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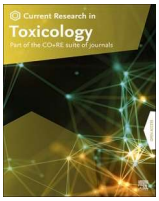

Chromatographic analysis of selected phytosterols from *Cyathea* and their characterization by *in silico* docking to potential therapeutic targets

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ABSTRACT

Separation and quantification of lupeol, stigmasterol and swertiamarin in ethanolic extracts of selected *Cyathea* species have been developed using HPTLC and an attempt is made to explore the biopotential of phytochemicals against various proteins by computational analysis. Compounds were separated using the specific mobile phase and the developed plates were sprayed with respective spraying reagents. The 3D structure of the receptor proteins viz., 1VSN, 5BNQ, 6HN8, 7DN4 and 3TJU, and the 3D SDF structures of ligands like lupeol, stigmasterol and swertiamarin were retrieved from the Protein Data Bank (PDB) and NCBI-Pub Chem Compound database respectively. The Argus 4.0.1 is computer generated drug design screening software is employed to analyze the binding affinity of test compounds against the selected proteins in the form of E-values versus potential drug targets. The docking result was saved and visualized using Discovery Studio Visualizer. The terpenoid band with R_f value 0.79 depicted the presence of lupeol in *C. gigantea* (0.04%) and *C. crinita* (0.02%). The steroid band with R_f value 0.41 confirmed the presence of stigmasterol with varied frequency viz., *C. nilgirensis* (0.33%), *C. gigantea* (0.29%) and *C. crinita* (0.52%). Lupeol, stigmasterol and swertiamarin showed the interaction against the studied proteins viz., 1VSN, 5BNQ, 6HN8, 7DN4, 3TJU with varied energy values and interacting residues. The results of the virtual screening and molecular docking analysis suggest that the phytochemical compounds of *Cyathea* species viz., lupeol and stigmasterol were identified as possible lead molecules to fight against cancer and cytotoxicity.

Introduction

Plant based medicines plays a pivotal role in health care systems. Around 80% of the world's inhabitants rely mainly on phytomedicines for various ailments (Van Andel et al., 2015; Ulian et al., 2017; Rahayu et al., 2020). Since ancient times, medicinal compounds from higher group of plants have sustained the life of human beings. Lower plants, especially pteridophytes are the least plant groups exploited for medicinal purposes. Despite the diversity of ferns and species richness, less attention has been given to pteridophytic research. Pteridophytes occupy the middle position between the lower cryptogams and seeded plants in the phylogeny of plant kingdom. It includes an extensive spectrum of biological types starting from small fern allies to arborescent tree ferns (Kumar, 1998). About 13,600 species of extant pteridophytes are recorded in the world flora (Moran, 2008).

Medicinal plants with potentially useful bioactive compounds are selected based on the taxonomic, phytochemical and ethnomedicinal approach (Cordell et al., 1991; Singh, 2016). Plants of a specific genus or

Abbreviations: HPTLC, High Performance Thin Layer Chromatography; NCBI, National Center for Biotechnology Information; PDB, Protein Data Bank; R_f, Retardation factor; SwissADME, Swiss Adsorption Distribution Metabolism Excretion; TLC, Thin Layer Chromatography; UV, Ultra Violet; XCH, XAVIERS COLLEGE HERBARIUM.

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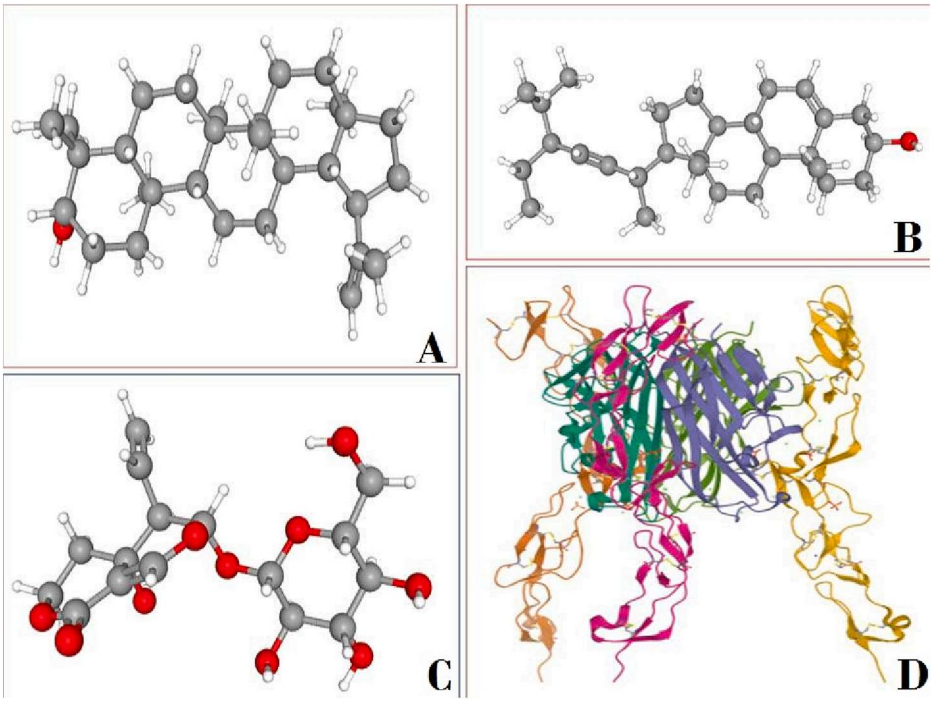


Fig. 1. Structure of Ligands - Lupeol, stigmasterol, swertiamarin and 5BNQ Protein. A - lupeol; B – Stigmasterol; C – Swertiamarin; D – 5BNQ - hRANKL-mRANK complex. (Source: National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 259846, Lupeol. Retrieved July 14, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Lupeol. National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 5280794, Stigmasterol. Retrieved July 14, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Stigmasterol. National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 442435, Swertiamarin. Retrieved July 14, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Swertiamarin. https://www.rcsb.org/structure/5BNQ)

family are collected from diverse locations based on a particular compound type which is of biological interest. Phytochemical and taxonomic approaches are closely related to each other and cannot be clearly divided. The ethnomedicinal approach is trustworthy because it provides the information about the medicinal uses of the plant. Based on this information, the particular plant is collected and evaluated (Farnsworth, 1991; Graham and Farnsworth, 2010).

Quantification of phytocompounds is very simple due to the recent developments in analytical instrumentation. Advances in the isolation, purification and structure elucidation of naturally occurring substances have made it possible to establish appropriate strategies for the process of standardization. HPTLC analysis provides highly reproducible results and traceable records through a standardized methodology. Srivastava et al. (2008) analyzed the HPTLC profile of *Lycopodium clavatum* stem using the mobile phase toluene: ethyl acetate: formaldehyde (6:3:1) and confirmed the presence of ferulic acid. Paul and Banerjee (2013) determined the HPTLC profile of flavonoids using the mobile phase ethyl acetate - formic acid - glacial acetic acid - water (10: 0.5: 0.5: 1.3) in *Pteris vittata*. Janakiraman and Johnson (2016) used different mobile phases and separated phenolic compounds, flavonoids and tannins from different *Cyathea* species using HPTLC. In addition, the HPTLC profiles are employed as pharmacognostical marker to distinguish the medicinally important plants. Johnson et al. (2020) employed the HPTLC profile as a marker to distinguish *Asplenium aethiopicum* from other species. Alkaloids, steroids, terpenoids, flavonoids and saponins of *Aerva lanata* are revealed using HPTLC by Yamunadevi et al. (2011; 2011a; 2011b; 2012; 2012a). Similarly, Selvamaleeswaran et al. (2013) determined the alkaloids profile of *Clitoria ternatea* using HPTLC. Bobby et al. (2012b; 2012a) quantified the phenyl propanoids and flavonoids profile of *Albizia lebbeck* using HPTLC.

