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EXTRACTION, ISOLATION, CHARACTERIZATION, *IN-SILICO* MOLECULAR DOCKING AND ANTICANCER EVALUATIONS OF *SOLANOPUBAMINE* FROM *SOLANUM PUBESCENS* WILLD

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ABSTRACT
The present work deals with the extraction, isolation, characterization, *in-silico* molecular docking and anticancer activity of Solanopubamine from leaf explants of *Solanum pubescens*. The extraction was carried out by Soxhlet apparatus using DMSO as solvent. The DMSO leaf extracts were purified by column chromatography. The functional groups and structure of the isolated Solanopubamine was confirmed by Fourier transforms infrared spectroscopy (FT-IR), ^{1}H-Nuclear magnetic resonance spectroscopy (^{1}H-NMR) and high performance liquid chromatography (HPLC). The isolated Solanopubamine was separately docked into two different active sites of EGFR and ERα. The docking results by the interaction with EGFR, showed only two hydrogen bond interactions; (i) the side chain hydrogen atom of the negative charged residue of ASP 776 were well interacted with oxygen atom (O-H) of Solanopubamine with bond length of 1.996Å and (ii) hydrogen atom (N-H) of Solanopubamine molecule were well interacted with hydrophobic residue of LEU 694. The results showed that the isolated Solanopubamine compound is potential for anticancer activity. *In-vitro* anticancer activities of the isolated Solanopubamine were screened against human breast cancer cell line (MCF-7) using MTT assay. The IC_{50} values were found to be 86.33µg/ml. The present study reveals that the isolated Solanopubamine shows a significant *in-vitro* anticancer activity against MCF-7 cells and also it is less toxic for human cells.

Keywords: Solanopubamine, Alkaloid, ^{1}H-NMR, HPLC, MCF-7.

1. INTRODUCTION
Solanum pubescens belongs to the family Solanaceae and very closely related to the Turkey berry (*Solanum torvum*). *Solanum pubescens* is commonly known as Kaattusundaikaai in Tamil (Fig.1). It is a medicinally useful plant and is particularly known for its therapeutically bio-active compound; alkaloids. A variety of compounds are reported in *S. pubescens* and some of them are myricetin methyl ethers [1] Solanopubamine [2] and solanopubamides A & B [3]. The tribal people used *S. pubescens* plants for the treatment of liver disorders, diarrhoea and cancer disorders [4], however, there is no report on the anticancer activity research in this plant.

Cancer is a major cause of death and breast cancer is one of the common malignancies leading to death in women around the world. The disease occurs almost entirely in women, but men are also frome to it. Radiation is a well documented risk factor for breast cancer and its exposure induces the formation of free radicals.

As a result, novel ligands for receptors of known structure were designed and their interaction energies were calculated using the scoring functions [5]. The three dimensional structure of the protein ligand composite could be served as a considerable source of understanding the way of interation of proteins with one another and the performance of their biological functions.

Computational homology based on the modeling methods, is the best and fast technique to predict the protein structure from a known 3D template when there is no solved structure [6]. Historically virtual screening of the drug development process is divided into the structure and ligand-based algorithms [7].

The functional groups and structure of the isolated Solanopubamine was confirmed by FT-IR, ^{1}H-NMR and HPLC. The isolated Solanopubamine was separately docked into two different active sites of EGFR and ERα. The docking results showed that the isolated Solanopubamine compound is potential for anticancer activity.

Finally, the *in-vitro* anti-cancer activity of isolated Solanopubamine from *Solanum pubescens* against breast cancer cell line (MCF-7) was confirmed.

Fig. 1: *Solanum pubescens* plant a) Plant habit; b) Fruits and Flower.

2. MATERIAL AND METHODS

2.1. Collection and authentication of plant materials:

The aerial parts of *Solanum pubescens* were collected from Sirumalai hills, a part of the Western Ghats region, Dindigul district, Tamilnadu. The species was authentically identified and compared with herbarium specimen available in BSI, Coimbatore, and Tamilnadu. Specimen No: BSI/SRC/5/23/2017/Tech/2985.

2.2. Preparation of Plant Extracts

The leaves of *S. pubescens* were dried in hot air oven at 40-50°C for a week. The dried plant material was powdered using mixer grinder and subjected to soxhlet extraction with 99% methanol for 24 hours.

2.3. Column chromatography

Solanopubamine was isolated from the condensed extracts using Column chromatography and TLC and structure was confirmed by FT-IR, ^{1}H NMR and HPLC. Separation of Solanopubamine was carried out using petroleum ether as a solvent, as this particular extract showed four different spots. The separation of Solanopubamine from the extracts was carried out by column chromatography [8].

