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Cancer selective cytotoxicity of *Sida acuta* extracts on *Artemia salina* and human breast adenocarcinoma cells

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ABSTRACT

Cancer is one of the dreaded diseases and is the foremost basis of morbidity and death worldwide. The interests in the use of plants or plant-derived compounds are increasing recently due to their promising results in chemoprevention. The present study investigates the anti-cancer potential of *Sida acuta*, a traditionally well-known medicinal plant. Accordingly the methanol (SAM) and aqueous (SAA) extracts of *Sida acuta*, were examined against *Artemia salina* nauplii for toxicity and on human breast adenocarcinoma cell lines (MDA-MB-231 and MCF-7) for cytotoxic and apoptotic properties. Both the extracts, SAM and SAA exhibited higher toxicity towards *Artemia salina*. Interestingly, the extracts exhibited minimal cytotoxicity in normal cells (VERO) than in human breast cancer cells (MDA-MB-231 and MCF-7). The highly active SAA effectively induced apoptosis in both the cells (MDA MB 231 and MCF-7) showing 17.81% and 4.27% of late apoptotic cells and 27.14% and 37.32% of early apoptotic cells, respectively. Most of the drugs being developed from plant sources had landed successfully in clinical trials. In conclusion, the observations clearly suggest that SAA may have possible therapeutic potential against human breast cancer-derived diseases specifically against ER-positive breast cancer.

KEYWORDS: *Sida acuta*, *Artemia salina*, human breast cancer, MDA-MB-231, MCF-7, apoptosis, chemoprevention.

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INTRODUCTION

Cancer, standing second to the cardiovascular disease, is one of the deadliest diseases that devour human life globally. The anticancer properties of plants have been recognized for centuries. According to WHO, few countries yet predominantly rely on traditional herbal medicines for treating their ailments. Of the available natural resources on the earth, only a small proportion was analysed scientifically for their potent therapeutic effects (Borris, 1996). Officially, the National Cancer Institute (NCI) had reported reproducible anticancer activity for 3000 species among the 35,000 screened plants (Desai *et al.*, 2008). *Sida acuta* belongs to Malvaceae family and has been documented as a common weed plant. It was well known for its medicinal uses. A decoction of the whole plant has been used as a treatment for fevers. The plant leaves were also believed to have anti-inflammatory potential. Alkaloids (cryptolepine, ephedrine and vasicine), saponosides, coumarins, steroids, phenolic compounds such as scopoletin, loliolid, 4-ketopinoresinol, evofolin-A and B, polyphenol, sesquiterpene, tannins and flavonoids were the few phytochemical compounds identified in this plant (Benjumea *et al.*,2016). Moreover, *S. acuta* was previously reported for its activity against BT-549 breast adenocarcinoma (Fadeyi *et al.*, 2013) and human hepatoma cells (HepG-2) (Pieme *et al.*, 2010). Based on the available literature evidence, the anticancer efficacy of *S. acuta* extracts (aqueous and methanol) were evaluated further in detail to support available literature.

MATERIALS AND METHODS

Plant Materials

The healthy plants of *S. acuta* were gathered from its natural habitat from Indur village of Dharmapuri district (Tamil Nadu, India). A voucher specimen (GACDPISA1) was preserved in the laboratory as reference. The whole plants were dried in shade and coarsely powdered in a mechanical mixer-grinder.

Preparation of Extracts

The plant powder obtained was subjected to hot extraction using a soxhlet apparatus with methanol and water as solvents in the

ratio of 1:6 [sample (g): solvent (ml)]. The resulting crude extracts were further concentrated to dryness in a rotavapor R-215 (BUCHI Labortechnik AG, Switzerland) under reduced pressure (72 mbar) at 40 °C. Further, the crude extracts were stored at room temperature in a vacuum desiccator until further use. The extracts were named as *Sida acuta* aqueous (SAA) and *Sida acuta* methanol (SAM).

