systems such as Ayurveda, the Indian System of Medicine, Unani System of Medicine, and Traditional Chinese Medicine.[3] In Ayurveda, it has been used for the treatments of limbs stiffness, snakebites, and chronic bronchitis. In Chinese medicine, it has been used for the treatment of rheumatism, nervous diseases, lumbago, and dropsy.[4] In Unani medicine, seeds were mentioned as tonic and used for the treatment of cancer.[5] Hence, considering the current prevalence of cancer, the purpose of this work was to investigate the anticancer effect of *C. halicacabum* oil and to identify the probable responsible anticancer compounds from this traditional medicine that effects on breast cancer cells growth.

MATERIALS AND METHODS

General

Sulforhodamine B colorimetric (SRB) assay was selected to evaluate the effect of oil fractions on three selected human breast carcinoma cell lines, namely MCF-7, MDA-MB-231, and MDA-MB-435. Four concentrations (10, 20, 40, and 80 µg/mL) were prepared for each test drug and compared with the standard drug of doxorubicin (Adriamycin), as a positive control compound. Each experiment was repeated three times and the means of the data were used. The most active fraction was subjected to analysis by gas chromatography-mass spectrometry (GC-MS) for the identification of present components.

Plant materials

C. halicacabum L. plant with its leafy flowering branches and mature seeds was collected from its natural habitat in Junagadh district of Gujarat, India. Herbarium of the plant was prepared and certified by Raw Material, Herbarium and Museum Division, CSIR-NISCAIR, Delhi, India. Voucher specimen (SU/DPS/263/2014) was kept at the Herbarium Unit of the Department of Pharmaceutical Sciences, Saurashtra University, for further references.

Extraction

Two hundred grams of the mature seeds was separated, air-dried, and subjected to mechanical pulverizer for size reduction and extraction. The coarse powders of seeds were extracted with *n*-hexane to collect the oil of the seeds. After collection of the extract, extra solvent present in the extract was removed by concentrating under reduced pressure using a rotary evaporator system, the remaining material was evaporated till complete removal of solvent, and the obtained oil was stored in an air-tight container for further fractionation.

Fractionation of plant extract by column chromatography

The bioactive *n*-hexane extracts in the form of greenish yellow oil (3 g) were pooled and subjected to column (50 cm × 1.5 cm) chromatography over silica gel (200–400 mesh). The column was eluted using 100% *n*-hexane solvent for saturation overnight. Oil sample was mixed with a small amount of silica and then transferred to the top of a prepared silica gel column. Sample was loaded, followed with apply of 100% *n*-hexane solvent, and continued elution with ratio change of 5 ml by addition of ethyl acetate to afford 40 fractions. The fractions eluted from the column were checked by TLC (precoated aluminum plate of 60-F_{254}) with *n*-hexane-ethyl acetate mobile phase (8:2) for their purity; similar fractions were merged and separately subjected for the removal of extra solvents and preserved on standard condition for cytotoxicity test with SRB assay. The following fractions were found to be similar and mixed and coded as C11 (F1–F8) from 100% *n*-hexane, C12 (F9–F16) from *n*-hexane-ethyl acetate (95:5), C13 (F17–F22) from *n*-hexane-ethyl acetate (90:10), C14 (F23–30) from *n*-hexane-ethyl acetate (85:15), and C15 (F31–F38) from *n*-hexane-ethyl acetate (80:20).

Cell lines and standard drug

Three different types of human breast cancer cell lines were selected in this study; breast carcinoma MCF-7 as a Luminal A group, MDA-MB-231 as a Claudin-low group, and MDA-MB-435 from human epidermal growth factor receptor 2 (HER2) group. They were grown in the RPMI1640 medium (Roswell Park Memorial Institute) which contains fetal bovine serum 10% and L-glutamine 2 mM. All the cell lines were incubated for 24 h at 37°C with 5% CO_2, 95% air, and 100% relative humidity before addition of experimental drugs. Doxorubicin was used as the standard drug.

Cytotoxicity test using sulforhodamine B colorimetric assay

The anticancer activities of fractions were carried out at Advanced Centre for Treatment, Research and Education in Cancer, Mumbai; as per our privies, reported method follows from the method reported by Skehan *et al.*[6] SRB assay used for cytotoxicity testing. All cells were inoculated into 96-well microtiter plates, depending on the doubling time of each cell line. Following the incubation for 24 hours, the cell lines were fixed *in situ* with trichloroacetic acid (TCA) for count the population of the cells when that drug was loaded (Tz). All the fractions were dissolved in dimethyl sulfoxide and further diluted with cell culture medium. Subsequently, different concentrations of each fraction were prepared and the final drug concentrations of 10, 20, 40, and 80 µg/ml were obtained. Known anticancer drug adriamycin (doxorubicin) used as a positive control for each test drugs on cell lines.