The column was packed by using wet packing technique with silica gel (300 g) as the adsorbent. Slurry was prepared using hexane and the slurry was poured in to the column. Fractions were concentrated and TLC was performed [9].

The R_f value of the spots were calculated using the formula (1)

R_f = Distance traveled by solute/Distance traveled by solvent (1)

2.4. Characterization:

2.4.1. FT-IR

Fourier transforms infrared (FT-IR) spectroscopy (Nicolet 6700 Thermo Fisher) was used to identify the functional groups present in the isolated sample (Solanopubamine) and the scanning rate was recorded in the range of 400-4000 cm^{-1}.

2.4.2. ^{1}H NMR

^{1}H-NMR analysis of the isolated Solanopubamine was performed on Varian Mercury 300 (300 MHz for ^{1}H) and Varian Inova 750 (750 MHz for 1H) instruments (Agilent Technologies, Palo Alto, CA), equipped with a 5 mm probe.

2.4.3. HPLC

Isolated *Solanopubamine* were subjected onto the HPLC analysis using Shimadzu Model LC2010 AHT Auto Sampler (UV-Vis Detector).

2.5. Cell Lines and Cytotoxicity

MCF-7 cells was obtained from the National Centre for Cell Science (NCCS), Pune, India, and grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-anti-mycotic solution (10000U per ml penicillin, 10,000 µg/mL streptomycin and 25µg/mL amphotericin B in the culture medium). The cells were maintained as monolayer in a 96 well microplate at a density of 4 x 104 cells in 100 mL of culture medium,

and incubated overnight at 37°C under 5% CO_2 atmosphere. The cell lines were maintained at 95% humidity in a CO_2 incubator and the cultures were allowed to grow till 80-90% confluency before drug treatment. Solanopubamine were loaded in each well at a final concentration of 10, 20, 40, 80, 160 and 320 µg/mL. After incubation for 24 and 48 h, unreacted and treated cells were washed with 1% Phosphate-Buffered Saline (PBS). After that, 10 µL of 12 mM MTT stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl-tetrazolium bromide was added to each well and incubated for 3h. Then the medium was carefully removed without disturbing the formazan crystals, and the intracellular purple formazan crystals were dissolved in 100µL of acidified isopropanol. The plates were read for measurement of absorbance at 590 nm.

2.6. Molecular Docking

2.6.1. Preparation of Ligand Structure:

Chemdraw assistance has been taken to sketch the plant isolated molecules (http://www.cambridgesoft.com). The sketched was then prepared by Ligprep 2.7 [10] where 2D structure gets organized into 3D structure and different tautomers were also the result of Ligprep 2.7. Ligands were ionized at a pH range about 7.0 ± 2.0 to preserve the qualities for molecular docking [11].

2.6.2. Qik Prop

QikProp [12] is a quick, accurate, easy-to-use absorption, distribution, metabolism, excretion and Toxicity (ADMET) prediction program designed by Professor William L. Jorgensen. QikProp predicts physically significant descriptors and pharmaceutically relevant properties of organic molecules, either individually or in batches. In addition to predicting molecular properties, QikProp provides ranges for comparing a particular molecule's properties with those of 95% of known drugs.

2.6.3. Selection of target protein EGFR and ERα

The PDB is a key resource in areas of structural biology, such as structural genomics. Most major scientific journals and some funding agencies such as the NIH in the USA, now require scientists to submit their structure data to the PDB. If the contents of the PDB are thought of as primary data, then there are hundreds of derived (*i.e* secondary) databases that categorize the data differently. For example both SCOP and CATH categorize structures according to type of structure and assumed evolutionary relations; GO categorize structures based on genes.

Prior to perform any molecular interaction study, preparing target molecule is significant. The crystal structure of cancer protein EGFR (PDB ID: 1M17) and ERα (PDB ID: 3ERT) were retrieved from protein data bank [13, 14]. Maestro is Schrodinger's powerful united multi-platform Graphical User Interface. Import a ligand/protein co-crystallized structure, typically from the Protein Data Bank, into Maestro. Locate any waters you want to keep then delete all others. Determine whether the protein-ligand complex is a dimer or other multimer containing duplicate binding sites and duplicate chains that are redundant. Adjust the protein, metal ions, and cofactors. If there are bonds to metal ions, delete the bonds, then adjust the formal charges of the atoms that were attached to the metal as well as the metal itself. Set charges and correct atom types for any metal atoms, as needed. Set bond orders and formal charges for any cofactors, as needed.

