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Research Article

EVALUATION OF ANTI-PROLIFERATIVE AND ANTIOXIDANT ACTIVITY OF *SWERTIA CHIRATA*: IMPLICATIONS IN BREAST CANCER

Homa Jilani Khan[1,2],Mohammad Kaleem Ahmad[2], Abbas Ali Mahdi[2], Namrata Rastogi[2], Jamal Akhtar Ansari[1,2], Uzma Shahab[2] , Abdul Rahman Khan[1]*

1. Department of Chemistry, Integral University, Lucknow, India, 226 026
2. Natural Products Research Lab, Department of Biochemistry, King George's Medical University, Lucknow, India, 226 003.

***Corresponding author's Email:** arahman@iul.ac.in, arahman.iun@gmail.com

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ABSTRACT

Background: *Swertia chirata* belonging to Gentianaceae family is a medicinal plant known to exhibit various pharmacological properties.
Objective: This study investigates the antiproliferative effects of the aerial parts of *S. chirata* methanol extract (SCME) against breast cancer cells.
Materials and methods: Antiproliferative effects of SCME were assessed on human breast cancer cell lines, MDA-MB-231 and MCF-7 using the MTT cell viability assay at concentrations ranging from 6.25 µg/mL to 400 µg/mL for 24 h, 48 h, and 72 h. Colony formation assay was performed to visualize the growth inhibition. Cellular apoptosis was determined by Hoechst staining. Additionally, enzymatic and non-enzymatic antioxidant activities were also assessed by DPPH, ABTS, TPC, SOD and CAT assays.
Results: The results obtained indicate that SCME has potent cytotoxic activity towardsMDA-MB-231 and MCF-7 with anIC50value of 112 ± 4.5 µg/ mL and 180 ± 3 µg/ mL, respectively. Microscopic assessment of cellular and nuclear morphology of SMCE treated breast cancer cells indicated that SCME induced apoptosis as a mode of programmed cell death in these cells. Antioxidant profiling of SCME also indicated a high concentration of polyphenols and presence of antioxidant enzymes SOD and Catalase with potent free radical scavenging activity.
Conclusion: Our study demonstrated that SCME has significant antiproliferative activity against human breast cancer cells, MDA-MB-231 and MCF-7 and showed features of apoptotic cell death. Thus, *S. chirata* can be an enthusiastic target for researchers to exploit its potential as a natural treatment of breast cancer.
Keywords: SOD, Catalase, MTT, DPPH, Colony formation assay.

INTRODUCTION

Cancer is one of the most frequent and major causes of mortality and is the third leading cause of death worldwide following cardiovascular and infectious diseases[1]. When talking about cancer in women, carcinoma of the breast is highly frequent worldwide. According to the World Health Organization report of 2013[2], it was estimated that over 1.7 million women worldwide were diagnosed with breast cancer in the year 2012. Free radicals are associated with initiation and progression of various chronic and deteriorating diseases including cancer [3,4]. Antioxidants are potent biochemical agents which protect normal as well as cancer cells from free radicals induced deleterious effects. The high antioxidant potential is one of the most significant

aspects of the pharmacological property of natural compounds. Therefore, traditional medicinal plants have acknowledged considerable attention as sources of biologically active constituents including antioxidants which signify their role in cancer prevention as well as management[5].

The effective treatment regimen of breast cancer comprises of surgical excision, radiotherapy, and chemotherapy. However, these treatment strategies exhibit many limitations such as several complications, adverse side effects and chemo-resistance thereby demanding identification and intervention of novel therapeutic agents that are more effective and have lesser side effects[6]. Various active compounds (or theirsemi-synthetic derivatives) derived from medicinal plants have been assessed for their efficacy and tolerability in the treatment of breast cancer and have been evaluated in clinical trials [7].A study with 453 cancer patients reported that 77% of patients using herbal medicines in combination with conventional chemotherapy got better treatment outcome[8].

S. chiratais a rich source of polyphenols and many other bioactive compounds such as xanthonoids, flavonoids, irridoids, terpenoids and secoiridoid glycoside[9]. Xanthonoids are the chief compounds of this genus which have been reported to show significant inhibitory property of proliferation of cancer cells [10]. Swertia chiratahas been reported to have anti-inflammatory, anti-viral, antihelmintic, anticarcinogenic, hepatoprotective, hypoglycemic, wound healing, antibacterial and antitumor properties[11-17].

