

Srinivasan Sowmyalakshmi · Mohammad Nur-e-Alam  
Mohammed A. Akbarsha · Subbiah Thirugnanam  
Jürgen Rohr · Damodaran Chendil

## Investigation on *Semecarpus Lehyam*—a *Siddha* medicine for breast cancer

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**Abstract** The use of complementary and alternative medicines for breast cancer patients has been increasing every year. Traditional Indian systems of medicine, such as *Siddha*, have been reported to benefit patients in India through herbal interventions for cancer. One such herbal medicine is *Semecarpus Lehyam* (SL), and this study aims at providing a scientific basis for the anti-tumor property of SL with respect to breast cancer. SL was subjected to serial extraction with four organic solvents of increasing polarity (*n*-hexane, chloroform, ethyl acetate, *n*-butanol and water). The solvents from all fractions were removed, dried and dissolved in dimethyl sulfoxide for testing their anti-tumor activity against two breast cancer cell lines, MCF-7 [estrogen receptor (ER)-positive] and MDA 231 (ER-negative) using cell viability and apoptosis assays. The most potent SL fractions were also combined with radiation and doxorubicin to determine the radio- and chemo-sensitizing effects of SL on these breast cancer cell lines. In terms of cytotoxicity as well as induction of apoptosis, the *n*-hexane and chloroform fractions of SL were more significantly active against MDA 231 cells than MCF-7 cells. The *n*-butanol fraction of SL showed some activity against

MCF-7 cells. When combined with radiation or doxorubicin, the *n*-hexane and chloroform fractions enhanced the radio-sensitivity (11.8-fold) and chemosensitivity (6.5-fold) of MDA 231 cells. This study demonstrated SL to be a potent anti-tumor agent against the ER-negative breast cancer cell line. The study is also the first step in the scientific validation of SL for use against breast cancer, particularly the ER-negative type.

**Keywords** Apoptosis · Breast cancer · Chemo sensitization · Complementary and alternative medicines · Herbal medicine · Radio sensitization

**Abbreviations** CAM: Complementary and alternative medicine · DMSO: Dimethyl sulfoxide · ER: Estrogen receptor · MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide · SL: *Semecarpus Lehyam*

S. Sowmyalakshmi · D. Chendil (✉)  
Department of Clinical Laboratory Sciences,  
College of Health Sciences,  
University of Kentucky, Room No. 209D,  
900 South Limestone Street, Lexington,  
KY 40536-0200, USA  
E-mail: dchen2@uky.edu  
Tel.: +1-859-323-1100x80851/80879  
Fax: +1-859-257-2454

M. Nur-e-Alam · J. Rohr  
Division of Pharmaceutical Sciences, College of Pharmacy,  
University of Kentucky, Lexington,  
KY 40536-0200, USA

M. A. Akbarsha  
Department of Animal Science, Bharathidasan University,  
Tiruchirappalli 620024, India

S. Thirugnanam  
Siddha Practitioner, Tiruchirappalli 620024, India

### Introduction

World wide, breast cancer is the most common cancer that affects women. It is estimated that one out of eight women is at risk of developing the disease during her lifetime (Greenlee et al. 2001). The most commonly used breast cancer treatment modalities are surgery, radiation, adjuvant chemotherapy and hormone therapy. However, these treatments are associated with increased risk of secondary cancers that may manifest several years after treatment (Matesich and Shapiro 2003). Although mortality rates in breast cancer patients have been decreasing in view of earlier detection and adjuvant therapies, once metastases develop the disease is incurable. In addition, the currently practiced treatments cause myelosuppression and immunosuppression, and also other toxic side effects such as nephrotoxicity and neurotoxicity (Vayalil et al. 2002). The biology of breast cancer is very complex; estrogen receptors (ERs) play a

major role. Though anti-estrogen therapy has shown some promising outcome, it is limited only to ER-positive breast cancer. Hence, the treatment of ER-negative breast cancer still remains a challenge. Apoptosis is an essential regulatory mechanism, and an imbalance between mitosis and apoptosis has been associated with malignant tumors (Cohen 1993). Development of therapeutic strategies is needed to induce apoptosis as well as to control cell proliferation and invasiveness in advanced stages of breast cancer.

The use of complementary and alternative medicine (CAM) for breast cancer patients has increased in the last decade (Boon et al. 2000). Reports indicate that 63–83% of breast cancer patients use at least one form of CAM during or after the conventional treatments (Ernst and Cassileth 1998). The use of CAM is increasing in women with breast cancer, especially in patients who have already undergone chemotherapy (Lengacher et al. 2002). The most commonly used CAM therapies are dietary counseling (94%), botanical medicines (88%), antioxidants (84%) and supplementary nutrition (84%; Standish et al. 2002). The objectives of CAM treatment are lower toxicity, improvement in cancer-related symptoms, stimulation of the immune system and even a direct anticancer effect (Tagliaferri et al. 2001). The Indian systems of medicine, *Ayurveda* and *Siddha*, which originated several centuries ago, are holistic approaches to health. These medicinal systems have identified more than 700 individual herbs, as well as several complex herbal drug preparations, as useful in the treatment and/or prevention of diseases including cancer, and improvement of quality of life of both healthy and diseased individuals (Joseph et al. 1999; Premalatha 2000; Diwanay et al. 2004). It is a continuing challenge to design a therapy which is both effective and also has a high specificity for the biology of a specific cancer and/or is efficiently targeted to tumor tissue. Our laboratories are engaged in the discovery and characterization of phytochemicals that may be useful in the treatment of breast cancer.