Finally the protein structure energy was minimized reached until the average root mean square deviation non bonded hydrogen atom 0.30Å [15], the prepared protein was input file for molecular docking. Grid files represent physical properties of a volume of the receptor (specifically the active site) that are searched when attempting to dock a ligand. The complex for this exercise is actually in two files, one containing the receptor and one containing the ligand. In the Van der Waals radii scaling section, we choose Scaling factor default value of 1.00 (no scaling). The grid box X=3.524, Y= 3.241, Z= -1.342 was set for protein.

2.6.4. Examining Glide Data

Glide is employed for the rapid docking of plant isolated molecules into the active site of the target receptor. Grid was prepared for protein with the exact same center and the size of the bounding box set on 30Å the Glide algorithm is operates with a systematic search of positions, orientations, and conformations of the ligand in the receptor binding site using funnel type approach and follows a unique scoring method. Glide score and glide energy was analyzed using XP visualizer [10, 12]. Glide results are examined with an emphasis on visual rather than numerical appraisal. The set of exercises uses the Glide XP Visualizer panel to display information on the terms in the Glide XP scoring function that contribute to the ligand binding. From the Glide Docking window panel we can view docked complex can analyses with its parameter for PI3K and generated conformers with ligands.

3. RESULTS AND DISCUSSION

3.1. Isolation of Solanopubamine

Column chromatography of plant extract sample endowed with number of fraction. The alkaloids isolated from leaf extracts of *Solanum pubescens* were subjected to a detailed evaluation. The chromatographic evaluation indicated that the isolated compound from *Solanum pubescens* was pure as a single spot was obtained in TLC, whereas the compound from *Solanum pubescens* was found to be alkaloids. The range of sample R_f value on 5.8 cm, 4.3 cm and 0.74, have been perfectly correlated with Compound exhibited positive response for Dragendorff's reagent which as indicates that the isolated compound was an alkaloid. The melting point of these compounds was found to be 157-158°C.

3.2. Functional group confirmation by FT-IR spectroscopy

FT-IR spectroscopy is a useful tool for the identification of presence/absence of functional groups in the isolated Solanopubamine and its obtained spectrum is shown in Fig.2. From the results, the sample contained a broad band which appeared at 3370 cm^{-1} indicates the hydroxyl stretching (-OH); the band at 2949 cm^{-1} is attributed to N-H (stretching vibration) group frequency. A band which appeared at 1113.99 cm^{-1} was attributed to C-N stretching vibration. This FT-IR study confirms the presences of functional groups such as hydroxyl, amino and methylene group in the prepared compound. The observed result matched very well with the literature values [16]. The functional groups corresponding to their wavelength were listed in table 1.

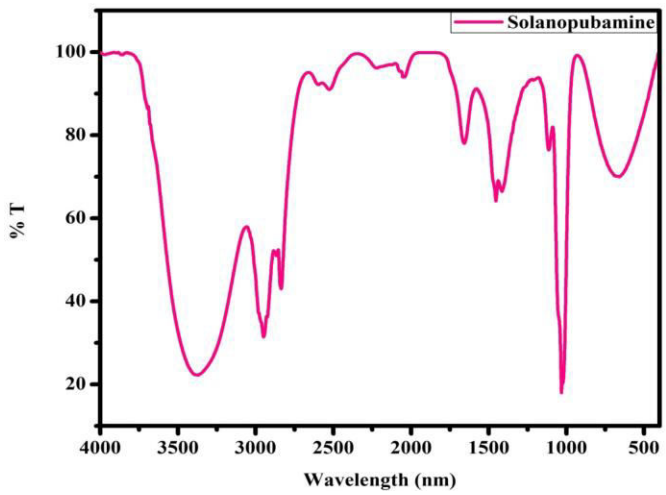

Fig. 2: FT-IR characterization of isolated compound (Solanopubamine)

Table 1: FT-IR characterization of isolated compound

Wavelength in (cm^{-1})	Name of the functional group	Functional group
3370	Alcohol	O-H
2949	Alkane	C-H
2835	Alkane	C-H
2866	Alkane	C-H
2525	Acid	O-H
2221	Alkyne	-C=C-
2025	Alkenes	-C≡C
1665	Alkene	C=C
1453	Aromatic	C=C
1414	Aromatic	C=C
1113	Amine	C-N
1031	Ether	C-O
657	Alkyl halide	C-C