In the light of the above explanations we for the first time explored the anticancer properties of *S. chirata* in breast cancer cells namely MDA-MB-231 and MCF-7. The crude form of *S. chirata* was prepared in methanol because polar bioactive compounds are easily soluble in methanol. Moreover, we also investigatedthe antioxidantactivity and total phenolic contents present in SCME through enzymatic and non-enzymatic methods.

MATERIALS AND METHOD

Chemicals and Reagents

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 1, 1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), dimethyl sulfoxide (DMSO), Folin-Ciocalteu phenol reagent, methanol (fisher scientific),gallicacid, trypan blue (0.4%), ascorbic acid, sodium carbonate anhydrous, phosphate-buffered saline (PBS), Roswell Park Media Institute (RPMI1640), penicillin/streptomycin (100X), trypsin-EDTA (1X) were purchased from Sigma-Aldrich, USA and fetal bovine serum (FBS) from Gibco.

Collection and identification of plant material

The well-known medicinal plant *Swertia chirata* was collected from National Botanical Research Institute Lucknow in April 2014. *S. chirata* (aerial part) were washed with sterile distilled water to remove the adhering dust particles and other unwanted materials and was shade dried for 5 days followed by grounding into fine powder. The powdered samples were stored in a clean, dry and sterile container for further use.

Preparation of extract

The powdered material (1 ± 0.03kg) was extracted in 100% methanol by percolation at room temperature (25°C). The extract was concentrated in a vacuum evaporator and kept in vacuum desiccators for complete removal of solvent and weighed 80g.The dried extract thus obtained was dissolved in the methanol solvents at the concentration of 1 mg/mL. This prepared *S. chirata* methanol extract (SCME) was used throughout the study.

Determination of the total phenolic content

Total phenol contents were determined by Folin-Ciocalteu reagent method[18] using gallic acid as a standard phenolic curve. The total phenolic contents were expressed as mg of gallic acid equivalent (GAE)/g.

Free radical-scavenging activity

DPPH radical scavenging activity was evaluated according to standardized protocol[19]. A stock solution of 0.052 mg/mL of DPPH was prepared in methanol and SCME with a concentration of 1mg/mL in methanol at various concentrations were mixed. Decolorization of DPPH was determined by measuring the absorbance at 517nm with a double beam spectrophotometer 2203 (Systronic). Ascorbic acid was used as reference material. DPPH radical scavenging activity was calculated as follows:

% inhibition = [(Absorbance sample - Absorbance control)/(Absorbance control)] x 100

Control = 1.0 mL methanol + 1.0 mL DPPH

Evaluation of antioxidant activity

The ability to scavenge the ABTS radical cation was determined according to the method of Re 20.TheABTS solution was prepared by the reaction of 5 mL of 7mM ABTS and 88 µL of 2.45mMpotassium persulphate after incubating the reaction mixture at room temperature in the dark for 16h. It was then diluted with methanol to obtain an absorbance at 734 nm. The ABTS•+solution (2.0 mL) was thoroughly mixed with 20µL of the SCME sample. The percentage of inhibition of ABTS•+ was calculated using the following formula:

%=(A control – A test)/ A control *100

Where A control = Absorbance of control, A test = Absorbance of test sample

ENZYMATIC ANTIOXIDANT ACTIVITY

Superoxide anion radicals scavenging activity

Superoxide radical scavenging was determined using PMS-NADH systems [21]. The superoxide anion scavengingactivity was determined as mg of ascorbic acidequivalents (AAE)/ g where one unit of SOD activity was defined as the amount of enzyme causing 50% inhibition of photochemical reduction of Nitro Blue Tetrazolium (NBT).

Assay of catalase

Catalase activity was determined by measuring the inhibition rate of hydrogen peroxide (H_2O_2) at 240 nm in accordance with the method described by Sinha[22].

Cell line and culture

MDA-MB231 and MCF-7 cancer cell linewas obtained from National Center for cell science (NCCS), Pune, India. MDA-MB231 and MCF-7 cells were cultured in RPMI medium supplemented with 5% FBS (MDA-MB-231)and 10% FBS(MCF-7)with 1% penicillin/ streptomycin solution and grown at 37°C, 5% CO2 in a humidified chamber.

Light microscopy

MDA-MB-231 and MCF-7 cells were grown to 70% confluence and treated with SCME for concentration ranging from 12.5 µg/ mL to 200 µg/ mL for 24 h. The photographs were taken at 40X magnification using a phase-contrast inverse microscope (Nikon).