All the calculated parameters were in a dose–response manner for the samples: the GI50 which is 50% of growth inhibition (the concentration the result in a 50% reduction in the net protein increase in control cells during the incubation of the drug); the TGI which is the concentration of the drug that results in total growth inhibition; and the LC50 which indicates a net loss of cells (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning).

For each of parameters, values were calculated if the level of activity was reached; however, if the effect was not reached or was exceeded, the values for that parameter were shown as bigger or less than the maximum or minimum tested concentration.

Gas chromatography-mass spectrometry analysis

Phytochemical analysis of the oil was carried out; GC-MS analyses were performed by GC ThermoFisher Company (United States) with Elmer system and flame ionization detector and injector to MS instrument employing the following conditions: column Elite-1 fused silica capillary column (15 mm × 0.25 mm × 0.25 µm, composed of 100% polysiloxane) and operating in electron impact mode at 70 eV; helium gas (99.999%) was used as a carrier gas at a constant flow of 1 ml/min, and an injection volume of 0.5 µl was employed an injector temperature of 250°C and ion source temperature of 200°C. The oven temperature was programmed from 110°C (isothermal for 2 min), with an increase of 5°C/min, to 280°C, ending with a 9-min isothermal at 280°C. Mass spectra were taken at 70eV, a scan interval of 0.5 s, and fragments from 25 to 900 Da. Total GC running time is 43 min. Software adapted to handle mass spectra and chromatograms was a Thermo XCalibur 3.0, Wiley, of Thermo Fisher.

Statistical analysis

Data were used from three independent experiments. GI50, TGI, and LC50 values were calculated from dose–response control growth curves by the mean graph.

RESULTS

As per the observation, the results indicated that the extraction of seeds of *C. halicacabum* L. with *n*-hexane solvent gave a high percentage yield of 24% w/w aromatic oil in light greenish color. As per the chromatography test on the oil, the *n*-hexane-ethyl acetate solvent was selected as a mobile phase for the preparation of column and fractionations of oil. The anticancer activity of *C. halicacabum* L. seeds oil and fractions was assessed with SRB assay method against three human breast cancer cell lines (MCF-7, MDA-MB-231, and MDA-MB-435) and GI50, TGI, and LC50 calculated are summarized in Table 1. All the fractions showed the activity against the MCF7 cell line from Luminal A type; the GI50 was observed on less dose of 10 µg/ml which indicated the activity; later, the four fractions (1, 2, 3, and 4) showed decrease in activity by increasing the dose. Among all fractions, the last fraction (fraction number 5) showed the highest activity compared to other fractions, and activity observed was inhibition of the cell growth in less and high concentrations. However, on two other types of breast cancer cells of Claudin-low-type and HER2 type (MDA-MB-231 and MDA-MB-435), the oil fractions did not show antiproliferative effect [Figure 1].

The fraction coded as C15 being the most active fraction was subjected to analysis with GC-MS to find out the probable compounds, which may play role in the activity and control the breast cancer cell growth. Data obtained from the analysis showed the presence of four main peaks in the graph, which are fatty acids such as palmitic acid, oleic acid, paullinic acid (omega 7), with squalene as the highest peaks [Figures 2 and 3].

DISCUSSION

In process of drug discovery, especially from natural and herbal origin, the extraction and fractionation for purification of selected compounds are the main parts of research to find out the responsible phytochemical for targeted activity. In the first stage of research for effect of active compounds on cancer cells, *in vitro* cytotoxic screening test was usually performed and different values were considered as the indication of cytotoxicity effect, as procedures described by the National Cancer Institute (NCI) and reported by Shoemaker.[7] In NCI-selective SRB assay, IC50 with the 12 concentrations that cause GI50, was replaced by the value of GI50 factor, to emphasize the correction for time zero

Table 1: Cytotoxic activity of *Cardiospermum halicacabum* L. seeds oil and various fractions against different type of human breast cancers cell lines

Cell line samples	Breast/MDA-MB-231			Breast/MDA-MB-435			Breast/MCF-7		
	LC50	TGI	GI50	LC50	TGI	GI50	LC50	TGI	GI50
C1 (oil)	-	-	-	-	-	-	>80	>80	12.8
C11	NE	NE	>80	NE	NE	>80	NE	NE	<10
C12	NE	NE	>80	NE	NE	>80	NE	NE	<10
C13	NE	NE	>80	NE	NE	>80	NE	NE	<10
C14	NE	NE	>80	NE	NE	>80	NE	NE	<10
C15	NE	NE	>80	NE	NE	>80	>80	48.7	<10
Doxorubicin	NE	<10	<10	NE	<10	<10	>80	<10	<10