Brine Shrimp Lethality Assay (BSLA)

Brine shrimp, *Artemia salina* nauplii was used as a preliminary model organism to evaluate the toxic effects of the plant extracts (Meyer *et al.*, 1982). The eggs of the shrimp were obtained from STORI Salt Lake *Artemia* Cysts, Fish Cave Seller, Howrah, WB, India and kept at 4 °C in a refrigerator. The *A. salina* cysts were hatched by sprinkling 1 g of the cysts into a glass container (1 L capacity) containing sterile artificial sea water (38 g of sea salt is dissolved in 1 litre of sterile water) and incubating them for 24 hrs under strong aeration and continuous light regime at room temperature (25-29°C). The freshly hatched pink-coloured free-swimming nauplii were collected in a small container from the bottom of the hatching vessel and used for the bioassay. Different dilutions of the plant extracts were prepared by dissolving 20 mg of the methanol and aqueous plant extracts in 2 mL of suitable solvents (stock solution) and further diluted to give a series of concentrations such as 1000, 100, 10, and 1ppm (µg/ml) (Mclaughlin & Rogers, 1998).

The experiment was performed by adding 0.5ml of the chosen concentration of the plant extracts with 4.5 ml of sterile seawater in a watch glass and left undisturbed for 24hrs under constant illumination of florescent lamp. After 24hrs, using a hand lens, the numbers of dead and alive nauplii were recorded. If the larvae didn't display any movement during several seconds of observation, it is considered as dead. The experiments were conducted in triplicates for each dose along with a solvent and a negative control. Percentage of mortality was calculated by the following formula: = No. of dead nauplii/ Total No. of nauplii×100. Probit regression analysis was used to calculate LC_{50} values (Finney, 1971) at 95% confidence intervals.

Cell lines and maintenance

The cell lines (MDA-MB-231, MCF-7 and VERO) were obtained from National Centre for Cell Sciences, Pune, India and maintained in their respective medium with 10% fetal bovine serum [MDA-MB-231 cells in L-15 (Leibovitz's) culture medium without 5% CO_2; MCF-7 and VERO cells in Minimum essential medium (MEM) (Eagle) with Non-essential amino acids and 5% CO_2] in a humidified atmosphere at 37 °C. The cell lines were maintained with regular passaging in their growing phase at 70% confluency.

Cytotoxicity Assay

This study used the MTT assay (Mosmann, 1983) with slight modifications to study the cytotoxicity of the selected plant extracts. Briefly, the cells were seeded in a 96- well plate at a concentration of 10,000 cells from column 2-11 and each well were made to 200 µl with respective fresh medium. Columns 1 and 12 were added with 200 µl of culture medium alone. The plate was sealed from all sides and was kept for incubation at 37 °C in a humidified atmosphere. After the incubation period of 24 hours the existing culture media was decanted and 200 µl of media containing different concentrations of plant extracts (12.5, 25, 50, 100, 200 µg/ml) were added. After 24 hours, the extract medium was removed and fresh medium was added along with 10 µl MTT reagent to achieve a final concentration of 0.45 µg/ml. The plate was then covered with aluminium foil and incubated at 37 °C for 3-4 hours. After incubation, the MTT medium was removed and each well was added with 100 µl of Solubilization solution (DMSO) to dissolve the formazan crystals. Absorbance was read at 570 nm in a Microplate Reader (Dynex Opsys MRTM, Dynex Technologies, VA, USA) having 630 nm as reference filter. The wells comprising cells without treatment served as the control. Percentage cytotoxicity of the extracts was calculated by using the formula: $A_c - A/A_c \times 100$. Where, A_c is the Absorbance of control cells and A is the absorbance of the extract treated cells. The linear regression equation ($Y = Mx + C$) is used to calculate the IC_{50} value. Here, Y = 50, M and C values were derived from the viability graph.