3.3. Structural conformation of Solanopubamine by ^{1}H-NMR

^{1}H-NMR spectra were recorded using DMSO-d6 solvent and the ^{1}NMR spectrum of isolated Solanopubamine presented in Fig.3. According to the literature survey, the proton NMR signal was assigned (ref). Ring A: 1.44 (1H, m, 2-H), 1.54 (1H, m, 2-H), 1.70 (1H, m, 4-H), 1.14 (1H, m, 4-H), 1.70 (1H, m, 1-H), 1.09 (1H, m, 1-H); 3.34 (1H, m, 3-H); Ring B: 1.30 (2H, m, 6-H), 1.91 (1H, m, 7-H), 1.23 (1H, m, 7-H); Ring A/B: 1.26 (1H, m, 5-H); Ring C: 1.54 (1H, m, 11-H), 1.44 (1H, m, 11-H); 1.98 (1H, m, 12-H), 1.63 (1H, m, 12-H); Ring B/C: 0.69 (1H, m, 9-H), 1.57 (1H, m, 8-H);Ring D: 1.95 (2H, m, 15-H); Ring C/D: 1.44 (1H, m, 14-H); Ring E: 2.27 (1H, m, 20-H); Ring D/E: 1.91 (1H, m, 17-H), 2.73 (1H, m, 16-H); Ring F: 2.80 (1H, m, 26-H), 2.22 (1H, dd,J= 3.5 Hz, 7.1 Hz, 26-H), 1.54 (1H, m,24-H), 1.95 (1H, m, 24-H), 4.1 (1H, m, 23-H); Ring E/F: 2.18 (1H, m, 22-H); Methyl: 0.83 (3H, s, 19-H) 0.87 (3H, s, 18-H), 0.98 (3H, d, J= 5.58 Hz, 21-H), 1.23 (3H, d, J= 7.3 Hz, 27-H); the observed ^{1}H-NMR spatral valus were matched with the repots suggested by Adnan *et al,* 2013[17]. The outcome of this reports which us similaer to our report.

Positive HRESI-MS: found for $C_{27}H_{47}N_2O$: 1.39 (1H, m, 11-H); 1.82 (1H, m, 1.61 (1H, m, 8-H); Ring D: 1.84 (2H, m, 15-H); Ring C/D: 1.24 (1H, m, 14-H); Ring E: 2.24 (1H, m, 20-H); Ring D/E: 1.57 (1H, m, 17-H), 2.81 (1H, m, 16-H); Ring F: 2.78 (1H, m, 26- H), 2.06 (1H, dd, J =3.5 Hz, 7.4 Hz, 26-H), 1.61 (1H, m, 24-H), 1.08 (1H, m, 24-H), 3.85 (1H, m, 23-H); Ring

E/F: 1.85 (1H, m, 22-H); Methyl: 0.90 (3H, s, 19-H) 0.97 (3H, s, 18-H), 0.99 (3H, d, J = 6.5 Hz, 21-H), 1.26 (3H, d, J=7.5 Hz, 27-H); and 13C NMR (CDCl3; dC): Ring A: 34.50 (C-2), 37.90 (C-1), 41.10 (C-4), 51.80 (C-3); Ring B: 29.60 (C-6), 32.20 (C-7); Ring A/B: 36.70 (C-10), 46.30 (C-5); Ring C: 22.10 (C-11), 37.80 (C-12); Ring B/C: 36.60 (C-8), 55.60 (C-9); Ring D: 28.0 (C-15); Ring C/D: 42.40 (C-13), 58.60 (C-14); Ring E: 31.90 (C-20); Ring D/E: 63.50 (C-17), 71.0 (C-16); Ring F: 28.60 (C-25), 33.30 (C-24), 59.70 (C-26), 67.40 (C-23); Ring E/F: 79.90 (C-22); Methyl: 12.60 (C-19), 17.40 (C-18), 18.70 (C-21), 22.10(C-27); 12-H), 1.61 (1H, m, 12-H); Ring B/C: 0.76 (1H, m, 9-H), [17].

Proton NMR spectroscopy was used for the confirmation of structure of isolated compound and it shows the presence of 50 hydrogens in the compound [18]. The aromatic protons are observed, and the five methoxy groups were also prominent ^{1}HNMR(300 MHz, DMSOd6) δ[ppm]: 8.02(2H,d,H2',H6');7.16 (2H,d,H3',H5');6.77 (1H,s,H3); 4.02 (3H,s,OCH_3); 3.97-3.78 (12H,m, 4xOCH_3) [19].

3.4. High Performance Liquid Chromatography (HPLC)

The quality control parameter studies clearly indicate the purified Solanopubamine. The total alkaloid fraction was also subjected to HPLC studies and results were presented in Fig.4. The qualitative HPLC profiles were detected at a wavelength of 254 nm due to sharpness of the peaks and proper baseline and recorded its percent area and heights. The Fig.4 shows one major peak at 3.725 retention time. The major peak at 3.725 retention time confirmed the presence of Solanopubamine. However, there is a need to carryout advanced hyphenated spectroscopic studies in order to elucidate the structure of these compounds. Furthermore, this data may be handy in probing of active bio compound of this plant in the future [20].