TGI: Total growth inhibition; NE: Not evaluable

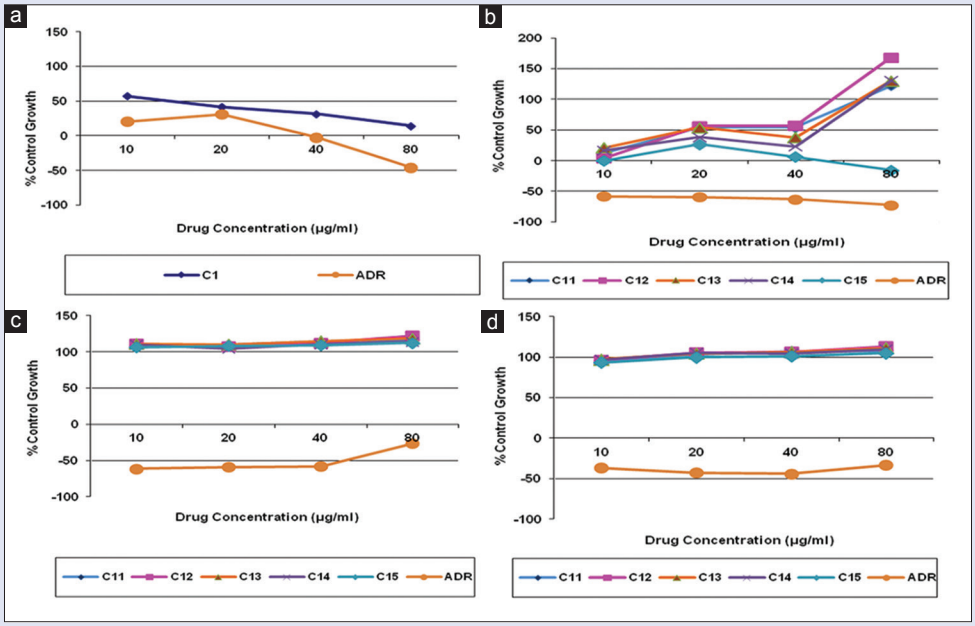

Figure 1: Cell cytotoxicity determined by sulforhodamine B colorimetric assay. Seed oil (C1) and different fractions of *Cardiospermum halicacabum* seed oil (C11, C12, C13, C14, and C15) tested with various concentrations on different types of human breast cancer cell line and compared with standard drug of adriamycin. Graph (a) showed growth curve of MCF-7 cell lines with oil; (b) growth curve of human breast cancer cell line MCF-7; (c) growth curve of human breast cancer cell line MDA-MB-435; (d) growth curve of human breast cancer cell line MDA-MB-231

count of the cells, and to measure the growth inhibitory power of the tested agent, as in TGI and LC50, as described in Materials and Methods. These parameters are interpolated values and can use the concentrations giving GI50 PRCNT values above and below the reference values to make interpolations on the concentration axis. If the level of activity reaches the level that inhibits the growth, the values are then calculated for these parameters; otherwise, they are considered to be not evaluable (NE). If the effect does not reach the level or exceeds, the value for the parameters is shown as more or less than the maximum or minimum tested concentration, which was > 80 and < 10 in our study, respectively.[6,8] Here, in this study, the decreasing trend was observed in cell growth in 10 µg/mL dose for all fractions; in the first four fractions, the cells show resistance to drug by increasing the dose; on the other hand, the fraction coded C15 showed significant differences compared to other fractions, which accepted as the most active fraction from seed oil. All the fractions were compared with reference standard drug adriamycin (doxorubicin), a known anticancer drug, in the same concentrations to examine their anti-proliferative effects.

