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ORIGINAL ARTICLE

Evaluation of cytotoxic activity of *Syringodium isoetifolium* against human breast cancer cell line - an *in silico* and *in vitro* study

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KEYWORDS

Syringodium isoetifolium;
MCF-7;

Abstract Seagrass is a natural, renewable, and much unexplored marine resource, which are capable, and reliable sources in the field of pharmaceuticals and drug discovery. The seagrass, *Syringodium isoetifolium* is our target plant for the study, which was collected from the

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Apoptosis;
Insilico;
HSP 90 and HER2 Kinase receptor;
Nootkatone and Zerumbone

Ramanathapuram district, Tamil Nadu. The current study is focused to evaluate the cytotoxic, cell cycle arrest, and apoptotic induction activities of the hydroalcoholic extract of *S. isoetifolium* against the human breast cancer (MCF-7) cell line. The cytotoxic evaluation revealed that the extract inhibited MCF-7 proliferation with IC_{50} value of 230.32 µg/ml. Evaluation of MCF-7 cell cycles demonstrated that the extract arrested the cell cycle in the S phase and continued to the G2/M phase at half of the IC_{50} value. The extract induced apoptotic of MCF-7 cells about 30.61% which was nearly the same with aripiprazole as a positive control (52.35%). Nootkatone significantly binds to the target proteins – HSP 90 and HER2 kinase with the least binding energy was predicted as the most active anticancer compounds by a molecular docking study. In light of the findings, it can be said that the hydroalcoholic extract of *S. isoetifolium* has the potential to be a therapeutic agent for breast malignancies by acting as an anticancer component. It can be used as an anticancer agent on its own and/or as a scaffold for analog synthesis to develop novel anticancer agents with improved therapeutic efficacy.

1. Introduction

Cancer ranks as the second highest cause of death globally, with mortality and morbidity rates on the rise due to new cases. (Liu et al., 2016). Among women worldwide, breast cancer stands out as the most commonly occurring cancer. Current treatment options for breast cancer include radiotherapy and chemotherapy. However, these therapies often come with various side effects such as nausea, vomiting, hair loss, changes in sexual function, and cognitive dysfunction. Therefore, it is crucial to emphasize the importance of complementary medicine as a natural approach to cancer treatment. (Smolarz et al., 2022).

A novel drug derived from marine sources has recently been developed and holds great promise as a future pharmaceutical resource. (Rhyaf et al., 2023, Singh et al., 2018). Extensive research has validated the efficacy and safety of these drugs. The marine environment is renowned for its breathtaking coral reefs, dolphins, ornamental fishes, sharks, and other fascinating marine life, captivating the interest of people worldwide. Among the marine flora, seagrass stands out as a submerged flowering plant that originated on land and successfully adapted to an underwater existence. (Deepak et al., 2019).

In India, researchers have identified 13 distinct types of seagrass, among which Syringodiumisoetifolium, known as tube grass, exhibits larvicidal and scavenging activities. (Venkataraman et al., 2015). S. isoetifolium holds significant pharmacological potential, displaying antibacterial, antifungal, antibiotic, tumor-inhibiting, anti-hemolytic, and cytotoxic properties attributed to its phytoconstituents. (Gono et al., 2022). Notably, phyto-compounds present in S. isoetifolium exhibit diverse biological activities, such as antiviral, antibacterial, anti-inflammatory, anti-allergenic, anticancer, and antioxidant effects. (Pietta, P.G. 2000).

Traditionally, the concept of "one drug, one target, one disease" has prevailed, but researchers have recently recognized the potential of multi-target medications in treating certain disorders. Molecular docking serves as a computational tool used by researchers to develop multifunctional drugs. This methodology, known as Computer-Aided Drug Design, involves creating a complex between the ligand (e.g., tannin) and the target protein specific to a particular disease. (Scottiet al., 2017).

The purpose of this research was to evaluate the cytotoxicity activity of hydroalcoholic extract of S. isoetifolium on breast cancer cell MCF-7 by MTT assay and analyze the inducing apoptosis and inhibiting cell cycle of these by flow cytometry. By LC-MS analysis, the bioactive components present in the hydroalcoholic extract of S. isoetifolium can be identified. To support the anticancer activity of S. isoetifolium, in silico molecular docking was employed, wherein the phytochemical compounds of the plant were docked against the HSP 90 and HER2 kinase proteins.

2. Materials and methods

2.1. Collection and authentication of *Syringodium isoetifolium*

Syringodium isoetifolium has been collected from Devipattinam, Ramanadhapuram District, Tamilnadu, India, on June 2019. The identification of seagrass *Syringodium isoetifolium* was confirmed and validated by Dr. P. Jeyaraman, Ph.D., Director of the Plant Anatomy Research Centre, Retd., Professor, Presidency College (Autonomous), Chennai-600005 and also get authenticated by the Regional Scientist, Southern Regional Centre, Botanical Survey of India, Agriculture University Campus, Coimbatore, Tamilnadu-641 003, India.

2.2. Preparation and extraction of Syringodium isoetifolium

One kilogram of dried, powdered seagrass was extracted with 30:70 proportion of hydro ethanol for maceration periods (24 hrs). The extraction was carried out at room temperature with 150 rpm agitation. The extracts were filtered through the Whatman filter paper after the maceration period. The extracts were concentrated by using the Rotary Evaporator and the dry weight of the crude extracts was weighed and stored at 4 °C in a dark place for further analysis.

2.3. Maintenance of cell lines

The MCF-7 (Human breast adenocarcinoma cell lines) were purchased from National Centre for Cell Sciences (NCCS), University of Pune Campus, Pune, Maharastra-411 007, India. The cells were maintained in DMEM high glucose media supplemented with 10 % FBS along with the 1% antibiotic–antimycotic solution in the atmosphere of 5% CO_2, 18–20% O_2 at 37 °C temperature in the CO_2 incubator and subcultured every 2 days.

2.4. MTT assay

MTT assay is a colorimetric assay used for the determination of cell proliferation and cytotoxicity, based on the reduction of the yellow-colored water-soluble tetrazolium dye MTT to for-

mazan crystals. In a 96-well plate, 200 µl of cell suspension was added without test reagent and allowed for 24 hrs. Now the test reagents were added and incubated at 37 °C in 5% CO_2 atmosphere, without light exposure incubate for 3 h, then the MTT reagent was removed with the addition of DMSO solution (100 µl). The reading was absorbed in 570 nm and IC_{50} values were calculated to check the cell viability percentage (Sulaiman et al., 2016, Al-jubori et al., 2021).

2.5. Cell cycle analysis

Culture the cells in 6 well plates at a density of 2×10^5 cells/2 mL and incubated in a CO_2 incubator overnight at 37 °C for 24 hrs. Now the spent medium is aspirated and 2 mL of cultured medium is treated with the required concentration of cells with incubation of 24 hrs. By PBS wash remove the medium from all wells and 200 µl of trypsin-EDTA solution was added, with 3–4 min incubation at 37 °C. The cells directly into 12 × 75 mm polystyrene tubes which were centrifuged for five minutes at 300g at 25 °C and the supernatant was decanted carefully with PBS wash. Finally, the pellet cells were stained with propidium iodide, incubated for 15–20 min in the dark, and analyzed by flow cytometry (Mohammed et al., 2021, Sulaiman et al., 2015).

2.6. Cell cycle arrest and apoptosis

With the above standard methods, 5 µl of FITC Annexin V were added and incubated for 15 min at RT (25 °C) in the dark, now 5 µl of PI were added and 400 µl of 1X Binding Buffer to each tube and vortex gently. Now analyzed immediately with the addition of PI by flow cytometry method. Apoptosis can be evaluated by inducing proteins like Caspase 9, Caspase 3, and Bcl-2 expression. After washing pellets with PBS, 0.5 mL BD Cytofix/Cytoperm solution was added and allowed for 10 min. Now, wash it with 0.5% bovine serum albumin (BSA) in 1X phosphate-buffered saline (PBS) and 0.1% sodium azide. Add 20 µl of FITC Rabbit anti-active Caspase 3 antibody/PE Mouse Anti-Human Bcl2 antibody/PE Anti-Human Caspase 9 antibody and mix thoroughly, incubate for 30 min in the dark at room temperature (20 °C to 25 °C). Finally, wash with 1X PBS with 0.1% sodium azide, add 0.5 mL of PBS, mix thoroughly, and analyze by Flow Cytometry with the excitation and emission of 494 nm and 520 nm for FITC or FL-1 channel and excitation and emission of 488 nm and 578 nm for PE or FL-2 channel respectively.

