwad, India. Harvested bark was chopped to small pieces and shade dried. Dry bark was pulverized (SS212R/2HP, Confider, India) using a 28 number mesh to a powder with 700 microns/0.028 inches particulate size.

Ultrasonicator Assisted Extraction (UAE)

UAE was carried out by using Athena Multifunctional Ultrasonicator Cleaner bath model (ATS-10L), 40KHz frequency, Ultrasonic power of 240W and heating power of 500W applying direct sonication method as described in the protocol in Liu et al., 2013 [13] and was standardized as per the needs. Phytochemicals were extracted by loading 300 g of *S. glauca* shade dried bark powder with 2.1 L of methanol (1:7 w/w) in a 5L autoclavable bottle stoppered with an air condenser. The mixture was kept 75 min for pre-extraction maceration and then subjected to UAE at 600 C temperature for 75 min with 100% sonication power. Extract was cooled to room temperature followed by 75 min of post UAE maceration, vacuum filtered twice using Whatman (No. 1) filter paper (Whatman PLC, Maidstone, UK) and concentrated in a Rotary flash evaporator (SuperFit - 2.0) further stirring on a magnetic stirrer and dried to pallets. Total yield in percentage was calculated by using the formula,

$$\% \text{ Yield} = \frac{\text{Weight of the fraction}}{\text{Weight of the plant material}} \times 100$$

Fractionation

Ethyl acetate fraction of *S. glauca* methanolic bark extract was taken out by solvent separation technique. 50 g of *S. glauca* UAE methanolic bark extract was loaded in a 2000 ml separatory funnel (SLW180/12M, Pyrex®) and subjected to the fractionation using 300 ml (1:7) of each solvent-Pet. Ether, Butanol, Ethyl acetate, and water in their increasing polarity. Ethyl acetate fraction was selected. Excess of solvent was distilled off in rotary evaporator, the concentrate was stirred on a magnetic stirrer to evaporate to a syrupy consistency and then evaporated to dryness. Total yield of the fraction was calculated using the formula,

$$\% \text{ Yield} = \frac{\text{Weight of the fraction}}{\text{Weight of the crude extract}} \times 100$$

Phytochemical analysis

The SG-fraction was qualitatively tested for different phytochemical constituents such as alkaloids, flavonoids, glycosides, anthraquinones, triterpenoids, tannins, and phenols, phytosterols, saponins, tannins, resins, carbohydrates, starch, volatile oil by following the standard procedure described in Deepti et al., 2012 [14].

Determination of total phenolic contents (TPC)

TPC of SG-fraction was estimated using the Folin-Ciocalteu method [15]. Briefly, five different concentrations of bark fraction were prepared and reacted with 0.4 ml Folin-Ciocalteu reagent and incubated for 90 min at room

temperature (BIOBASE, BJPX-H54BK). Methanol is used as blank and Gallic acid as standard. 7% sodium carbonate solution (4ml) was added and absorbance was measured at 765 nm (Visiscan 167, Systronics). Calibration curve was plotted and results were expressed as Gallic acid equivalent (mg GAE / 100 ml).

Radical-scavenging activity (DPPH assay)

Antioxidant activity was determined by DPPH method [15]. Five different concentrations 10, 20, 40, 100, and 200 µg/ml containing respective ppm volume of fraction were treated against 1ml of 0.002% DPPH and incubated for 30 min at room temperature in dark. Methanol was used as blank and Ascorbic acid as standard. Absorbance was measured at 517 nm for the discolouration of DPPH. Results were expressed as (%) DPPH scavenging activity, calculated using the formula,

DPPH scavenging activity (%)=[(OD of Blank─OD of Sample)/OD of Blank]×100

IC_{50} value was calculated by plotting the linear regression graph and using regression equation.

Cytotoxicity assay

MDA-MB-231 cells treated with SG-fraction and commonly used chemotherapeutic drug Camptothecin (#C9911, Sigma-Aldrich) as standard, incubated for 24 hrs. Medium was removed, 0.5mg/ml MTT reagent (#4060, HIMEDIA) was added. MTT formazan crystals were dissolved in DMSO and absorbance was taken at 570 nm [16].

% Cell viability was calculated using the formula:

% cell viability = [Mean abs of treated cells/Mean abs of Untreated cells] x 100

The IC_{50} value was determined by using linear regression equation i.e., Y=Mx+C. Where, Y=50, M and C values were derived from the viability graph.

Mitochondrial membrane potential (ΔΨm)

MDA-MB-231 cells treated with IC_{50} concentrations of SG-fraction and Camptothecin. After washing twice with PBS, cells were re-suspended in 0.5 ml JC–1 working solution and incubated for 15–20 min at 37° C in CO_2 incubator. Cells were washed in 1x Assay buffer and analysed in flow cytometer (BD FACSCalibur™, BD Biosciences, USA) in FL1 and FL2 channels using BD Cell Quest Pro-version 6.0 software [17].

DAPI nuclear staining

Cells were treated with IC_{50} concentrations of SG-fraction and Camptothecin. Spent medium was removed and washed with PBS and fixed. Rewashed with PBS and stained with 200µl of DAPI Staining solution (#TCL087, HIMEDIA) for 10 min in dark. Cells were observed under Confocal microscope (Carl Zeiss LSM 880 Fluorescence live cell imaging system, Germany) with filter cube Excitation 358 nm and Emission 461 nm for DAPI. Images were recorded and analysed using ZEN Blue Software [18].