Apoptosis Assay

The apoptosis inducing ability of the extract (SAA) was analysed by Annexin-V/PI Flow Cytometry. The cells, MDA-MB-231 and MCF-7 were seeded in a 6-well plate at a density of 3 x 10^5 cells/2 ml and incubated at 37°C in a CO_2 incubator. The existing medium was removed after 24 hours and the cells were treated with required concentration of the plant extract (IC_{50} value obtained from MTT assay). The medium in the control wells were replenished by fresh medium without extract. After the treatment hours, the cells were harvested directly in to the polystyrene centrifuge tubes (12 x 75 mm) after treating with trypsin-EDTA solution (200 µl) and centrifuged at 300 x g at 25°C. The cells were washed twice with PBS and 5 µl of FITC Annexin-V was added. Further, gently vortexed and incubated in dark at RT (25°C) for 15 minutes. Subsequently, the cells were treated with 5 µl of PI and 400 µl of 1X Binding Buffer and analysed immediately using BD FACS Calibur (San Jose, CA, USA) after gentle vortex. Camptothecin (Cat No: C9911, Sigma) was used as a standard control.

Statistical Analysis

All the experiments were carried out in triplicates. For each assay, data were presented as mean ± SD from three independent experiments ($n = 3$). One-way ANOVA was used to perform all the statistical analyses and significant differences between groups were determined at $P < 0.05$. The results were analysed using correlation to evaluate relationships between experimental parameters and Student's t-test ($P < 0.05$) is used to test the significance. Statistical and graphical evaluations were done using Microsoft Excel 2007 (Roselle, IL, USA), GraphPad Prism 5.0 (San Diego, CA, USA) and MATLAB ver. 7.0 (Natick, MA, USA).

RESULTS

Yield of Extracts

The whole plant powder (50 gm) of *S. acuta* up on hot extraction with methanol yielded 9 gm (percentage extract yield: 18% of dry weight) of crude extract (SAM). Similarly, sequential hot aqueous extraction of the powder yielded 8 gm (percentage extract yield: 16% of dry weight) of crude extract (SAA).

Brine Shrimp Lethality Assay

Both aqueous and methanol extracts of *S. acuta* exhibited mortality against the treated *Artemia* nauplii (Table 1 and 2). Figure 1 & 2 depicts the percentage mortality versus concentration of plant extracts for *S. acuta* whole plant aqueous and methanol extracts.

Cytotoxicity Assay

In this study, two extracts (SAA & SAM) were evaluated using MTT assay to check the cytotoxicity on MDA-MB-231, MCF-7 and VERO cell lines (Figure 3). SAA exhibited lower IC_{50} values, 50.05 and 22.07 µg/ml when compared to SAM, 184.93 and 94.81 µg/ml in MDA-MB-231 and MCF-7 cells respectively.

Apoptosis Assay

In the cytotoxicity analysis, SAA exhibited maximum cytotoxicity on the cell lines tested. Hence, it was subjected to cell cycle analysis on MDA-MB-231 and MCF-7 cells. Accordingly, the Annexin-V/PI expression analysis of SAA against MDA MB 231 (Figure 4) and MCF-7 cells (Figure 5) showed

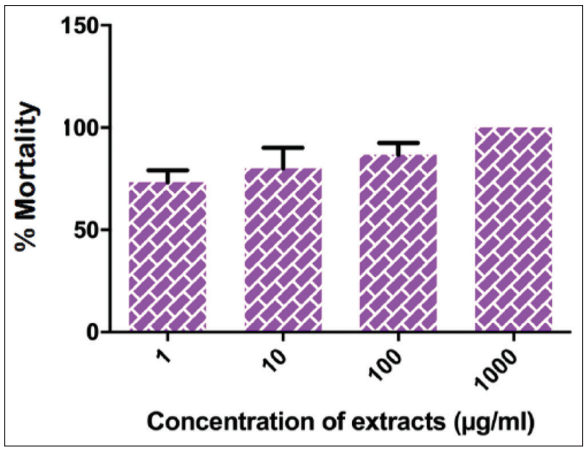

Figure 1: Percentage mortality of *Sida acuta* whole plant aqueous extract (SAA) on *A. salina nauplii*