Table 1 Percentage cell viability of the hydroalcoholic extract of *S.isoetifolium* treated MCF-7 cells after the treatment period of 24 hrs.

Concentration (µg/ml)	% of cell viability
0	100
25	95.96
50	91.13
100	74.47
200	53.95
400	14.84
Aripiprazole-35 mM	41.52

Table 2 Percentage of MCF-7 cells that get arrested in the different stages of the cell cycle.

Percentage of MCF-7 cells			
Cell Cycle stage	**Untreated**	**Aripiprazole**	**Plant Extract**
Sub G0/G1	2.06	3.51	1.58
G0/G1	68.46	26.87	56.73
S	1.95	7.82	8.75
G2/M	26.33	52.55	30.61
Total Events Selected per each group −10000			

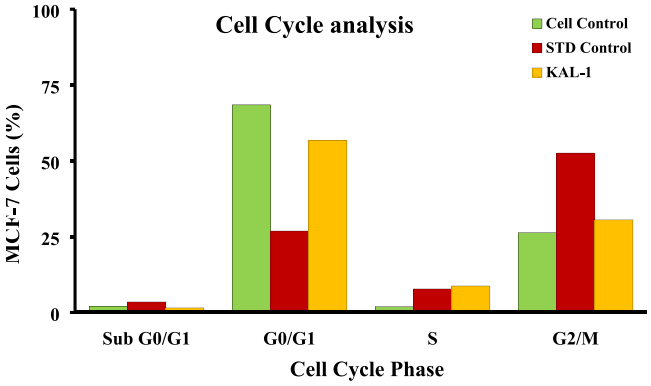

Fig. 1 Overlay showing the percentage of cells that get arrested in the different stages of the MCF-7 cell cycle.

2.7. Liquid chromatography-mass spectrometry analysis

All analytes were chromatographically screened using a ZORBAX Eclipse and a C18 column (2.1 × 100 mm, 5.0 µm molecule estimate; Agilent Technologies) at a flow rate of 0.5 mL/min and an infusion volume of 5 µl. To create the mobile phase, formic acid was combined with 10 mM ammonium formate, and 0.1% formic acid was added to methanol. (A: 80:20, B:10:90). The elution gradient started with 10% of B and progressed to 50% methanol after 0–7 min, 80% of B after 12–15 min, 100% of B after 15–18 min, 100% of B after 18–18.1 min, and 20% of B after 20–20 min. The oven was maintained at a temperature of 45 °C. The MS parameters were as follows: 325 °C for the drying gas; 11 L/min for the gas flow; 40 psi for the nebulizer; 350 °C for the sheath gas; 8 L/min for the sheath gas flow; 500 V for the delta EMV; and 4000 V for the capillary voltage. 3.2.5.1.

2.8. Molecular docking studies

In silico or molecular studies are to perform the interaction between the compounds identified from the HAE of S. isoetifolium. This study is to enhance the precision of biological tests, reliability and the compounds which interact are as follows: 7-Hydroxycoumarine, 4-Hydroxycoumarine, Phloretin, Zerumbone, Arecoline, and Nootkatone. The 3D structure

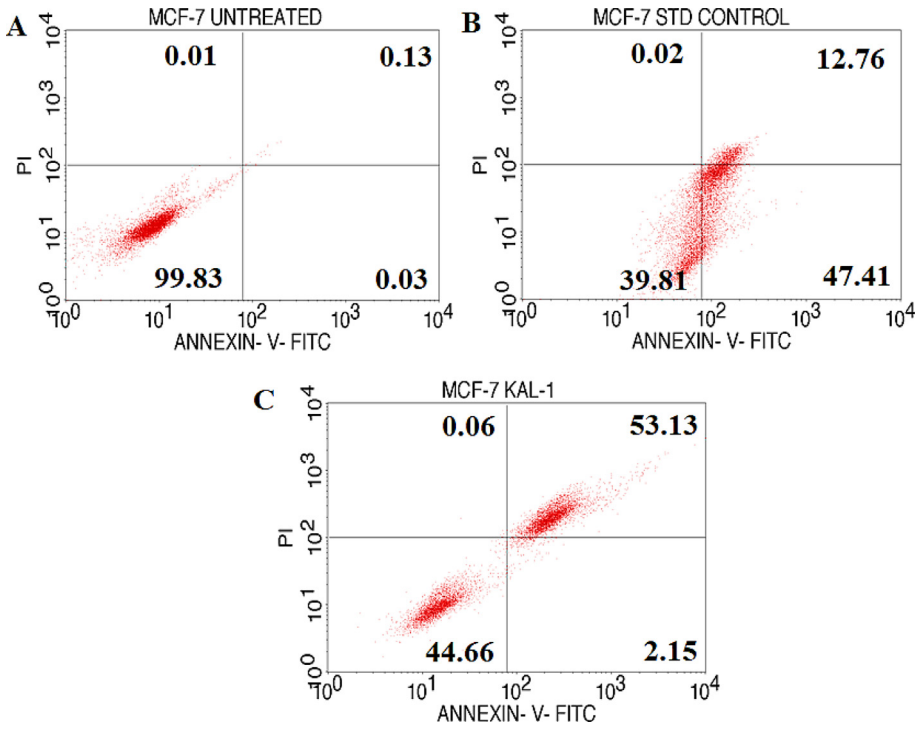

Fig. 2 Quadrangular figure illustrating the Annexin V/PI expression in MCF-7 cells upon culture in the presence and absence of test chemical, GA coupled with std control. Cell Quest Pro and BD FACScalibur were used for the analysis. (Version: 6.0). Here, Annexin V-FITC - Primary Marker, PI- Propidium Iodide (Secondary fluorescence Marker) A-MCF-7 Untreated, B-MCF-7 std control, C-MCF-7 hydroalcoholic extract of *S.isoetifolium*. Lower left: % Viable Cells; Upper left: % of Necrotic Cells; Lower right: % of Early apoptotic cells; Upper right: % Late Apoptotic Cells.

of the PDB ID: 3RCD; 3TUH was downloaded from the protein data bank. Following Sribalan et al.(Sribalan et al., 2019) the discovery studio is used for molecular docking and visualization. Molecular docking was done with the help of Autodock 4.2. ChemDraw 13.0 and MMFF 94 were used to optimize the 3D structure of compounds. (Maximum number of interactions: 5000, minimum RMS gradient: 0.100). The enzyme was cleared of any unnecessary ligands and water, and the default docking settings were adjusted and used.

2.9. Autodock

For the interaction of the ligand with bio macromolecular target, an automated procedure is predicted which is termed an auto dock. This method is used to analyze the three-dimensional structure of the drug binding to the receptor. For docking studies, Genetic Algorithms are used for confirmational search. Along with modeling studies, auto dock tools are employed in the preparation, execution, and analysis of docking simulations (Rauf et al., 2015).

2.10. Ligand for docking preparation

The ligands chosen for this study are 7-Hydroxycoumarine, 4-Hydroxycoumarine, Phloretin, Zerumbone, Arecoline, and Nootkatone were identified by LC-MS analysis. These compounds were planned to dock with two breast cancer proteins such as HER2 Kinase Receptor and HSP90. By using the PubChem database, the physicochemical and structural characteristics of these compounds were recovered. PubChem is an open database, used to search for wide properties which may include hydrogen bond donor, hydrogen acceptor, name of the compound, structure, molecular weight, fragments, chemical formula, and X Log P. The chemical was converted to PDB format using an online editor that uses the Simplified Molecular Input Line Entry Specification (SMILES) format.

2.11. Protein preparation for docking

Large biological macromolecules like proteins and nucleic acids are stored in the RCSB PDB (Research Collaborator for Structural Bioinformatics, Protein Data Bank), which is a repository for their 3D structural information. After researching its metabolic pathway, the target proteins from the National Centre for Biotechnology Information NCBI protein database (https://www.rcsb.org/pdb) were determined to be the HER2 Kinase Receptor with the accession number (PDB ID: 3RCD) and HSP90 with the accession number (PDB ID: 3TUH).