Annexin V-FITC/PI double staining analysis

$0.5x10^6$/2 ml MDA-MB-231 cells cultured and treated with IC_{50} concentrations of SG-fraction and Camptothecin and incubated for 24 hrs. Medium was removed and PBS washed cells were treated with 400µl of trypsin-EDTA (#TCL099, HIMEDIA) solution and incubate at 37°C for 3-4 minutes. 2 ml of culture medium was added and cells were harvested directly into 12x75 mm polystyrene tubes. Cells were centrifuged for 5 minutes at 300 x g at 25°C and washed twice in PBS. 5µl of FITC Annexin V (#51-65874X, BD Biosciences) was added and gently vortexed and incubated for 15 min at 25°C in the dark. 5µl of PI (# 51-66211E, BD Biosciences) and 400µl of 1X binding buffer were added to each tube and gently vortexed. Cells were analysed using FACS method [19].

Statistical data analysis

Results were expressed in terms of mean ± SEM. Differences between the groups were measured by applying student's t – test. The correlation coefficient of the obtained data was analysed by the regression analysis and Pearson's Correlation test using jamovi Desktop, Current version 2.4.5 (jamovi project, Sydney, Australia).

Results

Total yield of crude extract

UAE of *S. glauca* bark in methanol yielded 53g of crude extract for 300g of dry bark powder with a percentage yield of 17.6% w/w. Fractionation yielded 16g of Ethyl acetate fraction with a percentage yield of 32% w/w.

Preliminary Phytochemical Screening

The qualitative phytochemical analysis revealed the rich presence of alkaloids, flavonoids, phenols, tannins, triterpenoids, steroids in SG-fraction. Other metabolites like carbohydrates, proteins, glycosides, resins, saponins, starch were not detected in the fraction (Table 1).

Total Phenolic Content

TPC analysis of SG-fraction yielded the highest phenolic content 278.65±0.059 mg GAE/100 ml at 200 µg/ml concentration (Figure 1).

DPPH radical scavenging activity

Highest radical scavenging activity in terms of percentage inhibition by the SG-fraction was 66.38±0.532% at 200 µg/ml concentration, with an IC_{50} value of 36.94 µg/ml (Table 2).

Cell viability assay

The highest anticancer potentiality of SG-fraction was observed against MDA-MB-231 expressed in terms of cell viability, showing significantly low cell viability of 7.19% at 200 µg/ml concentration (Figure 2) with an IC_{50} value 101.72 µg/ml ($p \leq 0.05$). Camptothecin showed 25.64% at 200 µg/ml concentration with an IC_{50} value 101.72 µg/ml (Table 3).

Mitochondrial membrane potential (ΔΨm)

The reduction in mitochondrial membrane potential signifies the onset of apoptosis. Decrease in mitochondrial

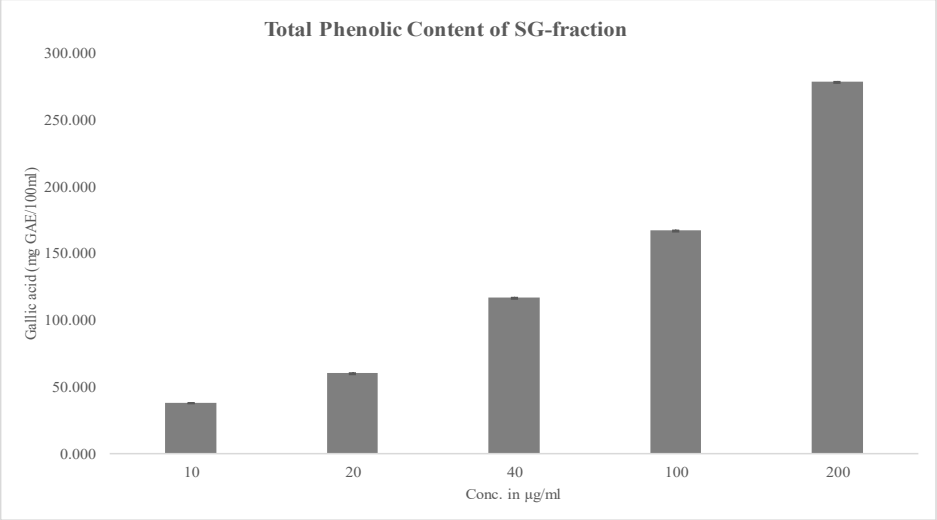

Figure 1. Total Phenolic Content of SG-Fraction.

membrane potential was observed in the MDA-MB-231 cells after treatment with SG-fraction (Figure 3). Intact mitochondria of the control group were highly polarized, reflecting electron transport and oxidative phosphorylation. However, numerous weakly polarized mitochondria are seen with in both test and positive control groups, showing a decrease in mitochondrial activity (Figure 4).

DAPI nuclear staining

Considerable number of MDA-MB-231 cells with apoptotic morphological changes such as nuclear blebbing, condensation, nuclear fragmentation due to the action of SG-fraction and Camptothecin were observed (Figure 5). Whereas, the untreated group showed negligible number of damaged cells.