The computer assisted molecular docking method is employed to explain the atomic level interaction between phytocompounds and protein. It helps the biologist and pharmacologist to define small molecular behaviour in target protein binding sites and assume the critical biochemical processes. It is the most important tool in structural biology, drug design process to understand protein–ligand interactions and biological activity prediction. The three-dimensional structure of the protein–ligand complex explains how proteins interact with one another in order to achieve biological functions. Iqbal et al. (2022) predicted the biocidal action of *Mentha piperita* derived chemical constituents using molecular docking. Paramashivam et al. (2015) performed the computational exploration of vicine – an alkaloid glycoside mediated pathological hallmark of adenosine kinase to promote neurological disorder. Krupanidhi et al. (2020) studied the *Tinospora cordifolia* phytochemical compounds inhibitory activity against SARS-CoV-2 through molecular docking. Arthur and Uzairu (2019) studied the molecular interaction of NCI anticancer analogs with human Phosphatidylinositol 4, l5-bisphosphate 3-kinase catalytic subunit by molecular docking. Khlaid et al. (2018) evaluated the antiplatelet and anticoagulant actions of the synthesized novel derivative 1, 2, 4 triazolehydrazone and sulphonamide using molecular docking. With this background, the present study was aimed to elucidate the presence of various phytochemical compounds (terpenoids, steroids and glycosides) of *Cyathea nilgirensis* Holttum, *Cyathea gigantea* (Wall. ex. Hook.) Holttum and *Cyathea crinita* (Hook.) Copel. using HPTLC. In the present study, an attempt is made to explore the biological potential of *Cyathea* species derived phytochemicals viz., lupeol, stigmasterol and swertiamarin against 1VSN, 5BNQ, 6HN8, 7DN4, 3TJU proteins by computational analysis.

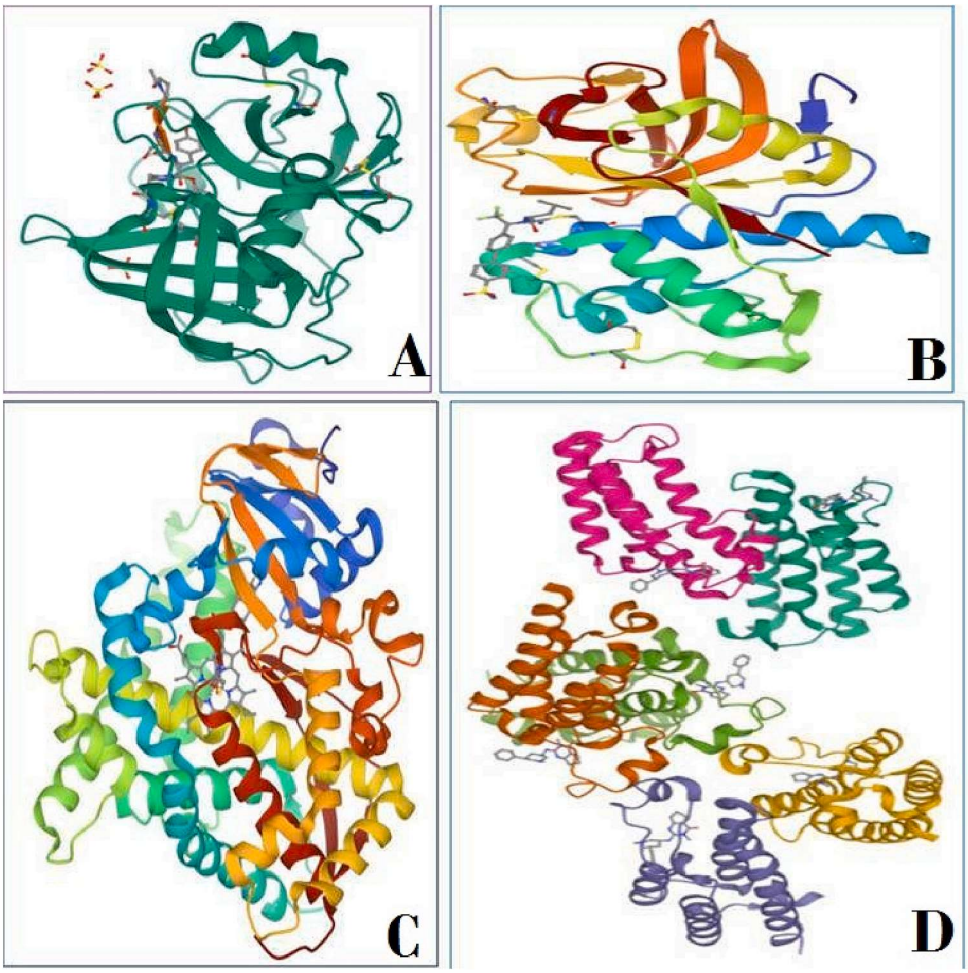


Fig. 2. Structure of Studied Proteins. A – 3TJU - Human granzyme H with an inhibitor (https://www.rcsb.org/structure/3TJU); B – 1VSN - Inhibitor bound to cathepsin K (https://www.rcsb.org/structure/1VSN); C – 6HN8 - BM3 heme domain in complex with troglitazone (https://www.rcsb.org/structure/6HN8); D –7DN4 - Cpd8 in complex with BPTF bromodomain (https://www.rcsb.org/structure/7DN4);

Materials and methods

Collection of plant materials

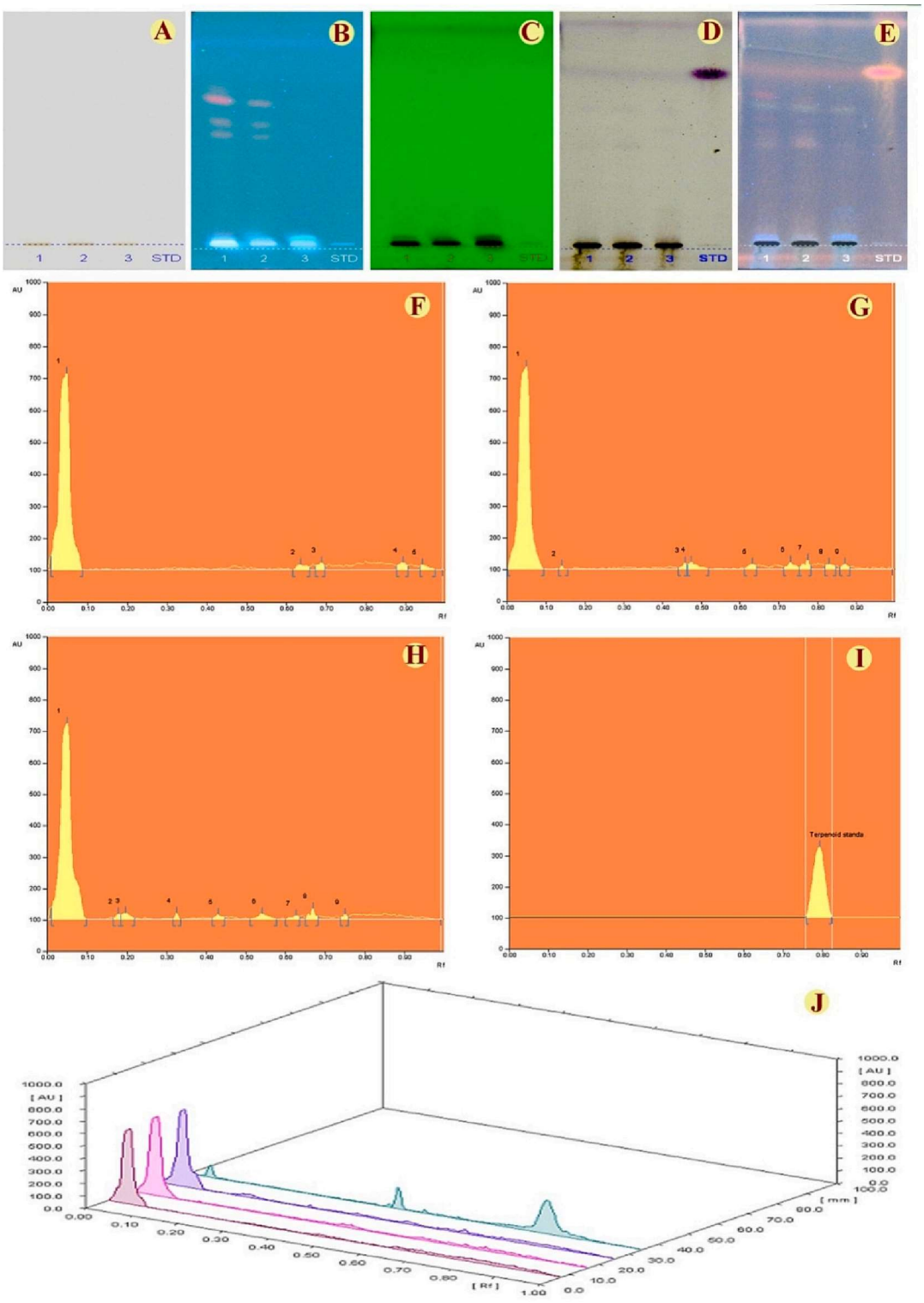
Specimens for the present study were collected from different localities of Tamil Nadu like *C. nilgirensis* was collected in and around Kakkachi stream (1,725 m), Kothayar, Tirunelveli hills (8°44′ N & 77°44′ E), *C. gigantea* from the road sides near Nadugani (2,637 m), Nilgiris hills (11°24′ N & 76°44′ E) and *C. crinita* from the Anglade Institute of Natural History, Shenbaganur, Kodaikanal (2,195 m), Palni hills (10°13′ N & 77°32′ E), Western Ghats, South India. The plants were identified based on the "Pteridophyte Flora of the Western Ghats, South India" by Manickam and Irudayaraj (1992). Herbarium specimens were prepared and the voucher specimens were deposited in the St. Xavier's College Herbarium (XCH), Palayamkottai, Tamil Nadu, India for further reference (*C. nilgirensis*- XCH 25423; *C. gigantea*- XCH 25422 and *C. crinita* - XCH 25424).