3. Results and discussion

3.1. Cytotoxic activity in MCF-7 cells

Hydroalcoholic extract of *S. isoetifolium* shows a more cytotoxic effect on MCF-7 cells. The findings from statistical analysis of the cell cytotoxicity study show that the hydroalcoholic extract of *S. isoetifolium* demonstrated significant cytotoxic

Table 3 Identification of bioactive compounds in hydroalcoholic extract of *S. isoetifolium* using LC-MS.

S.No.	Compound Name	Molecular Formula	Molecular Weight	Retention Time
1	4-Dodecylbenzenesulfonic acid	$C_{18}H_{30}O_3S$	326.19063	27.07
2	αα-trehalose	$C_{24}H_{38}O_4$	390.2777	23.57
3	Dibutyl phthalate	C_{16} H_0O_4	278.15236	19.92
4	9-Oxo-ODE	C_{18} $H_{30}O_3$	294.2202	20.757
5	Dioctyl phthalate	C_{24} $H_{38}O_4$	390.2777	27.203
6	Arecoline	C_8 H_{13} NO_2	155.09508	1.088
7	Reserpine	C_{33} H_{40} N_2O_9	608.27422	14.973
8	Choline	$C_5H_{13}NO$	103.09999	0.954
9	2-Aminoanthraquinone	$C_{14}H_9NO_2$	223.06403	17.403
10	Muramic acid	$C_9H_{17}NO_7$	251.1014	1.108
11	Betaine	$C_5H_{11}NO_2$	117.07925	1.062
12	Trigonelline	$C_7H_7NO_2$	137.04808	1.065
13	3-Hydroxybenzoic acid	$C_7H_6O_3$	138.03132	3.5
14	Caffeic acid	$C_9H_8O_4$	180.04263	19.923
15	Octyl decyl phthalate	$C_{26}H_{42}O_4$	418.30934	24.164
16	Myristyl sulfate	$C_{14}H_{30}O_4S$	294.18574	27.2
17	Monobutyl phthalate	$C_{12}H_{14}O_4$	222.08994	15.811
18	4-Hydroxycoumarin	$C_9H_6O_3$	162.03205	19.924
19	D-Glucosamine	$C_6H_{13}NO_5$	179.07982	1.079
20	L-Pyroglutamic acid	$C_5H_7NO_3$	129.043	1.167
21	3-Hydroxyfluorene	$C_{13}H_{10}O$	182.07389	16.773
22	4-Dodecylbenzenesulfonic acid	$C_{18}H_{30}O_3S$	326.19063	26.216
23	DL-Stachydrine	$C_7H_{13}NO_2$	143.09513	1
24	Polygodial	$C_{15}H_{22}O_2$	234.16277	19.183
25	2,2,6,6-Tetramethyl-4-piperidinol	$C_9H_{19}NO$	157.14722	16.081
26	4-Acetamidobutanoic acid	$C_6H_{11}NO_3$	145.07431	1.072
27	Diisopentyl phthalate	$C_{18}H_{26}O_4$	306.18407	21.109
28	1-Stearoylglycerol	$C_{21}H_{42}O_4$	358.30929	23.651
29	Hexadecanamide	$C_{16}H_{33}NO$	255.25691	22.728
30	4-Hydroxycoumarin	$C_9H_6O_3$	162.03205	23.584
31	Triphenyl phosphate	$C_{18}H_{15}O_4P$	326.07173	19.16
32	OPEO	$C_{16}H_{26}O_2$	250.19382	19.903
33	Diisodecyl phthalate	$C_{28}H_{46}O_4$	446.34093	24.817
34	4-Oxoproline	$C_5H_7NO_3$	129.04233	1.166
35	4-Pyridoxic acid	$C_8H_9NO_4$	183.05359	24.467
36	Bis(4-ethylbenzylidene)sorbitol	$C_{24}H_{30}O_6$	414.20485	18.325
37	Laurolactam	$C_{12}H_{23}NO$	197.17867	16.749
38	Glycerophospho-N-palmitoyl ethanolamine	$C_{21}H_{44}NO_7P$	453.28676	22.562
39	4-Hydroxycoumarin	$C_9H_6O_3$	162.03205	15.826
40	Cholest-4-en-3-one	$C_{27}H_{44}O$	384.3402	25.549
41	Citroflex A-4	$C_{20}H_{34}O_8$	402.22655	20.911
42	Ethyl myristate	$C_{16}H_{32}O_2$	256.23938	23.372
43	Stearamide	$C_{18}H_{37}NO$	283.28829	23.517
44	Oleamide	$C_{18}H_{35}NO$	281.27263	22.932
45	Betaine	$C_5H_{11}NO_2$	117.07925	29.507
46	Leucine	$C_6H_{13}NO_2$	131.095	1.161
47	L-Pyroglutamic acid	$C_5H_7NO_3$	129.043	29.086
48	β-Estradiol	$C_{18}H_{24}O_2$	272.1787	20.143
49	2,2,6,6-Tetramethyl-4-piperidinol	$C_9H_{19}NO$	157.14722	16.412
50	Phloretin	$C_{15}H_{14}O_5$	274.08483	12.82
51	Dodecyltrimethylammonium	$C_{15}H_{33}$ N	227.26196	17.415
52	Docosanamide	$C_{22}H_{45}NO$	339.35127	18.114
53	Pyridoxal	$C_8H_9NO_3$	167.05861	24.343
54	Nootkatone	$C_{15}H_{22}O$	218.1678	20.148
55	N,N'-Diphenylurea	$C_{13}H_{12}N_2O$	212.09581	14.854
56	Dimethyl sebacate	$C_{12}H_{22}O_4$	230.15251	14.892
57	Myristyl sulfate	$C_{14}H_{30}O_4S$	294.18574	28.249
58	O-Desmethyltramadol	$C_{15}H_{23}NO_2$	249.17352	20.619
59	Dibutyl phthalate	$C_{16}H_{22}O_4$	278.15236	27.648
60	4-Methoxycinnamic acid	$C_{10}H_{10}O_3$	178.06362	21.868
61	Astaxanthin	$C_{40}H_{52}O_4$	596.38738	23.313
62	4-Hydroxycoumarin	$C_9H_6O_3$	162.03205	21.112
63	Palmitoleic acid	$C_{16}H_{30}O_2$	254.22539	21.992
64	19-Nortestosterone	$C_{18}H_{26}O_2$	274.19426	19.639

(*continued on next page*)

Table 3 (*continued*)

S.No.	Compound Name	Molecular Formula	Molecular Weight	Retention Time
65	Monobutyl phthalate	$C_{12}H_{14}O_4$	222.08994	20.463
66	Zeatin-7-N-glucoside	$C_{16}H_{23}N_5O_6$	381.16451	1.018
67	Oleoyl ethanolamide	$C_{20}H_{39}NO_2$	325.299	22.847
68	Sucrose	$C_{12}H_{22}O_{11}$	342.11525	0.985
69	5α-Dihydrotestosterone	$C_{19}H_{30}O_2$	290.22508	21.26
70	6-Hydroxynicotinic acid	$C_6H_5NO_3$	139.02661	9.246
71	Meprednisone	$C_{22}H_{28}O_5$	372.19509	16.336
72	(±)-Abscisic acid	$C_{15}H_{20}O_4$	264.13713	13.707
73	Dihydrothymine	$C_5H_8N_2O_2$	128.05896	1.082
74	(+/-)-CP 47,497-C7-Hydroxy metabolite	$C_{21}H_{34}O_3$	334.2517	23.931
75	D-(+)-Pyroglutamic Acid	$C_5H_7NO_3$	129.043	28.773

