RESEARCH ARTICLE

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Antitumor Activity of *Choerospondias axillaris* Fruit Extract by Regulating the Expression of SNCAIP and SNCA on MDA-MB-231 Cells

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Abstract

Objective: Cancer is a huge problem of disease globally. Today, the percentage of people die from cancer is more than a combination of various diseases. In females, most common types of malignancies that occur are breast and cervical. The present focus has been shifted on medicinal plants as a form of therapy and there is a constant need to identify new therapeutic agents. *Choerospondias axillaris* (*C. axillaris*), an underutilized fruit, has been used in the remedy of various diseases. In the present communication, we evaluated the molecular mechanism of *C. axillaris* methanol extract in regulating cell death in human breast cancer cells (MDA-MB-231). **Methods:** Methanol extract of *C. axillaris* was prepared and compounds were screened by Gas chromatography-mass spectrometry. The effect of fruit extract was determined on MDA-MB-231 cells by MTT ((3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay and to analyse the molecular mechanism of human breast cancer cells after treating with fruit extract, protein profiling study was performed by two-dimensional gel electrophoresis. **Results:** A total 9 differentially expressed proteins were identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS/MS) analysis. Among 9 identified proteins, synphilin-1 protein was found to be significantly downregulated, validated by western blot and RT-qPCR analysis. Possible interacting partners of synphilin-1 (SNCAIP) were analyzed for their possible role in cancer by the in-silico method. **Conclusion:** Our data implicate that the presence of bioactive compound(s) in *C. axillaris* fruits might play an important role in inhibiting the proliferation of breast carcinoma cells and Synphilin-1 protein may play a role of apoptotic function.

Keywords: *Choerospondias axillaris*- Phytochemicals- GC-MS- proteomics- protein-protein interaction

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Introduction

Breast cancer ranks second to cervical cancer in women, according to the National Cancer Registry Program (Mathur et al., 2020). It is the most frequently diagnosed malignant tumours and reported to have the largest number of deaths due to breast cancer in India and worldwide [Ayob et al., 2014, Chimplee et al., 2019). Nearly 80% population worldwide relies on traditional medicine (Oyebode et al., 2016) for their primary health care. Efforts have always been focused on discovering new anti-cancerous agents from medicinal plants and have been successfully resulted in several experimental models using natural products for the treatment (Basri et al., 2016, Mann et al., 2015, Khan et al., 2020). Therefore, traditional medicine knowledge was used to discover novel anticancer drugs (Figueroa et al., 2012). Plant-based anticancer compounds may play a significant role in cancer therapy (Abdullah et al., 2014) and has been considered to be safe with fewer side effects in comparison to the available synthetic anticancer agents (Lichota and Gwozdzinsk, 2018).

Present paper has been focused on investigating an underutilized fruit *Choerospondias axillaris* (Anacardiaceae) for its anticancer property. It is commonly known as Lupsi or Lapsi and is available in North-East India from October to January and is a native of India, China, Japan and other Asian countries. The bark of *C. axillaris* tree is used for treating secondary burns (Nguyen et al., 1996). Recently, we have reported that *C. axillaris* fruit possesses anti-oxidant and anti-inflammatory properties (Mann et al., 2020). Earlier, total flavonoid content of *C. axillaris* was reported to have therapeutic potential for treating myocardial ischemia (Li et al., 2013). Tsantan Sumtag, a traditional Tibetan medicine also consists of *C. axillaris* (Roxb.) Burtt et Hill along with other ingredients, has been reported to be used as a folk medicine for cardiovascular diseases and heart

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failure (Dang et al., 2020).

The aim of the study was to evaluate the metabolic profile using Gas chromatography-mass spectrometry (GC-MS) and cytotoxic activities of methanol extract of *C. axillaris* fruits on human breast cancer cells and its mode of action.

Materials and Methods

Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 95% ethanol, methanol, dimethyl sulphoxide (DMSO), fetal bovine serum (FBS), phosphate buffer saline (PBS), antibiotics, trypan blue dye solution and trypsin ethylenediaminetetra acetic acid (EDTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Raw materials and preparation of crude extract

C. axillaris fruits were purchased from a local market in Kalimpong (West-Bengal), India and was recognized by a Botanist and voucher specimen from the Council of Scientific and Industrial Research (CSIR)- National Institute of Science Communication and Information Resources, Delhi, India. Fresh fruits of *C. axillaris* (100g) were washed, deseeded, grounded and 200ml methanol was added. The solution was centrifuged for 24h at 150rpm at 45˚C in an incubator shaker and filtered. The supernatant was recovered, dried under vacuum and stored at -20˚C for further analysis (Mann et al., 2016). *C. axillaris* fresh fruits were also extracted with a range of solvents depending upon the polarity (petroleum ether, n-hexane, dichloromethane, and methanol). Optimum results were obtained in methanol extract; hence only methanol extract was preceded for the present study (Mann et al., 2020).

Gas chromatography - mass spectrometry (GC-MS) analysis

Compounds present in the crude methanol extract of *C. axillaris* were identified by Agilent 6890 GC and 5975B MSD (Agilent Technologies#51832037). The extract was diluted in acetone and 1µl of the extract was injected into split injection (1:20) at 280˚C. Fused silica HP-5 ms capillary column was used for chromatographic separation. EI mode was used for eluents detection with ionization energy of 70eV. All the peaks of identified compound were compared with the spectra from the National Institute of Standards and Technology library (NIST'05) (Satpathy et al., 2011).