Induction of apoptosis in MDA-MB-231 cells

Annexin-V/FITC and propidium iodide (PI) double labelled cells were analysed by FACS to identify and quantify the drug induced apoptosis in MDA-MB-231 cells. Results showed that the SG-fraction effectively induced apoptosis in MDA-MB-231 cells. Exposure to the IC_{50} concentrations of SG-fraction and Camptothecin for 24 hrs increased the proportions of apoptotic cells from 0.02% (untreated) to 59.48% and 40.4% respectively, thus, signifying the ability of SG-fraction to induce the apoptosis in Triple Negative Breast Cancer cells (Figure 6, 7).

Statistical analysis

Paired Samples Student T-Test for DPPH radical scavenging ability of both SG-fraction and Camptothecin showed highest significance of $p<0.01$ and for MTT analysis results showed a significance of $p\leq0.05$. The Pearson's correlation analysis showed strong positive correlation of $r = 0.098^{**}$ between DPPH and TPC with a significance of $p=0.002$ (Table 4) and a stronger negative correlation of $r = -0.097^{***}$ between DPPH, TPC vs. MTT cell viability with a significance of $p<0.001$ (Figure 8,

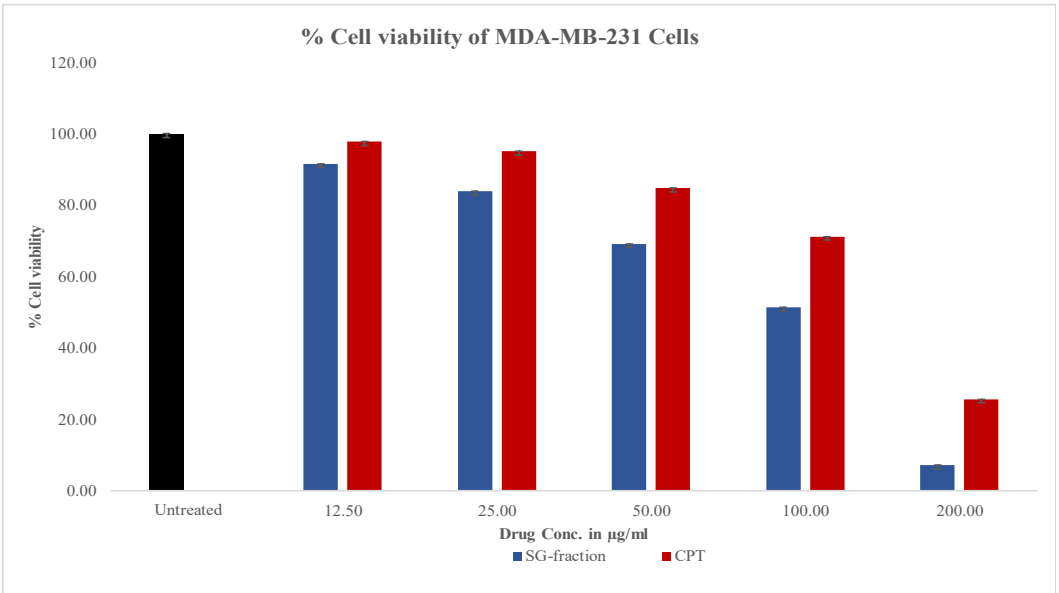

Figure 2. % Cell Viability Values of SG-Fraction against MDA MB 231 Cells after the Incubation Period of 24hrs.

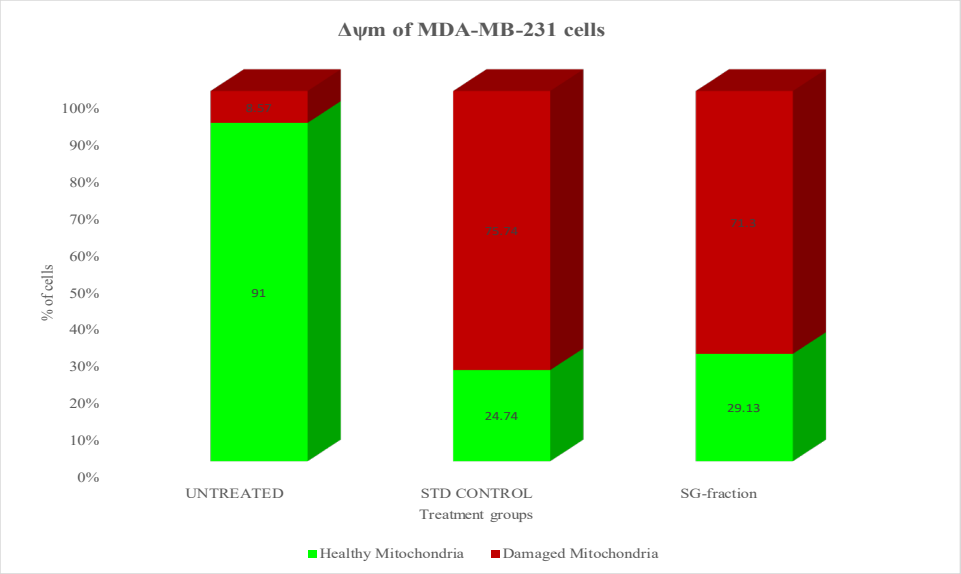

Figure 3. Overlaid Bar Graph Showing the Percentage of Cells with Healthy and Damaged Mitochondria in Different Treatment Groups.

Table 4).

