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Original Research Article

Chemical profiling and anti-breast cancer potential of hexane fraction of *Sphaeranthus indicus* flowers

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Abstract

Purpose: *The current study aimed to determine the phytochemicals and anti-breast cancer potential of Sphaeranthus indicus.*
Methods: *S. indicus flowers were extracted with methanol followed by fractionation using n-hexane. For the chemical composition of n-hexane fraction, qualitative phytochemical and GC-MS analysis were performed. The anti-proliferative activity was measured by MTT assay, whereas, cytotoxic and pro-apoptotic effects in MCF-7 (breast cancer) cells were determined using propidium iodide, 4',6-diamidino-2-phenylindole, dichlorofluorescin diacetate, and JC-1 staining through fluorescent microscopy.*
Results: *The phytochemical analysis indicated presence of phytosterols, oils and resins in the n-hexane fraction. GC-MS analysis showed that n-hexane fraction comprises of 11 compounds including methyl esters of caprylic acid, myristic acid, pentadecanoic acid, palmitic acid, margaric acid, stearic acid, oleic acid, elaidic acid, linoleic acid, linolenic acid and behanic acid. The tested fraction showed remarkable cytotoxic activity against breast cancer (MCF-7) cells while it was found less toxic towards non-cancerous (BHK-21) cells. Furthermore, morphological assessment through fluorescent microscopy revealed cytotoxic and apoptotic effects by improved cell membrane permeability, increased reactive oxygen species level, compromised mitochondrial activity and condensation of chromatin network.*
Conclusion: *The n-hexane fraction of S. indicus contains phytosterols, oils and fatty acid methyl esters and produced apoptotic effect against breast cancer cells.*

Keywords: *Apoptosis, DAPI staining, GC-MS, n-Hexane fraction, Reactive oxygen species, Sphaeranthus indicus*

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INTRODUCTION

Among cancers, breast cancer is the second leading cause of mortality after the lung cancer. Every year, about one million females are diagnosed with breast cancer worldwide with approximately 410,000 deaths [1]. Males are less prone to this disease and constitutes less than 1% out of the total diagnosed cases [2,3]. The exact pathophysiology of breast cancer is uncertain in males. The rate of breast cancer

related mortality continues to increase, especially in less developed countries [4].

Breast cancer is usually caused by the deregulation of molecular players including inflammatory cytokines, tumor suppressor genes, DNA repair genes, proto-oncogenes, growth factor and growth factor receptors. The abnormal increase in interleukins (ILs), nuclear factor kappa-B (NF-kB) and tumor necrosis factors (TNF) normally promote breast cancer. Moreover, down regulation of breast cancer gene 1 (BRCA1), breast cancer gene 2 (BRCA2), p53, ataxia-telangiectasia mutated (ATM) gene and up-regulation of vascular endothelial growth factor receptor (VEGFR), epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor (PDGFR) and HER2/neu also lead to breast cancer [5].

Recently, plant based natural compounds have shown promising anti-cancer activities at cellular, pre-clinical and clinical levels [6,7]. Some of these plants derived medicines like podophyllotoxin, vincristine, vinblastine and paclitaxil are in practice for the treatment of various cancers including breast cancer [7]. However, these medicines showed some limitations like non-selectivity, low bioavailability and efficacy, severe side effects or/and are costly.

Sphaeranthus indicus Linn. (Asteraceae) has been reported in traditional medicine for inflammation and breast cancer treatment [8]. Moreover, it is commonly used in Ayurvedic system for the treatment of nervine weakness (as food), epilepsy, mental diseases, diabetes, jaundice and leprosy [9]. This plant contains sterols, peptidal alkaloids, amino acids, essential oils and sugars [10]. The traditional uses of *S. indicus* were scientifically validated by observing its neuroleptic, anxiolytic, anti-inflammatory, immunomodulatory, anti-allergic, hepatoprotective, anti-hyperglycemic and renoprotective activities. Furthermore, anti-hyperlipidemic, bronchodilatory and larvicidal effects of this plant have been reported [8]. The plant is used in traditional system of medicine for the treatment of inflammation and cancer but no comprehensive scientific information are available on the n-hexane of *S. indicus* for anti-breast cancer activity.