In vitro assay-MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)

MDA-MB-231 cell line was obtained from the National Centre for cell science (NCCS), Pune, India. Cells were cultured in Dulbfecco's Modified Eagle's medium (DMEM), supplemented with 10% FBS and 1% antibiotics in an incubator at 37˚C with 5% CO_2. Cells were seeded in a 96-well plate having density 2×10^4 cells/well for 2h (Tominaga et al., 2015), incubated with various concentrations of methanol extract of the *C. axillaris* fruit (1,3,5,7,9 mg/ml) in triplicate at 37˚C in a CO_2 environment for 24h with not more than 1% final concentration of DMSO . The negative control was performed using growth medium with DMSO (1,3,5,7,9 mg/ml), while brucine (1,2,3,4,5 mg/ml) was used as the positive control. MDA-MB-231 cells without any treatment were used as control. Additionally, trauma cells (non-cancerous) were served as a healthy control (Makrane et al., 2018) generated from the tissues of trauma patients. Briefly, trauma tissues were chopped and transferred to DMEM containing collagenase (1mg/mL) and incubated for 12h at 37˚C in an incubator shaker. Cells were then transferred to T-flask after passing the digested tissues from the cell strainer of pore size-100µm and subcultured as per the needs. MTT solution (20µl) having a concentration 5mg/mL was added to each well and incubated again for 4h. After discarding the medium, solubilisation buffer was added to dissolve the formazan precipitate. The absorbance was recorded using a microtiter plate reader at 570 and 630nm (background) and the viability of the cells were determined as the percentage of live cells compared to control cells. The half-maximal inhibitory concentration (IC_{50}) of each treatment was estimated via dose-response curve.

Two-Dimensional Gel Electrophoresis (2-DE)

Protein estimation was performed by standard Bradford method. Protein (80µg) from cell lysate (*C. axillaris* treated cells and untreated cells) was solubilised for 30min with rehydration buffer (7M urea, 2M thiourea, 1% zwitterionic detergent ASB-14, 40mM Tris) and kept for 2h at room temperature (RT). The rehydrated sample was loaded on 7cm (Bio-Rad, USA) immobilized pH gradient (IPG) strip (pH- 4-7 NL strips) and kept for another 1h at RT followed by an overlay of mineral oil (Bio-Rad, USA) on the strip and overnight incubation. Proteins were then focused according to the isoelectric point using Protean Isoelectric focusing (IEF) System (Bio-Rad, USA), with the program: 250V rapid voltages ramping for 30min, 10,000V slow voltages ramping for 60min, and 10,000V rapid voltages ramping for 50KVh. After IEF, the strips were incubated for 20min in an equilibration buffer I (6M urea, 20% glycerol, 2% sodium dodecyl sulfate (SDS), 2% Dithiothreitol (DTT), and 0.375M Tris, pH 8.8) at RT followed by incubation in equilibration buffer II for an additional 20min by adding 2% iodoacetamide instead of DTT in the equilibration buffer I. The strips were then loaded onto 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and run in Mini protein gel assembly (Bio-Rad, USA) at 50V for 2.5h. A molecular standard was used to estimate the relative mass (Biswas et al., 2013). More than 3 sets of gel were run and silver stained.

Silver staining

After running 2-DE, gels were rinsed with water, followed by silver staining (Biswas et al., 2013) for spot visualization. Briefly, gels were fixed in fixative (50% methanol and 12% acetic acid) for 1h and then treated with 50% and 30% ethanol separately for 30min each. Gels were sensitized with freshly prepared sodium sulphite

(Na2S2O3) for 1min, washed with Milli Q and stained with 1M silver nitrate solution under dark. Again, gels were washed with Milli Q, and developed with sodium carbonate (60g/lt Na2CO3, 500µl/lt HCHO & 40µl//lt Na2S2O3). Once the spots appeared, further reactions were stopped by adding stop solution (6% acetic acid in MQ) (Biswas et al., 2013). All the sets of gel were silver stained and the three best sets of gel were used to make a master gel for each experimental group. The master gels were analyzed using "PDQuest" 2-D Analysis software (Bio-RAD) and significant differential protein spots with $p \leq 0.05$ were selected for further processing.

In-gel digestion and Matrix-Assisted laser Desorption/ Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS/MS) analysis

In gel digestion was carried out according to the method of Biswas et al., 2013. The screened and selected protein spots were subjected to in-gel protein digestion and proceeded for MALDI TOF MS/MS analysis as follows. Briefly, protein spots were excised manually, washed with distilled water and then destained with 30mM potassium ferricyanide (50µl of 1:1 solution) and sodium thiosulphate (100mM). The gel pieces were further washed and dehydrated with 40µl of 1:1 acetonitrile (ACN)/water for 15min, washed with 40µl of ACN, equilibrated with 100mM ammonium bicarbonate (50µl) followed by the addition of 50µl ACN for 15min. Gel pieces were then dried using vacuum centrifugation and then digested by incubating with trypsin (10µl; 0.1µg/µl trypsin) for 45min. After incubation, 25-60µl of trypsin digestion buffer was added (5µl of 1M $CaCl_2$, 25µl of 1M NH_4CO_3) and incubated again at 37˚C overnight. The collected supernatant was then dried in a vacuum centrifuge. Extraction of peptides from the gel pieces were carried out by altering ACN and trifluoroacetic acid (TFA) concentration, sonicated for 30min in a water bath, followed by drying in speed-vac to remove TFA/ACN. The peptides were then stored at -20˚C till further use.