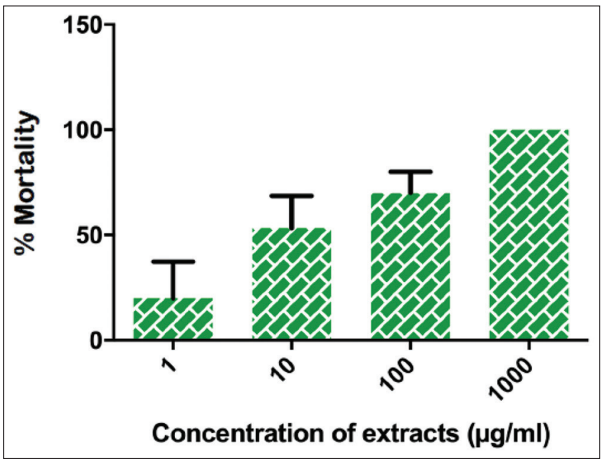

Figure 2: Percentage mortality of *Sida acuta* whole plant methanol extract (SAM) on *A. salina nauplii*

Table 1: Percentage mortality of shrimp nauplii after 24h post exposure to the *Sida acuta* whole plant aqueous extract (SAA)

Sl. No.	Conc. of Plant extract (ppm or µg/mL)	Log C Conc.	Number of Surviving Nauplii after 24 hrs (No. of nauplii taken in each trial=10)			Mean±SD	% Mortality	Probit % Mortality	LC_{50}
			T1	T2	T3				
1	1	0	2	3	3	2.67±2.3	73	5.61	0.75±1.6 µg/ml
2	10	1	3	2	1	2.00±3.2	80	5.84	
3	100	2	1	2	1	1.33±0.5	87	6.13	
4	1000	3	0	0	0	0.00±0.0	100	0	

*T=Trial; There is no probit value for 0% and 100%

Table 2: Percentage mortality of shrimp nauplii after 24h post exposure to the *Sida acuta* whole plant methanol extract (SAM)

Sl. No.	Conc. of Plant extract(ppm or µg/mL)	Log C Conc.	Number of Surviving Nauplii after 24 hrs (No. of nauplii taken in each T=10)			Mean±SD	% Mortality	Probit % Mortality	LC_{50}
			T1	T2	T3				
1	1	0	10	7	7	8.00±0.50	20	4.16	40.97±10.63 µg/ml
2	10	1	6	5	3	4.67±0.50	53	5.08	
3	100	2	3	4	2	3.00±0.50	70	5.52	
4	1000	3	0	0	0	0.00±0.0	100	0	