Preparation of extracts

The collected species of *Cyathea* were washed and blotted on the blotting paper. It was shade dried at room temperature under dark and ground to fine powder using mechanical grinder. 30 g powdered samples were successively extracted with 180 ml of ethanol using Soxhlet extractor for 8–12 h at a temperature not exceeding the boiling point. The extracts were concentrated in a vacuum at 40 °C using rotary evaporator (Janakiraman and Johnson, 2016a; Johnson et al., 2020).

HPTLC analysis

HPTLC analysis was carried out using the standard method described by Wagner et al. (1996). 25 mg of ethanolic extracts of selected *Cyathea* species were weighed accurately in an electronic balance (Shimadzu). It was dissolved in 0.5 ml of ethanol and centrifuged at 3000 rpm for 5 min. These solutions were used as test solution for terpenoids, steroids and glycosides. 2 μl of test solution



A - C: Before derivatization (A: Visible light; B: UV light - 366 nm; C: UV light - 254 nm) D and E: After derivatization (D: Visible light; E: UV light - 366 nm); F: Peak densitogram of *C. nilgirensis*; G: Peak densitogram of *C. gigantea*; H: Peak densitogram of *C. crinita*; I: Standard terpenoid - Lupeol; J: 3D display of all tracks

Fig. 3. HPTLC - Terpenoids profile of *Cyathea* species.

Table 1
HPTLC - Terpenoids profile of studied *Cyathea* species.

$R_f$values	*C. nilgirensis*	*C. gigantea*	*C. crinita*	Assigned substance
0.05	+	+	+	Terpenoid 1
0.14	–	+	–	Unknown
0.18	–	–	+	Unknown
0.33	–	–	+	Unknown
0.43	–	–	+	Unknown
0.46	–	+	–	Terpenoid 2
0.47	–	+	–	Unknown
0.54	–	–	+	Terpenoid 3
0.63	+	+	+	Terpenoid 4
0.69	+	–	+	Unknown
0.73	–	+	–	Unknown
0.75	–	–	+	Unknown
0.79	–	+	+	Lupeol
0.83	–	+	–	Unknown
0.89	+	+	–	Unknown
0.94	+	–	–	Unknown

and 2 μl of standard solution were loaded as 5 mm band length in the silica gel 60F_{254}TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The sample loaded plates were kept in a TLC twin trough developing chamber (after saturated with solvent vapour) with respective mobile phases viz., n-hexane - ethyl acetate (7.2: 2.9) for terpenoids, toluene - acetone (9: 1) for steroids and ethyl acetate - ethanol - water (8: 2: 1.2) for glycosides. The plate was developed up to 90 mm.

The developed plate was dried using hot air to evaporate solvents from the plate. The plate was kept in photo documentation chamber and the images were captured under visible light, UV 254 nm and UV 366 nm. The developed plate was sprayed with respective spraying reagents like anisaldehyde sulphuric acid reagent for terpenoids and steroids; Libermann - Burchard reagent for glycosides. The plates were dried at 100 °C in hot air oven. The plate was photo documented in visible light and UV 366 nm mode using photo documentation chamber. Before derivatization, the plate was fixed in scanner stage and scanned at UV 254 nm and UV 366 nm. After derivatization, the plate was fixed in scanner stage and scanned at UV 366 nm. The peak table, peak display and peak densitogram were noted. The software used was winCATS 1.3.4 version.

ADME and toxicity prediction

SwissADME and admetSAR were employed to study the *in silico* ADME and the toxicity properties of Lupeol, Stigmasterol and Swertiamarin from *Cyathea* species (Paramashivam et al., 2015; Daina *et al.,* 2017; Han et al., 2019; Indira *et al.,* 2020). To reveal the *in silico* ADME and toxicity of Lupeol, Stigmasterol and Swertiamarin (Fig. 1), the smile format of Lupeol, Stigmasterol and Swertiamarin were submitted into the SwissADME (http:// www. swiss adme.ch/) and admetSAR online server (http:// lmmd. ecust. edu.cn/ admet sar2/). SwissADME online server analyzes various parameters including physicochemical properties, lipophilicity, water-solubility, pharmacokinetics such as GI absorption, BBB penetration and drug-likeness. Similarly, the admetSAR server predicts toxicity factors such as mutagenicity, carcinogenicity, and hERG inhibition for Lupeol, Stigmasterol and Swertiamarin (Indira *et al.,* 2020).

In silico docking

The 3D structure of the receptor proteins viz., 3TJU - Human-granzyme H with an inhibitor, IVSN - Inhibitor bound to cathepsin K, 7DN4 - Cpd8 in complex with BPTF bromodomain, 6HN8 - BM3 heme domain in complex with troglitazone and 5BNQ - hRANKL-mRANK complex was retrieved from the protein data bank (PDB) and altered (Figs. 1 and 2). The active site was recognized after building the receptor. One of the active sites was designated because the receptor has many sites. Many water molecules and heteroatoms were removed. The Argus 4.0.1 is a computer generated drug design screening software is employed to analyze the binding affinity of test compounds viz., lupeol, stigmasterol and swertiamarin against the proteins 3TJU – Human granzyme H with an inhibitor, IVSN - Inhibitor bound to cathepsin K, 7DN4 - Cpd8 in complex with BPTF bromodomain, 6HN8 - BM3 heme domain in complex with troglitazone and 5BNQ - hRANKL-mRANK in the form of E- values versus potential drug targets (Das et al., 2020). A three dimensional grid with coordinates (x, y, and z) was designed to give the maximum surface area for chemical binding. The ligands lupeol, stigmasterol and swertiamarin 3D SDF structure were retrieved from the NCBI-Pub Chem Compound database (Fig. 1). Using GaussView 6.0.16, 3D SDF structure of lupeol, stigmasterol and swertiamarin was converted to PDB format. The ligand has been docked onto the receptor, and the interactions have been verified. The scoring function assigns scores based on the ligand with the best fit that was chosen.

Based on this binding energy between protein and the ligand, different forms of confirmations could be categorized into a cluster form, and also foremost docked confirmation was observed. The docking result was saved and visualized using Discovery Studio 4.0 Visualizer to record the varied interactions between the protein–ligand complexes (Karthick, 2015).

Cytotoxic activity - brine shrimp lethality bioassay

Cytotoxic activity of selected *Cyathea* species ethanolic extracts was evaluated using brine shrimp lethality bioassay method (Meyer et al., 1982). With the help of a Pasteur pipette, 20 nauplii were transferred to each test tube containing various concentrations (100, 200, 300, 400 and 500 mg/mL) of *C. nilgirensis*, *C. gigantea* and *C. crinita* ethanolic extracts (Janakiraman and Johnson, 2016). Five replicates were made for each concentration and a control DMSO was also maintained. The standard plumbagin was used as positive control. The setup was allowed to remain for 24 h under constant illumination. After 24 h, the dead nauplii were counted with a hand lens. Using the SPSS – 14, LC_{50}, 95% confidence limit, LC_{90} and chi square values were calculated.

