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Research article

Anti-proliferative potential of phytochemical fractions isolated from *Simarouba glauca* DC leaf

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ABSTRACT

Emerging advancements in anticancer drug discovery research are leaning towards the plant-based bioactive fractions, which is a cocktail of naturally abundant two or more substances with unique proportions, exhibiting greater potential to combat cancers than the individual molecules. Thus, isolation and characterization of anti-cancer activity enriched fractions from plants is gaining scientific attention. Consistent with this view, one of the evidence-based traditional medicinal plants, well known for its anti-cancer potential, *Simarouba glauca* (SG) leaf has been scientifically examined to identify and isolate the potent anti-cancer fraction. The dried SG leaves were extracted successively with the solvents of increasing polarity. The phytochemical characterization of obtained extracts and fractions were carried out to determine the phenolic acid composition. All fractions were individually examined for anti-cancer property in cancer cells representing lungs, cervix, breast, colon and rectum *in vitro*. Among all fractions tested, the chloroform (SGC) and ethyl acetate (SGEA) extracts showed potent anti-proliferative effects by triggering apoptosis. In summary, our findings demonstrate that the extracts SGC and SGEA have potent anti-cancer activities compared to other fractions of SG leaf and thus warrant further pre-clinical studies to establish scientific basis for the anticancer potentials of SG.

1. Introduction

Cancer is one of the most leading causes of death globally (Bray et al., 2018; Forouzanfar et al., 2016). According to recent estimates, more than 1.7 million new cancer cases were diagnosed in the year 2019, and of note, the cancer deaths are predicted to increase to 13 million by 2030 (Ferlay et al., 2019). This unprecedented increase in the incidence and mortality rates are due to lack of effective preventive strategies and poor therapeutics to combat advanced metastatic phenotypes (Delaney et al., 2005; Kularatne et al., 2019). Moreover, emergence of drug resistant phenotypes further exacerbates the cancer burden globally. Hence, recent strategies emphasize the focus on developing effective naturally occurring therapeutics. One such strategy is to prepare anti-cancer substances rich fractions from plants. Studies from recent publications demonstrated the efficacy of plant extracts for inhibiting carcinomas of breast and prostate (Kooti et al., 2017; Singh et al., 2016). Such success models prompted us to prepare anti-cancer molecules enriched fractions from traditional medicinal plants, which has well established ethno-medicine basis for their antitumor effects.

Simarouba glauca (*SG*), generally known as 'Laxmitaru' or 'Paradise Tree' belongs to the family Simaroubaceae. Since ancient times, SG has been well-known for its traditional use in treating cancer, malaria, dysentery, blood and gastric disorders and infectious diseases, especially in Southern Florida, West Indies and Brazil (Manasi and Gaikwad, 2011). The major phyto-constituent of SG was reported to be quassinoids, which is a group of triterpene lactones, including glaucarubin, glaucarubolone, and glaucarubinone (Cronquist, 1944; Almeida et al., 2007; Saraiva et al., 2006; Farnsworth et al., 1985; Govindaraju et al., 2009; Ham et al., 1954; Kupchan et al., 1976).

Among the various quassinoids, glaucarubin, a constituent of SG seeds was studied extensively (Ham et al., 1954). Reynertson et al. (2011) have isolated few compounds from the wood extract of SG and identified them as scopoletin and canthin-6-one and its dimethoxy derivative. It is also evident that SG contains canthin-6-one and its derivatives, limonoid, melianodiol, 14-deacetyleurylene (triterpenoid),

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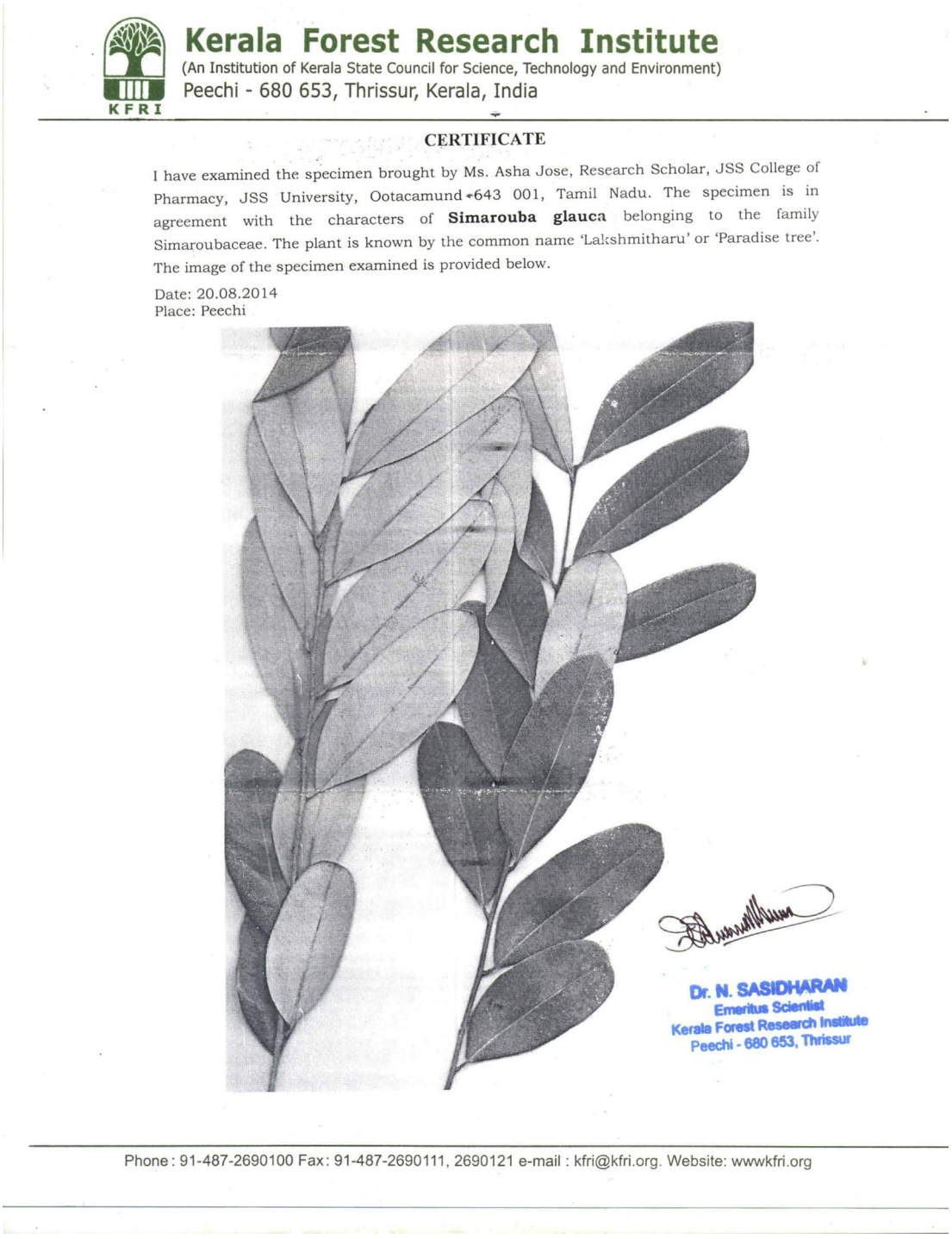


Figure 1. Plant authentification certificate. The picture representing the herbarium specimen of *Simarouba glauca*.

scopoletin and fraxidin (coumarin), triolein and trilinolein (triglycerides) (Jose et al., 2019; Rivero-Cruz et al., 2005). Recently, our group has isolated tricaproin from the leaf of SG and reported that tricaproin elicits potent cytotoxic effects in colon and rectum derived cancer cells *in vitro*. Further, the inhibition of histone deacetylases was reported to be the mechanism by which tricaproin elicits its cytotoxic effects (Jose et al., 2018).

Conversely, based on the traditional claims, it is likely that the phytoconstituents of SG are effective if given as extracts or fractions containing multiple compounds with varying proportions and perhaps target multiple pathways to combat cancer synergistically. Thus our study intended to prepare the extracts of leaf of SG with solvents of increasing polarity, characterize phytochemical properties of obtained fractions and examine cytotoxic activity using selective cancer cells *in vitro*.

2. Materials and methods

2.1. Materials

The cancer cell lines HCT-116 and HCT-15 (colorectal carcinoma cells), MDA-MB-231 (mammary/breast adenocarcinoma cells), SiHa (cervical carcinoma cells), A549 (lung carcinoma cells) were purchased

Table 1. Phytochemicals identified in the crude extracts of *Simarouba glauca* leaves.