*Semecarpus Lehyam* (SL), an herbal medicine in the *Siddha* tradition, is currently in practice as an effective CAM therapy for breast cancer in the southern parts of India (Veeraperumal Pillai). SL contains six herbals that are formulated into a paste with butter and palm sugar (Table 1). Scientific investigations on five of these herbals, *Semecarpus anacardium* (Indap et al. 1983; Phatak

et al. 1983; Smit et al. 1995; Premalatha 2000; Premalatha and Sachdanandam 2000; Sujatha and Sachdanandam 2002), *Strychnos nux vomica* (Salomi et al. 1992; Smit et al. 1995; Cai et al. 1998), *Nigella sativa* (Nair et al. 1991; Salomi et al. 1991; Farah and Begum 2003), *Smilax chinensis* or *S. china* (Lee et al. 2001) and *Plumbago zeylanica* (Krishnaswamy and Purushothaman 1980; Parimala and Sachdanandam 1993; Naresh et al. 1996; Prasad et al. 1996), have shown them to possess anti-tumor activity. As far as *Strychnos potatorum* is concerned, there is no scientific literature on its therapeutic use in cancer, but it is a plant listed in *Ayurveda* and *Siddha* pharmacopoeia.

We intended to test SL for scientific validation of its use in breast cancer therapy. This study was undertaken with two aims:

1. *Ayurveda* and *Siddha* are ancient medicinal systems, which are based on traditional, non-Western theories and philosophy (Ali 1998; Ashok and Ali 2003), so, we aimed at finding the therapeutic efficacy of this herbal remedy from Western medical perspectives. The major concerns about the formulated herbal medicines are quality control, with special reference to reproducibility of the results, and multiplicity of the phytochemicals. Hence, our study focused on breaking down the herbal mixture into a smaller number of phytochemicals, by extracting it in different solvents, and determining the most potent fraction(s) that would work against breast cancer.
2. Since many breast cancer patients use herbal medicines as complementary therapy along with the conventional treatments, we aimed at finding if SL fractions would enhance the radio- and/or chemosensitivity of the breast cancer cells. Thus, a preliminary investigation on SL was carried out in ER-positive and ER-negative breast cancer cell lines using SL fractions.

Although significant inhibition of growth and induction of apoptosis were found in both breast cancer cell lines, SL fractions inhibited the growth more significantly in ER-negative cells than ER-positive cells. In addition, SL fractions enhanced the radio-sensitivity (11.8-fold) and chemo-sensitivity (6.5-fold) of ER-negative cells.

## Materials and methods

### Extraction of SL and generation of fractions

Four hundred and eighty eight grams of SL paste was extracted with methanol. The methanolic extract was evaporated to dryness, yielding a solid gum-like residue (156.3 g), which was then suspended in water and extracted with various organic solvents of increasing polarity (starting with the lipophilic solvent *n*-hexane, ending with the more hydrophilic *n*-butanol). In this way, the lipophilic compounds were extracted first, then

**Table 1** Composition of SL ingredients

Ingredients	Weight (g)
<i>Nux vomica</i> seed	70
<i>Strychnos potatorum</i> seed	70
<i>Semecarpus anacardium</i> nut	140
<i>Smilax chinensis</i> root	70
<i>Plumbago zeylanica</i> root bark	70
<i>Nigella sativa</i> seed	105
Ghee	250
Jaggary from cane	1,000

the slightly less lipophilic and so on, and compounds were separated according to their solubility properties. The solvent from each extract was evaporated in vacuo to yield a lyophilized powder: 28.4 g from the *n*-hexane fraction, 5.8 g from the chloroform fraction, 2.5 g from the ethyl acetate fraction, 14.7 g from the *n*-butanol fraction and 102.5 g from the remaining aqueous (water) fraction (Fig. 1). The lyophilized fractions were then dissolved in dimethyl sulfoxide (DMSO) and tested for their anti-tumor activity against the breast cancer cell lines.