Cytotoxic activity - MTT cell proliferation assay

Cell line and culture

The cell line of MCF 7 (human breast carcinoma) was obtained from National Centre for Cell Science, Pune, India. The cells were cultured in

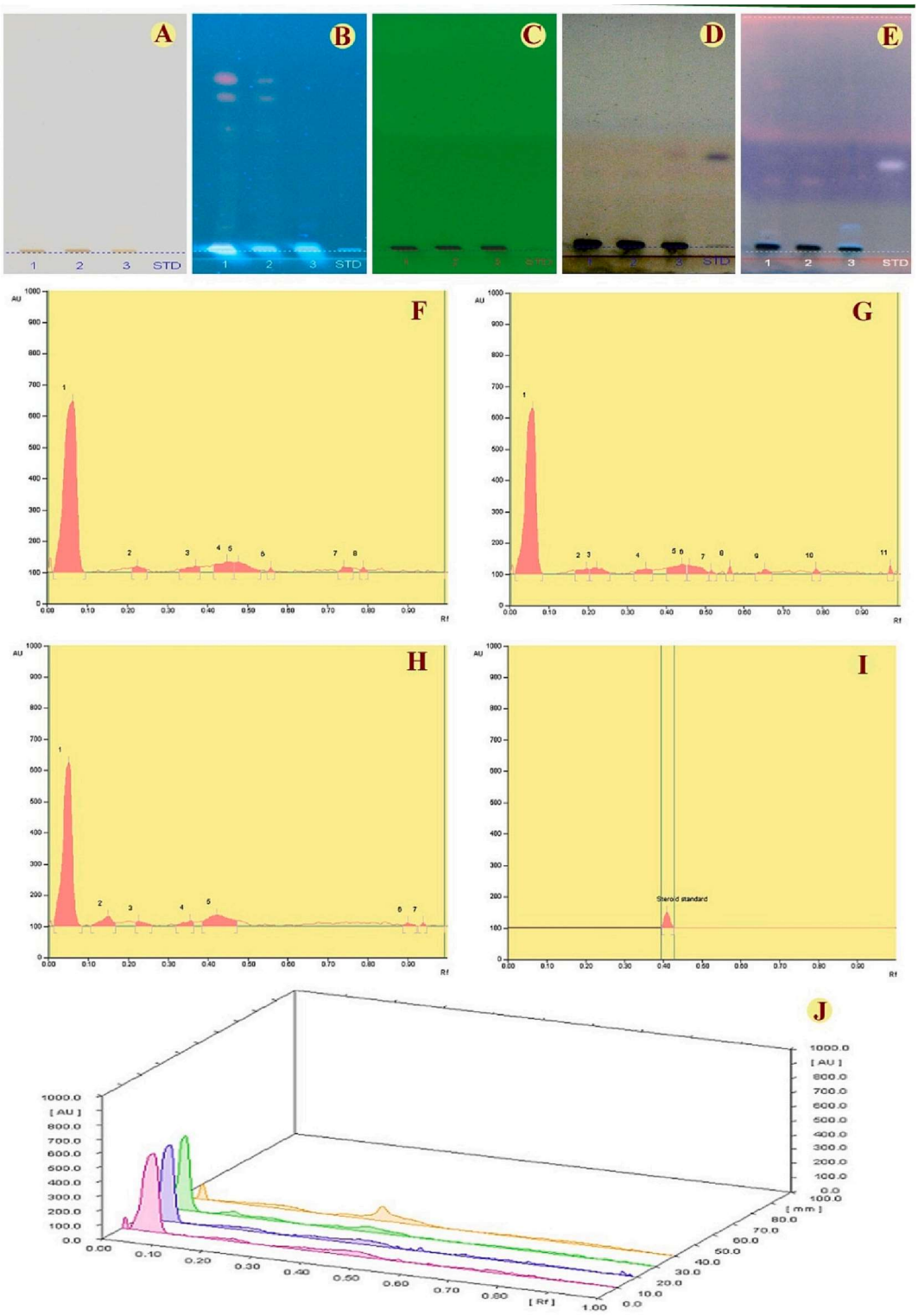


Fig. 4. HPTLC - Steroids profile of Cyathea species.

Table 2
HPTLC - Steroids profile of studied *Cyathea* species.

$R_f$values	*C. nilgirensis*	*C. gigantea*	*C. crinita*	Assigned substance
0.06	+	+	+	Unknown
0.15	–	–	+	Steroid 1
0.20	–	+	–	Unknown
0.23	+	+	+	Unknown
0.35	–	+	+	Steroid 2
0.37	+	–	–	Unknown
0.41	+	+	+	Stigmasterol
0.48	+	+	–	Steroid 3
0.52	–	+	–	Unknown
0.56	+	+	–	Unknown
0.65	–	+	–	Unknown
0.74	+	–	–	Unknown
0.79	+	+	–	Unknown
0.90	–	–	+	Unknown
0.97	–	+	+	Unknown

a growth medium (DMEM, pH 7.4), supplemented with 10% FBS and antibiotics, penicillin (100 units/ml) and streptomycin sulfate (100 µg/mL).

MTT assay

MTT assay is employed to determine the cytotoxicity of *C. nilgirensis*, *C. gigantea* and *C. crinita* ethanolic extracts (12.5, 25, 50, 100 and 200 µg/mL) against MCF 7- human breast carcinoma (Selvakumaran et al., 2003, Janakiraman and Johnson 2016). After 3 days of incubation at 37 °C under 5% CO_2, the medium was removed. 20 µl of 5 mg/ml MTT (pH 4.7) was added per well and cultivated for another 4 h, the supernatant fluid was removed. 100 µl of DMSO was added per well and shaken for 15 min. The absorbance at 570 nm was measured with a UV spectrophotometer, using wells without cells as blanks. All the experiments were performed in triplicates. The absorbance of untreated cells was considered as 100%. The IC_{50} value was determined graphically using MS - EXCEL 2007. The conventional anticancer drug, adriamycin was used as a positive control. The inhibition of cell growth was calculated as a percent anticancer activity using the following formula:

(Janakiraman and Johnson, 2016).

$$\%\text{of Cell Inhibition} = 100 - \text{Sample Absorbance/Control Absorbance} \times 100$$

Results

HPTLC separation of terpenoids determined high resolution and reproducible peaks in the mobile phase n-hexane - ethyl acetate (7.2: 2.9). The results showed the presence of 23 bands and confirmed 16 types of terpenoids with R_f values ranged from 0.05 to 0.94 (Fig. 3; Table 1). In general, more degree of terpenoid diversity was observed in *C. gigantean* and *C. crinita* compared to *C. nilgirensis*. *C. gigantean* and *C. crinita* revealed 9 terpenoid bands whereas *C. nilgirensis* showed only 5 terpenoid bands. Among the different terpenoids, the band with R_f value 0.05 and 0.63 was common to all the three studied species. *C. nilgirensis* confirmed the presence of one distinct band with R_f value 0.94 whereas the bands 0.14, 0.46, 0.47, 0.73 and 0.83 were present only in *C. gigantea*. The bands with R_f values 0.18, 0.33, 0.43, 0.54 and 0.75 displayed their unique presence in *C. crinita*. The terpenoid band with R_f value 0.79 depicted the presence of standard lupeol in *C. gigantean* (0.04%) and *C. crinita* (0.02%).

Various solvent compositions of the developing system were examined for HPTLC analysis of steroids in order to achieve high resolution and reproducible peaks. The desired aim was achieved using the mobile phase toluene - acetone (9: 1). The results displayed 26 bands and authenticated 15 types of steroids with R_f values ranged from 0.06 to 0.97 (Fig. 4; Table 2). In general, more degree of steroids diversity was observed in ethanolic extract of *C. gigantea* (11) followed by *C. nilgirensis* (8) and *C. crinita* (7). *C. nilgirensis* showed its unique steroidal expression with the R_f values of 0.37 and 0.74. The steroid bands with R_f values 0.20, 0.52 and 0.65 were present only in *C. gigantea*. *C. crinita* revealed its distinct identity with the R_f values 0.15 and 0.90. The bands with R_f value 0.06, 0.23 and 0.41 were present in all the three studied species. In particular, the steroid band 0.41 confirmed the presence of standard stigmasterol with varied frequency viz., *C. nilgirensis* (0.33%), *C. gigantea* (0.29%) and *C. crinita* (0.52%).