Discussion

The present study reported the anticancer activity of *S. glauca* bark on MDA-MB-231 cells. UAE method provides controlled temperature and energy conditions for the exhaustive and safe extraction of secondary metabolites, preserving phytochemicals from chemical and thermal degradation [20]. The UAE yielded 53 g, 17.6% w/w of crude extract in methanol for 300 g bark powder. With an optimum boiling point (77.10 C) and being moderately polar with polar carbonyl (C=O) group and oxygen atom, Ethyl acetate accumulates prominent phytochemicals like alkaloids, flavonoids, terpenoids and steroids responsible for anti-oxidant and anti-cancer activities [21]. Fractionation of methanolic *S. glauca* UAE bark extract yielded 16 g of semi solid Ethyl acetate fraction.

SG-fraction found to be rich in prominent secondary metabolites such as, alkaloids, polyphenols, flavonoids, steroids, saponins, anthraquinones, lignins, tannins, and glycosides (Table 1). These are responsible for potential antioxidant and anticancer activity due to which they have been employed in treating different types of cancers, inflammations, metabolic diseases

The total phenolic content of SG-fraction was determined as 278.65±0.059 µg GAE/100 ml by Folin-Ciocalteu (Figure 1) method. The phenolic content is prominently responsible for the antioxidant and anti-cancer effects of SG-fraction. These phytoconstituents are redox agents to scavenge reactive oxygen species that act as free radicals by their free hydroxyl ends. Thus, their concentration in the SG-fraction serves as the base for antioxidant properties. The percentage of free radical scavenging activity of the SG-fraction was 66.38±0.532% at 200 µg /ml concentration showing a similar trend of antioxidant effect compared to ascorbic acid (Table 2).

Table 1. Preliminary Phytochemical Screening of SG-Fraction.

Constituent	Test	Observation
Alkaloids	Dragandroff	+
	Mayer	+
	Hager	+
	Wagner	+
	Tannic acid	+
Flavonoids	Shinoda	+
	Alkaline Reagent	+
Glycosides	Keller-Killani	-
Anthraquinones	Borntrager	-
Triterpenoids	Salkowski's	+
Tannins and Phenolics	Ferric chloride	+
	Lead acetate	+
Phytosterols	Salkowski	+
	Liebermann-Burchard	+
Resins		-
Saponins		-
Carbohydrates	Molish	-
	Fehling	-
Starch		
Volatile oil		-

Table 2. DPPH Radical Scavenging Ability of SG-Fraction.

Concentration (µg/ml)	Percentage inhibition (%)	
	Ascorbic acid	SG-fraction
10	78.248	49.97
20	79.972	51.76
40	82.457	53.26
100	88.756	57.56
200	88.934	66.38

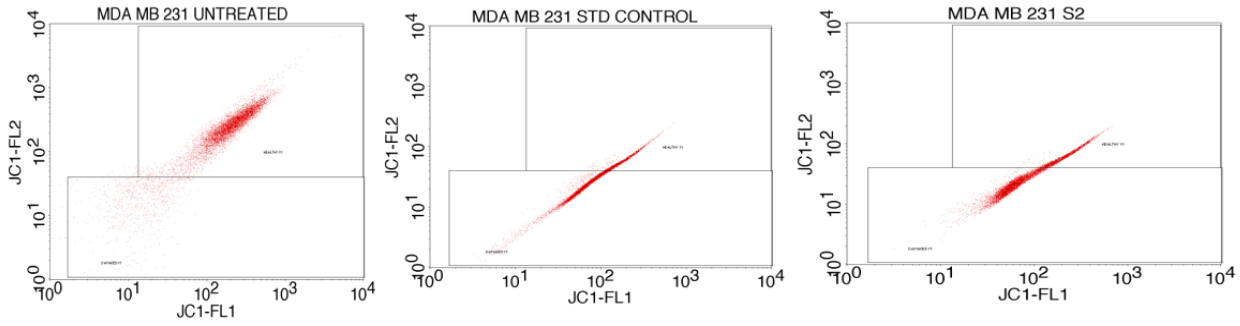

Figure 4. JC-1 MMP Study of Given Test Compounds with IC_{50} Concentration against the MDA MB 231 Cells along with Controls. Quadrants showing the expression of JC-1 stain in untreated cells (A), Camptothecin (B) and SG-fraction treated MDA MB 231 cells against the FL1-JC1 and FL2-JC1 channels by flow cytometry.

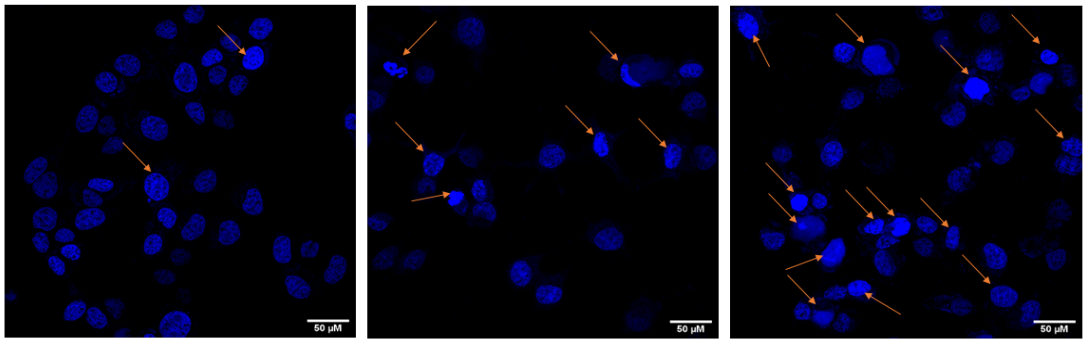

Figure 5. Fluorescent Microscope Images Depicting the Nuclear Damage of DAPI Stained MDA MB 231 Cells at the Magnification of 40X. Cell nuclei are stained in blue. Yellow arrows represent condensed and damaged DNA of cells.