Therefore, based on its ethnomedicinal uses in cancer and other scientific information, this study was designed to investigate the phytochemical and pharmacological potential of *S. indicus* n-hexane fraction for *in vitro* anti-breast cancer activity.

EXPERIMENTAL

Plant material, extraction and fractionation

The dried flowers of *S. indicus* were purchased from a local herbal store and the specimen was identified by a plant taxonomist, Hafiz Banaras Khan, Associate Professor, Department of Botany, Government College Attock, Pakistan. A voucher specimen (Akt/103/13) was deposited in the herbarium of the mentioned institute. The *S. indicus* flowers were ground into a fine powder and 10 Kg powdered plant material was extracted by cold maceration in methanol (25 L) at room temperature and concentrated under vacuum (Rotavapor R-300 system, Buchi, Switzerland) at 40°C to afford 1298 g extract [11].

Methanolic extract (800 g) was subjected to fractionation by suspending in distilled water and the suspension was further shaken with equal volume of n-hexane in separating funnel. The hexane layer was removed and concentrated using rotary evaporator (Rotavapor R-300 system) [12].

Phytochemical analysis

The n-hexane fraction was screened for various groups of natural products including alkaloids, carbohydrates, phenols, flavonoids, saponins, phytosterols, glycosides, tannins, oils and resins, and proteins as described by Khan *et al* [12].

Gas chromatography-mass spectrometry of n-hexane fraction

For the identification and quantification of n-hexane fraction of *S. indicus*, gas chromatograph - mass spectrometer (Shimadzu, Japan, Model # QP2010 plus) was used. GC was equipped with 0.25 mm (internal diameter) column with 30 m length and GC condition was set as follows: injector temperature, 240°C; carrier gas, helium; and flow rate, 1 mL/min. A 1 µL of the sample was injected into the GC system using a split method under the following oven conditions: 50°C for 1 min, then 5°C/min to 150 for 0 min, then 2°C/min to 175°C for 5 min, then 10°C/min to 220°C (end temperature) for 5 min.

In the mass spectrometry (MS) conditions, ion source temperature was set at 250°C and interface temperature was 240°C. Mass scan was started at 1.65 min and ended at 47 min at a range of 85–380 m/z. The obtained spectrum was compared with the fragmentation pattern available with the NIST (NIST 05) Library [13].

Cytotoxicity and anti-cancer activity

Cell culture

The cytotoxicity of *n*-hexane fraction was evaluated against normal fibroblast (BHK-21) cells. Similarly, the anti-cancer effect was evaluated against adherent breast cancer MCF-7 cells. These cells were acquired from American Type Culture Collection (ATCC, Rockville, MD, USA) and both activities were evaluated through MTT assay [14]. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100 U mL^{-1} penicillin, and streptomycin (all from Gibco/Invitrogen Life Technologies, US) at 37°C in a CO_2 incubator. Sub confluent cells were harvested with trypsin–EDTA and used for further experiments. Media were replaced every third day.

MTT assay

For MTT assay, cells at concentration of 1×10^4 cells/well were seeded into 96-well flat-bottom culture plates (Costar®, Corning Inc., Corning, NY, USA). Each well was supplied with 0.1 mL DMEM media. After 24 h culture (at about 70% confluence), cells were treated with 31.25-500 µg/mL of *n*-hexane fraction for 48 h in fresh medium. After that, media was replaced in each well with serum free media containing MTT reagent (BioShop Canada Inc. Burlington, Ontario, Canada) (1:10). The plates were further incubated at 37°C (under 5% CO_2) for additional 4 h in a humidified environment. After media aspiration, formazan crystals were dissolved with DMSO (100 µL). Absorbance of the formazan dye, generated by the reaction between dehydrogenase and MTT in metabolically active cells, was measured using a microplate reader (Tecan, Switzerland) at 492 nm. Experiments were performed at least thrice. Dimethyl sulphoxide (0.03-1%) was used as negative control. The percent inhibition of the cells was calculated using the formula:

$$\% \text{ Viability} = \frac{\text{Absorbance of sample}}{\text{Absorbance of negative control}} \times 100$$