#### High-performance liquid chromatography (HPLC)

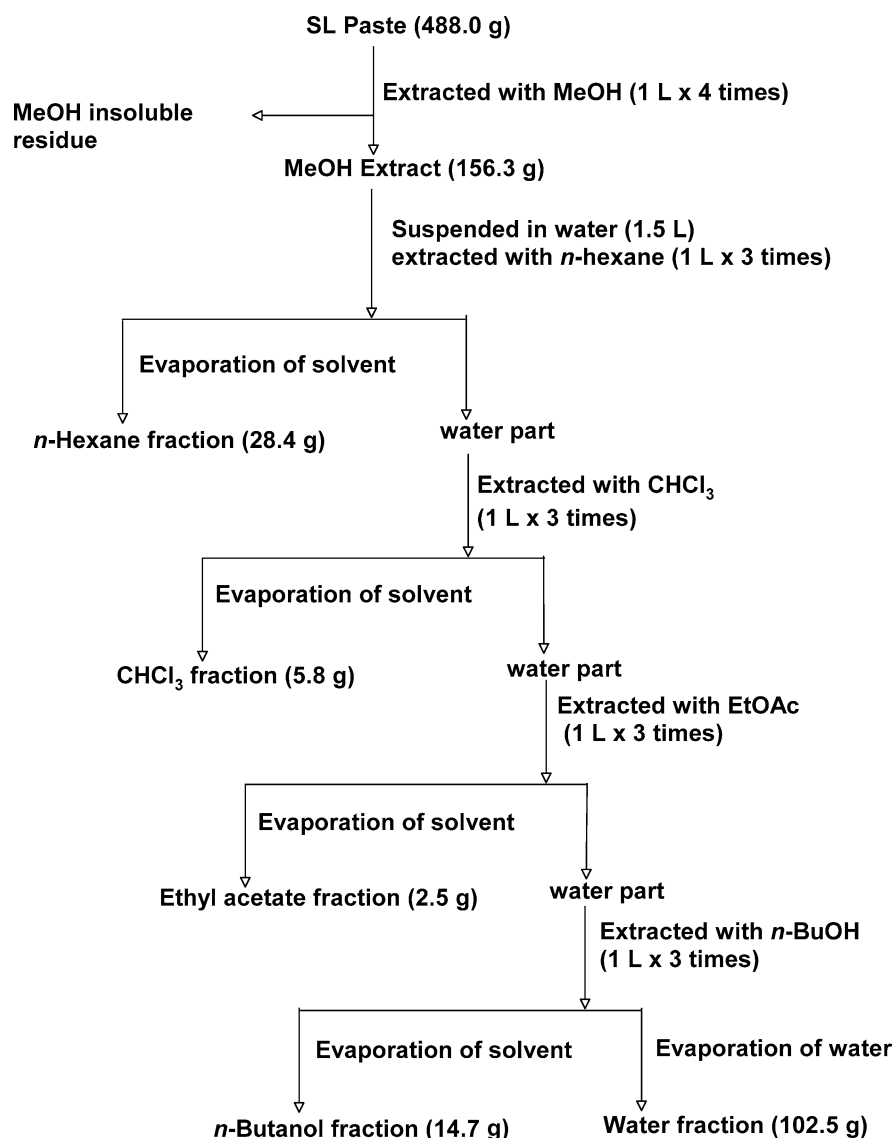
A sample of each the fraction was applied to a 'Waters' RP-18 silica gel column (250 mm long, 4.6 mm i.d.), which was eluted at a flow rate of 1 ml min<sup>-1</sup>, using acetonitrile and water as solvents. The following 1-h-

gradient liquid-phase mixture was used for the elution of all fractions: 100% H<sub>2</sub>O for 1 min, then within 4 min to H<sub>2</sub>O:CH<sub>3</sub>CN=7:3, then within 45 min to H<sub>2</sub>O:CH<sub>3</sub>CN=3:7, and finally within 5 min to 100% CH<sub>3</sub>CN. To prepare for the next run, the system was washed with 100% water for 5 min.

#### Cell culture

Two breast cancer cell lines, MDA 231 (ER-negative) and MCF-7 (ER-positive), obtained from the American Type Tissue Culture Collection, were used for testing the anti-tumor activity of SL extracts. Both the cell lines were maintained and propagated in 90% Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Cells were cultured as monolayers and maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>/95% air.

**Fig. 1** Extraction and solvent partitioning of SL. *BuOH*, *n*-butanol; *EtOAc*, ethyl acetate; *MeOH*, methanol



## Assay of cell viability

MDA 231 and MCF-7 cells were seeded in 96-well plates ( $10^4$  cells/well) in the presence of growth medium and incubated for 24 h for the cells to adhere to the surface of the well. The cells were then treated with different concentrations of the various fractions of SL (*n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water) dissolved in DMSO. Also, to determine the radio-sensitization and chemo-sensitization properties, the potent SL extracts were combined either with radiation (2 Gy and 5 Gy) or with 0.1  $\mu\text{M}$  doxorubicin (Sigma). The plates were incubated for 24 h at 37°C in a humidified atmosphere of 5%  $\text{CO}_2$ , 95% air. Then, 10  $\mu\text{l}$  of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT reagent) was added to each well and the plates were incubated for 4 h, after which 100  $\mu\text{l}$  of solubilizing solution (Roche) was added to each well and the plates were incubated overnight. Absorbance at 570 nm (ref. 650 nm) was then measured using a microplate reader, and the data were used as the measure of metabolically active cells.

## Assay of apoptosis

For analysis of apoptosis, the tumor cells were plated in 25-cm<sup>2</sup> tissue culture flasks ( $1 \times 10^6$  to  $1.5 \times 10^6$  cells/flask), treated as described above for 24 and 48 h, and apoptotic cell death was measured by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated annexin V and propidium iodide (BD Biosciences, San Jose, CA, USA). After the treatments, the cells were collected using trypsin, washed and re-suspended in binding buffer. Then 5  $\mu\text{l}$  of annexin V and 10  $\mu\text{l}$  of propidium iodide were added and the samples were incubated at room temperature for 10 min. They were then analyzed using FACS *Calibur* (Becton Dickinson, San Jose, CA, USA).