MALDI-TOF MS/MS (Applied Biosystems, Life Technologies, USA) was used for the determination of the molecular mass of polypeptides. Peptides (1µl) were dissolved with 1µl sinapinic acid matrix onto the MALDI target plate and dried. Spectra of MS/MS were found to be distributed within 10,000–20,000Da mass range in reflector positive mode having an intensity of 5600 laser shots. For the MS/MS precursor selection, minimum S/N (signal/noise) filter was set at 25 with an exclusion list for α-Cyano-4-hydroxycinnamic acid (CHCA) matrix peaks. Identifications of proteins were accepted with a statistically significant probability based Mowse score ($p \leq 0.05$). The protein score above 45 was considered a threshold score, having at least 2 unique peptide matches (Millares et al., 2012). However, a protein having a lower score was further validated using other technique such as Western-Blot and immunohistochemistry (IHC) (Llombart et al., 2017).

Western Blot Analysis

Protein (50µg) from cell lysate (*C. axillaris* treated cells and untreated cells) was run in 10% SDS gel using Mini protein gel assembly (Bio-Rad, USA) and transferred to the nitrocellulose membrane (NC) using Transblot semi-dry (Bio-Rad, USA). NC membrane was blocked with 5% BSA overnight. The membrane was washed 3 times with PBST (0.05% tween 20 in 1X PBS) for 10min each and then incubated for 3h with primary antibody Synphilin 1; 1:3,000 (monoclonal antibody, Santa Cruz Biotechnology; sc-365741). After washing again (3 times), the secondary antibody (polyclonal, Horseradish Peroxidase (HRP) conjugated anti-mouse (Jackson, USA)) was added in 1:5000 ratio and incubated for 1h. The immunoreactive bands were then visualized using enhanced chemiluminesc=-0980-nce (ECL) reagent (G-Biosciences). The densitometric analysis was carried out using Chemi Doc (BioRad, Image Lab 5.1).

Total RNA extraction and RT-qPCR

RNA was extracted from MDA-MB-231 cells (treated with 5mg/ml of *C. axillaris* methanol extract and untreated) using TRIzol reagent (G-Biosciences) and quantified using a nanodrop (Thermo Scientific, Nanodrop 1000). First strand cDNA was synthesised using kit (G-Biosciences) following the manufacturer's instructions. The sequences of the primers used for the RTPCR are shown in Table 1. The reverse transcription conditions were as follows 95°C for 5min for RT inactivation. The amplification was performed using the following conditions: 15s at 95°C for denaturation, 15s at 60°C for annealing, 15s at 72°C for extension.

Prediction of interacting partners

Interacting partners of SNCAIP (Synphilin-1) were identified using STRING database (http://string-db.org/). This database gives physical and functional protein-protein interaction network that may exhibit functional association of these partners with synphilin-1 (Khairon et al., 2016). The string network analysis was set at medium confidence (0.400). Proteins were linked to each other grounded on various criteria such as neighbourhood, gene fusion, co-occurrence, text mining, co-expression, experimental evidence, existing databases and protein homology (Khairon et al., 2016).

Results

In order to investigate anti-cancer property of *C. axillaris* fruit, following assays were conducted.

Bioactive Compound analysis via GC-MS

Eight bioactive compounds have been identified in the methanol extract of *C. axillaris* fruit by GC-MS analysis. Retention time, % area, molecular formulae and functions of these identified compounds has been shown in Table 2. The anti-cancerous property of *C. axillaris* extract has been investigated in this study by in-vitro experiments and protein profiling study by proteomic analysis.

Cytotoxic effect of methanol extract on control and cancer cells

The antiproliferative activity of methanol extract of *C. axillaris* fruit was evaluated against breast cancer cells

Table 1. Primer Sequences of RT-qPCR.

Gene	Forward Primer	Reverse Primer
SNCA	GGAGTGGCCATTCGACGAC	CCTGCTGCTTCTGCCACAC
SNCAIP	CATCAGGGGGACGCAGGTTT	TGTGGAGGTCCGCTGGAGAG
Bax	CCCGAGAGGTCTTTTTCCGAG	CCAGCCCATGATGGTTCTGAT
BCl2	TTGCCAGCCGGAACCTATG	CGAAGGCGACCAGCAATGATA
Caspase-3	CATGGAAGCGAATCAATGGACT	CTGTACCAGACCGAGATGTCA
Caspase-8	TAGGGACAGGAATGGAACACACTT	GAGAGGATACAGCAGATGAAGCAGT
ACTIN	CATCCGCAAAGACCTGTACG	CCTGCTTGCTGATCCACATC