Anticancer activity is the prominent and common ethno-medicinal claim by the traditional medicine practitioners in case of *S. glauca*. The consortium of diversified phytochemicals like bring higher levels of cytotoxicity in *S. glauca*. For 24 hrs exposure to the SG-fraction and standard there was no cell proliferation observed both in treatment and control groups. The cells of negative control group were 100% viable (Figure 2), while cells of both positive control and test groups recorded a

Table 3. IC_{50} Values of Test and Standard Compounds in µg/ml.

Cells	SG-fraction	Camptothecin
MDA-MB-231	101.72s	142.67

Table 4. Pearson's Correlation Analysis of TPC, DPPH, and MTT Results

		Correlation Matrix				
		TPC		DPPH		MTT
TPC	Pearson's r	—				
	df	—				
	p-value	—				
	N	—				
DPPH	Pearson's r	0.988	**	—		
	df	3		—		
	p-value	0.002		—		
	N	5		—		
MTT	Pearson's r	-0.997	***	-0.997	***	—
	df	3		3		—
	p-value	<.001		<.001		—
	N	5		5		—

Note. * p < 0.05, ** p <0 .01, *** p < 0.001

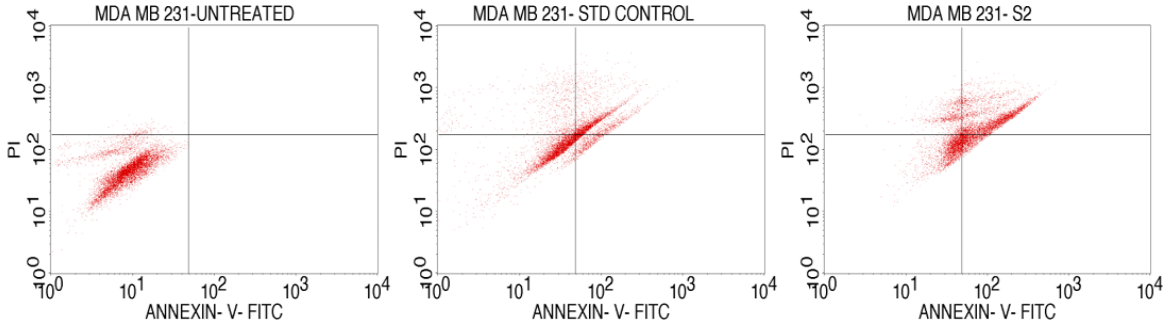

Figure 6. Quadrangular Plots Representing the Annexin V/PI Expression in MDA MB 231 Cells upon Culturing in the Presence and Absence of SG-fraction with IC_{50} Concentration along with Controls.

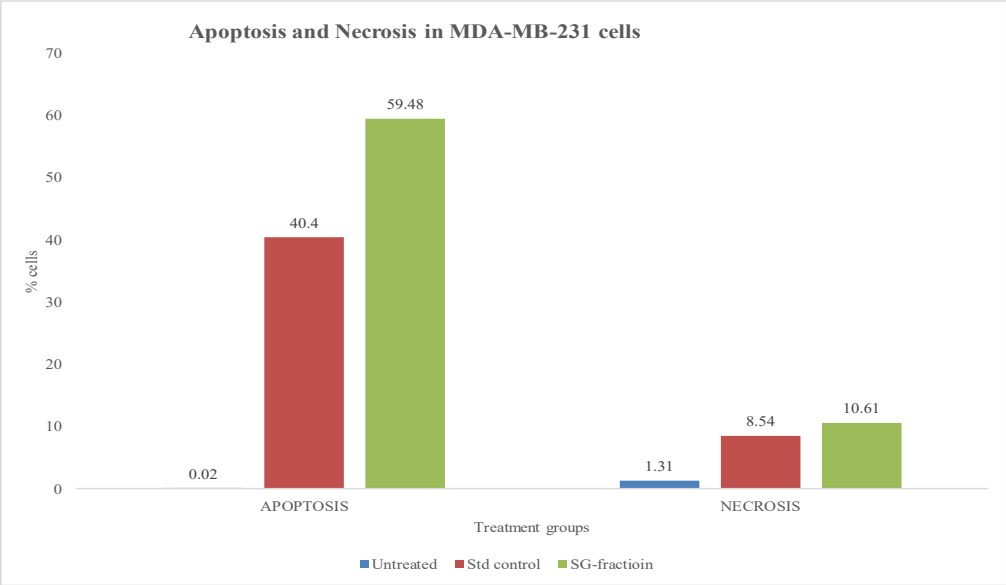

Figure 7. Bar Graph Showing Percentage of Apoptotic and Necrotic Cells.