Different compositions of the mobile phase were tested in order to obtain high resolution and reproducible peaks. The desired aim was achieved using ethyl acetate - ethanol - water (8:2: 1.2) as the developing system. The results showed the presence of 23 bands and authenticated 12 different types of glycosides with R_f values ranged from 0.05 to 0.96 (Fig. 5; Table 3). Maximum number (9) of glycosides has been observed in *C. crinita* followed by *C. nilgirensis* and *C. gigantea* (7). Among the nine different glycosides of *C. crinita*, two glycosides with R_f values 0.45 and 0.58 expressed their unique presence. The glycosidic band with R_f value 0.71 was present only in *C. nilgirensis* whereas *C. gigantea* showed the variation by the presence of glycosidic bands with R_f values 0.24 and 0.26. The glycosides with R_f value 0.05, 0.86 and 0.96 showed their common presence in all the three studied *Cyathea* species. The band with R_f value 0.62 indicates the presence of the standard glycoside swertiamarin. The glycoside swertiamarin failed to show its presence in the three studied *Cyathea* species.Fig. 5a..

In the drug discovery process, evaluation of ADME and toxicity properties of the drug molecules is an essential factor. SwissADME is employed to evaluate the *in silico* pharmacokinetic parameters viz., drug-likeness, physicochemical properties, lipophilicity, water solubility, and its potency of lupeol, stigmasterol and swertiamarin. *In silico* pharmacokinetics properties viz., GI absorption, blood–brain barrier (BBB) penetration, P-gp substrate, and cytochrome P450 of lupeol, stigmasterol and swertiamarin are exhibited in Table 4. Cytochrome P450 is one among the PK factors, which plays a vital role in drug metabolism and comprises a heme-containing protein family mediates several xenobiotic substances, drug molecules, and carcinogenic factors. CYP 1, 2, and 3 families are mainly governed the biotransformation of drugs and chemical substances. The physicochemical properties of lipophilicity, water-solubility, PK properties, and drug-likeness of lupeol, stigmasterol and swertiamarin were examined and reported in Table 4 & 5. The compounds possess drug-likeness and lead likeness potentiality as they obey Lipinski rule.

GI absorption, BBB penetration, and P-gp were found to be normal for all the phytoligands, but batimastat has less GI absorption and lack of

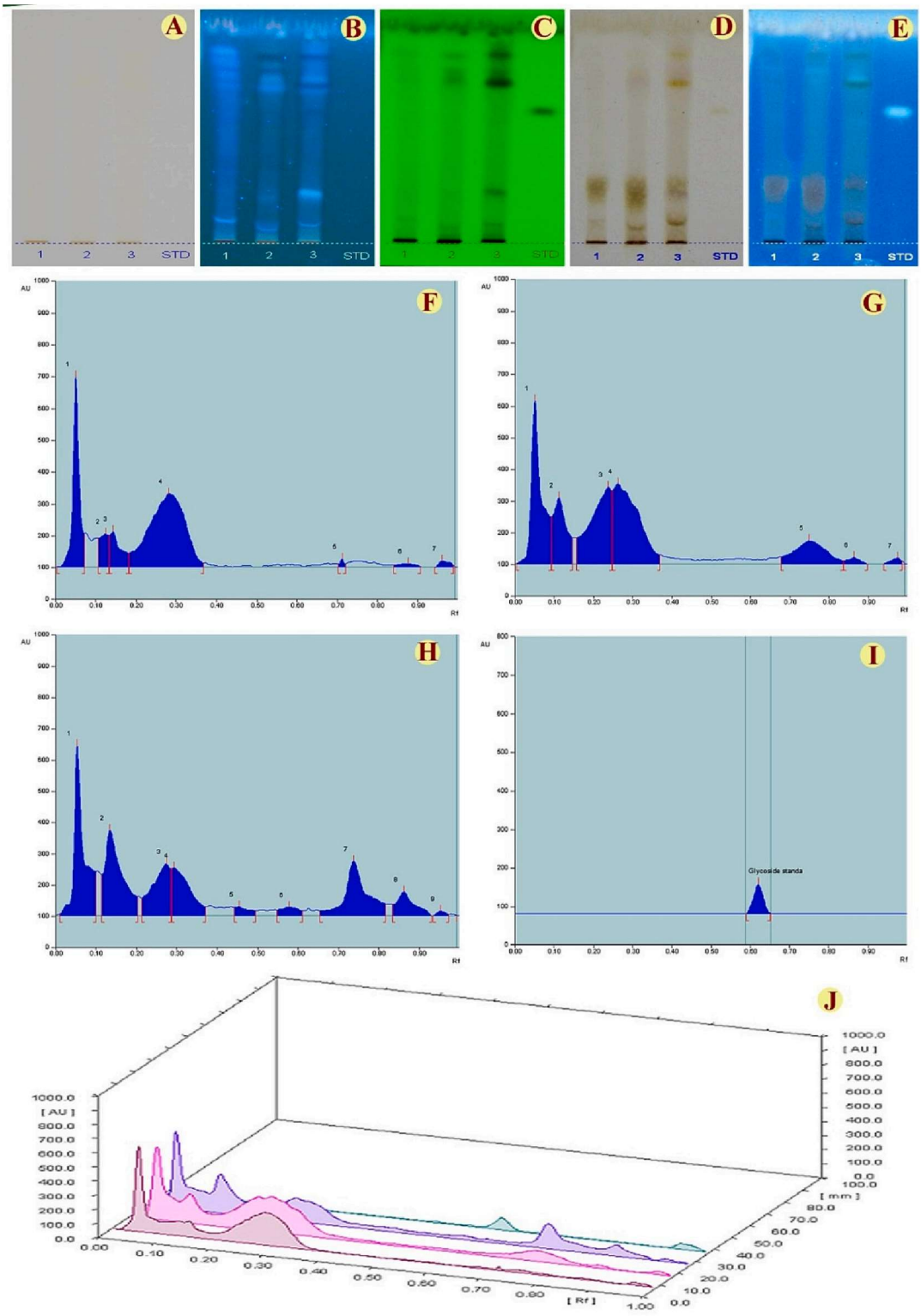


Fig. 5. HPTLC - Glycosides profile of Cyathea species.

Table 3
HPTLC - Glycosides profile of studied *Cyathea* species.

$_f$values	*C. nilgirensis*	*C. gigantea*	*C. crinita*	Assigned substance
0.05	+	+	+	Unknown
0.11	+	+	–	Glycoside 1
0.13	+	–	+	Glycoside 2
0.24	–	+	–	Unknown
0.26	–	+	+	Glycoside 3
0.28	+	–	+	Glycoside 4
0.45	–	–	+	Unknown
0.58	–	–	+	Unknown
0.62	–	–	–	Swertiamarin
0.71	+	–	–	Unknown
0.74	–	+	+	Glycoside 5
0.86	+	+	+	Glycoside 6
0.96	+	+	+	Unknown

BBB-permeability (Table 4). Lupeol, stigmasterol and swertiamarin do not have a carcinogenic effect and Ames mutagenicity. The outcome of SwissADME and admetSAR results suggest that compound lupeol, stigmasterol and swertiamarin possesses fine ADME properties and does not show any toxicity effects.