Name of the Phytochemical	SGH	SGC	SGEA	SGE	SGW
Alkaloids	-	+	+	+	+
Carbohydrates	-	-	+	+	-
Proteins	-	+	+	+	-
Glycosides	-	-	-	+	-
Phenols	-	+	+	+	+
Flavanoids	-	+	+	+	+
Tannins	-	-	+	+	+
Steroids and Terpenoids	+	+	+	-	-
Oils and resins	+	+	-	-	-

Qualitative analysis of SG extracts for various phytochemicals was carried out as detailed in materials and methods, and the data represented as presence (+) or absence (-) in the table. SGEA and SGE showed the presence of all the phytochemicals except glycosides, oils and resins in SGEA and steroids and terpenoids, oils and resins in SGE. As predicted the hexane extract SGH had steroids, terpenoids, oils and resins. Interestingly the water extract showed the presence of alkaloids, phenols, flavonoids, and tannins. No carbohydrates and proteins were present in water extract.

Table 2. Total phenols and flavonoid content in the extracts of SG leaves.

Phyto-constituent (g/100g)	SGH	SGC	SGEA	SGE	SGW
Phenols	0.07 ± 0.01	0.21 ± 0.02	0.65 ± 0.03	1.65 ± 0.09	0.59 ± 0.04
Flavonoids	0.06 ± 0.01	0.30 ± 0.01	0.28 ± 0.01	0.48 ± 0.04	0.43 ± 0.02

Total phenol and flavonoid content was estimated using F–C and Aluminium chloride method as detailed in methods section. SGE and SGEA extracts had high total phenol content compared to other extracts. Flavonoid content was high in SGE and SGW.

from National Centre for Cell Science, Pune, Maharashtra, India. The RAW267.4, noncancerous murine macrophage cell line was kindly gifted by Dr. Rajesh Thimmulappa, JSS Medical College, JSS AHER, Mysuru. The analytical (n-Hexane (H), Chloroform, Ethyl Acetate and Ethanol (E)) and HPLC grade (Methanol) solvents used in the study were obtained from Loba Chemie, Mumbai, Maharashtra, India. All cell culture reagents and disposable dishes were purchased from Life Technologies, Carlsbad, USA and Tarson's India Limited, Kolkata, India, respectively. Lactate dehydrogenase activity assay kit [cat#: K730–500] was procured from BioVision, Milpitas, CA, USA. Ethidium bromide, acridine orange and sodium butyrate were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, Maharashtra, India. Sulforhodamine-B and Standard phenolic acids (Benzoic acid, Mono, di and tri hydroxy benzoic acids, Dimethoxy cinnamic acid, Monohydroxy dimethoxy cinnamic acid, Dihydroxy cinnamic acid, Trimethoxy benzoic and cinnamic acids, Chlorogenic acid) were from Sigma Chemical Company, St. Louis, USA.

2.2. Methods

2.2.1. Collection, botanical identification and preparation of SG leaves for extraction

The healthy leaves of SG plant were collected from the rural areas of the district of Palakkad, which is located in one of the Southern states of India, Kerala. The specimen sample of the obtained leaves of SG (Figure 1) was identified and authenticated by the renowned plant taxonomist, Dr. N. Sasidharan from Kerala Forest Research Institute, Thrissur, Kerala, India. The collected leaves were washed with running water to remove any foreign adherent materials, including dust and debris. Following this, all leaves were thoroughly rinsed with distilled water and dried under shade to remove the moisture. The dried leaves were subsequently pulverised in clean electric blender and sieved through to generate a fine powder.

2.2.1.1. Sequential extraction of SG leaf powder. The successive extraction of dried leaf powder (100 gm batch) of SG with solvents of increasing polarity, starting from 500 ml of Hexane (SGH), Chloroform (SGC), Ethyl acetate (SGEA), 70% aqueous Ethanol (SGE) as per previously published method (Jose et al., 2018). Following this, the resulting spent was macerated with distilled water (SGW) for 24 h. The solvents and water from each fraction were evaporated in a rotary evaporator under reduced pressure and the concentrated fraction was then freeze-dried using a lyophilizer. The respective dried fractions and/or reconstituted fractions (50 mg/ml solution in cell culture grade dimethyl sulfoxide) were stored at 4 °C until use.

2.2.2. Qualitative phytochemical analysis of SG fractions

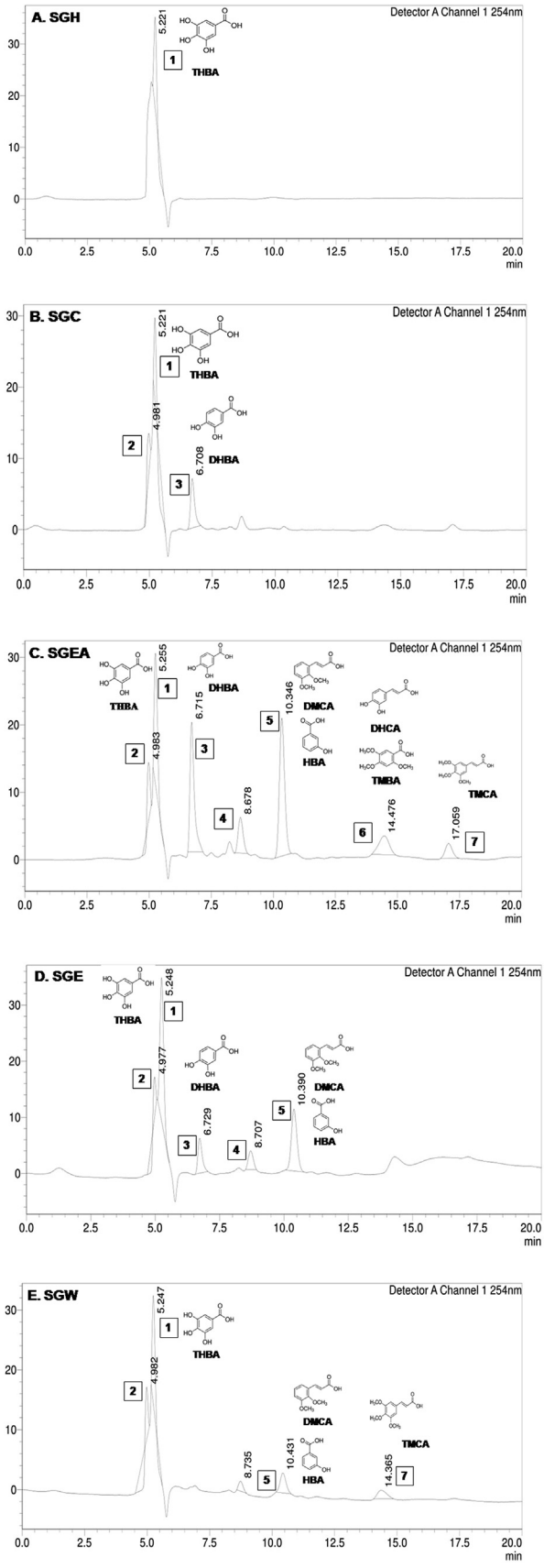
The qualitative phytochemical screening of the obtained fractions were performed to detect the presence of selective phyto-constituents as described earlier (Auwal et al., 2014; Harborne, 1998).

2.2.2.1. Test for alkaloids. Ten milligrams of each extracts were dissolved in 0.1N hydrochloric acid and filtered. The presence of alkaloids in the respective filtrates was tested by following Mayer's and Wagner's tests, as follows, *Mayer's test* was carried out by incubating filtrate (2.0 mg) with few drops of Mayer's reagent (5 g potassium iodide and 1.36 g mercuric chloride dissolved in 100 ml water). The presence of alkaloids was confirmed with the formation of yellowish creamy precipitate.

Wagner's test was performed by treating the filtrate (2.0 mg) with Wagner's reagent (1.27 g iodine and 2.0 g potassium iodide dissolved in 100 ml water). The filtrate with the formation of brown or reddish-brown precipitate indicate the presence of alkaloids.

2.2.2.2. Test for carbohydrates. The extract (0.5 mg each) was dissolved in 5.0 ml distilled water and filtered. The Molisch's reagent (10% α-naphthol in chloroform or alcohol) was then added to respective filtrates. The formation of a reddish violet ring at the junction of the filtrate and reagent indicated that the filtrate contains carbohydrates.

2.2.2.3. Test for protein & amino acids. Biuret test was carried out to determine the presence of proteins. Experimentally, 0.5 mg extract was incubated with equal volume of 40% NaOH solution and two drops of



(caption on next column)

Figure 2. Identification of phenolic compounds present in *Simarouba glauca* extracts using RP-HPLC. Phenolic compounds present in *Simarouba glauca* leaf extracts SGH (Figure 2A); SGC (Figure 2B); SGEA (Figure 2C), SGE (Figure 2D) and SGW (Figure 2E) were identified using reverse phase HPLC (RP-HPLC) as detailed in materials and methods. Analysis of the data showed the presence of 3,4,5-trihydroxybenzoic acid (THBA, Gallic acid) in SGH (Figure 2A), 3,4-dihydroxybenzoic acid (DHBA, protocatecheuic acid) and THBA in SGC (Figure 2B), THBA, DHBA and 2,3-dimethoxycinnamic acid (DMCA), 3-hydroxybenzoic acid (HBA, m-Salicylic acid). 3,4-dihydroxycinnamic acid (DHCA, Caffeic acid), 3,4,5-trimethoxybenzoic acid (TMBA, methylgallic acid), 3,4,5-trimethoxycinnamic acid (TMCA) in SGEA (Figure 2C); THBA, DHBA, DMCA and HBA in the SGE (Figure 2D); and THBA, DMCA, HBA and TMCA in SGW (Figure 2E) extracts. The identification was made by comparing the retention times (RTs) of sample peaks with that of standard phenolic acids.