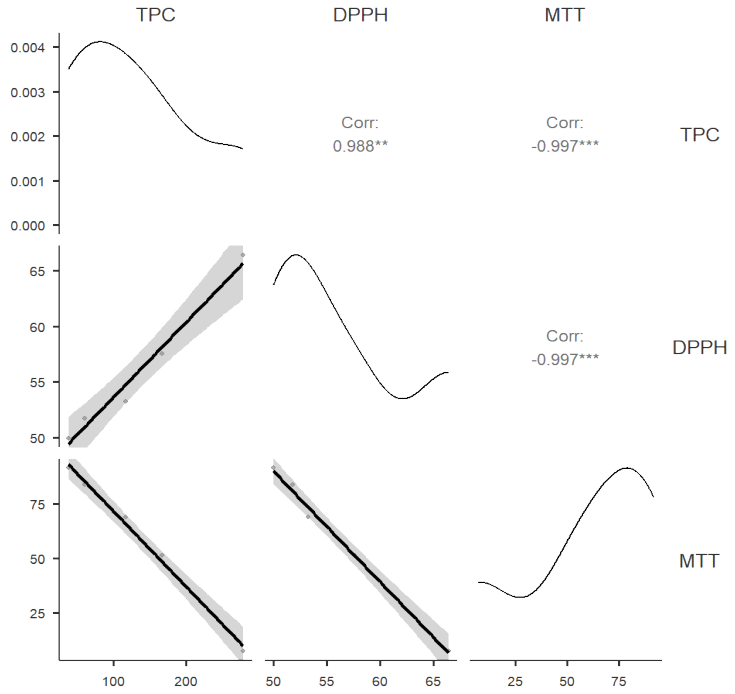

Figure 8. Pearson's Correlation Plot for TPC, DPPH, and MTT Assays, Showing Positive Correlation between TPC/DPPH, and Strong Negative Correlation between TPC/DPPH/MTT.

decline in the cell viability, where the cell viability stood inversely proportional to the concertation of the drug (Table 3). Student's T – Test (Paired Samples) indicated that the effects shown at respective concentrations were significant at $p < 0.05$. The IC_{50} value of MDA-MB-231 cell viability for the effect of SG-fraction (101.72 µg/ml) was significant than the IC_{50} value of Camptothecin (142.67 µg/ml) at $p < 0.05$, indicting the higher efficacy of SG-fraction over the standard drug. This might be due to the synergistic action of phyto-constituents in the fraction [22].

After treatment with SG-fraction, cells showed morphological changes like membrane blabbing and shrinkage. To understand the level and mechanism of cytotoxicity, DAPI nuclear staining and JC-1 dye based quantitative evaluation of mitochondrial membrane potential (ΔΨm) were performed. To corroborate the induction of apoptosis by the SG-fraction in triple negative breast cancer MDA-MB-231 cells, FACS based Annexin-V/FITC-PI double labelled study was performed.

ΔΨm is the summation of the effect of electron transport by the Electron Transport Chain (ETC) proteins across the inner membrane of the mitochondria. This is the driving force in bringing oxidative phosphorylation and ATP production. Thus, it takes part in bioenergetic stress signalling resulting in the release of apoptotic factors leading to cell death. Hence, the study of mitochondrial membrane potential (ΔΨm) is an important parameter in the determination of drug induced apoptosis in cancer cells [23]. The effect of SG-fraction on the ΔΨm of MDA-MB-231 cells using JC-1 (5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazol - carbocyanineiodide) dye was performed by FACS. In the living cells JC-1 formed aggregates emitting red fluorescence, and in dead cells, due to lower membrane potential, the dye remained as monomer emitting green fluorescence. Both JC-1 aggregates and monomers emitted fluorescence in the green region of spectrum analysed in the Green (FL-1) channel and only JC-1 aggregates showed a red spectral shift bringing higher levels of red fluorescence analysed in the red (FL-2) channel [17]. The SG-fraction damaged 71.3% of mitochondria, observed as depolarized ΔΨm, whereas, Camptothecin caused 75.74% damaged mitochondria in the cells among treatment group. Among test, positive and negative control groups 29.13%, 24.74%, and a highest 91% of healthy mitochondria respectively were observed in the cells exhibiting high polarized mitochondria (Figure 3). Upper Quadrant (UQ) represents living cells with healthy mitochondria stained with JC 1 stain (FL2). 91%, 24.74%, 29.13% of cells were found in Untreated and Treated with Standard drug and test compounds respectively. Lower Quadrant (LQ) represents the cells with depolarized ΔΨm by staining with JC1 Stain (FL1). 8.57%, 75.74%, 71.30% of cells were found in Untreated, Treated with positive control and Test compounds respectively (Figure 4). Our results showed that SG-fraction caused similar trend in ΔΨm damage to that of Camptothecin indicating the effective induction of apoptosis.

Cells were stained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) nuclear stain to analyse the apoptotic morphology due to the cytotoxic effects of SG-fraction at IC_{50} concentrations (101.72 µg/ml),. The stain binds to A-T rich regions of nucleic acids along the minor groove. DAPI stain being impermeant to the live cells, differentiates the intact cells from membrane-compromised cells. The fluorescence of the DNA bound-DAPI stain was increased approximately 20 folds than non-DNA bound DAPI. ds-DNA bound DAPI has an excitation wavelength maximum of 358 nm and emission maximum of 461 nm. Compared to the untreated cells, nuclear blebbing, condensation, and DNA damages were significantly observed in the cells of test group similar to positive control group. A very negligible number of cells with DNA damage were seen in the negative control group (Figure 5). This provides visual evidence for the initiation of apoptosis in test and positive control groups [24].