## Statistical analysis

The radio-sensitization and chemo-sensitization properties of SL on the breast cancer cell lines were analyzed statistically as described previously (Chendil et al. 2000, 2004; Dey et al. 2003).

## Results

### HPLC analysis of the SL fractions

Most of the activity was found in the *n*-hexane, chloroform and *n*-butanol fractions. HPLC chromatograms (RP-18, acetonitrile–water gradient,  $\text{UV}_{254 \text{ nm}}$ ) of the active fractions (Fig. 2) showed that the ingredients of SL could indeed be separated by such a series of

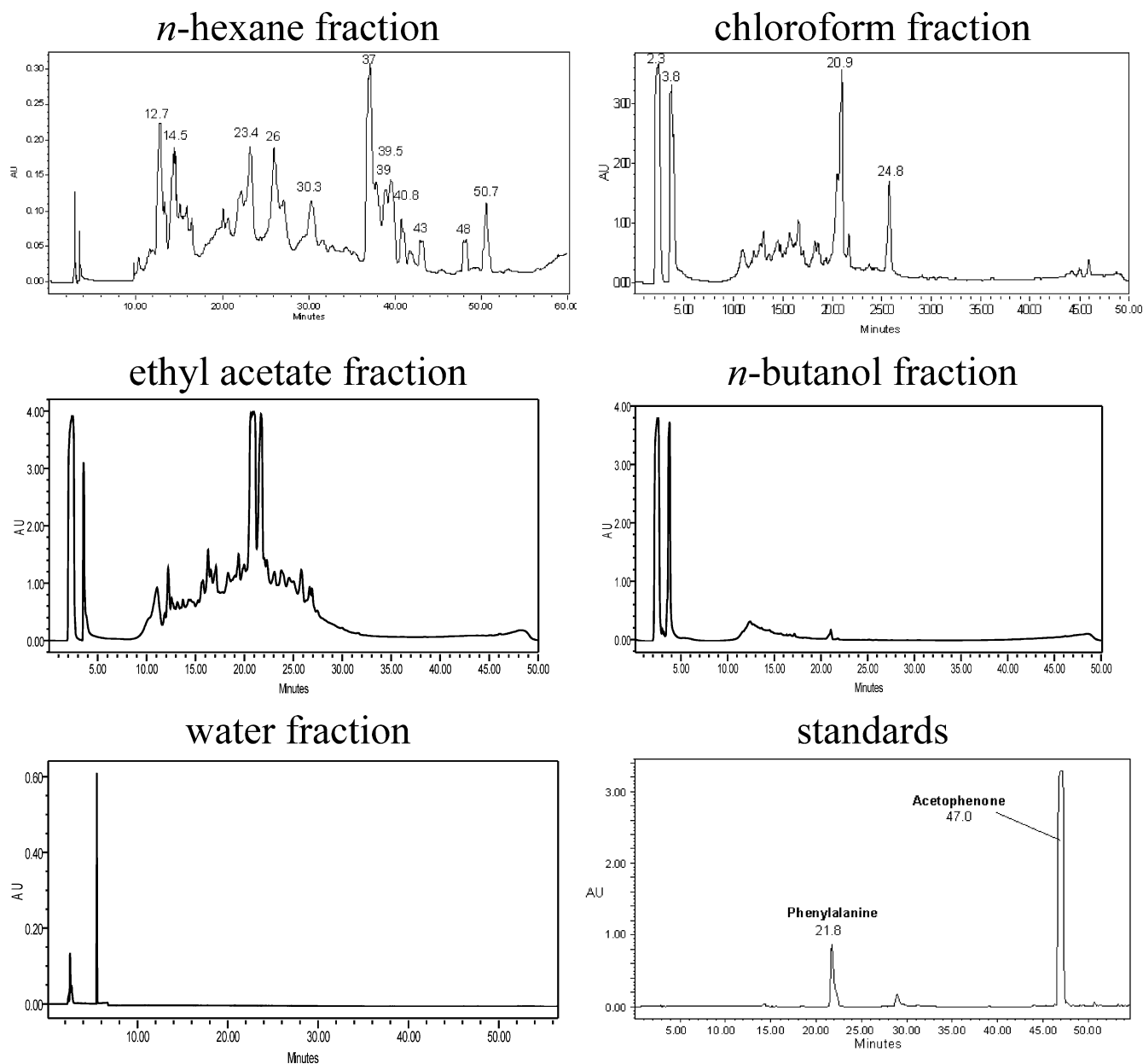
extractions. The most lipophilic extract, i.e. *n*-hexane, showed at least a dozen major compounds (eluting at 12.7, 14.5, 23.4, 26, 30.3, 37, 39, 39.5, 40.8, 43, 48 and 50.7 min), some of which also appeared as minor compounds in the chloroform fraction. Otherwise, the chloroform fraction showed only four major peaks (corresponding to compounds eluting at 2.3, 3.8, 20.9 and 24.8 min). The first two also appeared in ethyl acetate and *n*-butanol fractions. While *n*-hexane, chloroform and ethyl acetate fractions appeared to contain a plethora of diverse compounds, *n*-butanol and water fractions appeared to contain only two major compounds.