Table 6 explains the lupeol, stigmasterol and swertiamarin interaction against the studied proteins viz., 1VSN, 5BNQ, 6HN8, 7DN4, 3TJU with energy values and the details of interacting residues. Among the studied three compounds, stigmasterol binds more effectively to the 1VSN with the calculated binding free energy (ΔG) of − 10.217 kcal/mol, lupeol with calculated binding free energy (ΔG) of − 9. 917 kcal/mol and swertiamarin has lower calculated binding free energy (ΔG) of − 7.257 kcal/mol. With reference to 5BNQ, stigmasterol and swertiamarin showed the binding, lupeol failed to show the binding. Stigmasterol binds more effectively to the 5BNQ by the calculated binding free energy(ΔG) of −10.451 kcal/mol and swertiamarin with −8.312 kcal/mol. Stigmasterol and lupeol binds more effectively to the 6HN8 by the calculated binding free energy(ΔG) of −11.894 kcal/mol and −11.254 kcal/mol respectively and swertiamarin with −8.735 kcal/mol. Stigmasterol, lupeol and swertiamarin binds more effectively to the 7DN4 by the calculated binding free energy(ΔG) of −13.238 kcal/mol, −12.177 kcal/mol and −8.724 kcal/mol respectively. Lupeol binds more effectively to the 3TJU by the calculated binding free energy (ΔG) of −9.814 kcal/mol, stigmasterol with calculated binding free energy (ΔG) of −9.645 kcal/mol and swertiamarin has lower ligand calculated binding free energy (ΔG) of −7.289 kcal/mol. The protein–ligand interaction of the stable docked 1VSN, 6HN8, 7DN4, 3TJU and lupeol complex was visualized with ligand interaction diagram shown in Fig. 5 A - H (Table 6).

The protein–ligand interaction of the stable docked 1VSN, 5BNQ, 6HN8, 7DN4, 3TJU and stigmasterol complex was visualized with ligand interaction diagram shown in Fig. 6A–J (Table 6). The protein–ligand interaction of the stable docked 1VSN, 5BNQ, 6HN8, 7DN4, 3TJU and swertiamarin complex was visualized with ligand interaction displayed in Fig. 7A–J (Table 6). The docking studies confirmed the anticancer, cytoxicity and antidiabetic properties of lupeol, stigmasterol and swertiamarin.

The observed results of Brine Shrimps Lethality Bio Assay (BSLB) suggest that ethanolic extracts of the *C. nilgirensis*, *C. gigantea* and *C. crinita* are more effective against the brine shrimps naupli. The LC_{50} values of studied tree ferns ethanolic extracts were as follows *C. gigantea* 272.57 mg/mL > *C. crinita* 275.95 mg/mL > *C. nilgirensis* 302.34 mg/mL.

Fig. 8 explained the cytotoxicity of the studied ethanolic extracts of *C. nilgirensis*, *C. crinita* and *C. gigantea* against the MCF 7 Cell lines. The observed IC_{50} value of *C. crinita* was 375.94 μg/mL, *C. nilgirensis* 649.29 μg/mL and *C. gigantea* 714.29 μg/mL.

Discussion

In natural product drug discovery, the conventional approach of extraction, isolation, separation, identification, characterization and test for the desired biological activity suffers from problems like lower yields, de-replication, difficulty in separation and inconsistent biological activity (Chuprov-Netochin et al., 2016; De Diego et al., 2017). In the recent years, there has been an increasing interest in the application of chemical evidence to taxonomic problems (Chaabani et al., 2019; Grigore et al., 2019; Bhargava et al., 2021). Biochemical markers have their own significance and importance in chemical fingerprinting. Allozymes are the best biochemical markers in plants due to various strengths. However, modern and sensitive technologies for identifying markers based on biochemical / gene expression such as HPTLC have replaced allozymes. Chromatographic technique such as HPTLC can determine the homogeneity of a plant extract. They are powerful tools often used for standardization and to control the quality of both the raw material and the finished product (Dhanalakshmi et al., 2016; Senguttuvan and Subramaniam, 2016; Vinatha et al., 2017; Geethika and Sunojkumar, 2017). A plant during its life span produces various phytoactive compounds as secondary metabolites for its own growth and survival (Anandjiwala et al., 2007; Juszczak et al., 2019). In the present study, HPTLC separation of terpenoids, steroids and glycosides in *Cyathea* species showed high resolution and reproducible peaks. HPTLC pre-coated plates with various mobile phases developed chromatograms showed distinct phytochemical variations in ethanolic extracts of *Cyathea* species based on R_f values. HPTLC validated method for quantification of lupeol and stigmasterol showed significant level of presence in the studied *Cyathea* species. The results provide a chemical fingerprint to the nature of chemicals present in the plant extract. Information on these chemical constituents not only aid in discovering new therapeutic drugs, but such information can also help in disclosing new sources of economic materials which are precursors for the synthesis of complex chemical substances (Farnsworth, 1996; Gomathi et al., 2012; Nile and Park, 2015). Therefore the marker based on secondary metabolites should be able to discriminate one species from another species and one accession from other accessions.

The compounds lupeol, stigmasterol and swertiamarin possess drug-likeness and lead likeness potentiality as they obey Lipinski rule. With reference to Daina et al., (2014) Log Po/w (iLogP) is the n-octanol/ water partition coefficient is a main component of the physicochemical parameter in drug discovery. The log P value of lupeol and swertiamarin falls < 5 and for stigmasterol 5.08, which is found to be an acceptable value for the drug molecules for

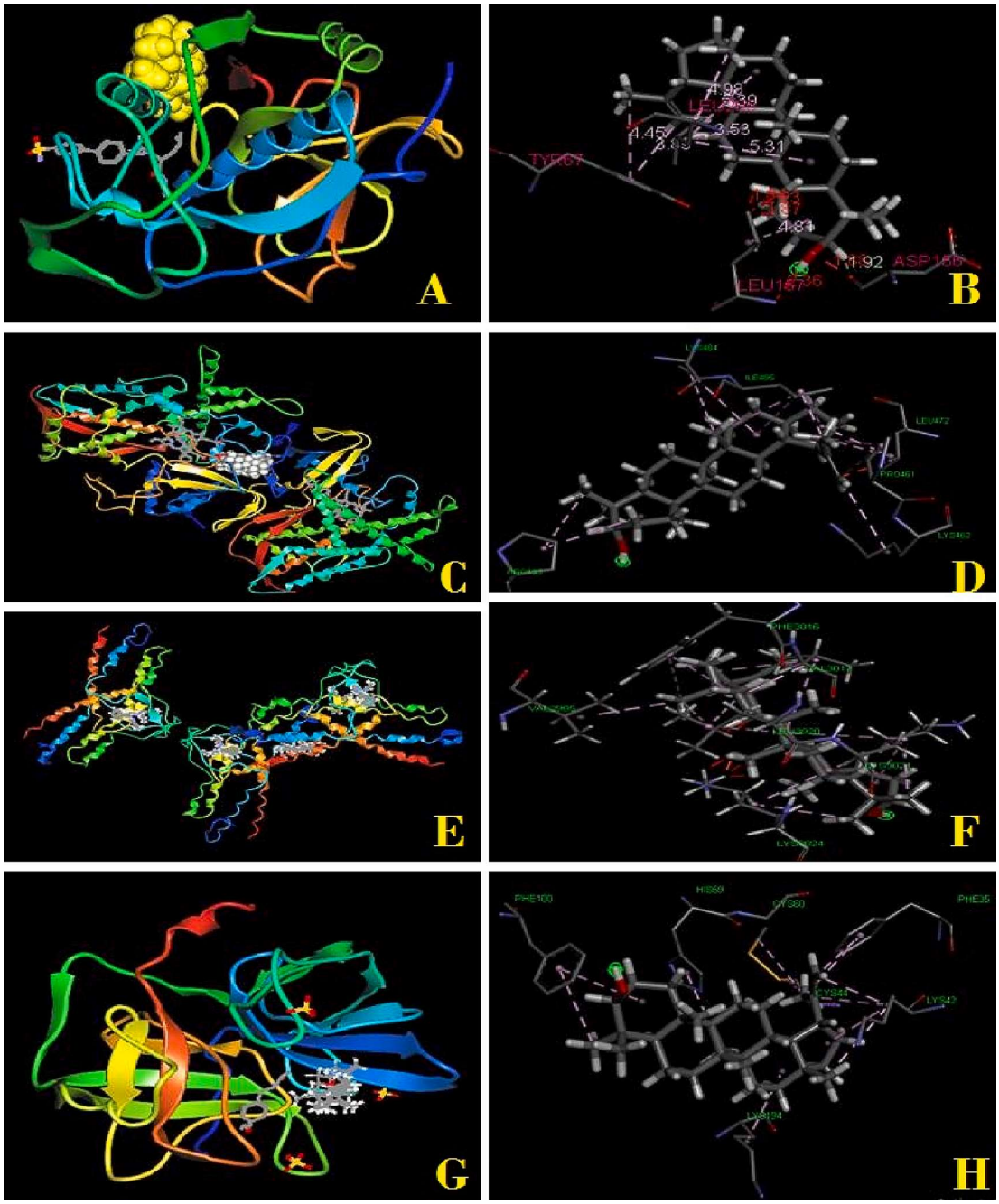


Fig. 5a. Glide docking pose of lupeol molecule in the active binding site of studied proteins. A & B – 1VSN; C & D – 6HN8; E & F – 7DN4; G & H – 3TJU. (The Argus 4.0.1 is employed to visualize the Glide Docking Pose Fig. 5 A, C, E, G (Das et al., 2020) and Discovery Studio 4.0 Visualizer is used to visualize active binding site of studied proteins Fig. 5 B, D, F, H. URL, 2023. http://accelrys.com/products/discovery-studio and Karthik (2015). https://doi.org/10.1155/2015/762716).