1.0% copper sulfate solution added. The presence of protein was confirmed with the formation of violet color in the solution.

To test the presence of free amino acids in the extracts, 0.5 mg extract was exposed to two drops of freshly prepared 0.2% Ninhydrin reagent and heated. The filtrate with aminoacids turned into pink or purple color.

2.2.2.4. Tests for glycosides. Presence of glycosides was tested by Liebermann's test. In brief, the extracts were mixed with acetic acid (2.0 ml) and chloroform (2.0 ml) and heated and then allowed to cool. Following this, 0.5 ml H_2SO_4 was added to the above reaction mixture. The presence of aglycone was confirmed with the formation of green color in the reaction mixture.

The presence of glycosides in the extracts were checked by Keller-Kiliani test. The extract was mixed with 4.0 ml of glacial acetic acid and 1.0 ml of sulphuric acid. A drop of 2.0 % $FeCl_3$ was then added to the above mixture. The presence of steroidal glycosides was confirmed with the formation brown ring at the junction of liquid layers of mixture.

The Salkowski's test was performed to identify the presence of steroidal aglycone by adding sulphuric acid (2.0 ml) to the crude extract. The formation of reddish-brown colour indicates the existence of aglycone moiety of the steroidal glycoside in the extract.

2.2.2.5. Test for phenols. The presence of phenol in the extracts was confirmed with the formation of bluish black color upon adding few drops of ferric chloride (1.0 %) to 10.0 mg of extract. Alternatively, the formation of yellow precipitate with the addition of lead acetate solution (10.0 %) to extract indicated the presence of phenols.

2.2.2.6. Tests for flavonoids. The Shinoda test detected the presence of flavonoids in the extracts. The crude extract was mixed with concentrated HCl and pieces of magnesium. The appearance of pink color in the above mixture indicated the presence of flavonoids. In addition, the extract was mixed with 2.0 % NaOH (2.0 ml), and dilute acid (added slowly). The disappearance of yellow color confirmed the presence of flavonoids.

2.2.2.7. Test for tannins. The disappearance of the color of bromine water (10.0 ml) upon the exposure of extract (0.5 g) indicates the presence tannins in the extract.

2.2.2.8. Test for steroids. The crude extract dissolved in water (5.0 ml), mixed with chloroform (2.0 ml) and concentrated H_2SO_4 results in the development of red color in the lower chloroform layer, which indicates the presence of steroids.

2.2.2.9. Test for terpenoids. The presence of terpenoids in the crude extract was tested by adding the chloroform (2.0 ml) to the plant extract, followed by evaporating the mixture on a water bath. Following this, 3.0 ml of concentrated sulfuric acid was added to the mixture and boiled. The grey color appearance of the mixture indicates presence of terpenoids.

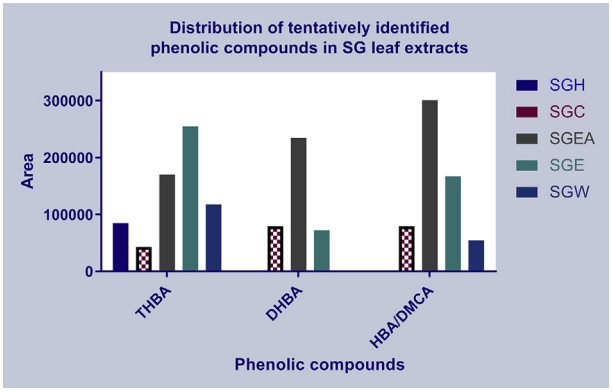


Figure 3. Distribution of major phenolic compounds present in the *Simarouba glauca* extracts. *Simarouba glauca* leaf was extracted sequentially with hexane (SGH), chloroform (SGC), ethylacetate (SGEA), aqueous ethanol (SGE) and water (SGW). The extracts were analyzed using RP-HPLC and phenolic compounds present identified by comparing with standards. Analysis of the data showed that the ethyl acetate fraction is rich in THBA, DHBA and HBA/DMCA, while ethanol fraction is rich in THBA.

2.2.2.10. Test for oils and resin. The presence of oils and resins are determined by the appearance of transparent region when a drop of crude extract applied to a filter paper.

2.2.3. Quantitative phytochemical analysis

2.2.3.1. Determination of total phenol content. The Folin-Ciocalteau method was used to determine the total phenol content as previously described by Singleton et al. (1999). To prepare a calibration curve, one milliliter of increasing concentration of gallic acid (2.5, 5.0,10.0, 20.0, 40.0, 50.0 μg/ml) solution was incubated with 1.0 ml of 50 % Folin--Ciocalteau's reagent and 0.8 ml of 4.0 % $NaHCO_3$ at room temperature for 30 min. The absorbance was measured at 765 nm in a UV-Visible Bio-spectrophotometer (Eppendorf). Ethanol was used as blank. The plant extracts were appropriately diluted and processed similarly as described for gallic acid. The concentration of total phenols in the plant extract was determined by extrapolating absorption of unknown samples in standard calibration curve, obtained with gallic acid.

Table 3. Identification of phenolic compounds based on relative retention time analysis using HPLC.

Peak number	Relative retention time with reference to benzoic acid	Tentative compound identified
1	0.28	THBA
2	Not identified	Not identified
3	0.36	DHBA
4	Not identified	Not identified
5	0.55	HBA/DMCA
6	0.76	TMBA/DHCA
7	0.96	TMCA

THBA: Tri hydroxy benzoic acid, DHBA: Di hydroxy benzoic acid, HBA: Hydroxy benzoic acid, DMCA: Dimethoxy cinnamic acid, TMBA: Trimethoxy benzoic acid, DHCA: Dihydroxy cinnamic acid, TMCA: Trimethoxy cinnamic acid.
Phenolic acid standards were analyzed on a C18 HPLC column as detailed in materials and methods section and retention times (in minutes) noted. Relative rentention time for each standard phenolic compound was determined by comparing with the retention time of benzoic acid. Phenolic compounds present in extracts were identified by comparing the relative retention times of peaks with that of standards.

2.2.3.2. Estimation of total flavonoid content. Quantification of total flavonoids present in the extract was carried out as per El Far and Taie (2009) with minor modifications. The standard curve was prepared by incubating 0.5 ml of increasing concentration of quercetin (10, 25, 50, 100, 200 μg/ml) and 1.5 ml of 95% methanol followed by sequential addition of 0.1 ml 10% aluminum chloride, 0.1 ml 1.0 M potassium acetate and 2.8 ml distilled water at ambient temperature for half hour. The absorbance of the above reaction mixture was measured at 415 nm. Distilled water was used as blank. The quercetin was replaced with appropriately diluted extract samples and processed as detailed above. The obtained absorbance of extract was extrapolated in standard curve to determine the concentration of total flavonoids.

2.2.3.3. Identification of phenolic acids using RP-HPLC. The phenolic compounds present in the extracts of SG were identified using RP-HPLC (Prominence-i, LC-2030C Shimadzu) as previously described with minor modifications (Subba Rao and Muralikrishna, 2001). Briefly, the extract (250 μg/ml) dissolved in mobile phase (water, acetic acid and methanol, 80:5:15, respectively) was filtered and injected in to a reverse phase C18 column (Shimpak, 4.6 × 250 mm) and eluted with isocratic mobile phase. The flow rate was maintained at 0.6 ml/min. An in-built UV detector was used to detect the compounds at 254 nm (Subba Rao and Muralikrishna, 2002). The presence of phenolic acids in the extracts were identified and quantified by comparing the retention time of following standard phenolic acids, benzoic acid, mono-, di-, and tri-hydroxy benzoic acids, dimethoxy cinnamic acid, monohydroxy dimethoxy cinnamic acid, dihydroxy cinnamic acid, mono hydroxy mono methoxy benzoic acid, trimethoxy benzoic, cinnamic acids, and chlorogenic acid at a concentration of 100.0 μg/ml.