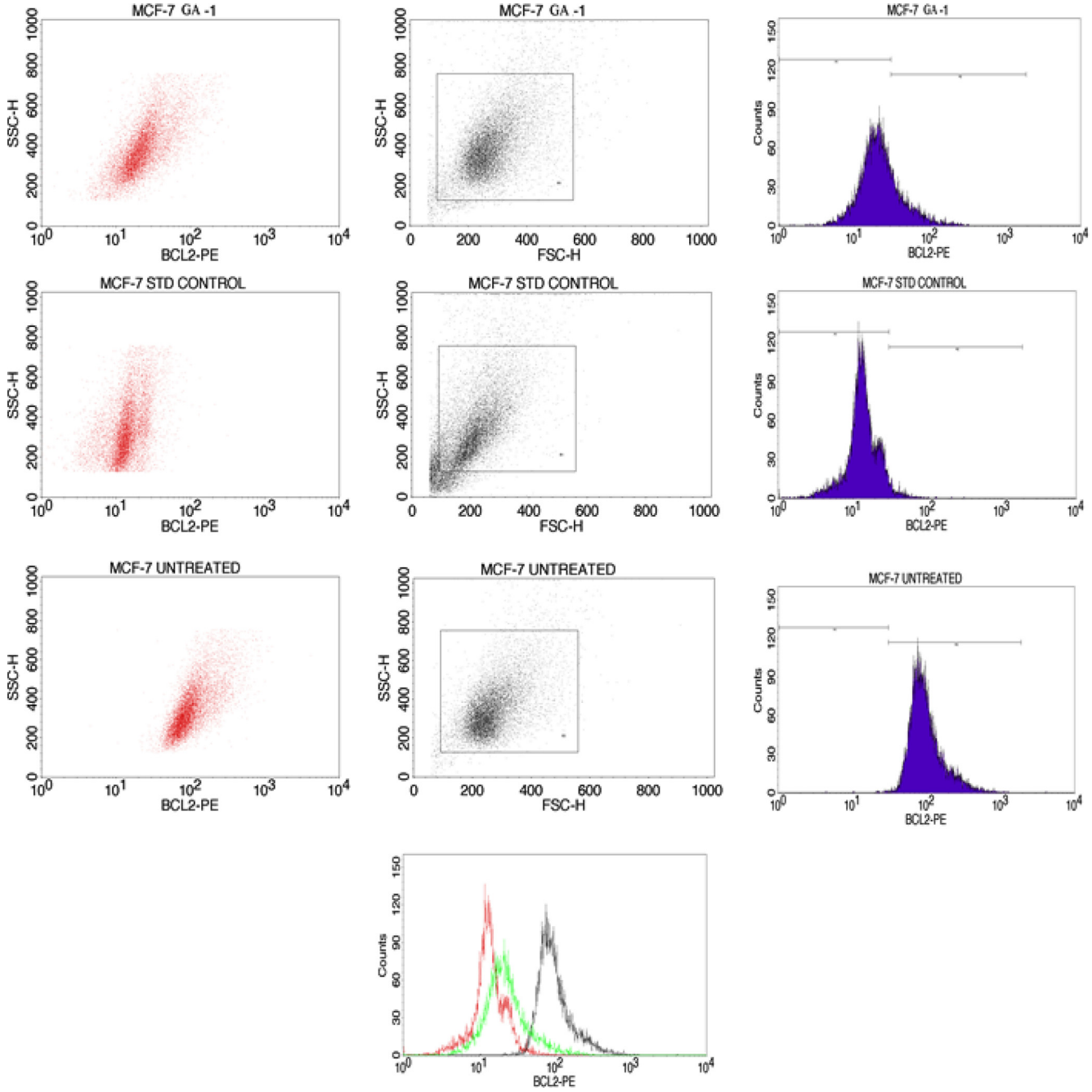

Fig. 3 Hydroalcoholic extract of *S.isoetifolium* induces apoptosis via Bcl-2 activation in MCF-7 cells.

potency against MCF-7 cells with an IC_{50} (50% cell viability) concentration at 230.32 µg/ml in comparison to the standard drug, Aripiprazole. The percentage of cell viability was displayed in Table 1.

The cells treated with standard, control, and test compound show IC_{50} concentrations are high % of cells at S and G2/M stage arrest when compared with untreated cells. So, the cell cycle got arrested at S and G2/M stages. Hence, we evaluated the cell cycle study by Flow Cytometry to check the stages of cell cycle arrest and obtained the results by flow cytometry were tabulated in Table 2 and the percentage of cell cycle arrest was shown in Fig. 1.

In the Sub G0/G1 phase (Apoptotic phase), 2.06%, 3.51%, and 1.58% of cells get arrested in untreated, standard, and hydroalcoholic extract of *S. isoetifolium* with IC_{50} concentration respectively. In G0/G1 phase (Growth Phase), 68.46%, 26.87%, and 56.73% of cells get arrested in untreated, standard, and hydroalcoholic extracts of *S. isoetifolium* with IC_{50} concentration respectively. In the S phase (synthetic phase), 1.95%, 7.82%, and 8.75% of cells get arrested in untreated,

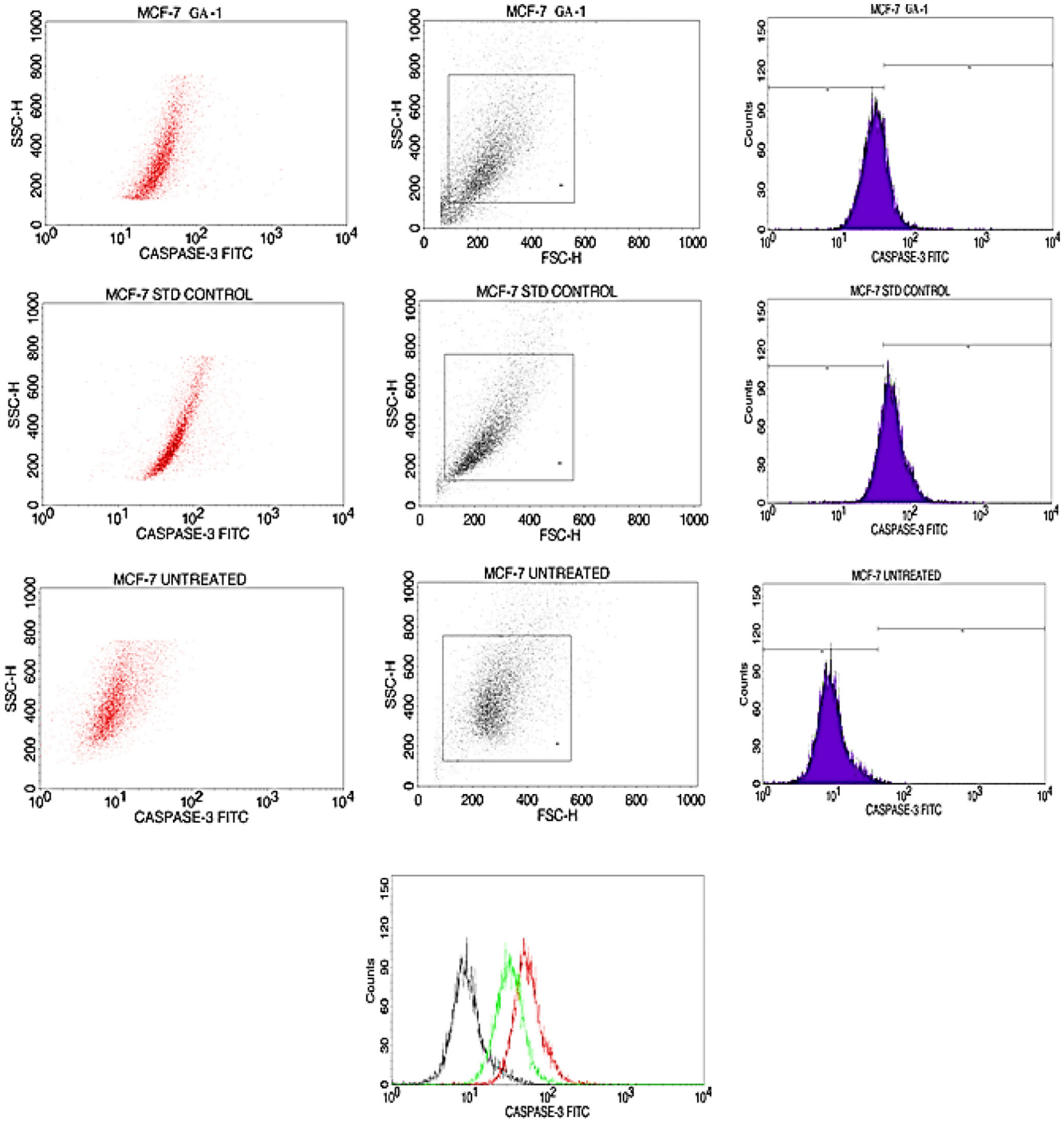

Fig. 4 Hydroalcoholic extract of *S.isoetifolium* induces apoptosis via Caspase-3 activation in MCF-7 cells.

standard, and hydroalcoholic extracts of *S. isoetifolium* with IC_{50} concentration respectively. On the other hand, in the G2/M phase, 26.33%, 52.35%, and 30.61% of cells get arrested in the untreated, standard, and hydroalcoholic extract of *S. isoetifolium* with IC_{50} concentration respectively. The efficacy of the hydroalcoholic extract of *S.isoetifolium* on cell cycle arrest is examined and shown in Fig. 1. Hydroalcoholic extract of *S. isoetifolium* may also cause poly ribose polymerase cleavage caspase 3, and caspase 9 additionally. (Agarwal et al., 2006).