MTT assay

The antiproliferative activity of the SCME was evaluated by MTT reduction assay. This assay is based on the enzymatic reduction phenomenon of MTT dye. The assay provides a direct relationship between the viable cells and color formation. In brief, MDA-MB231 and MCF-7 cells (5x103) were seeded in 96-well culture plate (100µL/ well) for 24h at 37oC and 5% CO2. Stocks of SCME were prepared in DMSO and diluted to the desired concentrations (6.25to 400µg/mL). After 24h, the 10µL of 5 mg/mL MTT solution was added to each well and the plates were further incubated for 2h at 37oC until formazan blue crystals developed. Supernatant was discarded and 100µL of DMSO was added to solubilize formazan crystals for 10 min at 37^{0}C. The absorbance was recorded at 540 nm by a microplate reader (BIORAD-680). The percentage inhibition was calculated by using the formula

% Growth inhibition = 100- % Cell viability

Where % Cell viability = [(OD of treated)/(OD of control)] X 100

Here OD = optical density

Colony formation assay

Briefly, cells at the initial density of 1x 103 in 2 mL medium were seeded in 6-well plates for 18 h-20h and the media was changed and cells were treated with varying concentration of SCME for 24h. Media was removed and cells were grown in complete growth media for next 4-7 days until distinct colonies were formed. Thereafter, colonies were washed with PBS and fixed with chilled 100% methanol for 10 min. Fixed cells were stained with 0.05% Coomassie blue stain for 10 min and washed with tap water. Plates were air dried and the number of colonies was determined by counting them under an inverted phase-contrast microscope at 10X-20X magnification and a group of ~50 cells were counted as a colony.

Hoechst staining

To determine changes in the nuclear morphology for apoptosis detection, we performed Hoechst 33342 staining followed by fluorescence microscopy. Cells were grown to 50% confluence on coverslip and left for 18h-20h in the incubator until full morphology is attained. Next day, media was replaced with fresh media and cells were treated with varying concentration of SCME. After 24h cells were fixed with 4% paraformaldehyde and permeabilized with 0.3% Triton-X100 in PBS. Cells were then stained with Hoechst 33342 at a final concentration of 0.5µg/ml in PBS and incubated for 5 min at 25°C. Brightly stained, condensed

nuclei with characteristic features of apoptotic cells were counted using a fluorescence microscope (Nikon). A minimum of 5 fields of at least 100 cells per field were counted. Images were captured at 20X magnification.

Statistical Analysis

The data presented as means SD were analyzed using ANOVA. Dunnett's multiple range test (DMRT) was used to determine significant differences between means. The results were considered statistically significant if the P values were 0.05 or less.

RESULTS

SCME induced growth inhibition of MDA-MB-231 and MCF-7 cells

In our preliminary experiment, we performed cell viability assay to evaluate the anti-proliferative activity of SCME against MDA-MB-231 and MCF-7 using MTT dye. Both the cells exhibited the antiproliferative activity in dose and time dependent manner (Figure 1). The SCME demonstrated a potent anti-proliferative activity against MDA-MB-231cells attaining the maximum % growth inhibition of 77.7% at 72h while the % growth inhibition at 24h and 48h are 67.2% and 72.9% respectively, at 400 µg/mL. However the IC50 values at 24h, 48h, and 72h are 112±0.5µg/mL, 75.5±1.5 µg/mL and 42.5±0.4 µg/mL. Moreover SCME showed the significant growth inhibition of MCF-7 cells also. The maximum growth inhibition being 77.17% at 400µg/mL in 72h with IC50 180± 0.3µg/mL (Table 1).

Table 1: IC_{50} value obtained for SCME against MDA-MB-231 and MCF-7 cancer cells after exposure of 24h, 48h and 72h. Values are expressed as mean ± S.D. (n = 3).

Time	MDA-MB-231 IC_{50} (µg/ml)	MCF-7 IC_{50} (µg/ml)
24h	112±0.5µg/mL	180±0.3µg/mL
48h	75.5±1.5µg/mL	99±1.3µg/mL
72h	42.5±0.4µg/mL	47.5±0.5µg/mL

SCME induced visual changes in cellular and nuclear morphology in MDA-MB-231 and MCF-7 cells

Treatment of MDA-MB-231 and MCF-7cells with varying concentrations of SCME exhibited typical features of programmed cell death (Figure 2A) including surface bleb formation, redistribution and compaction of cytoplasmic organelles, and formation of cytoplasmic vacuoles. The cellular changes were complimented by the changes in the nuclear morphology by Hoechst staining (Figure 2B). SCME treatment induced chromatin condensation and nuclear fragmentation which are considered as hallmarks of apoptotic cell death.