3.5. Biological Activities

3.5.1. In silico Anticancer analysis

The crystal structure of EGFR (PDB ID: 1M17) and ERα (PDB ID: 3ERT) was recovered from protein data bank. The plant isolated chemical compounds Solanopubamine were separately docked into active site of the EGFR and ERα. The crystal structure of the 3D docking simulation of Solanopubamine with EGFR, the following two residues were mainly involved in hydrogen bond interaction: (i) EGFR ASP 776 with bond length (1.996Å) and (ii) hydrophobic residue of LEU 694. Fig. 5(a) shows that the side-chain protonated oxygen atom (O-H) well interacted with negatively charged residue of ASP 776 and nitrogen atom (N-H) interacted well with hydrophobic residue of LEU 694. Fig. 5(b) shows the 3D docking simulation of Solanopubamine into ERα formed only one hydrogen bond interaction. The side chain hydrogen atom (O-H) interacted well with negative charged residue of ASP 351 with a bond length of 1.994 Å.

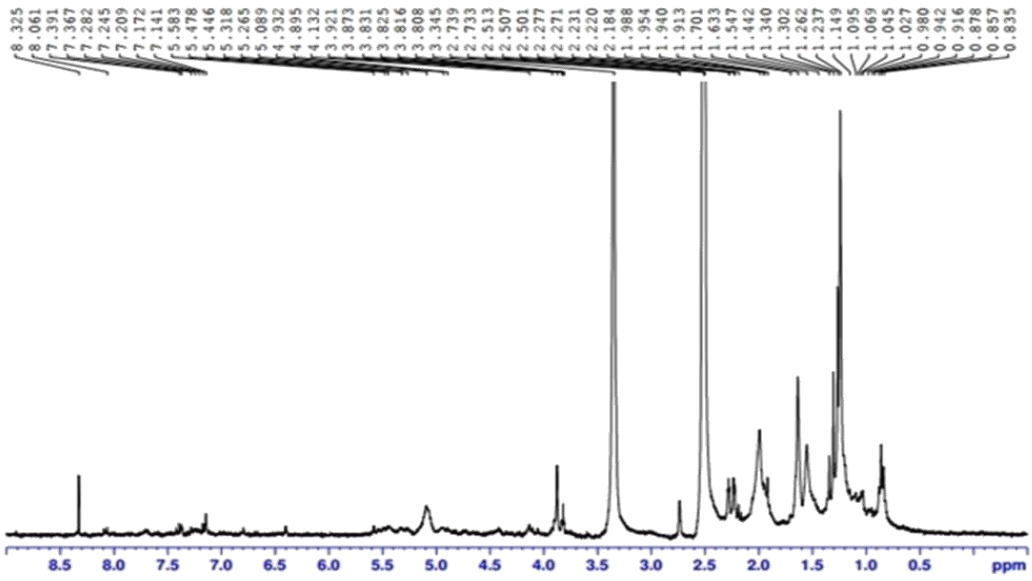

Fig. 3: ^{1}H-NMR Spectra of isolated compound Solanopubamine

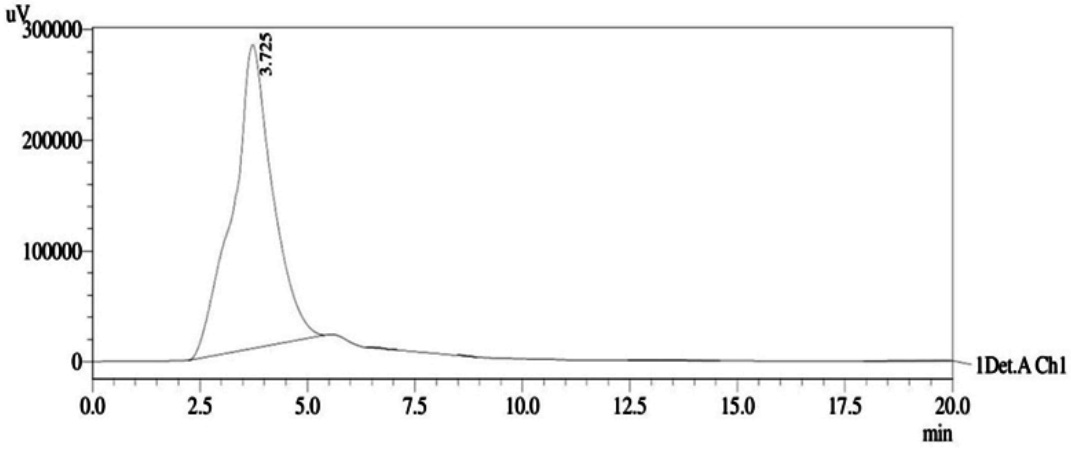

Fig. 4: HPLC- Spectra Conformation of Solanopubamine compound

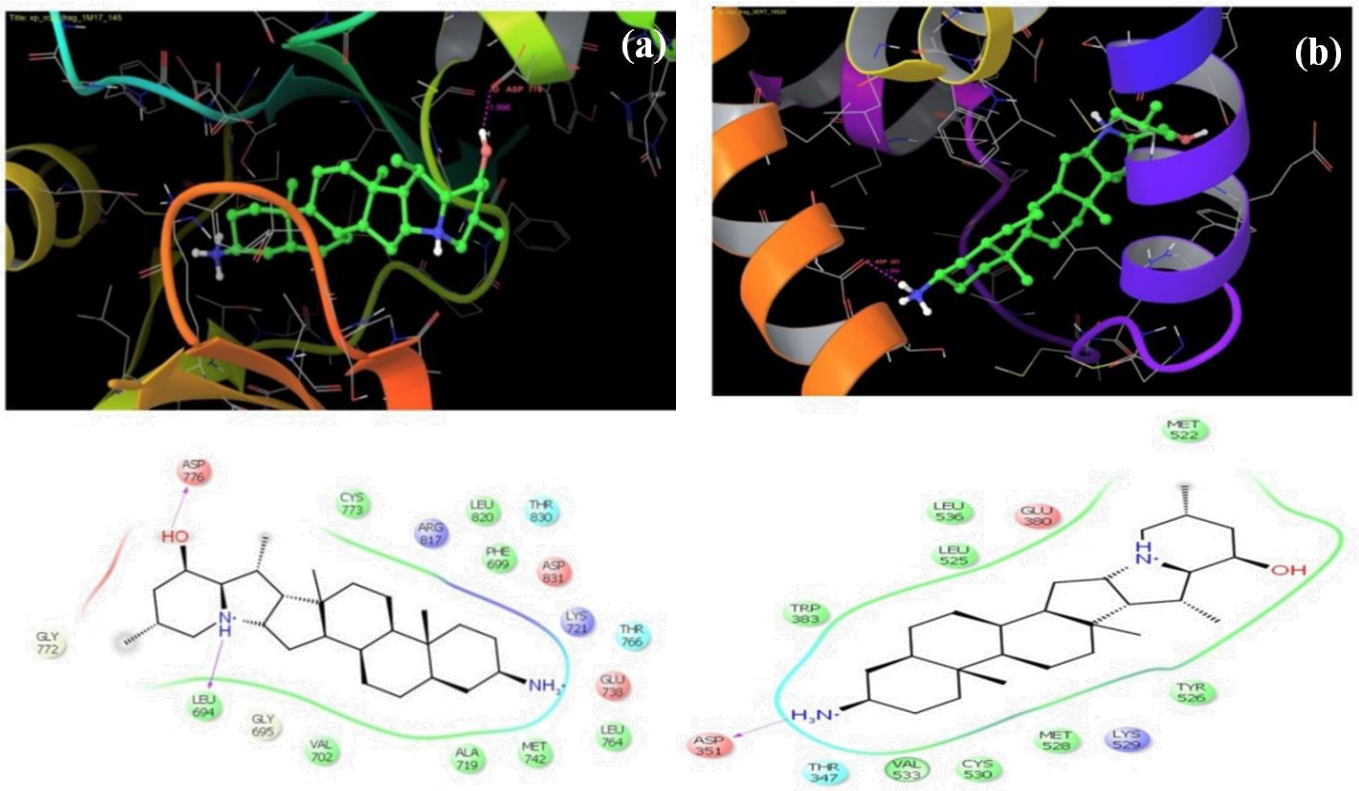

Fig. 5: 3D and 2D Docked structure of Solanopubamine target with (a) EGFR (PDB ID: IM17) protein and (b) ERα (PDB ID: 3ERT) Protein.

Interestingly, the residues TRP 383, LEU 536, MET 522, MET 528, CYS 530.CYS 773, LEU 820, MET 742 were mainly involved in hydrophobic interactions with the active site of the ERα and EGFR. The glide score and glide energy of the compounds were, as reported in Table 2. The glide score of EGFR and ERα are-4.421Kcal/mol, and -5.152Kcal/mol respectively, and the glide energy of EGFR and ERα are -30.13Kcal/mol and -32.61Kcal/mol respectively. The observed docking simulation was matched with the reports suggested by Bari *et al, 2012* [21].