*T=Trial; There is no probit value for 0% and 100%

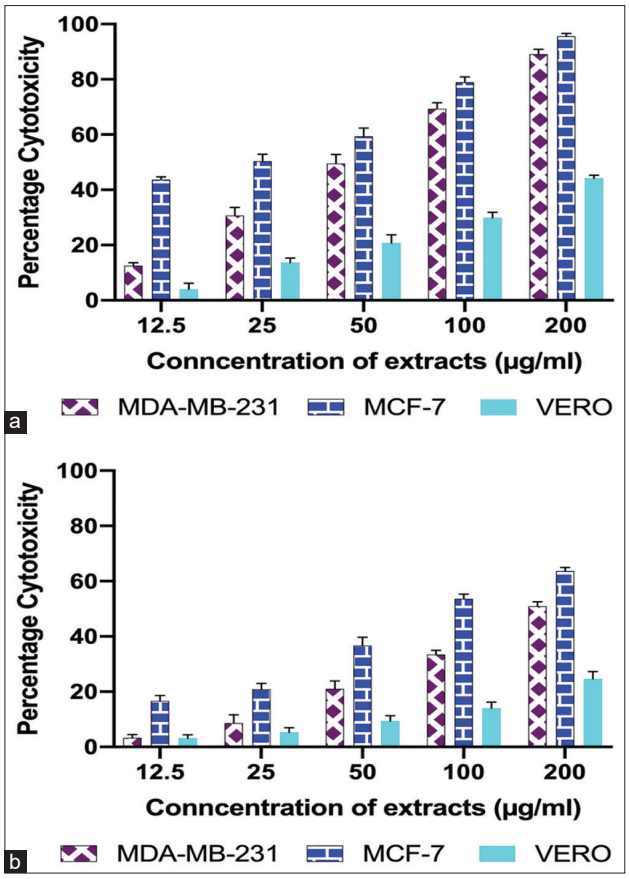

Figure 3: Cytotoxicity of SAA (a) and SAM (b) on MDA-MB-231 and MCF-7 breast adenocarcinoma cells, and VERO normal cell lines

17.81% and 4.27% of late apoptotic cells (UR) and 27.14% and 37.32% of early apoptotic cells (LR) respectively (Table 3).

DISCUSSION

Herbal medicines are used for years as the primary source of medical treatment in developing countries. They were well known for their natural antiseptic properties, leading to increased interest for their use in chemotherapy (Sivaraj *et al.*, 2014). World Health Organisation had estimated in 2007 that the drug trade including plant sources were worth US$100 billion. The trade was predicted to reach US$5 trillion by 2050 (Rao *et al.*, 2007). Brine Shrimp Lethality Assay is a simple and preliminary assay utilized in the natural products research and is expediently utilized to assess the cytotoxicity of the plant extracts (Meyer

Table 3: Annexin V-FITC and Propidium Iodide expression analysis on MDA-MB-231 and MCF-7 cells upon SAA treatment

Quadrant	% of Necrotic Cells	% Late Apoptotic Cells	% Viable Cells	% of Early apoptotic cells
Label	UL	UR	LL	LR
		MDA-MB-231		
Cell Control	0	0.83	98.69	0.48
Std Control	0.89	32.11	41.55	25.45
SAA	0.78	17.81	54.27	27.14
		MCF-7		
Cell Control	0.07	0	99.68	0.25
Std Control	6.13	15.07	50.3	28.5
SAA	0.7	4.27	57.71	37.32