The loss of membrane integrity that follows the most recent stages of cell death brought on by either necrotic or apoptotic processes is preceded by FITC Annexin V staining. To enable the researcher to recognize early apoptotic cells, staining with

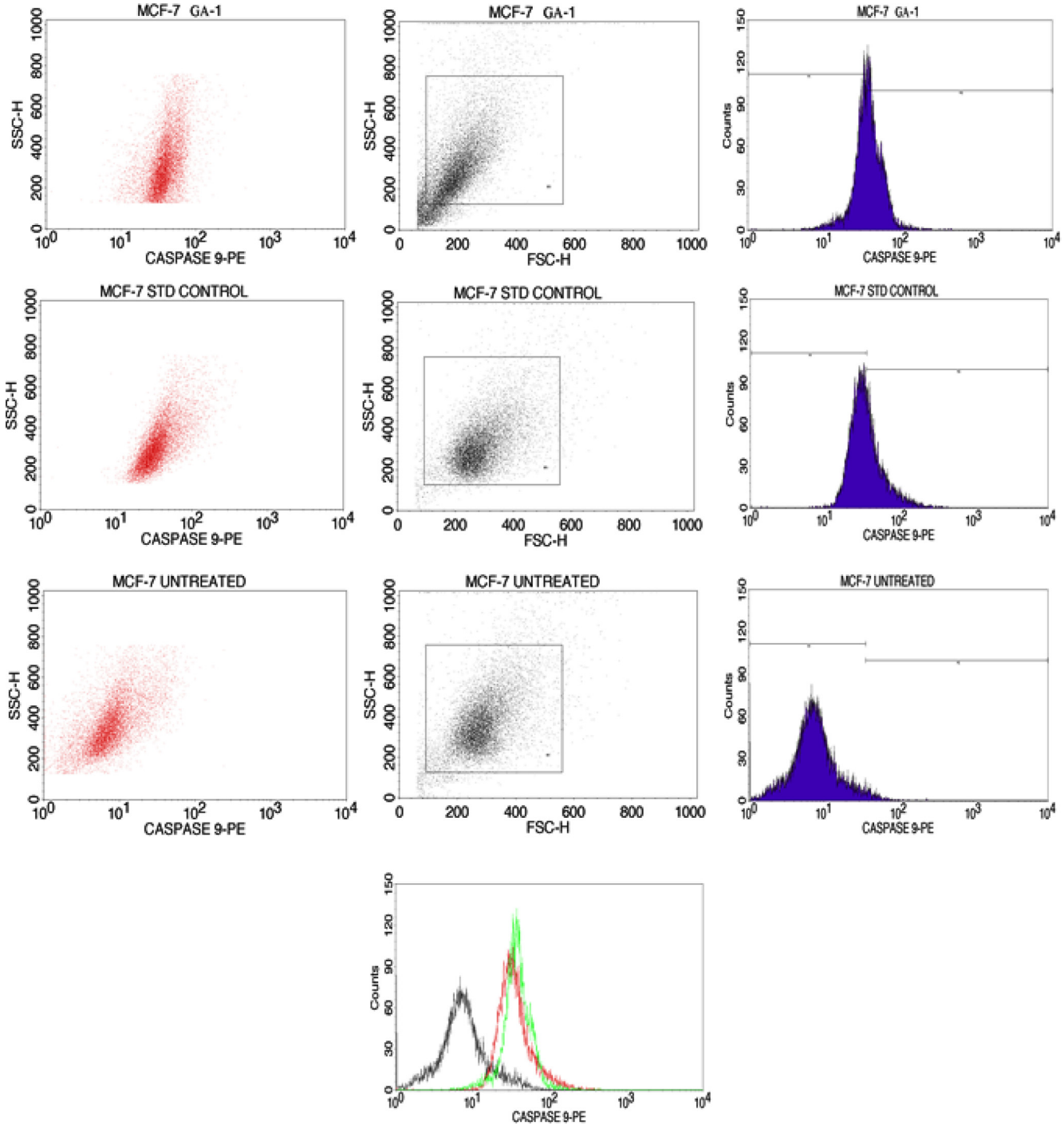

Fig. 5 Hydroalcoholic extract of *S. isoetifolium* induces apoptosis via Caspase 9 activation in MCF-7 cells.

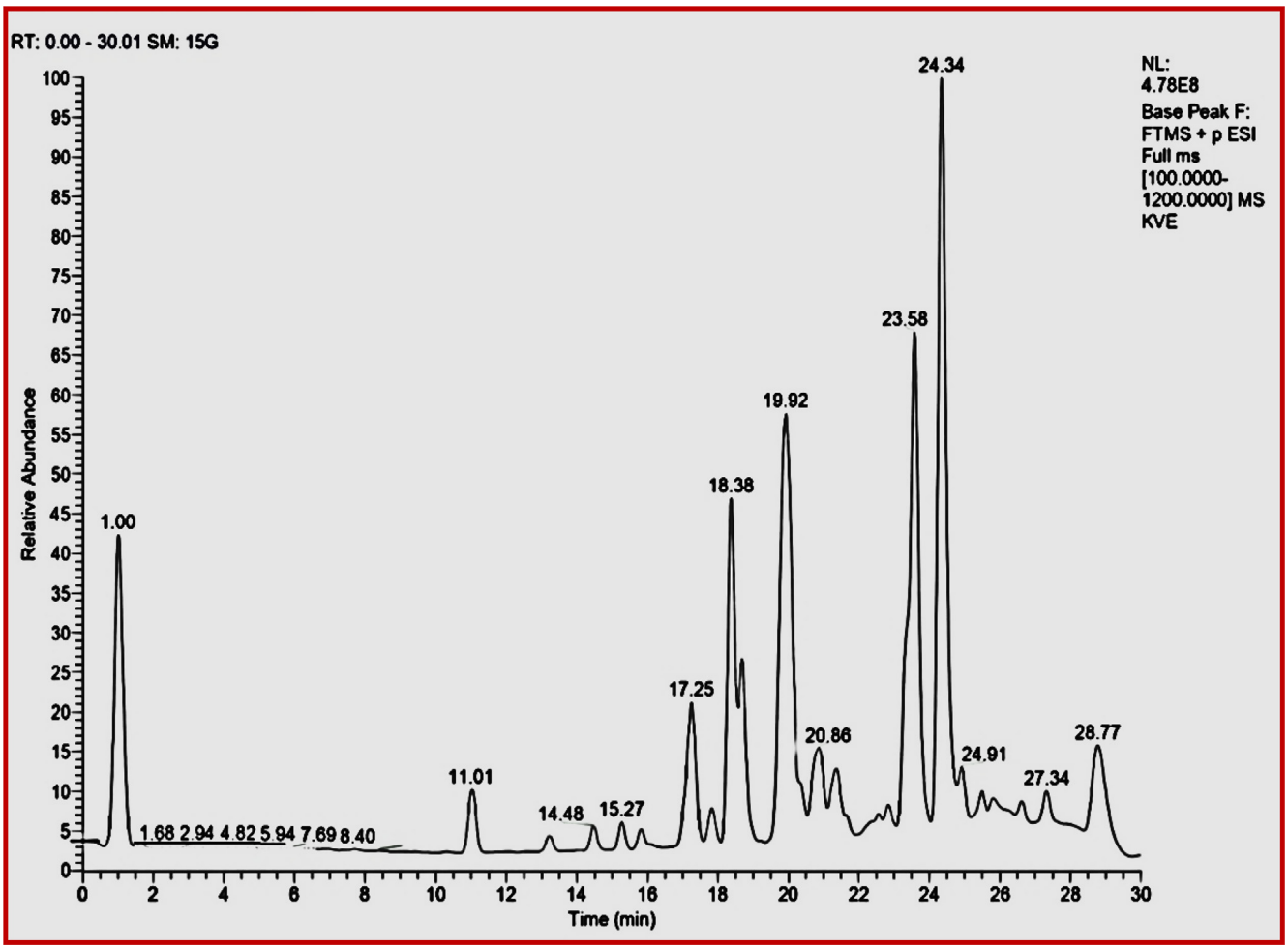

Fig. 6 LC-MS Chromatogram of the hydroalcoholic extract of *S. isoetifolium*.