2.2.4. Effect of crude extracts on selective cancer and non-cancer cells in vitro

The anticancer effects of prepared extracts were tested on the following cancer and noncancer cells, HCT-116 and HCT-15 (colorectal carcinoma cells), MDA-MB-231 (mammary/breast adenocarcinoma cells), HeLa and SiHa (cervical carcinoma cells), A549 (lung carcinoma cells) and RAW267.4 (noncancerous murine macrophage cells), as described earlier by Madhunapantula et al. (2008).

Briefly, the cells (0.5×10^4 cells) were seeded in a 96-well plate containing growth medium (DMEM supplemented with 10.0 % FBS, 5.0 % penstrep and 5.0 % glutamax) and incubated in a cell culture incubator maintained at 37 °C, 5% CO_2 and 90% relative humidity. Upon reaching ~70% confluency (after 48h), the cells were treated with increasing concentration of crude extracts (7.8–500 μg/ml prepared in DMSO and diluted with growth medium to ensure that DMSO concentration in sample is below 1%), positive control oxaliplatin (20 μg/ml) (Sigma-Aldrich) and vehicle control 1% DMSO. The 96-well plate was incubated for 24, 48 and 72 h and cell viability determined using SRB assay.

Sulforhodamine-B (SRB) assay as described by Skehan et al. (1990) was carried out to determine the cell viability of cancer and noncancer cells following the exposure with extracts. Briefly, the adherent cells were fixed in ice cold TCA (50 % w/v) and incubated at 4 °C for 1.0 h. Following this, the fixing media was aspirated and the cells thoroughly rinsed with ice cold water. The plate was gently tapped on the filter paper to dry the wells. The fixed cells were then incubated with 100 μl of 0.4 % SRB for 30 min. The cells were then washed with 1% acetic acid to remove the unbound SRB. The cells were lysed with 100 μl/well of 10.0 mM Tris Base and the absorbance was measured at 490 nm in multimode plate reader (Perkin Elmer, Germany). The percentage of growth inhibition by extract was calculated over the vehicle control using the below formula

Percentage Viability = [OD of Sample] / OD of Control X 100

Percentage inhibition = 100-Percentage viability

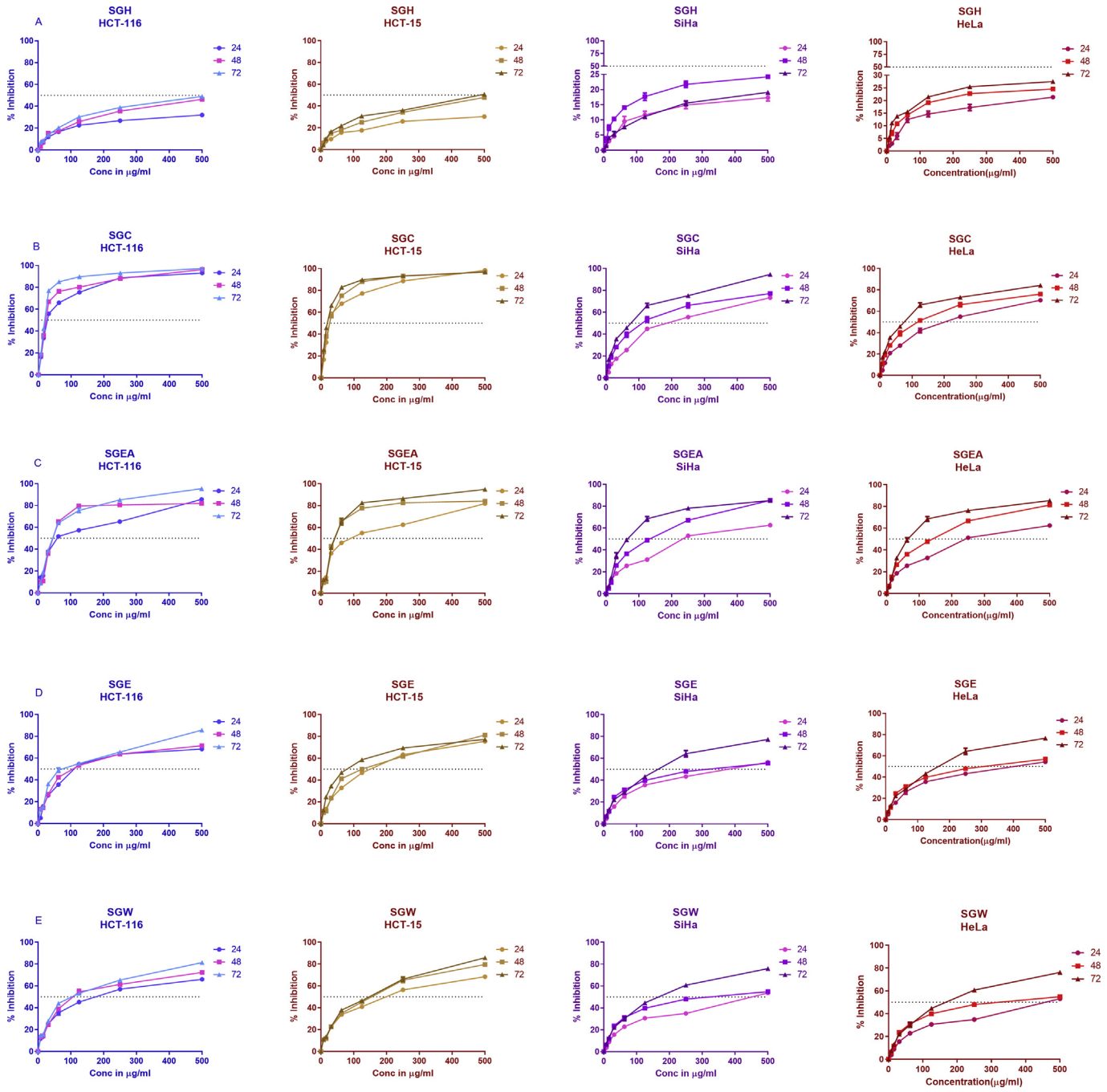


Figure 4. Cytototxic potential of SG extracts on cell lines representing carcinomas of colon and rectum and cervix. To test and identify the most effective extract of SG leaf, a cytotoxicity study was conducted by treating cell lines representing carcinomas of colon and rectum (HCT 116 and HCT-15), and cervix (HeLa and SiHa) with SGH (Figure 4A), SGC (Figure 4B), SGEA (Figure 4C), SGE (Figure 4D) and SGW (Figure 4E) for 24.0 h, 48.0 h and 72.0 h. Number of viable cells were determined using SRB and dose response curves plotted. IC50 values were calculated using GraphPad Prism. Among these extracts, SGC exhibited better cytotoxicity against all these cell lines tested.

2.2.5. Cell viability analysis using lactate dehydrogenase release assay

One of the markers of cellular damage is the amount of lactate dehydrogenase (LDH) enzyme released upon the exposure to stressors, as LDH release is proportional to the cell damage (Bacci et al., 1988). HCT-116 and SiHa cells (0.3×10^6) were seeded in 6-well plates containing 2.0 ml of growth media. Upon confluency the cells were treated with SGC 12.5, 25.0, and 50.0 µg/ml (in case of HCT-116) and 50.0, 100.0, and 200.0 µg/ml (in case of SiHa) for 48 h. The concentration of treatments was based on the IC_{50} values observed for each cell line. Following incubation, the media was collected and diluted (1:1) with LDH assay buffer. The LDH assay was performed using Picoprobe LDH assay kit (K730, Biovision) and the LDH activity of sample was calculated as per the manufacturer's guidelines (Anantharaju et al., 2017a, 2017b). LDH activity in samples was expressed in mU/mg of protein.

2.2.6. Estimation of intracellular reactive oxygen species (ROS)

The ROS assay was performed as described earlier by Shailasree et al. (2015) with minor modifications. Briefly, the HCT-116 and SiHa (0.5×10^4) were grown in 96-well plates and incubated with increasing concentrations of SGC for 24 and 48 h. The cells were then washed with ice-cold PBS and further incubated with 10.0 µM of 2′, 7′-

Table 4. IC_{50} (μg/ml) of *S.glauca* extracts on colorectal cancer cell lines HCT 116 and HCT-15.

Extract	HCT 116			HCT-15		
	24h	48h	72h	24h	48h	72h
SGH	ND	ND	ND	ND	ND	ND
SGC	30.98 ± 0.33	23.63 ± 0.37	18.08 ± 0.17	29.37 ± 0.12	23.66 ± 0.64	18.15 ± 0.11
SGEA	78.86 ± 1.94	49.65 ± 1.96	45.84 ± 1.10	92.29 ± 2.05	46.87 ± 1.36	42.74 ± 0.79
SGE	129.67 ± 0.91	116.30 ± 3.20	82.27 ± 4.23	137.06 ± 3.23	117.46 ± 3.78	79.55 ± 1.65
SGW	169.16 ± 0.96	123.00 ± 4.47	98.69 ± 4.31	179.20 ± 3.29	129.13 ± 1.73	114.86 ± 2.78

In order to determine and select the most potent extract among SGH, SGC, SGEA, SGE and SGW, colorectal carcinoma cell lines HCT 116 and HCT-15 were exposed to increasing concentration of extracts for 24h, 48h and 72h and number viable cells calculated by staining with SRB. Inhibitor concentration required for 50% growth inhibition (IC50) was calculated and the data represented in table. SGC exhibited better potency compared to all other extracts at all the time points tested. SGH failed to inhibit HCT 116 and HCT15 cell lines, hence, no IC50 could be calculated. ND: Not Determined.