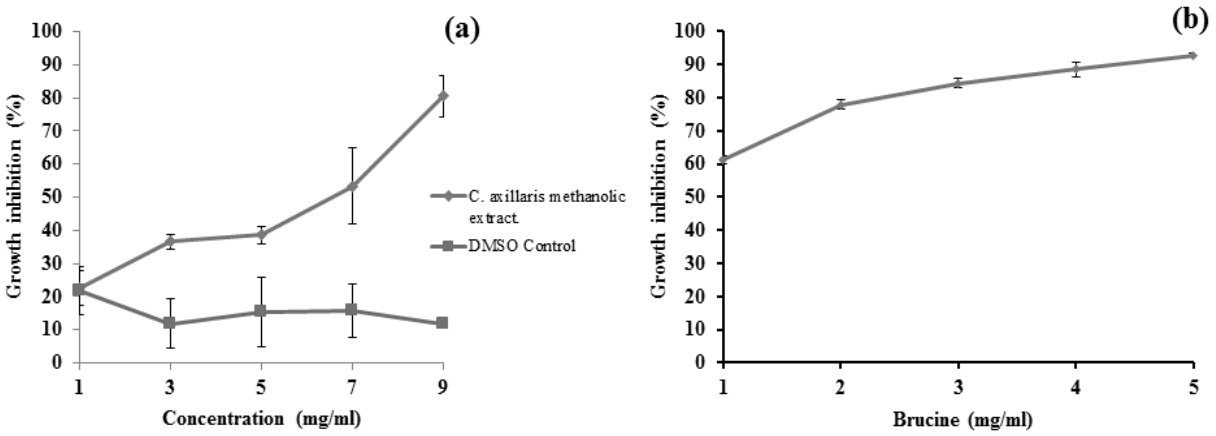

Figure 1. Cytotoxicity of (a) Methanol Extract of *C. axillaris* Fruit and DMSO Control on MDA-MB-231 Cells and (b) standard Brucine, seeded in 96 well plate (10,000 cells/well). Independent experiment was repeated thrice. Results are presented as mean ± S.D.

by MTT assay. MTT assay is a sensitive, quantitative and reliable colorimetric technique, most commonly used to determine cell viability (Ogbole et al., 2017). The human breast carcinoma cell line was used, which are estrogen receptor negative (ER-) MDA-MB-231 cells to determine the cytotoxicity of *C. axillaris* fruit extract. The MDA-MB-231 cell line is commonly used to model breast cancer (Conn 2013). The survival of the cells was determined after 24h treatment (*C. axillaris* extract, brucine and DMSO treatment). Brucine was used as a standard positive control whereas DMSO as negative control. The response of MDA-MB-231 cells with increasing concentrations of the methanol extract of *C. axillaris* fruit and DMSO has been shown in Figure 1a, and brucine has been shown in Figure 1b. Results showed that the proliferation of the cells (MDA-MB-231) were decreased significantly when treated with a methanol extract of *C. axillaris*, p-value ≤ 0.039 and when treated

Table 2. Secondary Metabolite Composition in *C. axillaris* Methanol Extract

RT	*Cas#	Compound Name	Molecular formula	**% Area	Function	Reference
6.194	000616-02-4	Succinic anhydride	C4H4O3	40.52	--	
8.605	000823-82-5	2,5-Furandicarboxaldehyde	C6H4O3	3.93	Antimicrobial, Preservative	[26]
9.805	028564-83-2	4H-Pyran-4-one	C5H4O2	4.15	Antimicrobial, Anti-inflammatory, Antiproliferative	[26]
11.274	000067-47-0	2-Furancarboxaldehyde, 5-hydroxymethyl	C6H6O3	29.73	Antimicrobial, Preservative	[26]
15.435	140224-88-0	6-Methyl benzobicyclo [2.2.1] heptenone-5	C12H12O	2.86	--	
16.545	001860-39-5	Hexanal	C6H12O	10.38	--	
19.932	000112-39-0	Hexadecanoic acid	C16H32O2	1.14	Antioxidant, Hypercholesterolemic, Lubricant, Pesticide, Antiandrogenic, Flavor, Hemolytic 5 alpha reductase inhibitor, Nematicide	[25]
21.637	000301-00-8	9,12,15-Octadecatrienoic acid	C18H30O2	0.68	Hepatoprotective, nematicide, insectifuge antihistaminic, antiarthritic, anticoronary, antieczemic antiacne,	[25]

*Chemical Abstracts Service (Identify the Chemical compound); ** % Matching with NIST library (mean 'Q value' is 91± 5.12%, n=3); RT Retention time of the compound, in minute; "area (%)" the percentages of the area of the total ion chromatogram represented by the peaks of each of the compounds identified. (+) means presence and (-) means absence

Table 3. Differentially Expressed Proteins on Treatment with *C. axillaris* Methanol Extract

Spot No.	Name of protein	Up/Down regulated in treatment	Protein Score	Theoretical mass (Da)	Experimental Mass (Da)	PI value*	Function**
1	Synphilin-1e-protein	↓	16	7744	7911	4.38	Cell death
2	HCG 1781285	↑	27	-	7247	6.31	-
3	General transcription factor IIB	↓	22	34833	25543	8.53	Gene expression
4	Cystic fibrosis transmembrane conductance regulator	↓	20	168142	4688	8.9	Transmembrane transport
5	Polyamine- modulated factor 1 isoform 3	↑	29	23339	20673	8.8	Chromosome segregation, mitotic nuclear division
6	Putative truncated protein	↓	19	-	15542	5.9	-
7	Interlukin 1 receptor like 1 isoform 2	↓	22	-	7807	6.1	Immune/inflammatory disorders; Upregulation in several conditions like inflammation, asthma, RA
8	HCG 2000618	↓	27	-	8853	4.75	-
9	Sodium channel type V alpha subunit	↓	26	226940	2018	6.02	Cellular response to calcium ion