### SL-induced cytotoxicity in MDA 231 and MCF-7 breast cancer cell lines

In the MTT assays, MCF-7 cells were sensitive to the *n*-butanol fraction; at 60  $\mu\text{g ml}^{-1}$  it reduced cell viability to 64%, compared with the other four fractions (Fig. 3a). In MDA 231 cells, the *n*-hexane and chloroform fractions (40  $\mu\text{g ml}^{-1}$ ) produced more significant cytotoxic effects than the other three fractions, reducing the cell viability to 2% and 47%, respectively (Fig. 3b), the *n*-hexane fraction being more potent than the chloroform fraction. Interestingly, there was no significant growth inhibition of MCF-7 cells by the *n*-butanol fraction even after 72 h of treatment, whereas the *n*-hexane (10  $\mu\text{g/ml}$ ) and chloroform (20  $\mu\text{g/ml}$ ) fractions of SL reduced the cell viability to 20% and 32%, respectively, in MDA 231 cells at 72 h of treatment (data not shown). Overall, SL produced a more significant cytotoxic effect in the ER-negative breast cancer cell line (MDA 231) than in the ER-positive cell line (MCF-7).

### SL-induced apoptosis in MDA 231 cells

Our earlier studies had indicated that chloroform and *n*-hexane fractions of SL produced a significant cytotoxic effect in MDA 231. Hence, we assayed cells to determine whether the cytotoxic property of SL is brought about through the induction of apoptosis. After 24 and 48 h of treatment of MDA 231 cells with chloroform and *n*-hexane fractions of SL, the incidence of apoptosis over the control (DMSO alone) population was calculated. At 5  $\mu\text{g ml}^{-1}$  the chloroform fraction induced 7% apoptosis at 24 h and 12% at 48 h, whereas at 15  $\mu\text{g ml}^{-1}$  the respective values were 16% and 32.1%. In *n*-hexane fraction-treated cells, the incidence of apoptosis was more than that in chloroform fraction-treated cells (5  $\mu\text{g ml}^{-1}$ : 12% at 24 h, 25% at 48 h; 15  $\mu\text{g ml}^{-1}$ : 29% at 24 h and 62.2% at 48 h; Fig. 4). Thus, the *n*-hexane fraction of SL induced more significant levels of apoptosis than the chloroform fraction in MDA 231 cells.



**Fig. 2** HPLC separation of the *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and water fractions of methanolic extract of SL

### SL-enhanced radio-sensitization and chemo-sensitization effects in MDA 231 cells

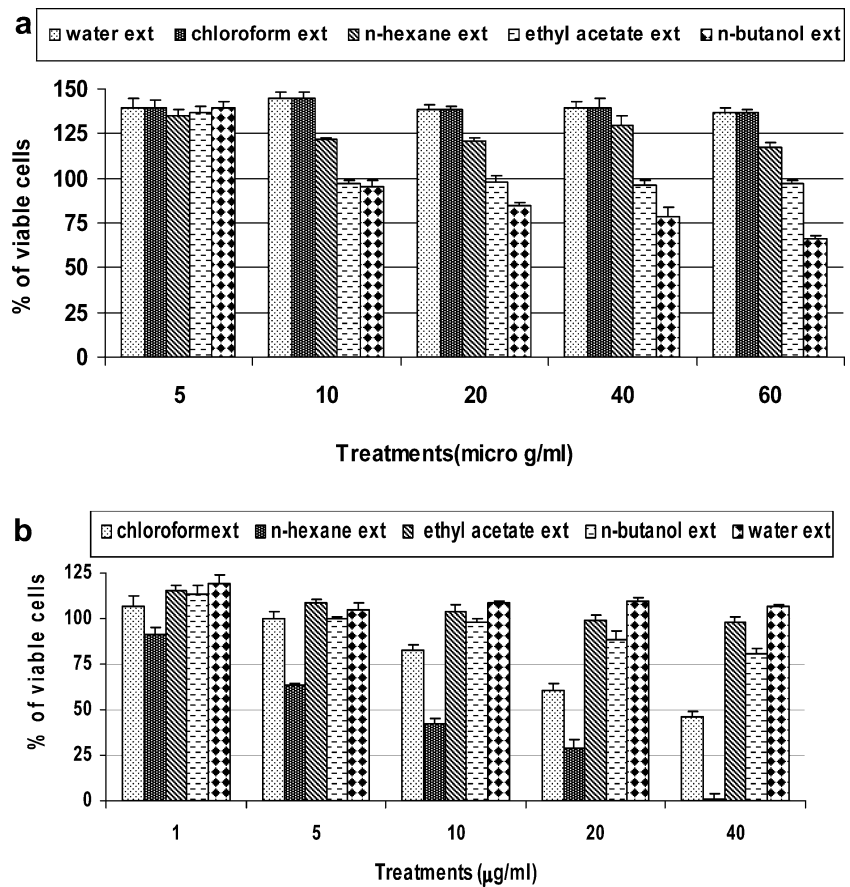
In order to determine whether the SL fractions would enhance radio-sensitivity in the ER-negative breast cancer cell line, different concentrations of chloroform and *n*-hexane fractions of SL were combined with radiation doses of 2 Gy and 5 Gy, and the combined cytotoxic effects were determined using the MTT assay. When compared to radiation alone (2 Gy: 94.5% cell viability; 5 Gy: 83.2%), the combination of SL fractions with radiation enhanced the cytotoxic effect significantly in MDA 231 cells. The chloroform fraction ( $20 \mu\text{g ml}^{-1}$ ) reduced the viability of the cells to 52%,