Propidium iodide staining

The *n*-hexane fraction was subjected to microscopic analysis for apoptosis using previously reported methods [15]. Confluent MCF-7 cells (2×10^5 cells/well) were treated with 125 and 250 µg/mL of the fraction, doxorubicin (5 and 10 µg/mL) (standard) and vehicle followed by incubation at 37°C (5% CO_2 incubator) for 48 h. Then, the media was aspirated and breast cancer cells were washed with phosphate buffer saline. After washing, cells were treated with formalin (4%) and fixed with Triton X–100 (0.1%) on glass slides. After 5 min of room incubation, propidium iodide (10 µL) dye was mixed and glass slides were further incubated for 10 min in dark. The images of slides were captured (at 20X magnification) at an excitation/emission wavelength of 493/632 nm using fluorescent microscope (Nikon, ECLIPSE Ni–U, Tokyo, Japan).

4′, 6-Diamidino-2-phenylindole (DAPI) staining

The DAPI staining was used to investigate induction of apoptosis by selected fraction and doxorubicin. Briefly, MCF-7 cells were seeded in 24-well plate. Confluent cells (80%) were exposed to different concentrations of selected sample for 48 h at 37°C. After this, cancer cells were gently washed with PBS and fixed with formalin. Then, the cells were immediately treated with DAPI stain for 5 min. Later, these cancer cells were imaged using florescence microscope using 20X lens (Nikon, ECLIPSE Ni–U) [15].

Investigation of intracellular reactive oxygen species (ROS) production

The ROS production in breast cancer (MCF-7) cells by different concentrations (125 and 250 µg/mL) of *n*-hexane fraction, doxorubicin (5 and 10 µg/mL) (standard drug) and vehicle was assessed using previously described method [16]. Briefly, MCF-7 cells (2×10^5) were seeded and grown on microscopic coverslips. These cells were treated with tested samples for 48 h. Then, the cells were washed and fixed, and were treated with dichlorofluorescin diacetate (H2DCF-DA) dye (10 µL). Cells treated were incubated at room temperature for 10 min in the dark. Images of the cells were captured (at 20X magnification) at 488/530 nm excitation/emission filters using fluorescence microscope (Nikon ECLIPSE Ni–U).

Measurement of mitochondrial membrane potential (ΔΨm)

The change in mitochondrial membrane potential of MCF-7 cells, after treating with n-hexane fraction, was observed using JC-1 staining. The JC-1 probe is dimerized in cells having functional mitochondria and form aggregates, which emits red fluorescence. On the other hand, it exists in monomeric form in cells that have lost mitochondrial activity (early apoptosis) and emits green fluorescence. Briefly, the MCF-7 breast cancer cells, at a density of 2×10^5 cells/well were seeded in 24-well plate with 1 mL complete growth medium and incubated for 12 h (cells

allowed to adhere). Next, the confluent cancer cells (70-80%) were treated with 125 µg/mL of n-hexane fraction and 10 µg/mL standard drug (doxorubicin). After treatment for 48 h, medium was removed and the cells were once washed with PBS. Then, cells were fixed with formalin and incubated with 10 µL JC-1 fluorescent dye for 30 min in the dark at 37°C. The cells were imaged for any change in mitochondrial potential using a fluorescence microscope (Nikon, ECLIPSE Ni–U) at 550 nm excitation and 570 nm emission wavelength [16].

Statistical analysis

Data are presented with mean ± standard error of the mean (SEM) with confidence interval (CI) of 95%. The results were statistically analyzed for three independent experiments using two-tailed Student's t-test by applying GraphPad prism (version 5.0) software.

RESULTS

Phytochemical analysis

The n-hexane fraction of *S. indicus* was subjected to phytochemical analysis, which indicated the presence of phytosterols, and oils and resins.