Our results recommend that SCME induced cell death is mediated by apoptotic induction in MDA-MB-231 and MCF-7 cells. However, further experiments are warranted to confirm these findings using more specific indicators of apoptosis.

SCME induced inhibition of colony formation of MDA-MB-231 and MCF-7 cells

The clonogenic assay is considered as the gold standard to determine the anticancer activity of drugs. Therefore, we performed colony formation assay on both the breast cancer cell lines at different concentrations. SCME significantly ($P \leq 0.05$) reduced the colony formation of both MDA-MB-231 and MCF-7 cells over a period of 10 days thereby signifying the long term antiproliferative consequence of SCME. As evident in Figure 3, treatment of human breast cancer cells, with SCME resulted in a significant decrease proliferation and colony formation as compared to untreated controls. A significant decrease in colony formation was noted in both the treated cells. A decrease of 28% to 95% at 12.5 µg/mL and 200 µg/mL for MDA-MB-231 and 20% to 89% at 12.5 µg/mL and 200 µg/mL for MCF-7 cells respectively, compared to control after exposure with SCME. Our results clearly suggested the anti-proliferative potential of SCME against breast cancer cells in vitro.

SCME contains high amounts of polyphenols

Most of the plant based anticancer compounds belong to the polyphenols sub-family of natural products and contribute to their anti-proliferative activity (Li et al., 2008). Therefore, we intended to determine the total phenolic content (TPC) of SCME using the Folin-Ciocalteau method. It is expressed in terms of Gallic acid equivalent (the standard curve equation: y = 7.026x – 0.0191, r2 = 0.999). The total phenolic content as measured was found to be 24.1mg which is expressed as mg of GAE/g of extract. Therefore, our results suggested that SCME contained high overall levels of

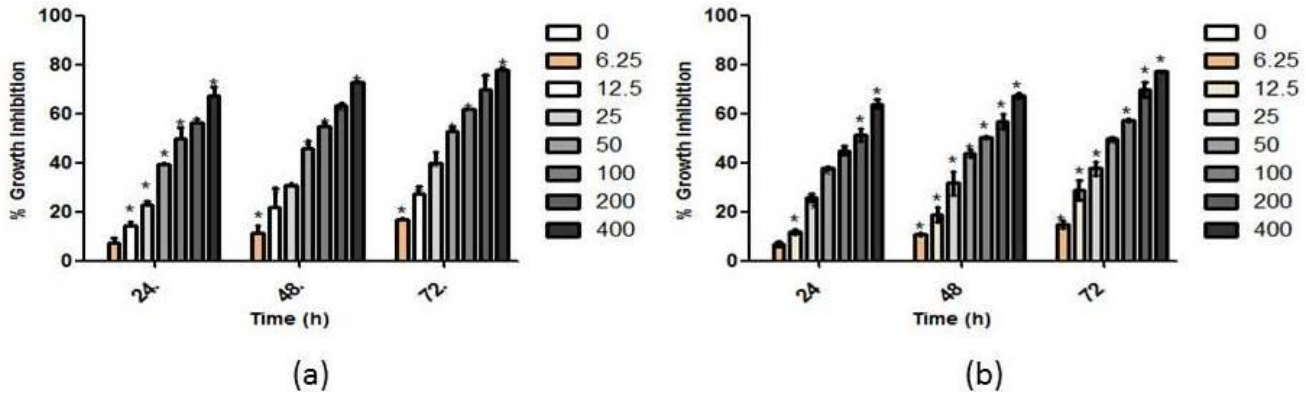

Figure1: Anti-proliferative effect of SCME against human breast cancer cells. Human breast cancer MDA-MB-231(a) and MCF 7(b) cells were exposed for concentrations range (6.25µg/mL - 400µg/mL) for 24, 48 and 72h, and the viability of the cells was determined by MTT assay. Cell viabilities are shown as percentages, and the untreated cells were regarded as 100% viable. Data represent the means of three experiments conducted in triplicate and were significant (P≤0.05).