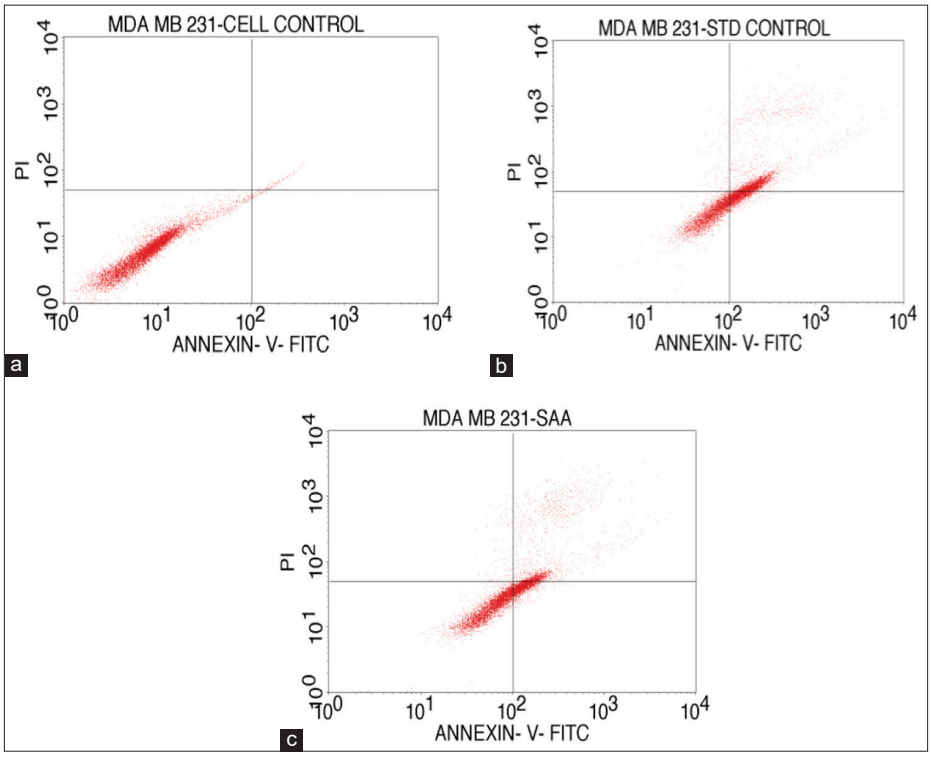

Figure 4: Quadrants showing the untreated (a), Std Control (b) and SAA treated MDA MB 231 cells expressing Annexin V-FITC and Propidium Iodide stains analysed using BD FACS calibur, Cell Quest Pro Software (Version: 6.0)

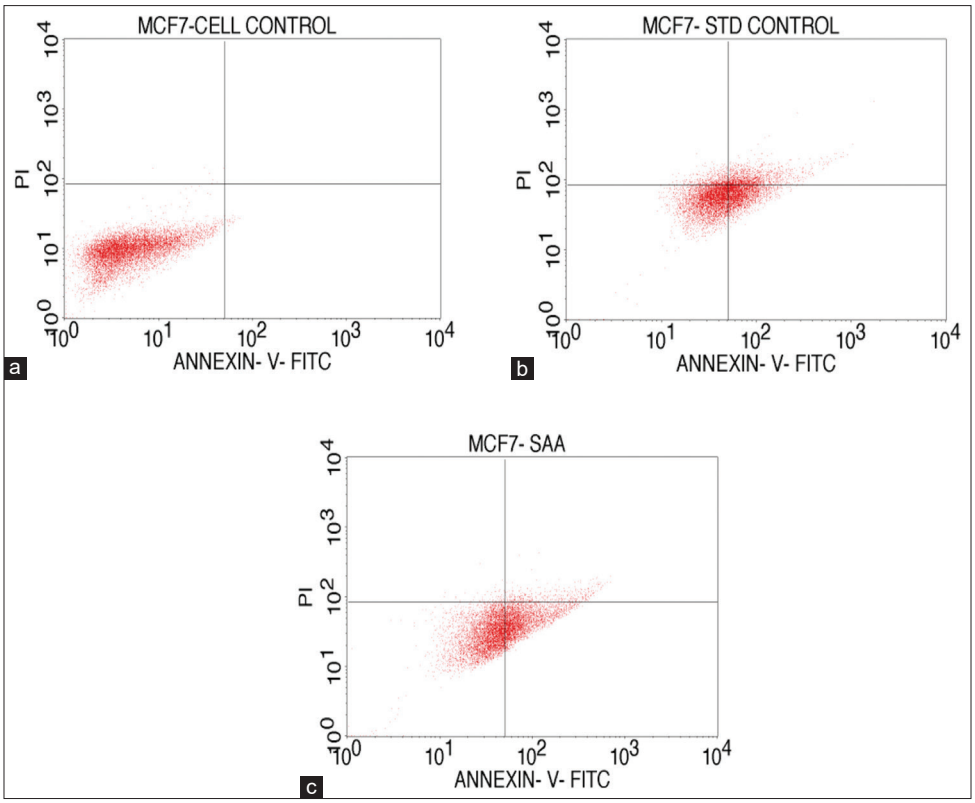

Figure 5: Quadrants showing the untreated (a), Std Control (b) and SAA treated MCF-7 cells expressing Annexin V-FITC and Propidium Iodide stains analysed using BD FACS calibur, Cell Quest Pro Software (Version: 6.0)