3.5.2. Anticancer activity

In vitro exposures of MCF-7 cells with different concentrations (10, 20, 40, 80, 160 and 320 µg/ml) of Solanopubamine DMSO extracts significantly sup-

pressed MCF-7 cancer cell growth and decrease in cells count was observed with increase in concentration of the extracts tested. A dose dependent increase in cytotoxic activity for all the concentrations was observed. The maximum inhibition of 90.3 % of MCF-7 cells was observed at a concentration of 320 µg/ml of the plant extracted tested. *In-vitro* anticancer activity of isolated Solanopubamine is shown in Fig. 5, 6 and Table 3. The isolated Solanopubamine were shown IC_{50} value of 86.33µg/ml. Anticancer drugs with minimal side effects on normal cells are highly desirable for therapeutic purposes [22] hence; the current study also emphasized that the efficiency of TC DMSO extract-mediated suppression of cell viability on cancer cells. Indeed the concentrations of DMSO extract of TC that was cytotoxic to human breast cancer cells failed to induce apoptosis cell death.

The main aim of analyzing crude plant extracts is either to isolate bioactive agents for direct use as drugs or to identify bioactive compounds that can be used as lead substances in the preparation of semi synthetic drugs [23]. A large number of novel anticancer drugs have been discovered from natural products in the past and new ones are continually being developed thereby improving their efficacy or reducing their toxicity.

Table 2: Docking score of Solanopubamine were retrieved by Glide.

Protein name	PDB ID	Compound Name	Glide Score	Glide Energy	No. of Hydrogen bond interaction	Interacting residue	Distance (Å)
ERα	3ERT	Solanopubamine	-5.152	-32.61	1	ASP 351 (Aspartate)	1.994
EGFR	1M17	Solanopubamine	-4.421	-30.13	2	ASP 776 (Aspartate) LEU 694 (Leucine)	1.996 -

Table 3: Determination of cytotoxicity of *Solanopubamine* on MCF-7 cell line by MTT Assay

Cell line: MCF 7				
Sample	**Conc. (µg/ml)**	**OD @ 590nm**	**%Inhibition**	**IC_{50}**
Control	control	0.69	0.0	
Solanopubamine	10	0.65	6.3	86.33µg/ml
	20	0.60	12.5	
	40	0.50	27.1	
	80	0.30	46.0	
	160	0.18	74.3	
	320	0.07	**90.3**	

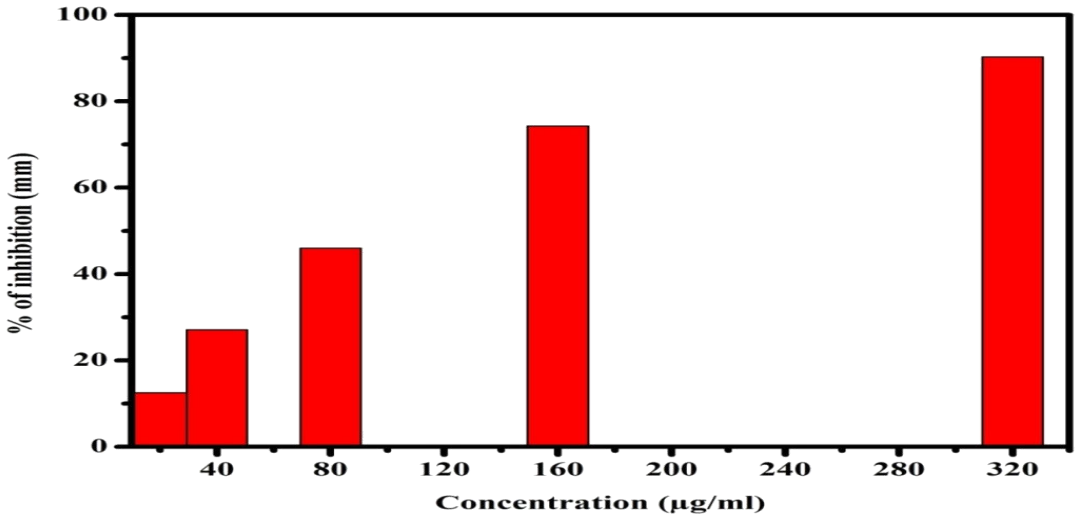

Fig. 6: Percentage (%) of Inhibition activity of isolated Solanopubamine compound with different concentrations (10-320 µg/ml)