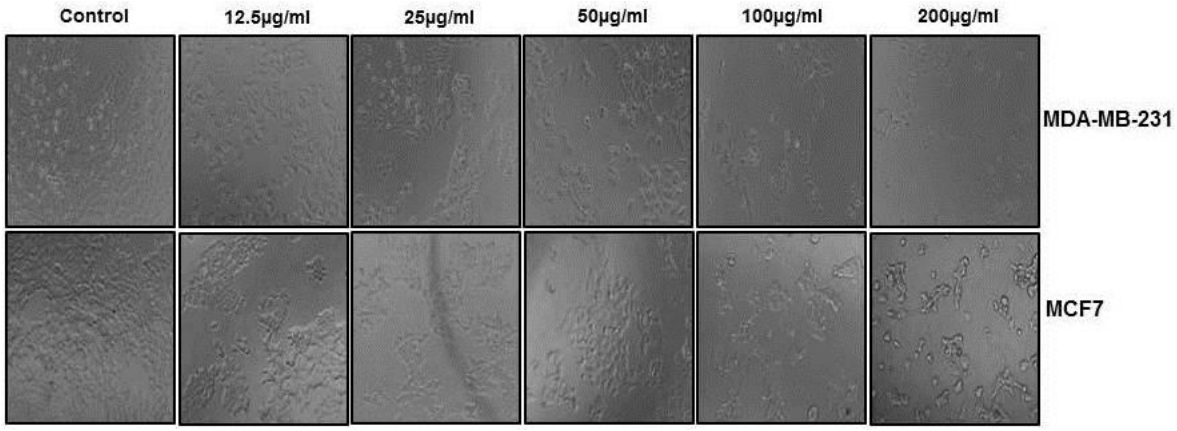

Figure2A. Microscopic examination of human breast cancer MDA-MB-231(a) and MCF 7(b) cells, after treatment with SCME for 24h.

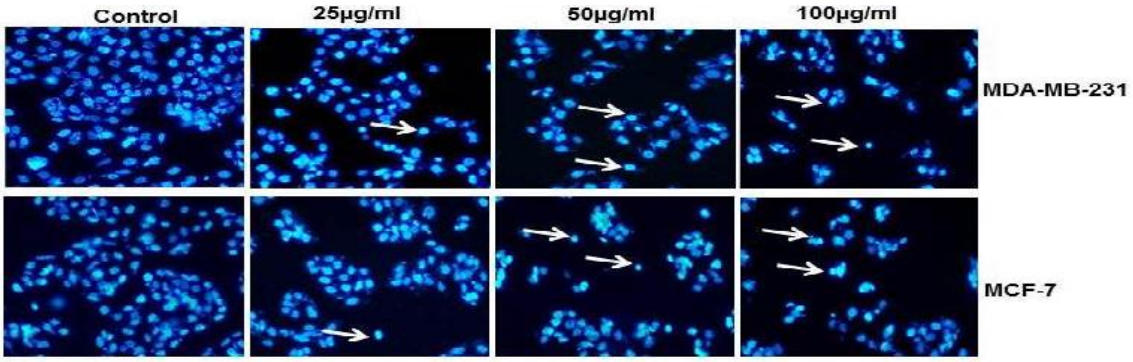

Figure2B. Induction of nuclear fragmentation by SCME in MDA-MB-231(a) and MCF-7(b) cells: 2 × 104 cells/well were seeded in 12-well culture plate and allowed to grow for 24h and then treated with different concentrations of SCME for 24h and stained with Hoechst 33242 stain following standard protocol and image was captured by inverted microscopy at 20X magnification.