et al.,, 1982). The advantages of this assay are rapidness, easiness and minimum requirements (Hamidi *et al.*, 2014). As stated by Meyer et al. (1982), LC_{50} values less than 1000 µg/mL, is considered to be toxic and if it is greater than 1000 µg/mL it is said to be non-toxic. The criterion for the toxicity assessment of plant extracts mentioned by Clarkson et al. (2004), denotes that the LC_{50} values between 0 - 100 µg/ml are highly toxic, 100 - 500 µg/ml are medium toxic, 500 - 1000 µg/ml are low toxic and above 1000 µg/ml is non-toxic. Both extracts tested in this study exhibited high toxicity towards A. *salina* nauplii with LC_{50} values of 0.75 ± 1.6 µg/ml and 40.97 ± 10.63 µg/ml for aqueous and methanol extracts respectively. According to National Cancer Institute (NCI), America the cytotoxicity criteria is an IC_{50} < 30 µg/ml for the crude plant extracts in the preliminary test (Suffness & Pezzuto, 1991). SAA had lower IC_{50} values in MDA-MB-231 and MCF-7 cells, 50.05 and 22.07 µg/ml, close to the reported values by NCI when compared to SAM 184.93 and 94.81 µg/ml. Earlier studies on S. *acuta* demonstrated moderate cytotoxic activity with IC_{50} values of 41.1, 42.3 and 37.1 µg/ml against BT-20, JURKAT and PC-3 cells respectively (Fadeyi *et al.*, 2013). S. *acuta* was also reported to inhibit the proliferation of HepG-2 cells (human hepatoma) by 51.62% at 250 µg/ml (Pieme *et al.*, 2010). However, its effect on breast adenocarcinoma cells has not yet been reported.

Apoptosis is a programmed cell suicide in which cell death naturally occurs during tissue turnover and helps to maintain homeostasis (George *et al.*, 2012). The induction of cytotoxicity by a plant extract alone may not be sufficient to support the existence of anticancer activity. Apoptosis induction is considered as a proof-of-concept approach for identifying a chemopreventive compound and this has always been the accepted approach for exterminating cancer cells (Kumar *et al.*, 2012). Most antineoplastic drugs follow this kind of strategic action (Shawi *et al.* 2011; Hasan *et al.*, 2011). Flow and image cytometry is a versatile technique through which most of the classical hallmarks of apoptosis can be scrutinized. Hence this technique has become the choice for diverse studies of cellular demise (Wlodkowic *et al.*, 2009). The apoptotic cells can be studied by utilizing a commonly used Annexin V/ PI staining method (Cornelissen *et al.*, 2002). Propidium iodide (PI) in conjunction with Annexin V is extensively used to determine whether the cells are viable or apoptotic or necrotic by observing the differences in the integrity and permeability of the plasma membrane (Vermes *et al.*, 1995; Vermes *et al.*, 2000). Hence this technique is utilized in this study to examine the efficacy of SAA extract to induce apoptosis. The cytotoxicity results of S. *acuta* aqueous extract was well attributed by the Annexin V/PI flow cytometry analysis. SAA was found to induce apoptosis successfully in MDA-MB-231 and MCF-7 cells. These results were satisfactory in comparison to the standard. Moreover, the results suggest that the extract was more efficient in inducing apoptosis in MCF-7 (ER-positive) cells and less efficient in MDA-MB-231(ER-negative) cells comparatively.

CONCLUSION

The study concludes that the extracts exhibited concentration dependent toxicity towards the Brine shrimp, A. *salina* nauplii

where the extracts were able to kill all the napulii in its highest concentration. In cytotoxicity studies, the extracts exhibited lower cytotoxicity in normal cells than in cancer cells and were able to induce apoptosis in both the cancer cells examined. Most of the drugs being developed from plant sources had landed successfully in clinical trials. Most importantly their specific cytotoxicity towards cancer cells and non-toxic effects on normal cells put them in high demand. The observations clearly suggest that SAA may have possible therapeutic potential against human breast cancer and specifically against ER-positive breast cancer.

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