↑, Up regulated; ↓, Down regulated; * Iso Electric Point; **, http://www.rcsb.org/

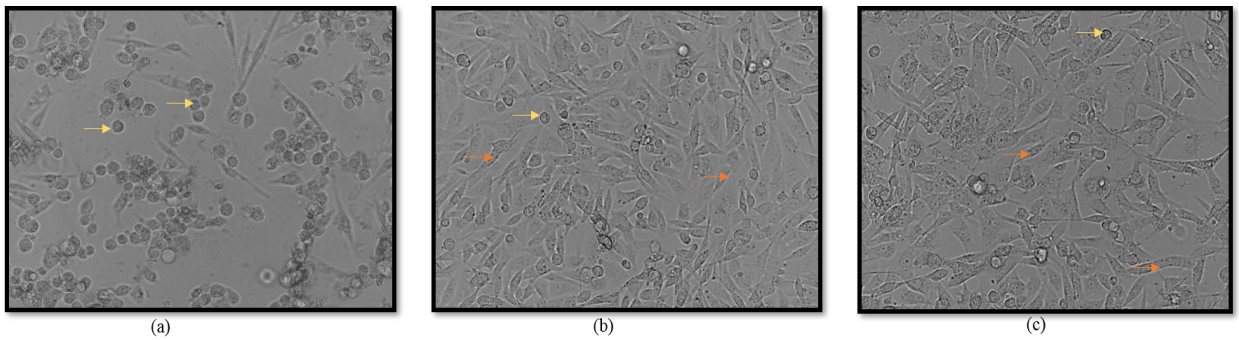

Figure 2. Representative Images of MDA-MB-231 Cells Showing; (a) MDA-MB-231 cells treated with methanol extract of *C. axillaris*, (b) DMSO treated MDA-MB-231 cells, (c) Healthy MDA-MB-231 cells at 10x resolution under inverted microscope (Nikon ECLIPSE TE2000-s). Dead cells are indicated with yellow colour arrows and viable cells with red arrows.

with standard brucine (p-value ≤ 0.0001), indicating that the results were statistically significant (Figure 1). DMSO treated cells were observed to have non-significant results as p-value ≥ 0.05. Brucine is a natural plant alkaloid that was reported to have IC_{50} at 116.4µM concentration against MDA-MB-231 cells (Habli et al., 2017, Serasanambati et

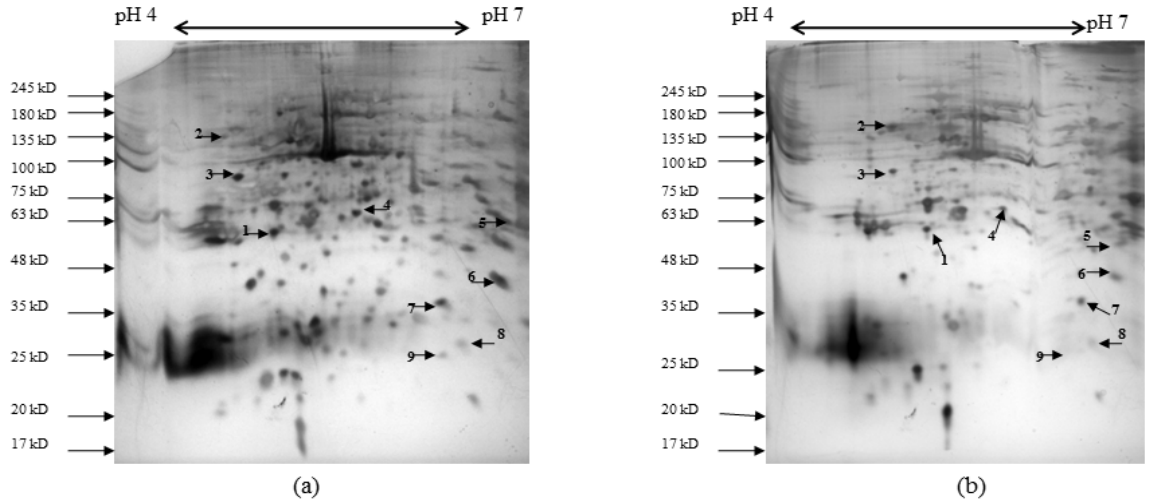

Figure 3. A Representative 2D Image of (a) MDA-MB-231 control cells without treatment and (b) cells treated with *C. axillaris* methanol extract (5 mg/ml); showing differentially expressed proteins.