GC-MS analysis of n-hexane fraction

The GC-MS analysis led to the identification of eleven chemical constituents (99.99%). Methyl esters of linoleic acid (42.58%), palmitic acid (36.43%), stearic acid (8.14%), behanic acid (4.30%), oleic acid (3.45%) and linolenic acid (1.62%). The others were methyl esters of myristic acid (0.94%), margaric acid (0.90%), pentadecanoic acid (0.67%), caprylic acid (0.56%) and elaidic acid (0.40%) (Table 1).

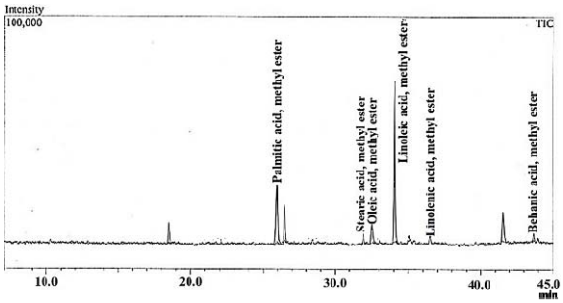

Figure 1: GC-MS chromatogram of some identified constituents in the *n*-hexane fraction of *S. indicus*.

Cytotoxicity assessments and anti-cancer activity

In the current study, *n*-hexane of *S. indicus* showed remarkable anti-proliferative effect against breast cancer cells (Figure 2A). The treatment with fraction for 48 h showed significant reduction (90.36±0.12) in MCF-7 cells viability (% cell death) in comparison to control (0) and doxorubicin, a standard drug (97.16±0.28) (Figure 2B). The respective IC_{50} values of fraction and doxorubicin after 48 h of treatment are 2.39 and 1.89 µg/mL. However, it was observed that *n*-hexane fraction induced lower toxicity to BHK-21 (normal) cells in comparison to standard doxorubicin as shown in Figure 2C and 2D, respectively.

Determination of cytotoxic activity through fluorescence microscopy

The appearance of yellowish red fluorescence, after PI staining, confirmed the cytotoxic effect of *n*-hexane fraction in apoptotic breast cancer cells (Figure 3).

Table 1: Chemical composition of *n*-hexane fraction from *S. indicus* flowers analyzed by GC-MS. RT is indicated for the retention time on chromatogram

Compound name	RT (min)	Area	Concentration (%)
*C8:0; Caprylic acid, methyl ester	7.803	707	0.56
*C14:0; Myristic acid, methyl ester	21.182	1189	0.94
*C15:0; Pentadecanoic acid, methyl ester	23.387	846	0.67
C16:0; Palmitic acid, methyl ester	25.962	45919	36.43
*C17:0; Margaric acid, methyl ester	28.810	1138	0.90
C18:0; Stearic acid, methyl ester	31.962	10260	8.14
C18:1c; Oleic acid, methyl ester	32.502	4353	3.45
*C18: 1n9t; Elaidic acid, methyl ester	32.704	499	0.40
C18:2c; Linoleic acid, methyl ester	34.080	53675	42.58
C18:3n3; Linolenic acid, methyl ester	36.520	2042	1.62
C22:0; Behanic acid, methyl ester	43.644	5417	4.30

*Constituents detected by the instrument but not visible in the chromatogram due to lower concentration (<1%). Calculation of percentages were based on the compounds detected

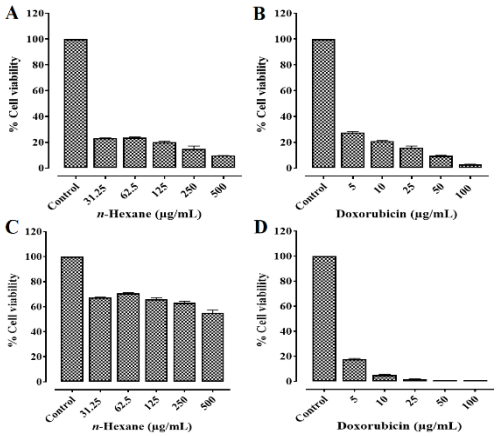

Figure 2: Graphical presentation of respective inhibitory effect of *S. indicus n*-hexane fraction (31.25-500 µg/mL) and doxorubicin (5-100 µg/mL) on MCF-7 (**A**, **B**) and BHK-21 (**C, D**) cells proliferation in a dose dependent manner. The results are expressed as the percentage of control