and the *n*-hexane fraction ( $20 \mu\text{g ml}^{-1}$ ) reduced it further to 12%, in combination with radiation (2 Gy) of MDA 231 cells (Fig. 5a). Similarly, in combination with 5 Gy irradiation, chloroform ( $15 \mu\text{g ml}^{-1}$ ) and *n*-hexane ( $15 \mu\text{g ml}^{-1}$ ) fractions reduced the viability of the cells to 39% and 13.2%, respectively (Fig. 5b).

In order to determine whether SL fractions would enhance chemo-sensitivity of the ER-negative breast cancer cell line, different concentrations of chloroform and *n*-hexane fractions of SL were combined only with a single dose ( $0.1 \mu\text{M}$ ) of doxorubicin, and the cytotoxicity was assayed. Doxorubicin alone ( $0.1$ – $0.5 \mu\text{M}$ ) did not alter cell viability, even at  $0.5 \mu\text{M}$  (Fig. 6a). However, when doxorubicin ( $0.1 \mu\text{M}$ ) was combined with chloroform or *n*-hexane fractions ( $2.5$ – $15 \mu\text{g ml}^{-1}$ ) for 24 h there was a significant cytotoxic effect. The chloroform and *n*-hexane fractions of SL ( $15 \mu\text{g ml}^{-1}$ ) in



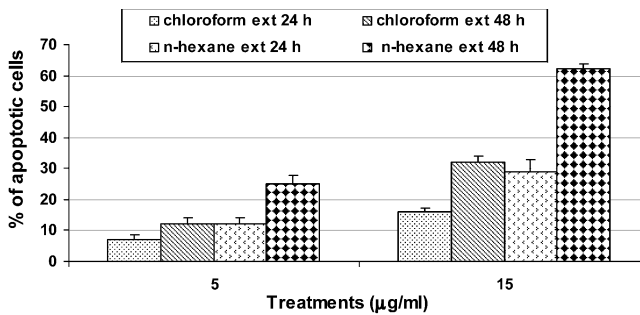
**Fig. 3a,b** Effect of SL fractions (*n*-hexane, chloroform, ethyl acetate, *n*-butanol and water) on breast cancer cell lines. **a** MCF-7 (ER-positive); **b** MDA 231 (ER-negative). The cells were treated with various concentrations of SL fractions for 24 h, and cell viability (expressed relative to the control) was measured by MTT assay. Each data point represents the mean  $\pm$  SE for four wells from three independent experiments



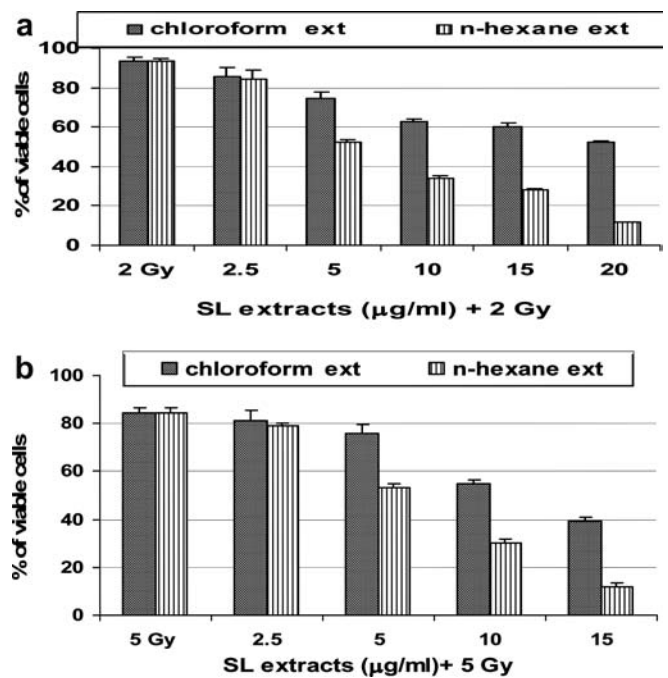
combination with doxorubicin (0.1  $\mu$ M) reduced the number of viable cells to 27% and 18%, respectively, in MDA 231 cells (Fig. 6b). These results demonstrated that SL fractions enhanced the cytotoxic effect of radiation or doxorubicin, in MDA 231 cells.