FITC Annexin V is often employed in conjunction with a crucial dye like propidium iodide (PI) or 7-Amino-Actinomycin (7-AAD). (PI negative, FITC Annexin V positive). Without inhibiting the healthy cells hydroalcoholic extract of *S. isoetifolium* shows a more cytotoxic effect on cancer cells. It may also possess good anti-inflammatory and anti-malarial activity (Subramanian et al., 2015). In recent research, the authors prove the efficacy of gallic acid with the results obtained, it can induce cell cycle arrest at G2/M phase via Ch2K mediated phosphorylation in bladder carcinoma cell line (Ou et al., 2010). At an earlier stage, Annexin V/PI dye can identify apoptosis. The membrane integrity has been lost due to the stain. The study proves that Annexin V binds with the cells to inhibit the proliferation of cells and also detect apoptosis at different phases the expression of Annexin V with MCF 7 cells was shown in Fig. 2 and the meaning for quadrant was given in Table 3 (Kalpana et al., 2020). The percentage of cells that undergo apoptosis was shown in Fig. 2, which shows the live and apoptotic cell percentages.

Apoptotic investigation revealed that the hydroalcoholic extract of *S. isoetifolium*, effectively triggered programmed cell death, or apoptosis, in human breast cancer cells. This observation suggests that the hydroalcoholic extract may possess therapeutic potential against human breast cancer. To confirm the mechanism behind the anti-cancer effects of the test compound on human breast cancer cells, further preclinical research is needed. The study found that the hydroalcoholic extract of *S. isoetifolium* induces apoptosis in human breast cancer cells (MCF-7) through the activation of specific signaling pathways, namely caspase 3, caspase 9, and Bcl2 apoptotic signaling pathway. These pathways play a critical role in promoting programmed cell death, which is essential to prevent the development and progression of cancer cells. By measuring the amount of activated caspase(s), one can detect whether or not apoptosis is occurring. It is necessary to choose in advance the caspase(s) to be assayed. Among the various caspase(s), caspase 3 and 9 both initiate the cascade of apoptosis events. Caspase 9 is usually activated by cytotoxic agents that damage mitochondria, allowing cytochrome *c* leakage into the cytosol. Caspase 3 is a common downstream effector caspase associated with some forms of β-cell apoptosis (Yamada et al., 1999). It is worth noting that a previous study demonstrated that vinculin, another compound or factor under investigation, similarly induces apoptosis through the intrinsic caspase 9 pathway. By elucidating the underlying mechanisms by which these compounds induce apoptosis, we can gain a deeper understanding of their potential as anti-cancer agents in the context of human breast cancer (Lee et al., 2020).

A recent study proves that Bcl2 function in the antioxidant pathway and can inhibit lipid peroxidation. Bcl2 can be identified at the chromosomal translocation breakpoint and was mainly studied in lymphoma as well as in leukemia (Haldar et al., 1994). Fig. 3,4, and 5 show the activation of the apoptotic pathway via Bcl-2, caspase 3, and caspase 9 in the MCF-7 cells. On treating crude extract with MCF- 7 cells, Bcl2 may interfere with cytochrome *c* while the same cytochrome can be induced by the expression of Bax. Bcl2 which is an anti-apoptotic protein can induce the apoptotic expression in MCF- 7 breast cancer cells via an intrinsic apoptosis pathway (Rosse et al., 1998, Ekins et al., 2007).(See Figs. 4 and 5).

3.2. Liquid chromatography-mass spectroscopy analysis

About seventy-five chemical components were recognized in HAE of *S. isoetifolium*, along with their retention time, molecular weight, and molecular formula were listed in Table 3 and the chromatogram was represented in Fig. 6. The biological activity of selected compounds was presented in Table 4. The prevalent compounds were 4-Dodecylbenzenesulfonic acid showing a retention time of 27.07 min. The mass spectrum of Arecoline shows the peak at RT 1.088 with the ESI- MS spectrum at *m*/*z* 155 revealing the occurrence of this compound. Nootkatone showed the peak at a retention time of 20.148 and ESI-MS spectrum showed at *m*/*z* 19.924 indicating the presence of compound 4-Hydroxycoumarin. Similarly, the mass spectrum of 3-Hydroxybenzoic acid shows RT at 3.5. While the compound Reserpine shows the band at 14.973 and at 22.932 the compound present is Oleamide. Dibutyl phthalate showed the highest retention time peak at 27.648; the substance Choline showed the lowest retention time peak at 0.954. Compounds such as 9-oxo-ODE, Dioctyl phthalate, Myristyl sulfate, Diisodecyl phthalate, and Betaine show the highest value of RT at 20.757, 27.203, 27.2, 24.817, and 29.507, while lowest RT 1.018, 1.161, 1.166, 1.072 and 1 shows with the compounds Zeatin-7-N-glucoside, Leucine, 4-oxo proline, 4-Acetamidobutanoic acid, respectively.

Table 4 Pharmacological activities of the identified compounds from HAE of *S. isoetifolium* by LC-MS analysis.

S. No.	R. T	Name of the Compounds	Pharmacological activities
1	23.57	αα-trehalose	Antitumor effects, suppression of bone loss, and migration of insulin resistance (Kapetanovic, 2008)
2	16.336	Meprednisone	Anti-inflammatory, neuroprotective agent, antiemetic, and androgenic agent (Shaker et al., 2021)
3	9.246	6-Hydroxynicotinic acid	Antitumor effects, anticancer activity
4	24.794	Cholecalciferol	suppress NF-kB activities, slowing down cancer growth
5	21.992	Palmitoleic acid	Antioxidant activity (Wada et al., 2014)
6	22.728	Hexadecanamide	Down-regulation of mast cell activation and inflammation
7	0.985	Sucrose	Antimicrobial and cytotoxic activity
8	23.313	Astaxanthin	Anti-oxidant, Anti-inflammatory, Anti-apoptotic activity
9	27.648	Dibutyl phthalate	Antifungal activity
10	19.923	Caffeic acid	Anti-inflammatory, neuroprotective, hepatoprotective, and cardioprotective effect
11	1.079	D-Glucosamine	Mineralization of mature osteoblasts, reduction in expression of receptor activator- NF- *k*b
12	12.82	Phloretin	Anti-inflammatory, Anti-oxidative (Brodkiewicz et al., 2020)

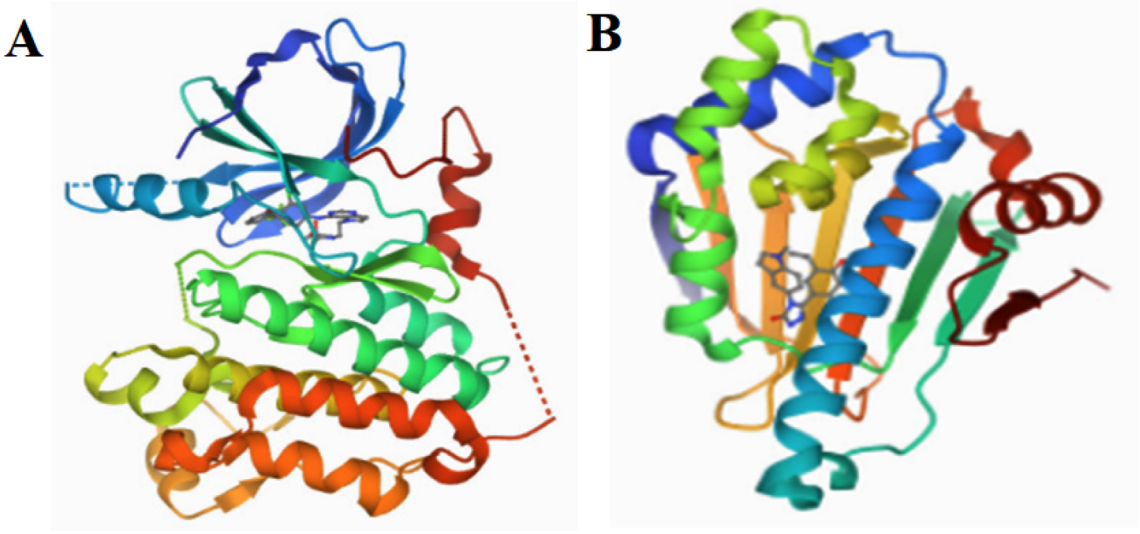

Fig. 7 (A) 3D Structure of HER2 kinase (B) 3D Structure of HSP90.