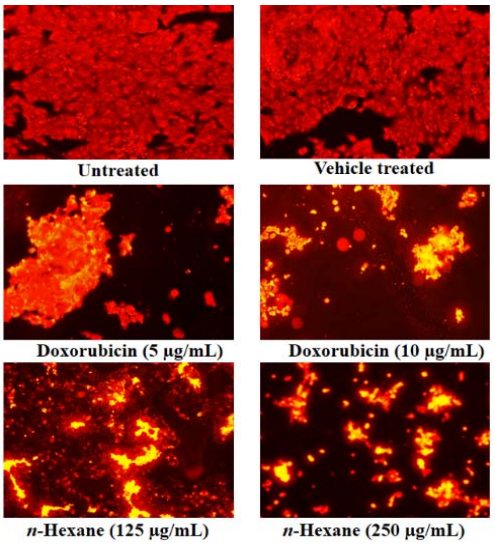

Figure 3: Representative images of changes in the nuclear morphology of MCF-7 cells induced by 125 and 250 µg/mL of *S. indicus n*-hexane fraction in comparison to standard drug (doxorubicin 5 and 10 µg/mL), vehicle treated (0.5% DMSO) and control (untreated) after PI staining.

Determination of apoptosis by DAPI staining

Breast cancer cells were stained with DAPI (a fluorescent dye) to assess the apoptosis after 48 h of treatment with fraction and standard drug. The images obtained through fluorescent microscopy revealed the binding of DAPI with the DNA of apoptotic cells (Figure 4). Moreover, apoptotic features like cellular shrinkage and condensation of nuclear material were observed as shown in Figure 4.

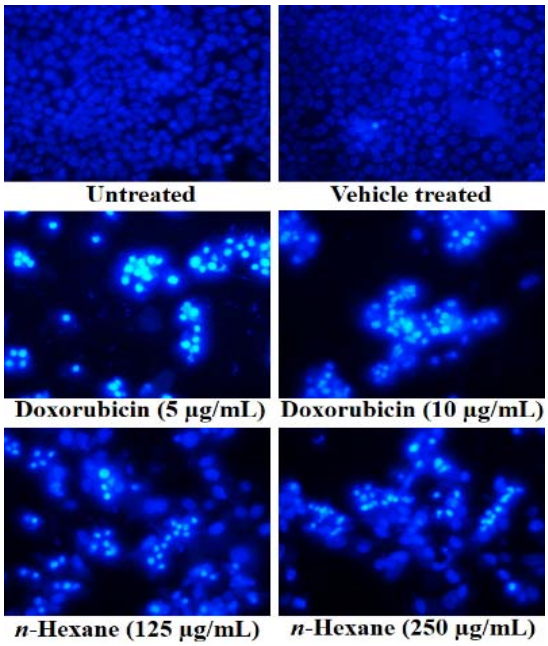

Figure 4: Representative images of changes in the nuclear morphology of MCF-7 cells induced by 125 and 250 µg/mL of *S. indicus n*-hexane fraction in comparison to standard drug (doxorubicin 5 and 10 µg/mL), vehicle treated and control (untreated) after DAPI staining

Intracellular ROS production

In the current study, 125 and 250 µg/mL of *n*-hexane fraction produced ROS comparable to doxorubicin (5 and 10 µg/mL) in MCF-7 (breast cancer) cells, which was detected by staining with 2',7'-dichlorofluorescin diacetate (H2DCF-DA). A difference in treated and untreated cells by integrity of cell membrane and intense green fluorescence can be observed in Figure 5.

Mitochondrial membrane potential

There was considerable loss in mitochondrial membrane potential in MCF-7 cells after treatment with tested sample in comparison to control. Figure 6 shows the fluorescence conversion from red to green in response to treatment with *n*-hexane fraction and standard drug, which indicates early apoptosis in cancer cells.