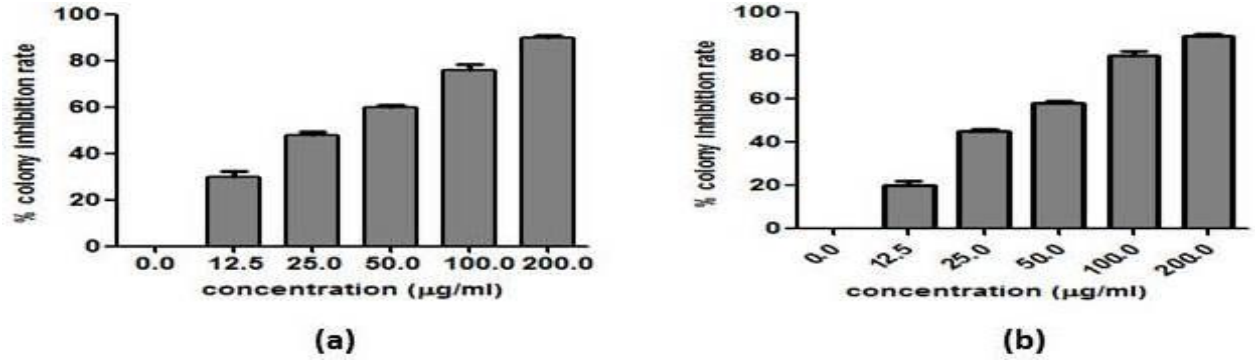

Figure3. Effect of SCME on growth assay estimated by colony formation.Human breast cancer MDA-MB-231(a) and MCF-7(b) cells were grown in media along with specified concentration of the SCME. The number of colonies was recorded after 7 days of treatment. Data represents the mean ± SD of three different assays. *P ≤ 0.05 versus control.

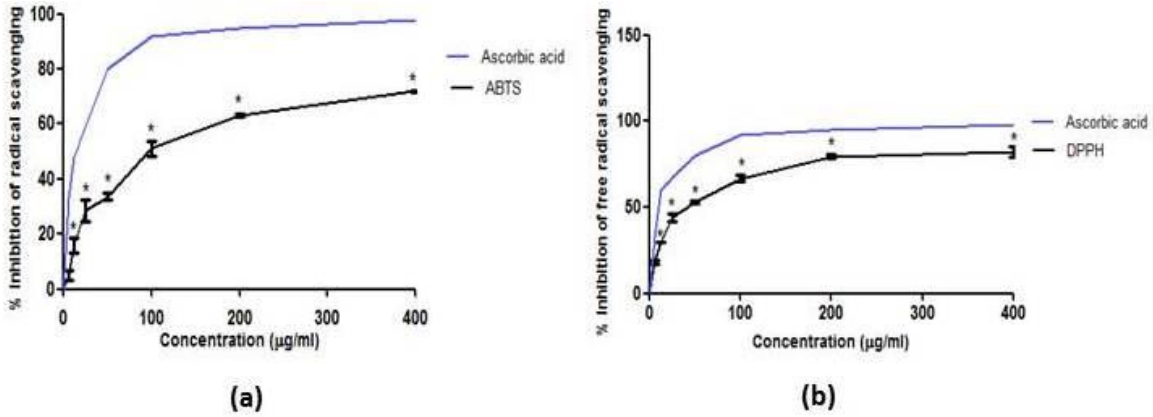

Figure 4: (a) Free radical scavenging effect of SCME in DPPH assay, **(b)** Free radical scavenging effect of SCME in ABTS assay.

biochemically active polyphenols.

SCME exhibits potent free radical scavenging activity

In order to determine the free radical scavenging activity of SCME, we performed DPPH and ABTS assays. SCME demonstrated dose-dependent scavenging activity of DPPH free radicals as shown in (Figure 4a). The highest free radical scavenging activity of 81.5% was observed at 400µg/mL with an IC50 of 41.7±0.2µg/mLand this activity was comparable to that of standard ascorbic acidIC5010±0.1µg/mL.The ABTS assay measures the ability of plant extracts to scavenge the cationic ABTS.+ radical produced by the oxidation of ABTS. It exhibited potent scavenging effects against ABTS.+ with concentration ranging from 6.25 µg/mL to 400µg/mL with inhibition 6.98 % to 71%. The IC50 value of SCME was found to be 194±1.2µg/mLwhile that of ascorbic acid IC50 value was found to be 18±0.1µg/mL(Figure 4b).

Table 2: Non-enzymatic and Enzymatic antioxidant contents in SCME, values are expressed as mean ± S.D. (n = 3).

Non-Enzymatic antioxidant activity SCME	
TPC (mg of GAE/g)	4.1
ABTS (IC_{50} µg/mL)	194 ± 1.2
DPPH (IC_{50} µg/mL)	41.7 ± 0.2
Enzymatic antioxidant activity SCME	
SOD (Unit/mg)	64± 0.6
CAT (Unit/mg)	26 ± 0.5

Our experimental results of ABTS.+ assay indicated that the SCME was fast and effective scavengers of the ABTS radical.