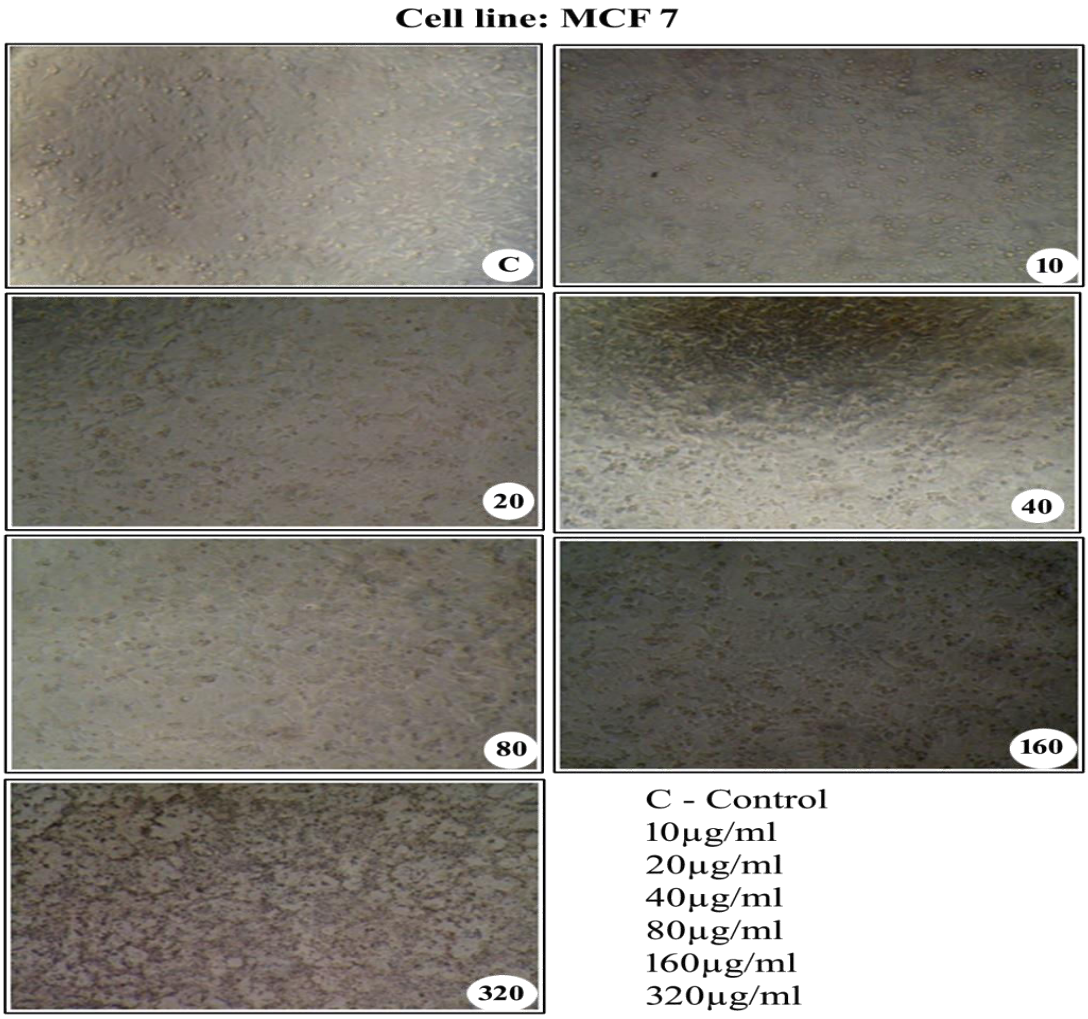

Fig. 7: *In-vitro* anticancer activity of isolated Solanopubamine compound with different concentrations (10-320 µg/ml)

4. CONCLUSION

On the basis of preliminary characterization studies, the isolated Solanopubamine compound has good physical and chemical properties (colour, state, solubility, melting range and R_f value) which are identical to the standard Solanopubamine. Spectral data shows that the isolated compound is mostly similar to standard Solanopubamine. IR peaks of various functional groups of Solanopubamine are found in this isolated compound**.** 50 protons were found in the ^{1}H-NMR spectra of isolated compound. So, it can be concluded that the isolated compound has the molecular formula $C_{27}H_{47}N_2O$ which corresponds to the molecular formula of Solanopubamine. The above results confirmed that the isolated compound is Solanopubamine. The isolated Solanopubamine was separately docked into active site of the EGFR and ERα. Further, the isolated Solanopubamine showed a significant *in-vitro* anti cancer activity against MCF-7 cells.

Conflict of interest

No potential conflict of interest was reported by the authors.

5. ACKNOWLEDGEMENT

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