The cytotoxic potential effects of most plants were usually related to their different phytochemicals present in different parts of them. Therefore, making the analysis to find out the range of present phytoconstituent in the targeted herbal medicine is required to get the profile of plant and find the responsible compound for the activity.[9] In this study, seeds were selected for investigation based on their traditional clime, the basic standardization of this seed has been done, and investigation reported the presence of various phytoconstituents such as alkaloids, flavonoids, and tannins as well as the amount of total phenols and the content of flavonoids in various extracts including the oil.[10] Other reported study indicate the presence of erucic acid, eicosanoic acid, oleic acid, tetradecanoic acid, octanoic acid, and *n*-hexadecanoic acid as fatty acids in *C. halicacabum*.[11] Here, in this work, we find out the presence of three fatty acids namely palmitic acid, petroselinic acid (omega-12 fatty acid), and paullinic acid (omega 7 fatty acid), and the squalene as possible responsible compound for the activity. Different secondary metabolites, such as phenolics and flavonoids compounds, and essential fatty acids that are available in many plants are fundamental for humans in the diet and help to lower the risk of heart disease, inflammation, and healing the lipid barriers. They play a role in oxidative stress conditions, cancer prevention, and treatment of cancerous cells.[12,13] However, many actions such as inflammatory responses, hormonal changes, cell cycle, and apoptosis can be responsible for these activities and multiple cancer-related processes that may account for the ability of plants to inhibit cancer. In this study, squalene as the main peak and presented compound in the fractions of *C. halicacabum* L. may play a key role in this activity. Squalene present in vegetable oils such as olive oil and animal tissue such as shark liver. It has been claimed that sharks are resistant to cancer.[14] They have unusually high tissue levels of squalene. Shark liver oil contains 40% or more squalene.[15] and dogfish liver oil is very high, reported to be over 90% squalene.[16] Studies of breast and pancreatic cancer in Mediterranean populations have shown that increased dietary intake of oils with squalene such as olive oil is associated with less or no increased risk of cancer, despite a higher proportion of overall lipid intake. It is suggested that the high squalene content in some oil, as compared to other human foods, is a major factor in the cancer risk-reducing effect. Squalene in humans is supplied by both endogenous biosynthesis and dietary sources. Some *in vitro* experiments and animal models suggest a tumor-inhibiting role for squalene. A mechanism is proposed for the tumor-inhibitory activity of squalene based on its known strong inhibitory activity of β-hydroxy-β-methylg1utaryl CoA reductase catalytic activity *in vivo*, thus reducing farnesyl pyrophosphate availability for prenylation of the *ras* oncogene, which relocates this oncogene to cell membranes and is required for the signal-transducing function of *ras*.[17] The findings obtained in the present work are similar to those obtained previously. Our study had limitations; we did not evaluate the effect of fractions on normal breast epithelial cells. Moreover, using MCF-10A cell as normal breast epithelial cell line cannot substitute normal cell characteristic completely. MCF-10A cell line has a near-diploid karyotype with the modest genetic modifications.[18]

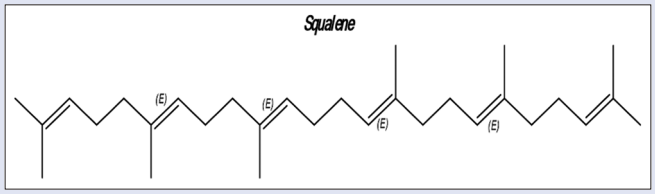

Figure 2: Structure of squalene: Active compound of *n*-hexane extract of *Cardiospermum halicacabum*

CONCLUSION

We reported that *C. halicacabum* L. seeds oil fractions with the presence of fatty acids and squalene inhibited the proliferation of a breast cancer cell line MCF-7 and can be considered as a medicinal plant with potential activity, which supports the traditional claim. However, the more chemistry research needs to be conducted to identify and characterize the single bioactive compound and find out the exact mechanism of action.

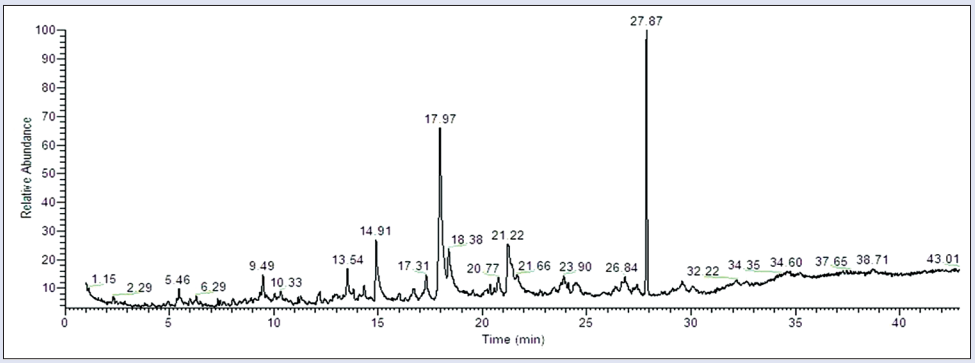

Figure 3: Chromatogram of gas chromatography analysis of *Cardiospermum halicacabum*

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Nil.

Conflicts of interest

There are no conflicts of interest.

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