Apoptosis induction is an advantageous strategy in chemotherapy drug discovery. Plant derived phytochemicals bring cell death by inducing apoptosis in cancer cells. In this study Annexin-V/FITC-PI double labelled flow cytometry was employed to quantify the apoptosis in MDA-MB-231 cells. Annexin-V/FITC is used to quantitatively determine the percentage of cells actively undergoing apoptosis. It is dependent on the property of cells to lose membrane asymmetry in early apoptosis. During apoptosis, the membrane phospholipid phosphatidylserine (PS) is shifted from inner leaflet to the outer leaflet, exposing PS to extracellular environment. Having high affinity towards PS, Annexin-V protein binds to this exposed PS detecting apoptotic cells. Propidium Iodide (PI) is a live/dead cell detection probe, only dead and membrane damaged cells are permeable to PI. It is important to note that the cells that stain positive for FITC Annexin-V and negative for PI are undergoing apoptosis. Cells that stain positive for both FITC Annexin-V and PI are either in the end stage of apoptosis, or undergoing necrosis, or already dead. Cells that stain negative for both FITC Annexin-V and PI are alive and not undergoing measurable apoptosis [25] (Figure 6). SG-fraction at its IC_{50} concentration induced the significant apoptosis in triple negative breast cancer cells (MDA-MB-231). Among the treatment groups, cells of negative control, positive control, and test groups showed apoptosis with 0.02%, 40.4%, and 59.48% respectively and percentage of necrosis as 1.31%, 8.54%, 10.61% respectively (Figure 7).

As a next step, student's T-Test was applied to compare the effect and to identify the difference between groups at respective concentrations. SG-fraction showed quite good and similar trend of antioxidant activity whereas the antioxidant activity of Camptothecin increased with an increase in drug concentration, showing higher results than the fraction with significance of $p<0.01$. SG-fraction was found to be more significantly cytotoxic than Camptothecin with $p\leq0.05$, by leaving very less percentage of viable cells [26]. The enhancement in the antioxidant and anticancer activity is directly proportional to the quantity and accumulation of total phenolic compounds in the fraction. To test this, the results of TPC, DPPH, and MTT cell viability assays were correlated by using Pearson's correlation analysis. It showed a strong

positive correlation of r = 0.0988** between TPC and DPPH with p<0.05 significance indicating that TPC is responsible for the higher antioxidant activity. Also, a stronger negative correlation of r = -0.097** between the results of TPC, DPPH, and MTT cytotoxicity assays with a significance of p<0.01 was found (Figure 8, Table 4), suggesting that the quantity of TPC and their antioxidant activity are inversely proportional to the percentage of cell viability exhibited. It also shows that the higher anticancer activity of SG-fraction was due to the synergistic effect of different phenolic compounds [27]. Thus, the overall results of this investigation revealed the antioxidant and anticancer potentiality of *Simarouba glauca* bark and can be considered for the search for new, potential, less toxic drugs for the development of anti-breast cancer. In conclusion, the present study provides strong evidence for the therapeutic potential of *S. glauca* bark against triple negative breast cancer cells.

Author Contribution Statement

The first author RMRP designed the study, performed the experiments, managed the analyses of the study, and wrote the first draft of the manuscript. The second author TCT, guided and approved the study, corrected, finalized, and communicated the manuscript. Both authors have read and approved the final manuscript.

Acknowledgements

The authors express their sincere gratitude to - the Chairman, P. G. Department of Studies in Botany, Karnatak University, Dharwad, Karnataka, India, for the laboratory facility; to the SAIF-USIC and DST-PURSE Program Phase-II, Karnatak University, Dharwad, for providing the required instrumentation facility; to Dr. K. Kotresha, Professor and Taxonomist, Taxonomy and Floristic Laboratory, Department of UG, PG, and Research in Botany, Karnatak University's Karnataka Science College, Dharwad, Karnataka, India, for plant identification and authentication. One of the authors (RP) is thankful to Karnatak University, Dharwad, for providing financial support as University Research Studentship (KUD/SCH/URS/2019/365).