Table 5. IC_{50} (μg/ml) values of *S.glauca* extracts on cervical cancer cell lines SiHa and HeLa.

Extract	SiHa			HeLa		
	24h	48h	72h	24h	48h	72h
SGH	ND	ND	ND	ND	ND	ND
SGC	175.70 ± 1.77	105.8 ± 4.2	61.9 ± 1.5	185.66 ± 6.5	109.7 ± 4.7	66.7 ± 1.9
SGEA	255.03 ± 6.26	113.03 ± 2.9	66.08 ± 3.9	260.46 ± 7.6	116.5 ± 1.86	68.48 ± 3.6
SGE	336.1 ± 3.75	286.1 ± 2.7	145.3 ± 2.66	360.56 ± 6.2	275.9 ± 4.12	146.43 ± 5.1
SGW	460.4 ± 5.71	291.7 ± 3.1	149.7 ± 1.8	472.6 ± 10.4	291.26 ± 3.44	149.46 ± 2.14

To determine whether the most potent extract identified ie., SGC, show efficacy against cervical cancer cell lines HeLa and SiHa, the cells were exposed to increasing concentration of SGC and other extracts for 24h, 48h and 72h and number viable cells calculated by staining with SRB. Inhibitor concentration required for 50% growth inhibition (IC50) was calculated and the data represented in table. SGC exhibited better potency compared to all other extracts at all the time points tested. The hexane extract SGH failed to inhibit the growth of SiHa and HeLa by 50% at the concentrations tested. Hence, the IC50 values could not be calculated. ND: Not Determined.

Table 6. IC_{50} (μg/ml) values of *S.glauca* crude extracts on lung cancer cell line A549 and breast cancer cell line MDA-MB-231.

Extract	A549			MDA-MB-231		
	24h	48h	72h	24h	48h	72h
SGH	ND	ND	ND	ND	ND	ND
SGC	ND	ND	481.13 ± 7.07	301.83 ± 0.81	267.23 ± 1.72	113.70 ± 0.42
SGEA	ND	ND	ND	408.57 ± 3.57	351.43 ± 4.68	175.90 ± 4.38
SGE	ND	ND	ND	ND	409.93 ± 3.98	374.77 ± 3.84
SGW	ND	ND	ND	ND	402.70 ± 2.44	225.77 ± 2.98

ND: Not Determined.

To determine whether the most potent extract identified ie., SGC, show efficacy against lung cancer cell line A549 and breast cancer cell line MDA-MB-231, the cells were exposed to increasing concentration of SGC and other extracts for 24h, 48h and 72h and number viable cells calculated by staining with SRB. Inhibitor concentration required for 50% growth inhibition (IC50) was calculated and the data represented in table. SGC exhibited better potency compared to all other extracts. Interestingly none of these extracts could kill A549 cells at 24h and 48h. At 72h of treatment SGC showed a moderate efficacy with an IC50 of 481.13 ± 7.07.

dichlorodihydrofluorescein diacetate (H2DCFDA, prepared in PBS) for 30 min at 37 °C in the CO_2 incubator. The intensity of fluorescence was measured at an excitation of 435.0 nm and emission of 520.0 nm in a microplate reader.

2.2.7. Cell death analysis using double staining method

A double staining method using acridine orange and ethidium bromide was used to count the number of cells undergoing cell death as previously described by Shailasree et al. (2015).

Experimentally, 0.3×10^6 HCT-116, and SiHa cells in 2.0 ml media/well were seeded in 6-well plates and exposed to increasing concentrations of SGC (12.5, 25.0, 50.0 μg/ml in case of HCT-116 and 50.0, 100.0, 200.0 μg/ml in case of SiHa) for 48 h. Oxaliplatin at 20.0 μg/ml served as positive control for inducing cell death. The single cell suspension was prepared by trypsinizing and homogenising the cells. Following neutralisation, 20.0 μl cell suspension was mixed with 10.0 μl of 100.0 mg/ml solutions of ethidium bromide and acridine orange separately and incubated for 5.0 min. The fluorescent images were then captured in a fluorescence microscope using TRITC and FITC. The respective images obtained with these filters were then overlapped each other. The cells exhibited green fluorescence denoted the viable cells and the one that exhibited orange fluorescence denoted the dead cells.

2.2.8. Statistical analysis

All samples were analysed at least in triplicates and the results expressed as mean ± SEM of at least 3.0 independent experiments. IC_{50} value for each cell line and extract was calculated by first determining the percentage inhibition at each concentration followed by subjecting the data to IC50 estimation. The constraints fixed were: bottom value = 0.0 and top value = 100.0. The data were subjected to two-way ANOVA followed by the Bonferroni *post-hoc* test to measure the difference between various extracts and controls/positive controls. A "*P*" value of <0.05 was considered significant. Graph Pad Prism 5 was used to construct the graphical presentation of results and Statistical Package for the Social Sciences (SPSS) Version 22.0 was used to conduct all statistical analysis.

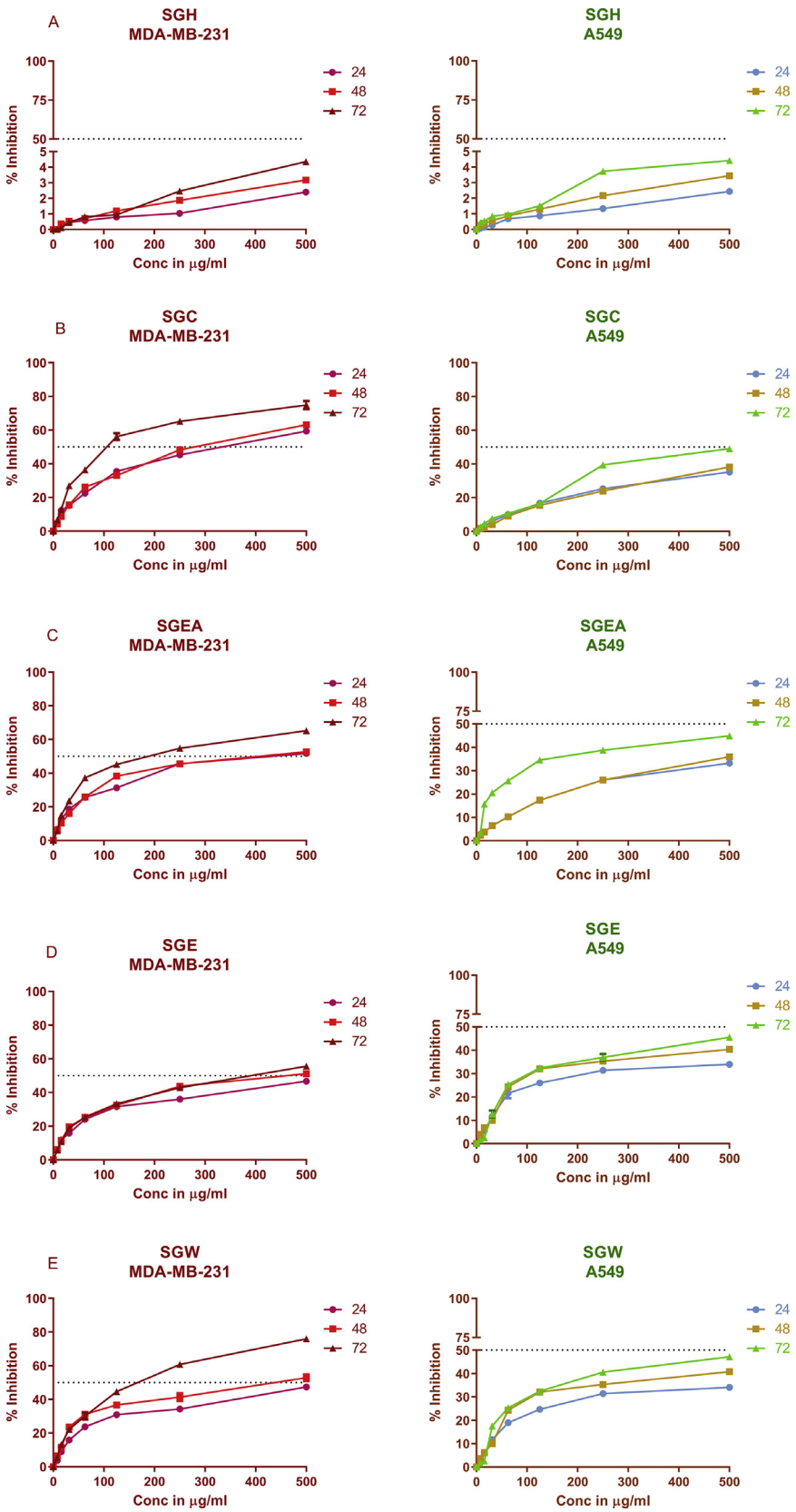


Figure 5. Cytotoxic potential of SG extracts against MDA-MB-231 and A549 cell lines. The ability of SG extracts SGH (Figure 5A), SGC (Figure 5B), SGEA (Figure 5C), SGE (Figure 5D) and SGW (Figure 5E) for inhibiting breast cancer cell line MDA-MB-231 and lung cancer cell line A549 was tested by treating the cells with increasing concentration of extracts for 24.0 h, 48.0 h, and 72.0 h. SGC and SGEA exhibited better cell killing when tested against MDA-MB-231. However, all the extracts exhibited poor cell growth inhibition against A549 cells.