Enhancement of radiation- and doxorubicin-induced apoptosis by SL fractions in MDA 231 cells

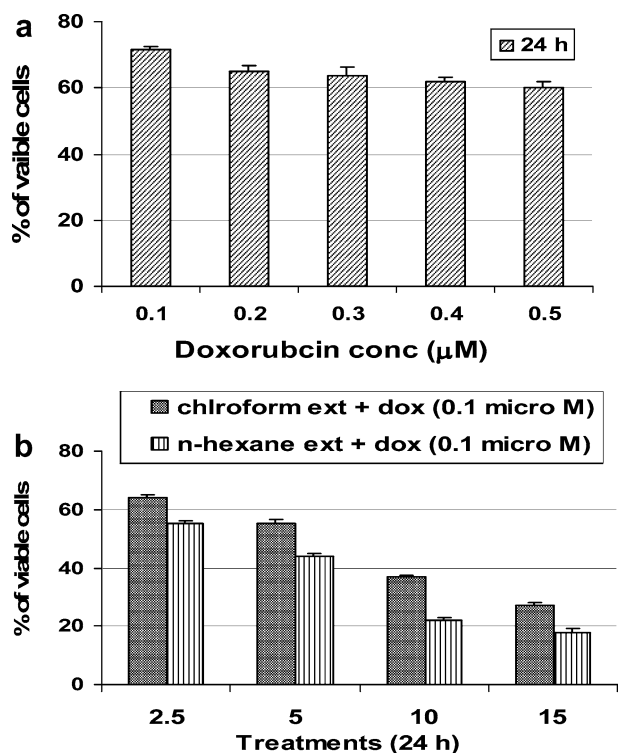
After finding that SL fractions enhance radio- and chemosensitization in MDA 231 cells, we performed apoptotic



**Fig. 4** SL-induced apoptosis in MDA 231 cells. Cells were treated with chloroform and *n*-hexane fractions of SL dissolved in DMSO, and the apoptosis assay was performed after 24 and 48 h of exposure. Bar graph shows the percentage of apoptotic cells. The base-line apoptosis in the untreated group was normalized with data on the treated group. The data shown are representative of the combined means  $\pm$  SE from three independent experiments



**Fig. 5a,b** Radiation-induced cytotoxicity in MDA 231 cells. The cells were treated with the chloroform and *n*-hexane fractions of SL dissolved (suspended?) in DMSO, combined with radiation [2 Gy (a) or 5 Gy (b)] for 24 h, and the cell viability was measured by MTT assay. Each data point represents the mean  $\pm$  SE for four wells

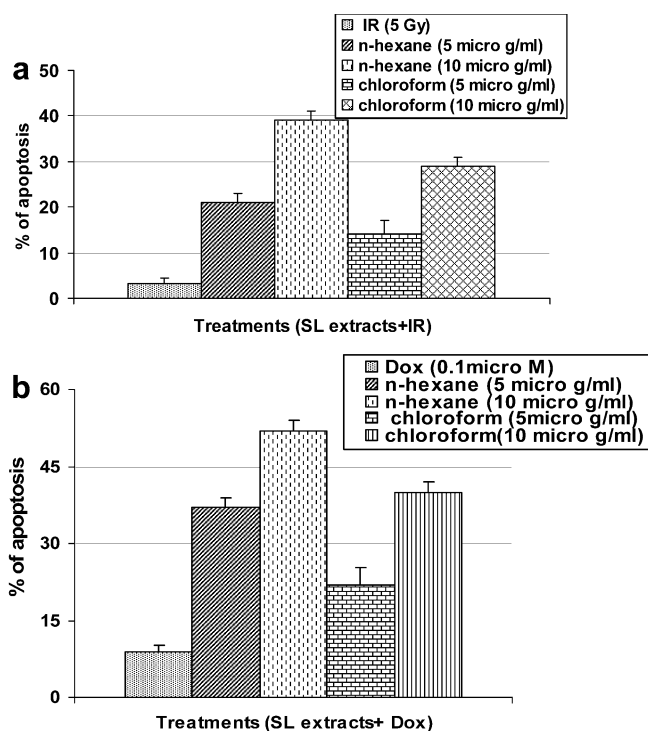


**Fig. 6a,b** Cytotoxicity of doxorubicin alone or in combination with SL extracts, induced in MDA 231 cells. The cells were treated with doxorubicin alone (a) or in combination with the chloroform and *n*-hexane extracts of SL (b), for 24 h, and the cell viability was measured by MTT assay. Each data point represents the mean  $\pm$  SE for four wells

assays adopting the annexin V-FITC method. There was marginal induction of apoptosis by radiation (5 Gy) and doxorubicin (0.1  $\mu$ M) alone, which induced 3.3% and 8% apoptosis, respectively, within 24 h in MDA 231 cells. However, in combination with SL fractions, the induction of apoptosis was enhanced [with radiation: *n*-hexane fraction at 5  $\mu$ g ml<sup>-1</sup> = 21%, at 10  $\mu$ g ml<sup>-1</sup> = 39%, and chloroform fraction at 5  $\mu$ g ml<sup>-1</sup> = 14%, at 10  $\mu$ g ml<sup>-1</sup> = 29% (Fig. 7a); with doxorubicin: *n*-hexane fraction at 5  $\mu$ g ml<sup>-1</sup> = 37%, at 10  $\mu$ g ml<sup>-1</sup> = 52%, and chloroform fraction at 5  $\mu$ g ml<sup>-1</sup> = 22%, at 10  $\mu$ g ml<sup>-1</sup> = 40% (Fig. 7b)]. Thus, SL fractions enhanced both radio-sensitization (up to 11.8-fold) and chemo-sensitization (up to 6.5-fold) in MDA 231 cells, as determined using the apoptotic assays.