SCME possess high enzymatic SOD and Catalase activity

In our next set of experiments, we evaluated the enzymatic activity of SOD and Catalase enzymes in SCME. The

scavenging capacity of SCME on superoxide radicals showed highest superoxide radicals scavenging activities (64 ± 0.6U/mg) in the reaction mixture, which is comparable to Ascorbic acid(81 ± 0.3U/mg). Catalase level of the active antioxidant enzymes of the SCME of the plant was found to be (26± 0.5 U/mg). Altogether our above results (Table II) suggested that the free radical scavenging activity of SCME as mentioned in our previous experiments can be attributed to the presence of antioxidant enzymes SOD and Catalase.

DISCUSSION

In this study, we found that SCME possessed a significant anti-proliferative effect on breast cancer cells in vitro. Since our previous study indicated that aqueous extract of S. chirata exhibits moderate growth inhibitory activity in T47D breast cancer cells **23**. We next sought to examine the anticancer property of its methanol extract in more detail. The result of the present study showed that SCME induced significant growth inhibition of two phenotypically different breast cancer cells MDA-MB-231 and MCF-7 in MTT assay. Clonogenic assay results further confirmed results and indicated that SCME treatment greatly reduced the number of colonies when compared with the control group. Interestingly SCME was found to be more effective on MDA-MB-231 cells as compared to MCF-7 cells in inducing cell death. Moreover, many studies have also shown that natural agents have the potential to kill different cancer cell types owing to their multiple modes of action [24]. These properties of natural agents can be explored for treatment of cancers with heterogeneous nature. Therefore,growth inhibitory activity of SCME on MDA-MB-231 and MCF-7 cells provides aninitial lead that it can be an effective anticancer agent.

Cancer is one of the state where too little apoptotic occurs, resulting in live malignant cells. The mechanism of apoptosis is complex and involves many pathways[25]. Like many other plants extract[26] SCME was found efficient in inducing apoptosis in MDA-MB-231 and MCF-7 cells. The cellular and nuclear morphology of SCME treated MDA-MB-231 and MCF-7 cells reveals the characteristic features of apoptosis. Thus SCME provides an important strategy like many other studies where apoptosis is targeted for cancer treatment [25].

Free radicals are formed naturally in the body and play an important role in many normal cellular processes[27,28]. At high concentration, however, free radicals can be hazardous to the body and damage all major components of cells, including DNA, proteins, and cell membranes. The damaged to cells caused by free radicals, especially the damage to DNA, may play a role in the development of cancer[27,28]. Therefore natural compounds with free radical scavenger activity have been found to play a protective role in maintain normal healthy cells and can also be taken as an adjuvant to the therapy resulting in symptomatic relief [29]. Our results showed that SCME contained high polyphenolic content which was comparable with the earlier studies in which medicinal plant extracts have high levels of polyphenols as their major bioactive ingredients [30]. For non-enzymatic components, we assessed free radical scavenging activity using the DPPH assay and ABTS•+. Previous report and our experimental findings have shown that SCME was very effective in inhibiting DPPH and reducing ABTS free radicals in a dose-dependent manner comparable to that of ascorbic acid[31]. Enzymatic components like SOD and Catalase activity were not previously reported in SCME. We determined the activities of SOD and Catalase enzymes in SCME and found significant enzymatic activity. Similar results were found in other studies wherethe presence of both enzymatic, as well as non-enzymatic components; confirm potent antioxidant potential to plant extracts[32].

Cumulatively our experimental findings suggest that the antiproliferative effect of SCME is partly due to its polyphenolic content and other bioactive compounds. These results are in conjunction with previous studies where natural compounds with high polyphenolic content are known to exhibit potent anticancer activity[33]. However, its detailed biochemical analysis, isolation of active ingredients as well as evaluation of its pharmacological profiles requires research attention. Altogether our preliminary antiproliferative property of SCME against hormone-dependent and independent breast cancer cells underscores its potential for more detailed pre-clinical evaluation as antibreast cancer agent.

CONCLUSION

Overall our findings suggest that SCME not only possess potent antioxidants but also as an effective antiproliferative and apoptotic agent for breast cancer cells, MDA-MB-231 and MCF-7. Thus, this Indian traditional plant, S. chirata can open new door for researchers to find an effective treatment

for breast cancer with no side effects. Therefore, further investigations are underway to isolate compounds from its different bioassay guided fractions and to identify specific phytochemicals responsible for their antioxidant and anti-proliferative activities. Characterization of these active ingredients in SCME may help to determine their mechanism of action responsible for their anti-proliferative activity against breast cancer cells.

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Declaration of interest

The authors declare no conflict of interest.

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