DISCUSSION

In this study, GC-MS analysis of n-hexane fraction from *S. indicus* indicated the presence of 11 fatty acids methyl esters. The n-hexane fraction exhibited significant cytotoxic and pro-apoptotic effects in MCF-7 cells. Similarly, an increased oxidative stress and mitochondrial dysfunction in MCF-7 cells treated with n-hexane

fraction revealed the involvement of intrinsic pathway for the induction of apoptosis.

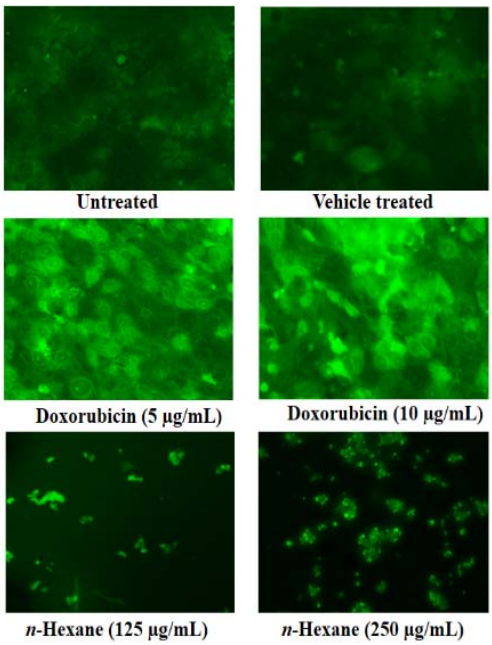

Figure 5: Representative images of oxidized dichlorofluorescein fluorescence of MCF-7 cells induced by 125 and 250 µg/mL of *S. indicus n*-hexane fraction in comparison to standard drug (doxorubicin 5 and 10 µg/mL), vehicle treated and control (untreated) after treatment with 2', 7'dichlorodihydrofluorescein diacetate.

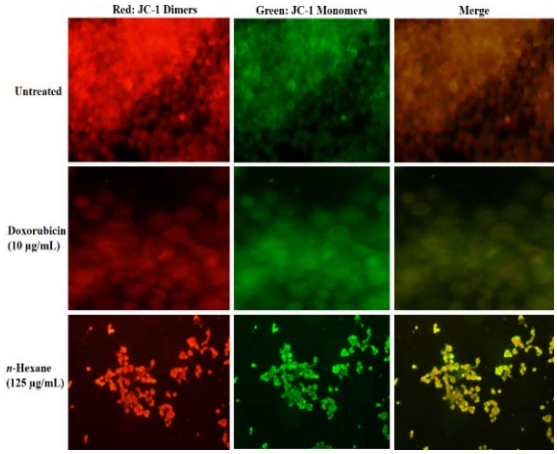

Figure 6: Representative images of morphological changes in MCF-7 cells induced by 125 µg/mL of *S. indicus* n-hexane fraction in comparison to standard drug (doxorubicin 10 µg/mL) and control (untreated) after JC-1 staining.

Using GC-MS analysis, fats and oils, previously reported in the n-hexane fraction of *S. indicus* flowers [17], were also detected in the current study. However, detection of hexose sugars in the same fraction [17] could not be confirmed in this study. This variation in chemical composition of secondary metabolites might be due to various factors like extraction procedure, solvents, habitat, edaphic factors and climate conditions [13]. In GC-MS analysis, most of the identified constituents have been reported to possess important biological activities. For example, linoleic acid, palmitic acid, linolenic acid, pentadecanoic acid, oleic acid and stearic acid have cytotoxic activity against various cancer cell lines [18-22]. On the basis of the available information; it is assumed that the anti-breast cancer effect of n-hexane fraction may be due to the presence of fatty acid methyl esters. Natural compounds may have beneficial or deleterious effects depending on their chemical nature and mode of utilization. In disease management, safety is the main concern for development of novel therapeutics [23]. In this study, a less cytotoxic activity of *n*-hexane fraction against non-cancerous cells demonstrated a good safety margin of the tested sample. Based on earlier report [24], selectively targeting of cancer cells with minimum toxicity to normal cells prevents damaging effects on body organ or tissues. However, significant reduction in viable breast cancer cells revealed tremendous anti-proliferative effect that may be attributed to the presence of linoleic acid and steric acid (major constituents), which have been reported earlier for their anti-breast cancer activities [18-20]. These results indicated the potential of *n*-hexane fraction for the development of anti-breast cancer molecules. However, to validate these *in vitro* studies, pre-clinical and clinical investigations are needed to have an insight into further details regarding the efficacy and safety of *S. indicus* flowers.