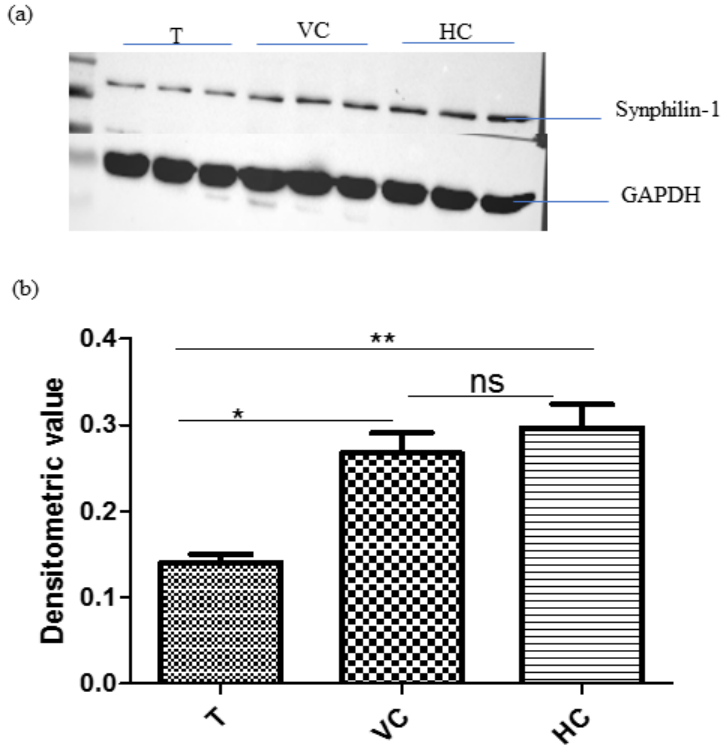

Figure 4. Western Blot Analysis (a) Representative image of protein expression level of Synphilin-1e. MDA-MB-231 cells were treated with 5mg/ml of *C. axillaris* methanol extract for 24h. (b) Densitometric analysis of Synphilin-1e protein over expression revealed 2.1fold change with $p<0.04$ after normalization with loading control GAPDH. T=Treated cells of MDA-MB-231 with 5mg/ml of *C. axillaris* methanol extract, VC=Vehicle control and HC=Control MDA-MB-231 cells without any treatment.

al., 2014). The IC50 value of *C. axillaris* extract was found to be 6.1±0.49mg/ml as compared to brucine (0.83mg/ml) indicating that *C. axillaris* extract possesses less cytotoxic property then brucine. Brucine being a pure compound may be highly active and hence may be more cytotoxic than the crude methanol extract. Similar results were also observed when an extract of Allium atroviolaceum flower was used to treat cells (MDA-MB-231) and was reported to have an anti-cancer property (Khazaei et al., 2017). Thus, to confirm our cytotoxic results, morphological

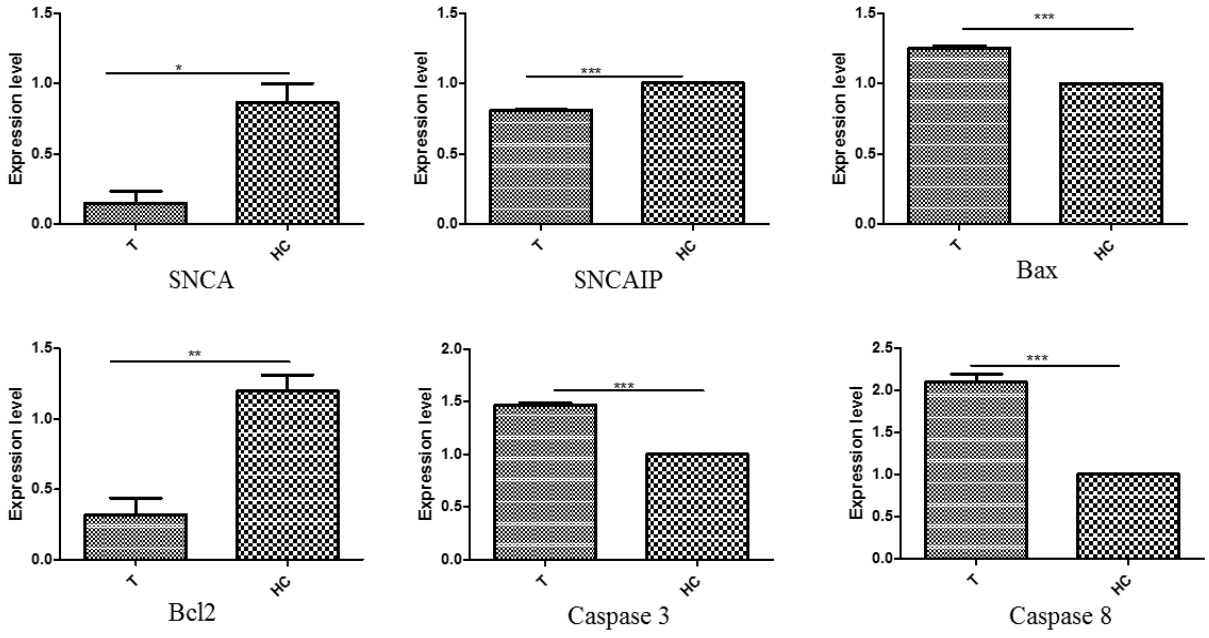

Figure 5. Effect of *C. axillaris* Methanol Extract on Apoptosis-Related Gene Expression in MDA-MB-231 Cells as Determined by RT-qPCR. T =Treated cells of MDA-MB-231 with 5mg/ml of *C. axillaris* methanol extract and HC=Control MDA-MB-231 cells without any treatment. * = Pvalue ≤ 0.0455, ** = Pvalue ≤0.0064, *** = Pvalue ≤ 0.0001.