Table 5 Docking results of HAE of *S. isoetifolium*-derived compounds against HER2 kinase receptor.

Compound Name	Binding Energy (Kcal/mol)	Inhibition constant (μM)	Hydrogen bond Interactions	Distance	Binding residues (Around 5 Å)	Binding region of Ligand
4-Hydroxycoumarine	−5.79	56.64	Thr862(O)...H-OSer783 (O)...H-OThr798(N)-H...O	1.8 3.03.0	Leu796, Met774, Leu785, Thr798, Arg784, Asp863, Phe864, Ser783, Thr863	chromen-2-one4-hydroxy
7- Hydroxycoumarine	−5.32	125.07	Thr862(O)...HOSer783(O) -H...OThr798(O) H...OPhe864(O)...H-O	3.3 2.7 2.82.1	Leu796, Met774, Leu785, Thr798, Arg784, Ser783, Asp863, Ser783, Phe864, Lys753, Thr863	chromen-2-one7-hydroxy
Arecoline	−4.41	590.04	Thr862(O)H...NLys753(N)-H...O	3.03.1	Leu796, Leu785, Val797, Thr798, Arg784, Ser783, Asp863, Ser783, Thr862, Phe864, Lys753, Thr863	pyridinecarboxylate
Nootkatone	−7.72	2.2	–	–	Leu796, Met774, Leu785, Val797, Thr798, Arg784, Ser783, ASP863, Ser783, Thr862, Phe864, Lys753, The863	prop-1-en-2-ylhexahydronaphthalen-2-one
Phloretin	−6.1	33.58	Thr862(O)...H-OSer783(O) -H...OLys753(N) -H...OAsp863(O)...H-O	2.6 2.0 3.03.4	Leu785, Arg784, Ser783, ASP863, Ser783, Phe864, Met774, Lys753, Thr798, Leu796, Thr862	4-hydroxyphenyl2,4,6-trihydroxyphenyl
Zerumbone	−7.7	2.27	–	–	Leu796, Met774, Leu785, Val797, Thr798, Arg784, Ser783, Asp863, Ser783, Thr862, Phe864, Lys753, Thr863	tetramethylcycloundeca-2,6,10-trien-1-one

Table 6 Docking results of HAE of *S. isoetifolium*-derived compounds against HSP90 protein.

Compound Name	Binding Energy (Kcal/mol)	Inhibition constant (μM)	Hydrogen bond Interactions	Distance	Binding residues (Around 5 Å)	Binding region of Ligand
4-Hydroxycoumarine	−5.9	47.56	Thr109(O)-H...OGly135(O)...H-O	2.62.1	Asn106, Ile26, Thr109, Ile110, Ala111, Thr115, Lys112, Ser113, Phe134, Gly136, Asn51, Phe138, Tyr139, Leu107	chromen-2-one4-hydroxy
7- Hydroxycoumarine	−5.69	67.52	Asn51(O)-H...OPhe138(N)...H-O	3.02.8	Asn106, Ile26, Thr109, Ile110, Ala111, Thr115, Lys112, Ser113, Phe134, Gly136, Asn51, Phe138, Tyr139, Leu107	chromen-2-one7-hydroxy
Arecoline	−5.26	139.49	Phe138(N)...H-OAsn106(O)-H...N	3.43.0	Asn106, Ile26, Thr109, Ile110, Ala111, Thr115, Lys112, Ser113, Phe134, Gly136, Asn51, Phe138, Tyr139, Leu107	pyridinecarboxylate
Nootkatone	−7.5	3.19	–	–	Asn106, Ile26, Asn51, Asp54, Thr109, Ile110, Ala111, Thr115, Lys112, Ser113, Phe134, Gly136, Asn51, Phe138, Tyr139, Leu107	prop-1-en-2-ylhexahydronaphthalen-2-one
Phloretin	−6.18	29.43	Ser52(O)...H-OAsn51 (N)...H-OGly135(O)...H-O	2.7 2.92.0	Asn106, Ile26, Thr109, Ser113, Val136, Lys112, Ser113, Phe134, Gly135, Asn51, Ser52, Val186, Asp93, Phe138, Tyr139 Leu107	4-hydroxyphenyl2,4,6-trihydroxyphenyl
Zerumbone	−7.45	3.38	–	–	Asn106, Ile26, Thr109, Ser113, Val136, Lys112, Ser113, Phe134, Gly135, Asn51, Asp93, Phe138, Tyr139, Leu107, Met98	tetramethylcycloundeca-2,6,10-trien-1-one

3.3. Molecular docking studies

7-Hydroxy coumarin, 4-Hydroxy coumarin, Phloretin, Zerumbone, Nootkatone, and Arecoline are the ligands used for *in silico* study (Gao et al., 2021) from the LC-MS analysis against breast cancer target proteins HER2 Kinase and HSP90 respectively. Fig. 7(A and B) shows the three-dimensional structure of HER2 Kinase and HSP90 respectively. Monitoring characteristic features including docking score, binding energy, Van der Waals interactions, hydrophobic interactions, and unusual charge interactions can help to determine how well a ligand will bind to a receptor. The bigger the binding energy's negative value, the stronger the molecule's affinity for the receptor (Shamsee et al., 2019).

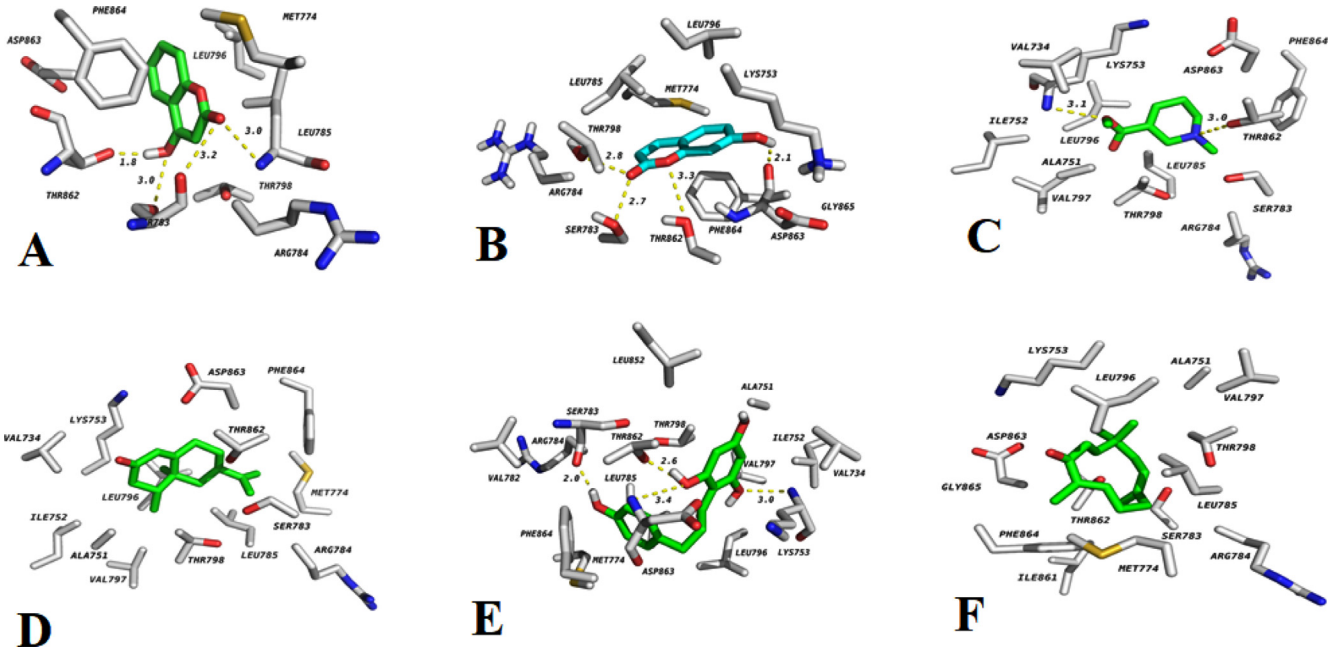

Fig. 8 Interaction of 7-Hydroxycoumarine (A),4- Hydroxycoumarine (B), Nootkatone (C), Arecoline (D), Zerumbone (E), and Phloretin(F) with HER2 Kinase.