penetration into the membranes (Table 6). Swertiamarin is found to be highly water-soluble, stigmasterol is moderately soluble and lupeol is poorly soluble in water. Among the cytochrome P450, CYP3A4 metabolizes the drug to the target site and the drug executes its mechanism of action (Roy and Roy, 2009). Lupeol, stigmasterol and swertiamarin do not have a carcinogenic effect and Ames mutagenicity (Ujan et al., 2019).

Prasan and Jongkon (2016) conducted a docking study with

Table 4
SwissADME – Pharmcokinetics Properties and Physiological Properties of Lupeol, Stigmasterol and Swertiamarin.

Compounds	Pharmcokinetics Properties							Physiological Properties				
	GI-Absorption	BBB Permeability	P-gp	CYP1A2 Inhibitor	CYP2C19 Inhibitor	CYP2C9 Inhibitor	CYP2D6 Inhibitor	CYP3A4 Inhibitor	Mol. Wt. (g/mol)	No. of rotatable bonds	No. of H Bond Acceptors	No. of H Bond Donors (Å)	TPSA Å2
Lupeol	Low	No	No	No	No	No	No	No	426.72	1	1	1	20.23
Stigmasterol	Low	No	No	No	No	Yes	No	No	412.69	5	1	1	20.23
Swertiamarin	Low	No	No	No	No	No	No	No	373.34	4	10	5	155.14

ArgusLab 4.0.1, free molecular docking software, on tyrosinase inhibitors comparing with AutoDock 4 and AutoDock Vina. The best linear correlation coefficient of 0.8865 was observed in ArgusLab while AutoDock 4 and AutoDock Vina showed 0.6849 and 0.7805 respectively. Kaneria et al. (2019) employed the ArgusLab for the computational evaluation of compounds from *Couroupita guianensis*. Das et al. (2020) identified antisickling agent from *Carica papaya* using molecular docking approach by employing ArgusLab 4.0.1. In the present study also ArgusLab 4.0.1 was employed and identified the cytotoxicity, anti-inflammatory, anti-diabetic properties of lupeol, stigmasterol and swertiamarin. Lolok et al. (2022) identified the antidiabetic properties of β-sitosterol and stigmasterol using AutoDock tools. Masfria et al. (2022) performed the computational study by Autodock Tools against KEAP1 targeted macromolecule and identified antioxidant properties of Sitosterol, Stigmasterol, Campesterol and 28-Isofucosterol. Osafoa et al. (2023) determined the antileishmanial activity of lupeol using molecular docking studies. Molecular docking studies of the lupeol and monostearin showed that lupeol and monostearin established important interactions with key amino acid residues against trypanothione reductase (TR) and pteridine reductase 1 (PTR1). Koirala et al. (2017) studied the inhibitory potential of lupeol and lupenone and observed notable or moderate BACE1 inhibitory activity. The AutoDock 4.2 was employed to predict the binding free energies of enzyme-inhibitor complexes. The computer aided docking studies revealed that hydroxyl group of lupeol formed two hydrogen bonds with the ASP32 (catalytic aspartic residue) and SER35 residues of BACE1 with the binding energy of (−8.2 kcal/mol), while the ketone group of lupenone did not form any hydrogen bonds with BACE1 giving evidence for less binding affinity. The molecular docking confirmed the probable remedies for Alzheimer's disease. Swertiamarin is a multipotent compound with varied pharmacological activities viz., hepatoprotective, analgesic, anti-inflammatory, antiarthritis, antidiabetic, antioxidant, neuroprotective and gastroprotective activities (Muhamad Fadzil et al., 2021). The results of the present study confirmed the anti-inflammatory, antidiabetic and cytotoxic properties of lupeol and Stigmasterol. The results of the present docking studies confirmed the anti-inflammatory, antidiabetic and cytotoxic properties of swertiamarin. The results of the present study supplemented to the observations of Koirala et al. (2017), Lolok et al. (2022), Masfria et al. (2022) and Osafoa et al. (2023). Arthur and Uzairu (2019) confirmed the use of molecular docking in the discovery of innovative tiny drug-like scaffolds with the best binding selectivity and affinity for the target. The results of the present study directly coincided with their observations.

Table 6 explained the binding energy values and residual interaction between the compounds and studied protein. These binding energy values indicate the leading confirmational position of these molecules. The negative value of (ΔG) signifies robust favorable bonding between the compounds and the studied proteins. The observed results confirmed various interactions between lupeol, stigmasterol, swertiamarin and the studied proteins with varied binding efficiency.

The HPTLC studies confirmed the presence of lupeol, stigmasterol and swertiamarin in the ethanolic extracts of *Cyathea nilgirensis, Cyathea crinita* and *Cyathea gigantea.* The outcome of SwissADME and admetSAR results suggest that compound lupeol, stigmasterol and swertiamarin possesses fine ADME properties and does not show any toxicity effects. Janakiraman and Johnson (2016a) observed the cytotoxicity and anticancer potential of *Cyathea nilgirensis, Cyathea crinita* and *Cyathea gigantea* ethanolic extracts using Brine Shrimp Lethality Bio-assay and MCF 7 cell line. In the present study also similar trends was observed. The observed results suggest that *C. nilgirensis*, *C. gigantea* and *C. crinita* ethanolic extracts were found to be more effective against brine shrimps with LC_{50} values of 302.34 mg/mL, 272.57 mg/mL, and 275.95 mg/mL, respectively. The observed IC_{50} value of *C. crinita* was 375.94 μg/

Table 5
SwissADME – Drug Likeness Properties and Solubility Properties of Lupeol, Stigmasterol and Swertiamarin.

Compounds	Drug Likeness Properties				Solubility Properties			
	Lipinski Rule	Violation	Bioavilability score	Leadlikeness	LogPo/w (iLOGP)	Consenus Log Po/w	Log S (ESOL)	Class – Water Solubility
Lupeol	Yes	1	0.55	No	4.72	7.27	−8.64	Poorly soluble
Stigmasterol	Yes	1	0.55	No	5.08	6.98	−7.46	Moderately Soluble
Swertiamarin	Yes	1	0.11	No	1.66	−1.32	−0.64	Soluble

Table 6
Lupeol, Stigmasterol and Swertiamarin interaction with studied proteins.