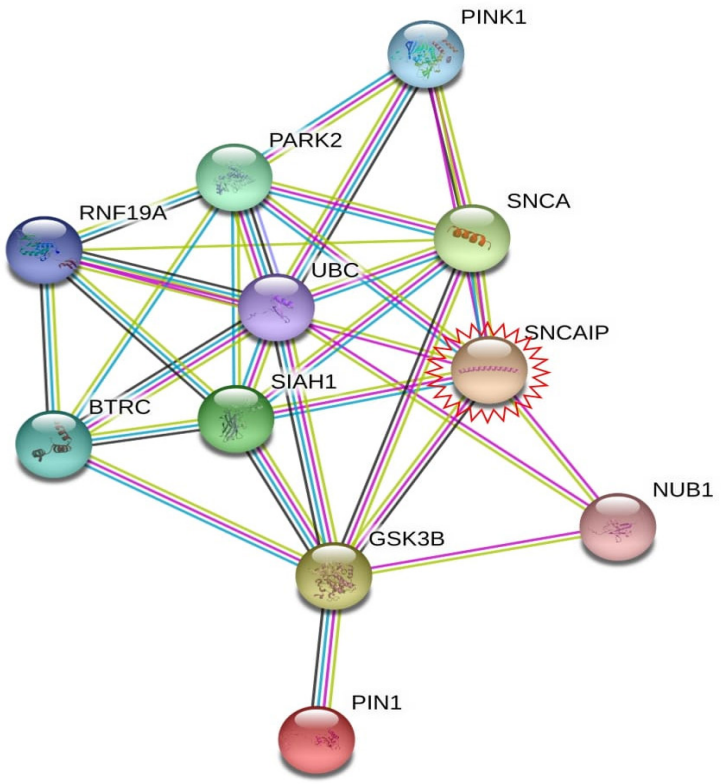

Figure 6. Interacting Partners of Synphilin-1 Protein (STRING network). Alpha-synuclein (SNCA), Glycogen Synthase Kinase 3 Beta (GSK3B), seven in absentia homolog (SIAH1), Ubiquitin C (UBC) and NEDD8 ultimate buster 1 (NUB1) showed close association with synphilin-1.

changes of cells (MDA-MB-231) were observed under an inverted microscope (Nikon ECLIPSE TE2000-s) at 10x magnification. Untreated and DMSO treated cells were observed to grow uniformly in high density monolayer with inherent morphology (Figure 2b and 2c). Whereas, treated (*C. axillaris* methanol extract treated) cells were observed to show the features of apoptosis such as shrinking, irregular nuclei and nuclear fragments (Figure 2a) than untreated cells, indicating that the treated cells were affected by cytotoxic property of extract (*C. axillaris*).

Further, experiments were conducted using additional control of human cells, generated out of trauma patients. The results revealed that there was no statistically significant (p-value ≤ 0.7231) cell death amongst control trauma cells (Supplementary Figure 1s, Figure 2s).

Proteomic analysis

In order to check the effect of the extract on the protein profile of cells (MDA-MB-231), total protein was extracted after the treatment of cells with methanol extract of *C. axillaris* fruit (5mg/ml). Nine proteins were identified after the differential protein profile analysis of control and experimental cells (Table 3, Figure 3). Proteins were well separated within the range of 10-70kDa and isoelectric points between pH 4 and 7.

Synphilin-1 protein was selected further among all identified proteins to study the effect on breast cancer. To further strengthen our studies, validation of Synphilin-1 protein was carried out by western blot analysis and 2.1 fold higher expression of Synphilin-1 protein was revealed in *C. axillaris* treated cells as compared to control cells ($p<0.005$) (Figure 4).

RT-PCR analysis of apoptosis related genes

Compared to untreated cells, the expression of Bax, Caspase-3 and Caspase-8 increased while the expression of BCl-2 decreased significantly in treated cells ($p<0.05$). Expression of SNCA and SNCAIP was also found to be decreased significantly ($p<0.05$) in treated cells as compared to untreated cells (Figure 5).

STRING analysis

Additionally, to elucidate more cancer-related roles, interacting partner study of SNCAIP (Synphilin-1) was carried out using the STRING database. The analysis revealed that this protein has 10 interacting partners (Figure 6).

Discussion

Breast cancer is the most frequent cancer among women in the world. There is a constant rising demand for new therapies to treat cancer because of toxicity of conventional drugs. Report shows that the naturally-derived compounds has less toxic side effects compared to presently available chemotherapy treatments at low cost (Greenwell and Rahman, 2015). Bioactive compound occurs in less quantity in fruits and vegetables, and provide various health benefits beyond nutrition (Singh et al., 2016). It also possesses therapeutic potential such as reducing inflammation, oxidative stress

and metabolic disorders (Singh et al., 2016). Amongst all the eight identified compounds via GC-MS in methanol extract of *C. axillaris*, 4H-Pyran-4-one was reported to have antiproliferative activity (Mahdavia et al., 2018) and other identified compounds (2,5-Furandicarboxaldehyde, hexadecanoic acid and 5-hydroxymethyl) were reported to have antimicrobial, anti-inflammatory and antioxidant activity (Tyagi et al., 2017, Ramalakshmi et al., 2011). Herbal/natural medicinal plants are producing secondary metabolites that are playing a role in the prevention and treatment of cancer (Greenwell and Rahman, 2015). Earlier studies also demonstrated the use of total flavonoid content (possessing good antioxidants) of *C. axillaris* extract for the treatment of cardiovascular diseases in clinic (Li et al., 2013). Studies have also reported the use of *C. axillaris* in aquaculture over drugs and antibiotics as the drug causes several side effects to the consumer (Shakya and Labh, 2019). The methanol extract of *C. axillaris* has been demonstrated to have good cytotoxic activity against breast cancer cells, suggesting that the extract might have great potential as an anti-cancer agent. To strengthen our results further, proteomic analysis was carried out.