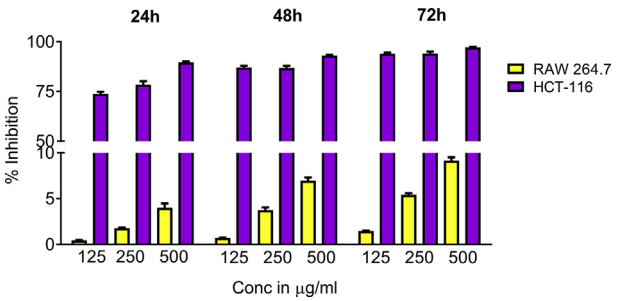


Figure 6. SGC is more specific to HCT 116 cancer cells compared to murine macrophages RAW 264.7: In order to test whether the most potent extract of SG ie., SGC exhibit selectivity to cancer cells or kill the cells non specifically, human colorectal cancer cell line HCT 116 and mouse macrophage cell line RAW 264.7 were exposed to increasing concentration of SGC for 24.0 h, 48.0 h and 72.0 h and cell viability determined. SGC selectively killed HCT 116 at the concentrations tested without effecting much the normal cells. Therefore, SGC is selective to cancer cells.

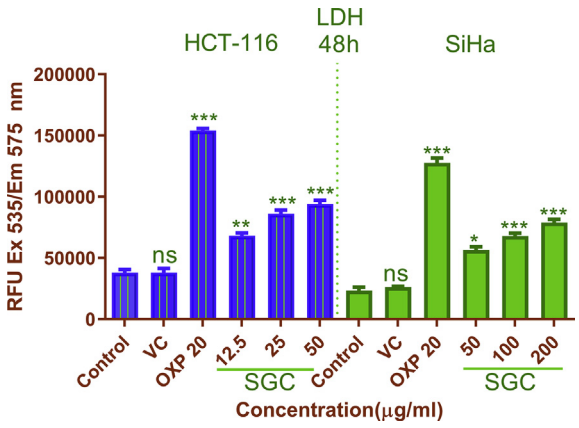


Figure 7. SGC induces the release of LDH from HCT 116 and SiHa cell lines. LDH assay was carried out to determine whether cytotoxicity induced by SGC is due to cell membrane damage. The media from control and treated cells was collected and LDH activity estimated. A dose dependent and significant increase in LDH activity was observed with increasing SGC concentration compared to untreated or vehicle 1.0 % DMSO treated cells (mean ± SEM, $P < 0.05$, One-Way ANOVA). 20.0 µg/ml Oxaliplatin served as positive control for LDH release.

3. Results

3.1. Yield and phytochemical characterization of SG extracts

The dried leaves of SG were extracted successively with solvents of increasing polarity, including n-hexane (SGH), chloroform (SGC), ethyl acetate (SGEA), hydro-alcoholic (70 % aqueous alcohol; SGE) and water (SGW). Among various extracts obtained the SGE and SGW has highest yield of 8.5 and 5.5 g/100 g, respectively. The qualitative phytochemical screening of extracts showed the presence of alkaloids, phenols and flavonoids as major contents in the obtained extracts (Table 1).

Total phenol content was analyzed by first comparing the absorbance values with standard gallic acid concentration, followed by converting the value in to gram percentage of extract (expressed as gram percentage equivalent of gallic acid/100.0 g crude extract). The data showed a range varying from 0.07 ± 0.01 g percentage in SGH to 1.65 ± 0.09 g percent in SGE (Table 2). Very low total phenol content was observed in chloroform (0.21 g% ± 0.02) and hexane (0.07 g% ± 0.01) extracts. The order of total phenol content was found to be SGE > SGEA > SGW > SGC > SGH.

The content of flavonoids was expressed as quercetin equivalents per 100.0 g of dried leaves (Table 2). The concentration of flavonoids varied from 0.06 ± 0.01 percent in SGH to 0.48 ± 0.04 percent in SGE. The order of total flavonoids was observed as SGE > SGW > SGC > SGEA > SGH (Table 2).

Subsequent analysis of these extracts using RP-HPLC revealed the presence of 2,3,4-trihydroxy benzoic acid (THBA, commonly known as gallic acid), 3,4-dihydroxy benzoic acid (DHBA, commonly known as protocatecheuic acid) and 3,4-dimethoxy cinnamic acid (DMCA, commonly known as caffeic acid dimethyl ether). SGEA and SGE had higher levels of these phenolic compounds compared to other extracts (Figures 2A to 2E and 3 and Table 3).

3.2. Cytotoxic effects of SG extracts in vitro

To determine whether the SG extracts rich in anti-cancer molecules such as phenolic compounds and flavonoids, exhibit potent anti-proliferative activity compared to conventional drug, oxaliplatin in cancer cells, a concentration and time-dependent cytotoxicity assay was performed on cell lines, HCT-116 and HCT-15 (colorectal carcinoma cells), MDA-MB-231 (mammary/breast adenocarcinoma cells), HeLa and SiHa (cervical carcinoma cells), A549 (lung carcinoma cells) and RAW267.4 (noncancerous murine macrophage cells).

The cells were treated with increasing concentration of SGH, SGC, SGEA, SGE, SGW for 24, 48 and 72 h. The effect of extracts on cell viability was determined using SRB assay (Figure 4A to 4E and Tables 4, 5, and 6). Based on the analysis, SGC has greater potency to inhibit the cell growth in all tested cancer cells compared to all other extracts and this effect was concentration and time-dependent. Of note, the cytotoxic effect of SGC was prominent in colon carcinoma cells (HCT-116 & HCT-15) (Figure 4B and Table 4). For instance, at 62.5 µg/ml, SGC inhibited the growth of HCT-116 and HCT-15 cells by 75.0 % at 48 h. Similarly, SGC inhibited the growth of cervical cancer cell lines SiHa and HeLa (Figure 4B). For instance, an about 40.0 % cell growth inhibition was observed at 62.5 µg/ml SGC (Figure 4 and Table 5). However only 9.0 % and 25.0 % inhibition was observed when exposed to A549 and MDA-MB-231, respectively (Figure 5 and Table 6).

To determine whether or not the cytotoxic potential of SGC in cancer cells (for example HCT-116) is similar to normal noncancerous cells, RAW 264.7 (mouse macrophage cell line), was used (Anantharaju et al., 2017a). The results demonstrate that SGC inhibited only 9.0 % of murine macrophage cells RAW264.7 at the highest dose tested (500.0 µg/ml) even after 72 h of exposure (Figure 6). This observation suggests that SGC elicits cytotoxic effects specific to cancer cells. Furthermore, as the observation that the cytotoxic potential was greater in colon carcinoma cells, it would have been appropriate if the specificity of SGC also tested parallel with normal colon epithelial cells, including CCD18Co or FHC (CRL-1831). However, these normal cells lines were currently not available and that limited us to use RAW 264.7 cells.

3.3. SGC trigger the release of lactate dehydrogenase in vitro

The abrupt increase in the release of lactate dehydrogenase from cells is regarded as an indicator of cell death (Bacci et al., 1988). A number of anti-cancer agents has been shown to induce cell death by promoting the release of LDH in to the culture media (Xie et al., 2009). Hence, assessing the release of LDH in the culture media is an easy way of estimating cell death. Therefore, the SGC at varying concentration was added to exponentially growing colorectal carcinoma cells, HCT 116 and cervical cancer cell, SiHa. As illustrated in (Figure 7), a concentration-dependent increase in LDH content in media was observed with SGC treatment. The positive control, oxaliplatin (20.0 µg/ml) increased the LDH release by 3-fold compared to control vehicle treated cells (Figure 7).

3.4. SGC induces ROS generation in cancer cells

Reactive oxygen species (ROS), which are produced due to incomplete oxidation of biomolecules or insufficient quantities of reducing substances in cells, are known to induce cell death (Hwang et al., 2008).