PI, a light sensitive, membrane impermeable and hydrophilic fluorescent dye, binds to nucleic acids inside the apoptotic or dead cells [25]. PI staining is used to differentiate live from dead cells. In the current study, the presence of a large population of cells with intense yellowish fluorescence indicated the cytotoxic potential of the tested fraction. Apoptotic cells have characteristic features like chromatin condensation, nuclear fragmentation, membrane blebbing and formation of apoptotic bodies [26]. In DAPI staining, an increase in bluish fluorescence indicated the binding of the dye with DNA after penetration into the cells due to increased membrane permeability. Additionally, nuclear condensation and cell shrinkage was observed in treated cancer cells as compared to the control cells, which confirmed the pro-apoptotic potential of *n*-hexane fraction. These data supported the results of PI staining.

Reactive oxygen species are produced in mitochondria normally in a low amount and play vital roles in different signaling pathways [27]. Cancer cells have a moderately increased ROS level due to higher metabolic rate and hypoxic conditions, which helps these cells to proliferate and progress rapidly [28]. However, a much higher ROS levels are lethal to cells. Thus, cancer cells are more prone to apoptosis as compared to normal cells when treated with ROS inducing compounds [29]. 2',7'-Dichloro-dihydrofluorescein diacetate (H2DCF-DA) is a non-fluorescent, cell permeable and most commonly used reagent for measuring ROS in cells [30]. After diffusion into the cells, H2DCF-DA is converted into a highly fluorescent product 2',7'-dichloro-fluorescein (DCF) by ROS [31]. Treated cancer cells, in present study, showed dose dependent DCF fluorescence. This confirmed the apoptosis in these cells due to overproduction of ROS in a similar manner as reported previously [32]. These results showed that the tested sample has an ability to induce ROS generation in breast cancer cells for apoptotic activity.

JC-1 is an excellent fluorescent stain and it is commonly used for the detection of mitochondrial membrane potential in tissues or cells [33]. When the mitochondrial membrane potential is increased, JC-1 accumulates in mitochondria forming J-aggregates (red fluorescence) [34]. Contrarily, when mitochondrial membrane potential is reduced, JC-1 cannot accumulate in mitochondrial environment and JC-1 monomers with the green fluorescence are produced [35]. Therefore, mitochondrial membrane potential can be easily determined based on this fluorescence color change. A decrease in mitochondrial membrane potential is a sign of an early apoptosis [36]. The current study revealed an intense green fluorescence as compared to red fluorescence in fraction treated cancer cells, which is an indicative of early apoptosis in breast cancer MCF-7 cells due to mitochondrial dysfunction.

CONCLUSION

The GC-MS analysis results suggested that *n*-hexane fraction of *S. indicus* is highly rich in fatty acids. Moreover, current study demonstrated that *n*-hexane fraction induced apoptosis in MCF-7 cells possibly through nuclear condensation, ROS generation and mitochondrial dysfunction pathways. Anti-cancer and apoptotic properties of *n*-hexane fraction may be attributed to linoleic acid (a major constituent). Therefore, phyto-constituents identified in this study may serve as potential candidates for the development of anti-breast cancer agents for clinical applications.

DECLARATIONS

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Conflicts of interest

Authors have no conflict of interest.

Availability of data and material

Full data is available on reasonable request.

Ethics approval

The study was approved by the ethical committee of Department of Pharmacy, CUI, Abbottabad Campus, Pakistan.

Authors' contributions

All authors reviewed the manuscript and give consent for publication. TK, FW and AJS conceived the project, supervised the research, and reviewed the manuscript. HMR, MI and MQ designed and performed the experiments, analyzed the results, wrote the manuscript as well as prepared the figures. We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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