References

1. Poorkiani M, Hazrati M, Abbaszadeh A, Jafari P, Sadeghi M, Dejbakhsh T, et al. Does a rehabilitation program improve quality of life in breast cancer patients? Payesh (Health Monitor). 2010;9(1):61-8.
2. Ataollahi MR, Sharifi J, Paknahad MR, Paknahad A. Breast cancer and associated factors: A review. J Med Life. 2015;8(Spec Iss 4):6-11.
3. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin. 2009;59(4):225-49. https://doi.org/10.3322/caac.20006.
4. Aysola K, Desai A, Welch C, Xu J, Qin Y, Reddy V, et al. Triple negative breast cancer - an overview. Hereditary Genet. 2013;2013(Suppl 2). https://doi.org/10.4172/2161-1041.S2-001.
5. Kulkarni SS, Kulkarni BB, Vandana T, Kulkarni SS, Shepur VB, Kulkarni ML, et al. A comparative study of cdna microarray analysis of familial and sporadic breast cancer in india. J Med Genet Genomics. 2012;4(1):6-12.
6. Vasanthi VJ, Radhjeyalakshmi R, Nasrin F. Evaluation of anticancer activity using hexanic extract of vitex trifolia on two different cancer cell lines. Int J Pharmacognosy Phytochem Res. 2014;6(3):449-53.
7. Gerson-Cwilich R, Serrano-Olvera A, Villalobos-Prieto A. Complementary and alternative medicine (cam) in mexican patients with cancer. Clin Transl Oncol. 2006;8(3):200-7. https://doi.org/10.1007/s12094-006-0011-2.
8. Gordaliza M. Natural products as leads to anticancer drugs. Clin Transl Oncol. 2007;9(12):767-76. https://doi.org/10.1007/s12094-007-0138-9.
9. Armour RP. Investigations on *simarouba glauca* dc. In el salvador. Econ Bot. 1959;13(1):41-66.
10. Rivero-Cruz JF, Lezutekong R, Lobo-Echeverri T, Ito A, Mi Q, Chai HB, et al. Cytotoxic constituents of the twigs of *simarouba glauca* collected from a plot in southern florida. Phytother Res. 2005;19(2):136-40. https://doi.org/10.1002/ptr.1642.
11. Manasi PS, Gaikwad DK. A critical review on medicinally important oil yielding plant laxmitaru (*simarouba glauca* dc.). J Pharm Sci Res. 2011;3(4):1195.
12. Wlodkowic D, Skommer J, Darzynkiewicz Z. Flow cytometry-based apoptosis detection. Methods Mol Biol. 2009;559:19-32. https://doi.org/10.1007/978-1-60327-017-5_2.
13. Liu B, Ma Y, Liu Y, Yang Z, Zhang L. Ultrasonic-assisted extraction and antioxidant activity of flavonoids from adinandra nitida leaves. Trop J Pharm Res. 2013;12(6):1045-51.
14. Deepti K, Umadevi P, Vijayalakshmi G. Antimicrobial activity and phytochemical analysis of morinda tinctoria roxb. Leaf extracts. Asian Pac J Trop Biomed. 2012;2(3):S1440-S2.
15. Puranik SI, Ghagane SC, Nerli RB, Jalalpure SS, Hiremath MB. Evaluation of in vitro antioxidant and anticancer activity of *simarouba glauca* leaf extracts on t-24 bladder cancer cell line. Pharmacogn J. 2017;9(6).
16. Chua LS, Lau CH, Chew CY, Dawood DAS. Solvent fractionation and acetone precipitation for crude saponins from eurycoma longifolia extract. Molecules. 2019;24(7):1416. https://doi.org/10.3390/molecules24071416.
17. Sivandzade F, Bhalerao A, Cucullo L. Analysis of the mitochondrial membrane potential using the cationic jc-1 dye as a sensitive fluorescent probe. Bio Protoc. 2019;9(1):e3128-e3128. https://doi.org/10.21769/BioProtoc.3128.
18. Pradhan B, Patra S, Behera C, Nayak R, Patil S, Bhutia SK, et al. Enteromorpha compressa extract induces anticancer activity through apoptosis and autophagy in oral cancer. Mol Biol Rep. 2020;47(12):9567-78. https://doi.org/10.1007/s11033-020-06010-4.
19. Koduru S, Grierson DS, Van de Venter M, Afolayan AJ. Anticancer activity of steroid alkaloids isolated from solanum aculeastrum. Pharmaceutical Biology. 2007;45(8):613-8.
20. Kumar K, Srivastav S, Sharanagat VS. Ultrasound assisted extraction (uae) of bioactive compounds from fruit and vegetable processing by-products: A review. Ultrason Sonochem. 2021;70:105325. https://doi.org/10.1016/j.ultsonch.2020.105325.
21. Babbar N, Oberoi HS, Sandhu SK, Bhargav VK. Influence of different solvents in extraction of phenolic compounds from vegetable residues and their evaluation as natural sources of antioxidants. J Food Sci Technol. 2014;51(10):2568-75. https://doi.org/10.1007/s13197-012-0754-4.
22. Prajapati C, Reddy M, Bhatt M. Evaluation of anticancer

activity using leaf extract of *simarouba glauca* on leukemic cancer cell lines. Evaluation. 2018;3(2):52-6.
23. Zorova LD, Popkov VA, Plotnikov EJ, Silachev DN, Pevzner IB, Jankauskas SS. Functional significance of the mitochondrial membrane potential. Biochem (Mosc) Suppl A: Membr Cell Biol. 2018;12:20-6.
24. Peram MR, Jalalpure S, Kumbar V, Patil S, Joshi S, Bhat K, et al. Factorial design based curcumin ethosomal nanocarriers for the skin cancer delivery: In vitro evaluation. J Liposome Res. 2019;29(3):291-311. https://doi.org/10.1080/08982104.2018.1556292.
25. Eray M, Mättö M, Kaartinen M, Andersson L, Pelkonen J. Flow cytometric analysis of apoptotic subpopulations with a combination of annexin v-fitc, propidium iodide, and syto 17. Cytometry. 2001;43(2):134-42.https://doi.org/10.1002/1097-0320(20010201)43:2<134::aid-cyto1028>3.0.co;2-l.
26. Soltanian S, Sheikhbahaei M, Mirtadzadini M, Kalantari Khandani B. Evaluation of anticancer, antioxidant and antibacterial properties of methanol extract of three acantholimon boiss. Species. Avicenna J Phytomed. 2020;10(6):641-52.
27. Eddebbagh M, Messaoudi M, Abourriche A, Berrada M, Attaleb M, Benbacer L, et al. Correlation of the cytotoxic and antioxidant activities of moroccan pomegranate (punica granatum) with phenolic and flavonoid contents. J Pharm Pharmacol. 2016;4(9):511-9.