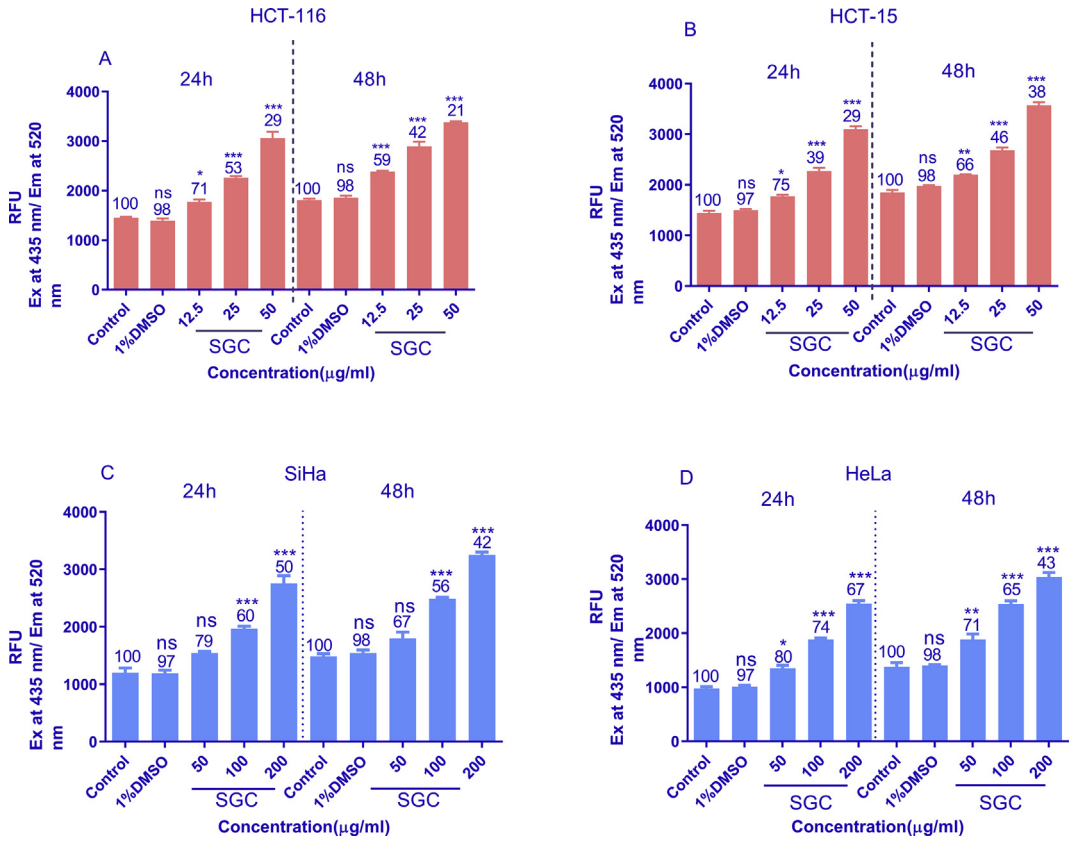


Figure 8. SGC triggered reactive oxygen species production in cancer cells. In order to determine whether treatment with SGC triggered the production of ROS, colorectal cancer cell lines HCT 116 (Figure 8A), HCT-15 (Figure 8B) and cervical cancer cell lines SiHa (Figure 8C) and HeLa (Figure 8D) were exposed to increasing concentration (12.5 µg/ml, 25.0 µg/ml and 50 µg/ml) of SGC for 24.0 h and 48.0 h and levels of ROS determined using H2DCFDA method. SGC triggered the production of ROS ($P < 0.05$ when compared treated ones with control untreated and vehicle treated cells, One-Way ANOVA) in all these cell lines (Figures 8A to D). A slight increase in ROS was observed upon 48.0 h treatment compared to 24.0 h treatment.

Mechanistically, ROS promotes the oxidation of cellular lipids, proteins, and DNA thereby induce cell death (Trachootham et al., 2009). Induction of ROS by pharmacological agents is one of the viable approaches to kill cancer cells (Kopetz et al., 2009). Therefore, in this study, the efficacy of SGC for inducing ROS in cancer cells was determined by treating colorectal (HCT 116 and HCT-15; Figures 8A and 8B) and cervical (SiHa and HeLa; Figures 8C and 8D) carcinoma cells. SGC has elicited concentration and time-dependent increase in ROS levels in both types of cells (Figure 8), suggesting that SGC induced cancer cell death could be in part mediated by elevated ROS.

3.5. SGC induces apoptosis in cancer cells

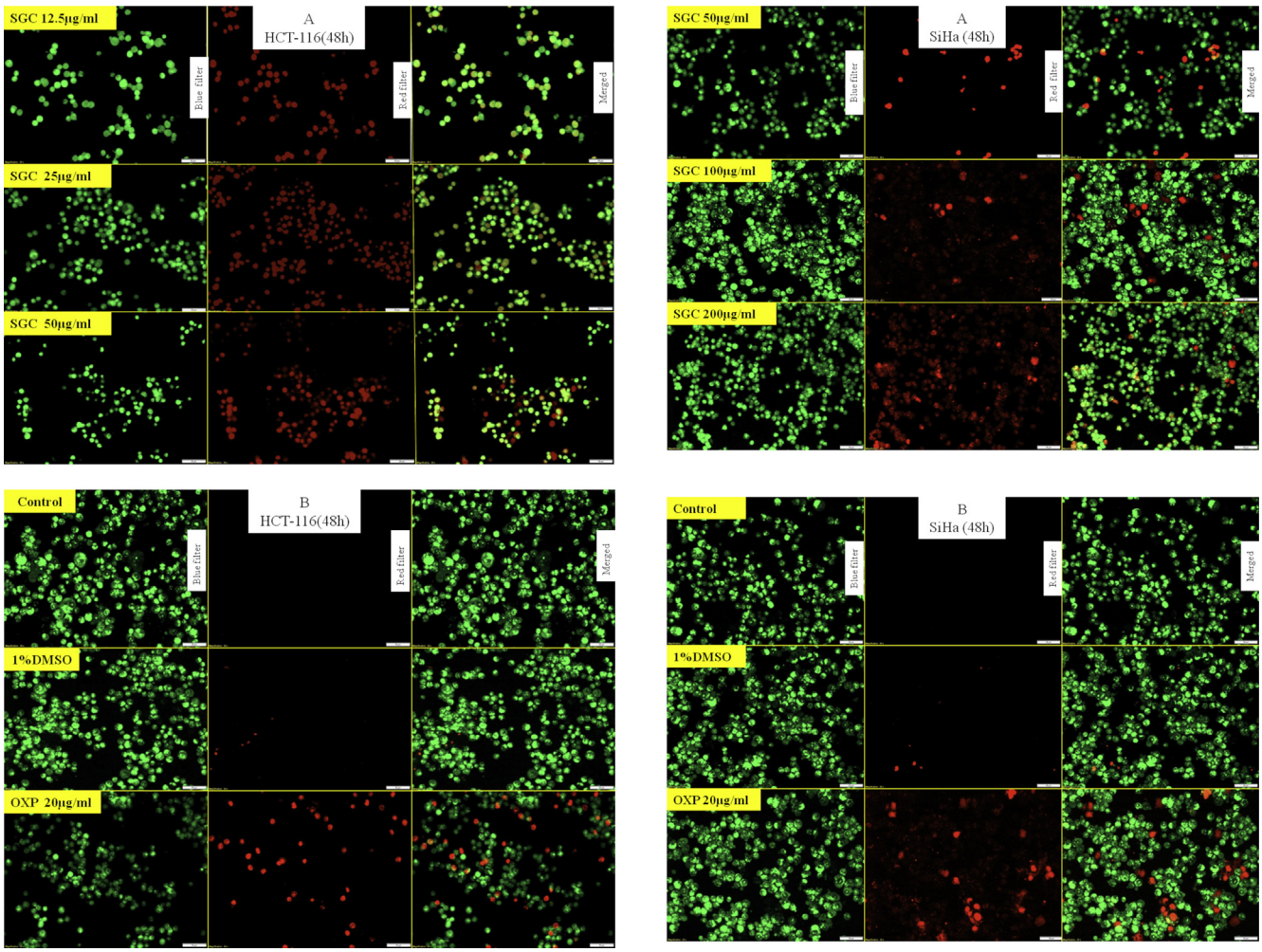
Dual staining method using acridine orange (AO) and ethidium bromide (EtBr) is a widely used method to detect cellular apoptosis (Kasibhatla, 2006). In principle, AO easily permeates to live cells and emits green fluorescence when binds to double stranded DNA (indicating no apoptotic DNA breakage in the cells). However, AO emits red fluorescence when bind to single stranded DNA or RNA (an indicator of apoptotic DNA breaks in cells).