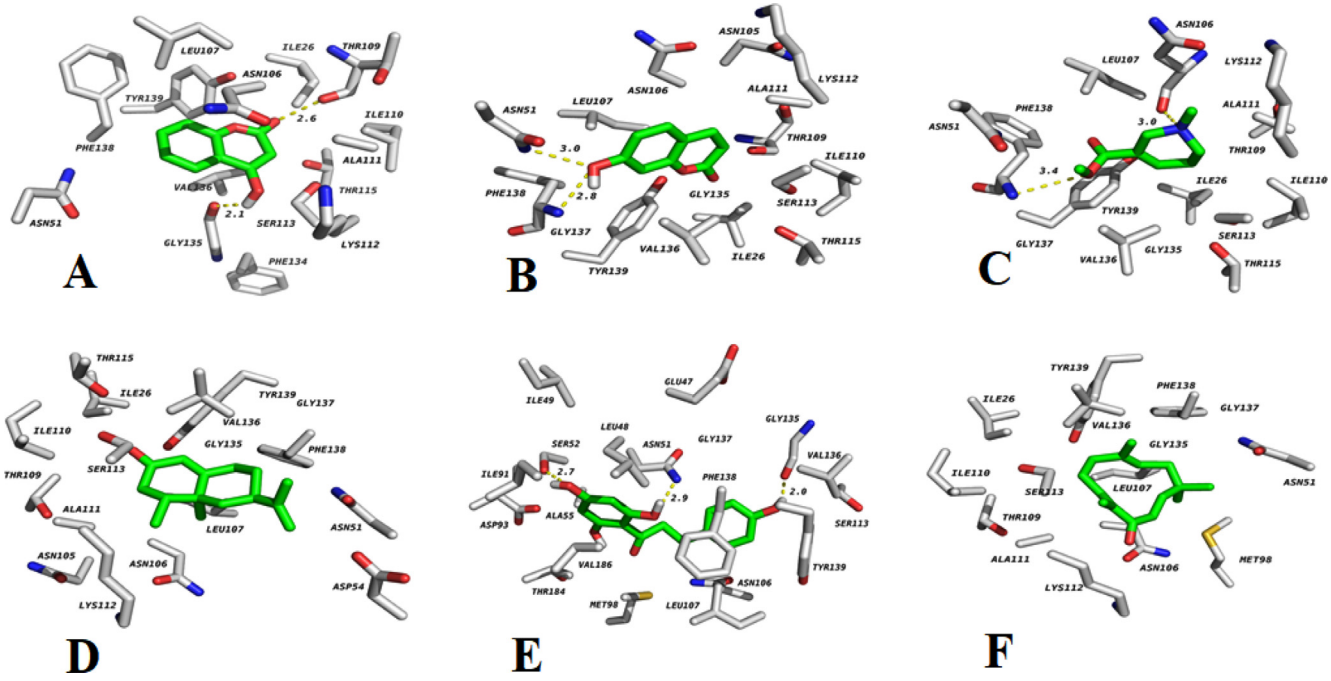

Fig. 9 Interaction of 4-Hydroxycoumarine (A), 7-Hydroxycoumarine (B), Arecoline (C), Nookatone (D), Phloretin (E), and Zerumbone (F) with HSP90.

The Protein Data Bank was used to obtain the receptor structures for molecular docking. In addition to the prioritized list of docked ligands and their binding poses, the docking positions were sorted based on their docking scores (Shaliza et al., 2007). Their binding energy was used to rank them. The outcomes for both breast cancer proteins are shown in Tables 5 and 6 and were determined by docking energy of 7-Hydroxycoumarine, 4-Hydroxycoumarine, Nootkatone, Arecoline, Zerumbone, and Phloretin Interactions with HER2 Kinase and HSP90.

3.4. Interaction of ligands with HER2 Kinase

The binding affinity of 4-hydroxy coumarin, 7-hydroxy coumarin, arecoline, nootkatone, phloretin, and zerumbone was identified through docking investigations, which supported the target protein HER2 Kinase receptor's restraint. The outcomes of the docked compound with HER2 kinase receptor were shown in Table 5 and Fig. 8. The docking score of 4-Hydroxycoumarine, 7-Hydroxycoumarine, Arecoline, Nookatone, Phloretin, Zerumbone was found to be −5.79, −5.32, −4.41, −7.72, −6.1, −7.7 Kcal/mol respectively. The order of binding energy was Nootkatone > Zerumbone > Phloretin > 4-Hydroxycoumarine > 7-Hydroxycoumarine > Arecoline. Among all the other compounds, Nootkatone was found to have more affinity. Nootkatone compounds possess both anticancer and antiplatelet effects which might be of therapeutic benefit for the prevention of platelet-associated cardiovascular diseases (Yoo et al., 2020). It is the most abundant component and possesses a wide range of beneficial effects mainly antiproliferative and anti-inflammatory activities. It possesses anticancer activity especially in lung cancer via AMPK pathway and shows more activity against colorectal cancer (Zhu et al., 2020). Along with the ROS production nootkatone induce the cell cycle arrest at S-phase, it may also inhibit the retinoblastoma by inhibiting the Nf-kB signaling pathway and cell migration.

3.5. Interaction of ligands with HSP90

The docking score of Nootkatone, Zerumbone, 7-Hydroxycoumarine, 4-Hydroxycoumarine, phloretin, and arecoline against HSP90 protein was found to be −7.5, −7.45, −6.18, −5.9, −5.69, and 5.26 respectively. The order of binding energy was Nootkatone > Zerumbone > Phloretin > 4-Hydroxycoumarine > 7-Hydroxycoumarine > Arecoline. Heat Shock Proteins (HSP90) possess anti-parasitic and anticancer activity. The outcomes of the docked compound with HER2 kinase receptor were shown in Table 6 and Fig. 9 which shows the interaction of 4-Hydroxycoumarine, 7-hydroxycoumarine, arecoline, nookatone, phloretin, zerumbone. The inhibition of protein expression related to metastatic cancer and the induction of autophagy is attributed to the effects of nootkatone (Zho et al., 2020). Therefore, gaining a deeper understanding of the molecular mechanisms of nootkatone in anti-tumor activity could enhance our comprehension of metastatic cancer treatment and potentially improve therapeutic approaches. The docking studies confirmed the suppressive activity through suppression of target protein HER2 Kinase and HSP90. Among the various compounds, Nootkatone has more potential binding interactions than other compounds.

4. Conclusions

The hydroalcoholic extract of *S. ifolium* was found to be strong anticancer potential against human breast cancer cells. It exhibits prominent cell cycle phase arrest similar to the standard control, aripiprazole on MCF-7 cells. It may induce apoptosis via the activation of caspase 3, caspase 9, and Bcl-2 pathway. The *in silico* docking studies demonstrate the binding activity of the compound present in the HAE of *S. isoetifolium* to the breast cancer receptor proteins such as HER2 Kinase and HSP90, respectively. It supports the use of *S. isoetifolium* for the possible treatment of breast cancer. Further *in vivo* research, the success of this additional set of investigations will help to clarify how it is possible to mix the most potent extracts with the existing medication without running into problems with drug resistance and negative side effects. This study is the first scientific report that provides convincing anticancer and rich antioxidant sources as evidence for the relevance of *S. isoetifolium* thus providing scientific validity to its medicinal uses such as an anticancer agent.

CRediT authorship contribution statement

P. Kalaivani: Conceptualization, Methodology. **P. Amudha:** Conceptualization, Methodology. **A. Chandramohan:** . **R. Vidya:** Conceptualization, Methodology. **M. Prabhaharan:** Supervision, Conceptualization, Methodology. **P. Sasikumar:** Project administration, Supervision, Conceptualization, Methodology, Formal analysis. **Salim Albukhaty:** Writing – review & editing, Formal analysis, Investigation, Data curation. **Ghassan M. Sulaiman:** Project administration, Writing – review & editing, Formal analysis, Investigation, Data curation. **Mosleh M. Abomughaid:** Writing – review & editing, Investigation, Data curation, Validation. **Mohammed Abu-Alghayth:** Visualization, Validation, Investigation, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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