## Discussion

Natural plant products have recently gained attention because of their intriguing biological activities, especially for women who prefer alternative therapies. In spite of increasing visits to CAM practitioners by cancer patients, several factors limit CAM research. Among them are insufficient number of patients with the same clinical condition and difficulty in undertaking and interpreting the systematic reviews and methodological issues related



**Fig. 7a,b** Enhancement of radiation and chemotherapy-induced apoptosis in MDA 231 cells by SL fractions. Cells were treated with SL extracts and radiation (5 Gy; a) or doxorubicin (0.1  $\mu$ M; b) and apoptosis was assayed after 24 h of exposure. The data shown are representative of the combined means from two independent experiments

to the patient's response to treatment. Even with these barriers to research, many patients use at least one form of CAM along with the conventional therapy (Giordano et al. 2002).

Although investigation of the use of herbal medicines has gained importance in the recent years, many concerns still persist among clinicians. The major concerns that limit the use of herbal medicines are the quality control of the products, batch-to-batch variability, safety and stability (Fong 2002). For these reasons, in this study, three different batches of SL were subjected to serial extraction, and the HPLC peaks were found to be similar. To further substantiate this point, bioassays (MTT and apoptotic assays) were performed on the fractions from all the three batches, and the results were reproducible. Thus, there was no significant batch-to-batch variability as far as SL was concerned. The possibility of heavy-metal contamination in the herbals was ruled out by the extraction with organic solvents. SL, when stored at room temperature, was stable for a year without degradation. Further, the HPLC peaks (number of peaks as well as intensity of peaks) that were obtained soon after the extraction matched those obtained from a year-old paste. These results demonstrated the stability of SL.

The present study is an attempt to find the efficacy of the formulated drug SL, tested from Western perspective

of extraction with appropriate solvents and in vitro test systems. Since the phytochemicals from different sources are capable of producing synergistic and/or additive effects, the toxic effect, if any, from any one of the ingredients may be neutralized by the effect of the others. The extraction, in addition to rendering it possible to test the crude formulated SL, has formed a working mechanism for the elimination of heavy-metal contamination, if any, which would otherwise produce toxic side effects (Tagliaferri et al. 2001; Sujatha et al. 2000).

The present study indicates that the *n*-hexane and chloroform fractions of SL effectively inhibit MDA 231 cells, and the *n*-butanol fraction inhibits MCF-7 cells in culture. The results of these studies show that the more-lipophilic fractions of SL (*n*-hexane and chloroform) inhibit the growth of the ER-negative breast cancer cell line and a more-hydrophilic fraction (*n*-butanol) inhibits growth of the ER-positive breast cancer cell line. However, ethyl acetate and aqueous fractions do not appear to have any anti-tumor activity. Further, radiation alone was neither cytotoxic nor induced apoptosis in either of the cell lines (MCF data not shown). This is in correlation with a report by Watson et al. (1997) who barely found any detectable radiation-induced apoptosis in MDA 231 and MCF-7 cells. The combination of SL fractions with radiation (2 or 5 Gy) induced the cytotoxic effect and apoptosis in MDA-231 cells, which indicates that SL enhances radio-sensitivity of the ER-negative breast cancer cell line. Similarly, SL fractions are also capable of chemo-sensitizing the ER-negative breast cancer cells that are treated with doxorubicin. These results indicate that SL, apart from having a direct anti-tumor activity by itself, has the potential to enhance the sensitivity of ER-negative breast cancer cells to radiotherapy and chemotherapy.

Estrogens are potent mitogens, and can play a major role in stimulating cell proliferation in hormone-responsive tissues by promoting the G1/S phase transition (Dobrzycka et al. 2003; Keen and Davidson 2003). Though anti-estrogen therapy has shown some promising results, it is limited to ER-positive breast cancers. Hence, ER-positive breast cancers have a better prognosis than ER-negative breast cancers. Yet, high systemic toxicity and drug resistance limit the successful outcome in most cases (Sharma et al. 2004). Therefore, the findings in the present study should be a panacea, particularly for ER-negative breast cancer patients.

Thus, this preliminary study scientifically validates the use of SL, a polyherbal formulation in the *Siddha* tradition, as a CAM for the treatment of breast cancer. However, to bring this efficacious drug into the mainstream of cancer therapy, future studies will focus on the active principles of SL and their modes of action, as well as their possible synergistic or additive effects. The outcome of such studies is expected to lead to the production of an effective anti-tumor agent, based on SL, against breast cancer.

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