Unlike AO, the EtBr permeates cells only when there is a membrane damage and stains nucleus red (Kononov et al., 2001). Consequently, the cells appear with following staining patterns, live cells: green nucleus, early apoptotic cells: bright green nucleus but condensed or fragmented chromatin, late stage apoptotic cells: condensed and fragmented orange chromatin and necrotic dead cells: structurally normal orange nucleus (Byczkowska et al., 2013). In conclusion, dual staining procedure using AO and EtBr is an inexpensive and reliable protocol to determine the cellular apoptosis. Hence, in the present study, the efficacy of SGC to induce apoptotic cell death was determined by treating HCT-116 (Figure 9) and SiHa (Figure 10) cells for 48.0 h followed by staining with EtBr and AO. The image analysis showed significantly high orange and red stained cells only when treated with SGC (Figures 9A & 10A). Control cells exposed to just media and vehicle 1.0 % DMSO showed predominance in green cells, suggesting no cell death. However, the cells exposed to positive control 20.0 µg/ml oxaliplatin exhibited red cells, indicates the cell apoptosis (Figures 9B & 10B). The number of orange stained cells increased with increasing SGC concentration (Figures 9A & 10A), suggesting necrosis.

In summary, results of this study demonstrated that among all extracts the chloroform extract of SG leaves (SGC) has shown potent anticancer activity against a variety of cancer cell lines. Furthermore, our findings illustrate that SGC induces cell death through apoptosis induction, increasing cellular oxidative stress and DNA breakage. Further studies should evaluate SGC in animal models for safety and efficacy.

4. Discussion

Recent studies have demonstrated the success of combination therapies over monotherapies, necessitating the development of pharmacological agent-cocktails, or identification of potent anti-cancer phytochemical-rich fractions from natural source (Carney et al., 2010; Sartorelli, 1964; Uchida et al., 2005). Adding to this recent development, several studies have shown that targeting carcinomas of colon and rectum, and many other cancers with monotherapies lead to the development of drug resistance and failure of drugs (Cunningham et al., 2004). Therefore, recent research is focussing more on developing



Figures 9. and 10Treatment with SGC induced apoptotic cell death in colorectal- and cervical cancer cells. To check the ability of SGC to induce apoptosis in colorectal (Figure 9) and cervical (Figure 10) cancer cells, a dual staining method using acridine orange (AO) and ethidium bromide (EtBr) was implemented and the cells photographed using a fluorescence microscope. HCT 116 cells treated with SGC (12.5 µg/ml, 25.0 µg/ml and 50.0 µg/ml) showed a dose dependent increase apoptotic (orange) and necrotic (red) cells. Number of necrotic cells were more at higher dose (Figure 9A). Control untreated cells and cells exposed to vehicle DMSO exhibited very minimal number of apoptotic cells (Figure 9B). The positive control 20.0 µg/ml Oxaliplatin induced apoptosis and necrosis in majority of the cells (Figure 9B). To check the apoptosis inducing effect in a second cell line, cervical cancer cells SiHa were treated with 50.0 µg/ml, 100.0 µg/ml and 200.0 µg/ml SGC and stained with AO and EtBr. A dose dependent increase in apoptotic and necrotic cells were observed with SGC treatment (Figure 10A). Similar to HCT 116, very minimal apoptotic cells were observed in untreated and vehicle DMSO treated cells (Figure 10B). Oxaliplatin 20.0 µg/ml induced apoptosis and necrosis in SiHa cells (Figure 10B).

phytochemical-rich fractions that exhibit better safety and efficacy profiles for treating cancers. Consistent to this regard, one of the recent studies by (Tizziani et al., 2017) showed that crude extracts of medicinal plants of Polygala genus are more effective in killing breast- and colorectal cancer cells.

Simaroubaceae family is well known for the presence of quassinoids, which are a class of bitter substances responsible for a variety of biological activities including anticancer activity (Bhatnagar et al., 1984; Monseur and Motte, 1983; Polonsky et al., 1978). To date about 200 quassinoids have been isolated and structures elucidated (Jose et al., 2019). Structurally, quassinoids are the products of oxidative degradation of triterpenes and have been divided in to 5.0 different groups (C18, C19, C20, C22 and C25) based on the number of carbons present in the backbone (Jose et al., 2019). A recent study by Tung et al., (2017) demonstrated the anti-proliferative activity of quassinoids (in particular eurycomanone) isolated from the roots of *Eurycoma longifolia* against various cancer cell lines. In addition to quassinoids, β-carboline alkaloids, canthin alkaloids, triglycerides, coumarins, squalene type triterpenoids and fatty acids are purified from Simaroubaceae (Polonsky et al., 1978; Reynertson et al., 2011; Rivero-Cruz et al., 2005).

A study from our group has purified and characterized tricaproin from the chloroform extract of *S. glauca* (Jose et al., 2018). Tricaproin inhibited colorectal cancer cells growth by promoting apoptosis through HDAC inhibition. Another report from Shimoga Cancer Treatment suggested the use of decoction prepared from *Simarouba glauca* leaves for treating cancers. The ethanolic extract of *Simarouba glacua* leaf reported to contain secondary metabolites such as phenolic compounds, and exhibit potent anticancer activity against bladder cancer (Puranik et al., 2017). In addition, in a recent review, we have addressed the complexity of phytochemicals present in *Simarouba glauca* and their anti-cancer potential (Jose et al., 2019). Hence, an attempt was made to extract phytochemical-rich fractions from *Simarouba glauca* and study the isolated fractions for their anti-cancer potential.

In general, the solvent extracts of plants are known to contain phytochemicals such as phenolic compounds, flavonoids, terpenoids etc, which exhibit potent anti-cancer activity (Mojab et al., 2003). For instance, the phenolic acids such as benzoic acid and cinnamic acid derivatives have been shown to retard colorectal carcinoma cells proliferation *in vitro* by inhibiting HDAC2 (Anantharaju et al., 2017a, 2017b). In this study, we have demonstrated that the chloroform and ethyl acetate

extracts contain gallic acid, gentisic acid and other phenolic compounds, hence, SGC and SGEA exhibited potent anticancer properties against a variety of cancer cells. Of note, our findings are in accord with a study by Umesh (2014), which reported that the methanolic extract of SG leaves inhibits the cell growth of SCC9 and HCT 116 cells at a concentration of 312.2 μg/ml. However, the water extract showed no significant inhibitory effect on lung cancer cell line A549 (Umesh, 2014).

Mechanistically, phenolic compounds induce death in cancer cells through (a) activation of apoptotic signaling cascades; (b) inhibition of proliferative pathways; (c) increasing oxidative stress (Anantharaju et al., 2016). However, these events are concentration and time dependent, hence, caution should be executed while recommending the phenolic compounds rich fractions to treat cancer patients. For instance, low doses of phenolic compounds promote cell proliferation by protecting cells from ROS induced damage (Phan et al., 2001). Moreover, phenolic compounds are known to prevent the transformation of normal cells in to cancer cells through protection of DNA and key proteins involved in cellular transformation (Anantharaju et al., 2016). Since oxidative stress plays a major role in the initiation and development of cancers, neurodegenerative diseases, autoimmune disorders, mitigating cellular ROS using phytochemicals is considered a viable chemopreventive strategy. While lower doses of phenolic compounds mitigate cellular ROS, the higher doses can act as pro-oxidants and promote oxidative stress, which ultimately induce apoptosis thereby killing cancer cells (Anantharaju et al., 2016).

Induction of apoptosis is one of the key aspects of anti-cancer agents and anti-cancer agent rich phytochemical fractions (Katdare et al., 1998; Surh, 2003). Apoptosis is in general categorized in to early and late stage events (Hengartner, 2000). Whereas the early apoptosis is characterized by changes in plasma membrane, mitochondrial signaling and activation of caspase-3 signaling, the late stage apoptosis is marked by damage to plasma membrane, expression of phagocyte recognition molecules, and release of intracellular contents (Wyllie et al., 1980). The most potent extract SGC has induced the release of LDH, a marker for membrane disintegration, and promoted the breakage of DNA as evidenced by elevated number of cells fluorescing red in acridine orange and ethidium bromide staining.

In summary, our data convincingly demonstrated that SGC contains the mixture of phenolic compounds, which elicits cytotoxic effect against most prevalent cancer types by increasing oxidative stress, LDH release and DNA damage. In addition, the exposure of SGC to normal noncancerous cells has none to very minimal cell damage even at a very high concentration, indicated that the cytotoxic effects of SGC is specific to cancer cells. Base on these observations, it is warranted that further studies should focus on evaluating the SGC fraction in appropriate preclinical models and pave the scientific advancement of anticancer potentials of SGC.

Declarations

Author contribution statement

Asha Jose: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Elango Kannan, SubbaRao V. Madhunapantula: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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