Functional analysis indicated that the majority of the identified proteins were related to binding and transport mechanisms. The cell surface proteins are mainly phosphoproteins (DAVID Bioinformatics Resources, 6.7) and are playing various roles in different pathways. Amongst several differential proteins, dysfunctional cystic fibrosis transmembrane conductance regulator (CFTR) was found to be responsible for increased blood adenosine 5′-triphosphate (ATP) concentration in mice and in cystic fibrosis (CF) patients. Increased extracellular ATP concentration was demonstrated to inhibit tumor growth (Qiaoa et al., 2008). The upregulation of human chorionic gonadotropin (HCG) was observed in breast cancer. Studies reported that the overexpression of HCG decreases the proliferation and invasion of breast cancer (Toprak et al., 2017). Further, we have identified the synphilin-1 protein as a downregulated protein compared to control, in breast cancer cells after treatment with methanol extract of *C. axillaris*. But the role of this protein in disease pathogenesis has not been documented well, negligible reports are available. Literature report shows that the expression of α-synuclein (SNCA) was observed to have a close relationship with Synphilin-1 (SNCAIP), which has been reported to be related with various tumors. Downregulation of SNCA demonstrated potential tumor suppressor function and has a role in regulation of p53 gene, showing defects in case of cancer (Li et al., 2018, Szargel et al., 2009). Studies have also reported that knocking out SNCA in melanoma cells suppresses tumor growth (Shekoohi et al., 2021). As, SNCA and Synphilin-1 are significantly corelated, it can suggest that the protein (Synphilin-1) might control the growth of cancer cells, hence decreased cell proliferation.

Apoptosis is a gene related cell death process known as programmed cell death. Two types of signalling pathways lead to apoptosis i.e. extrinsic and intrinsic (Jang et al., 2020). The extrinsic pathway follows caspase cascade which finally leads to cell death, whereas intrinsic pathway is mitochondria-dependent and responds to various stress conditions, such as cytosolic calcium, genetic damage, and oxidative stress (Adams and Cory, 2007, Jonges et al., 2001, Llambi and Green, 2011, Liang et al., 2011). In the regulation of cell apoptosis Bcl-2 family plays an important role, which includes a sequence of antiapoptotic and proapoptotic markers. Apoptotic signalling pathway via Bcl-2 further stimulates the apoptotic pathway through various intercellular and intracellular signals. Bax is a proapoptotic protein which falls in the Bcl family, and it enhances apoptosis by activating caspases. On the contrary, Bcl-2 is an antiapoptotic protein, and it prevents apoptosis by inhibiting the mitochondrial apoptogenic factors release into the cytoplasm. Apoptosis is controlled by the comparative concentration of Bax and Bcl-2. Loss of MMP occurs when there is an imbalance of Bax and Bcl-2 proteins, which leads to release of cytochrome C, and activation of Caspase-3 and further results in apoptosis. SNCA and SNCAIP were also found to be significantly decreased as compared to untreated group, and studies have reported that downregulation/knocking out of SNCA supressed tumor growth (Shekoohi et al., 2021). As, SNCA and SNCAIP are positively corelated (Li et al., 2018) and in present study *C. axillaris* methanol extract treated cells have shown downregulation of SNCA and SNCAIP which might indicate that downregulation of Synphilin-1(SNCAIP) also helps in tumor suppression.

String analysis revealed 10 interacting partners of Synphilin-1, beta-transducing repeat containing E3 ubiquitin protein ligase (BTRC), Glycogen Synthase Kinase 3 Beta (GSK3B) and NEDD8 ultimate buster 1(NUB1) showed a close association with synphilin-1. BTRC was found to be involved in ubiquitination of protein, whereas GSK3B, is an anti-apoptotic protein, playing an important role in p53 binding (Jacobs et al., 2012). This protein phosphorylates Mucin 1(MUC1) and interferes in the interaction of Catenin beta-1(CTNNB1) in breast cancer cells (Adams and Cory, 2007). Another partner NUB1 includes tumour suppressor that includes growth arrest and apoptosis (Yang et al., 1999, Hosono et al., 2010). Results obtained from our analysis implicate that synphilin-1 may exhibit its antiproliferative function in association with identified interacting partners and presence of various bioactive compounds. Further studies are required to understand the effect of interacting partners on synphilin-1 function to use *C. axillaris* in the regulation of apoptosis during the cancerous conditions.

In conclusion, the investigation of our research towards the health beneficial properties of underutilized (*C. axillaris*) fruits may contribute to enhance its utilization. The findings of the present study indicated that *C. axillaris* fruit has various bioactive compounds that exhibit anti-oxidant, anti-microbial, and anti-inflammatory activities. The methanol extract of *C. axillaris* fruit also exhibit anti-cancerous property and the down-regulation of Synphilin-1 protein. Synphilin-1 protein may play a role in apoptotic function along with various protein interacting partners. Thus, this study implicates that *C. axillaris* fruit extract may be considered for the design and development of novel drug for treating cancer in the near future.

Author Contribution Statement

SM: work design, experimental and manuscript writing; AS and AS: Experimental and writing; RKG: data analysis and SB: data analysis, manuscript writing and overall approval of the work.

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Availability of data and materials

The authors confirmed that the data supporting the findings of this research are available within the article.

Conflict of interest

The authors declare no conflict of interest, financial or otherwise.

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