Protein ID	Name of the Compound	Compound ID	Energy Value (Kcal/mol)	Amino Acid Residues Interactions
1VSN	Lupeol	CID_259846	−9.917	TYR67, LEU205, LEU157, ASP156
	Stigmasterol	CID_5280794	−10.217	LEU157, LEU205, TYR67
	Swertiamarin	CID_442435	−7.257	TYR67, THR69, ASN70, GLN73, TYR110
5BNQ	Stigmasterol	CID_5280794	−10.451	PHE270, PHE272
	Swertiamarin	CID_442435	−8.312	LEU88, HIS253, SER251, TYR77, LEU78, ASP79, THR80
6HN8	Lupeol	CID_259846	−11.254	PRO193,LYS462, PRO461,LEU472, ILE485,LYS484
	Stigmasterol	CID_5280794	−11.894	ARG849, LEU539, VAL540, ALA850, ILE852,CYS851, PHE712, PHE844, THR719, PHE560, ILE606, PHE856, ALA857
	Swertiamarin	CID_442435	−8.735	LYS69, ARG398, VAL87, LEU86, CYS400, PHE393, ALA328, THR260, ALA399, ILE401, ALA406, PHE405
7DN4	Lupeol	CID_259846	−12.177	VAL2985, PHE3016, VAL3017, LEU3020, LYS3021, LYS3024
	Stigmasterol	CID_5280794	−13.238	PRO3358, PHE3359, VAL2798, LEU2799, TRP3296
	Swertiamarin	CID_442435	−8.724	PRO3301,THR3322, GLU3325,GLU3300, ARG3326
3TJU	Lupeol	CID_259846	−9.814	PHE100, HIS59, CYS60, PHE35, CYS44, LYS42, LYS194
	Stigmasterol	CID_5280794	−9.645	PHE35, LYS42, ARG43, LYS194, LYS216
	Swertiamarin	CID_442435	−7.289	TYR144, LYS194, ARG43, PHE35, LYS42

mL, *C. nilgirensis* 649.29 μg/mL and *C. gigantea* 714.29 μg/mL. The observed results and Janakiraman and Johnson (2016) observations confirm that ethanolic extracts are bioactive with less toxicity. The observed results and Janakiraman and Johnson (2016) observations on cytotoxicity and anticancer potential of *Cyathea nilgirensis, Cyathea crinita* and *Cyathea gigantea* ethanolic extracts using Brine Shrimp Lethality Bio-assay and MCF 7 cell line validated and supplemented the present ADMET observation. Stigmasterol and its derivatives are one of the anticancer compounds (Street et al., 2013; Al-snafi, 2016) which show anti proliferative activity against various cancer cell lines and tumors (Ali et al., 2015). In the present study also stigmasterol showed the good binding energy of −10.217 against IVSN and −13.228 against 7DN4. The observed results of the present *in silico* analysis validated and supplemented the Janakiraman and Johnson (2016a) observation on the cytotoxicity and anticancer potential of *C. nilgirensis*, *C. crinita* and *C. gigantea* ethanolic extracts. The cytotoxic and anticancer potential of *C. nilgirensis*, *C. crinita* and *C. gigantea* ethanolic extracts may be due to the existence of stigmasterol.

Lupeol is one of the plant derived secondary metabolite (triterpenoid) and found to have effective herbs immense biological activity against several diseases including its cytotoxic effect on cancer cells. *In silico* molecular docking analyses of lupeol with target protein viz., BCL-2, Topoisomerase, PTK, mTOR and PI3K showed good dock score with best binding energy, ligand efficiency and minimum inhibition constant. The *in-silico* molecular docking analysis showed that the lupeol may be considered as good inhibitor of proliferating cancer cells (Mahalakshmi et al., 2022). In the present study also lupeol showed the good binding energy −9.917 against IVSN and −12.177 against 7DN4. The outcome of *in silico* analysis and Janakiraman and Johnson (2016) reported cytotoxicity and anticancer potential of *C. nilgirensis*, *C. crinita* and *C. gigantea* ethanolic extracts may be due to the presence of lupeol. The stigmasterol and lupeol presence in the ethanolic extracts of *C. nilgirensis*, *C. crinita* and *C. gigantea* may be responsible for cytotoxic effect and anticancer properties.

Conclusion

The developed HPTLC profile for *Cyathea* species provides a simple, accurate and precise analytical method for the identification and quantification of lupeol and stigmasterol. This provides chromatographic fingerprint of phytochemicals and is helpful for confirming the identity and purity of *Cyathea* species. Further characterization of active principles in the studied *Cyathea* species can be used in generating species specific fingerprint. The results of the virtual screening and molecular docking analysis suggest that the phytochemical compounds lupeol, stigmasterol and swertiamarin of *Cyathea* species were identified as possible lead molecules to fight against cancer and diabetics. The present *in silico* study proved stigmasterol and swertiamarin as potent, selective and nontoxic compounds with anticancer properties.

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.

Data availability

Data will be made available on request.

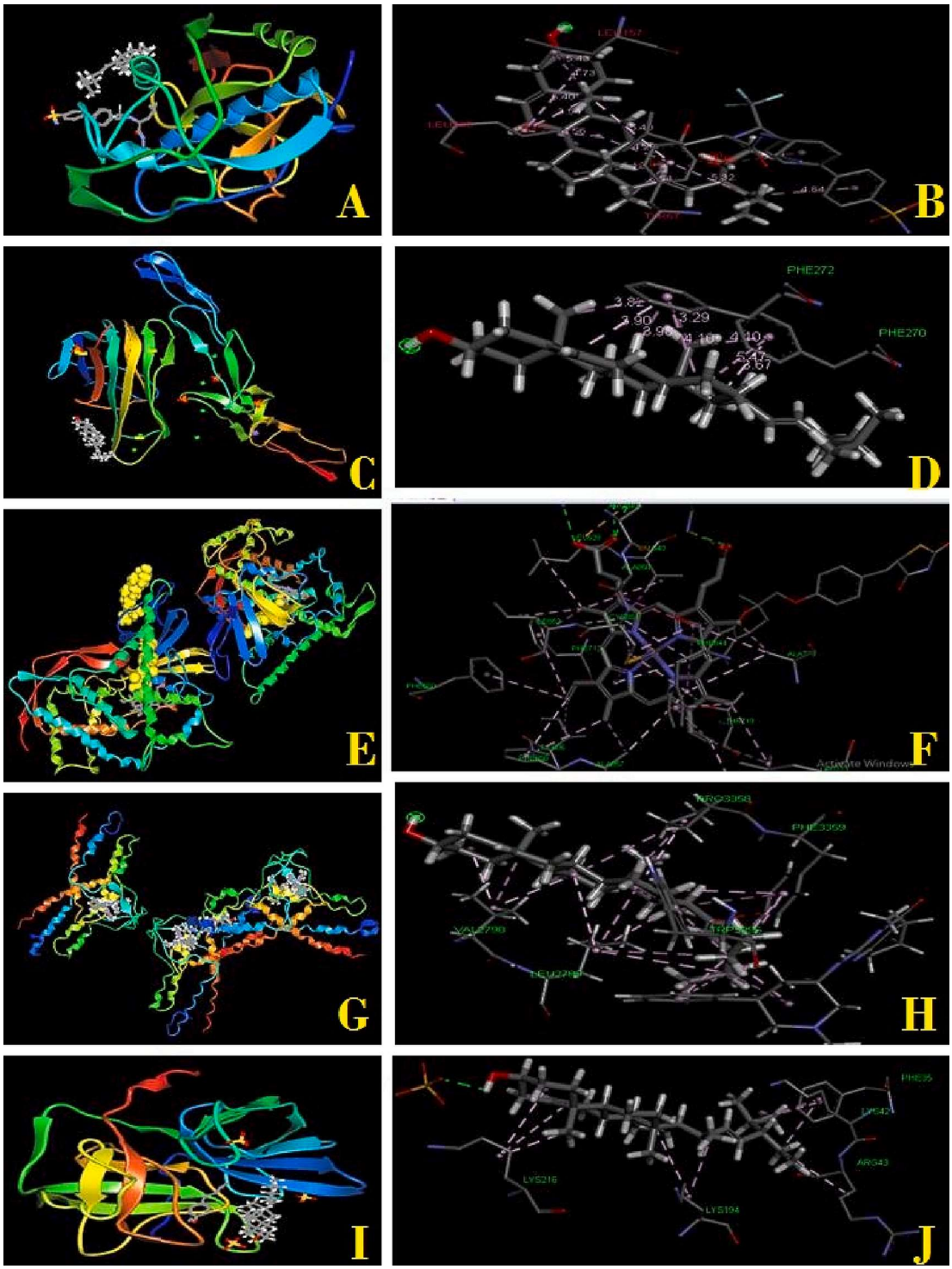


Fig. 6. Glide docking pose of stigmasterol molecule in the active binding site of studied proteins. A & B – 1VSN; C & D – 5BNQ; E & F – 6HN8; G & H – 7DN4; I & J – 3TJU.

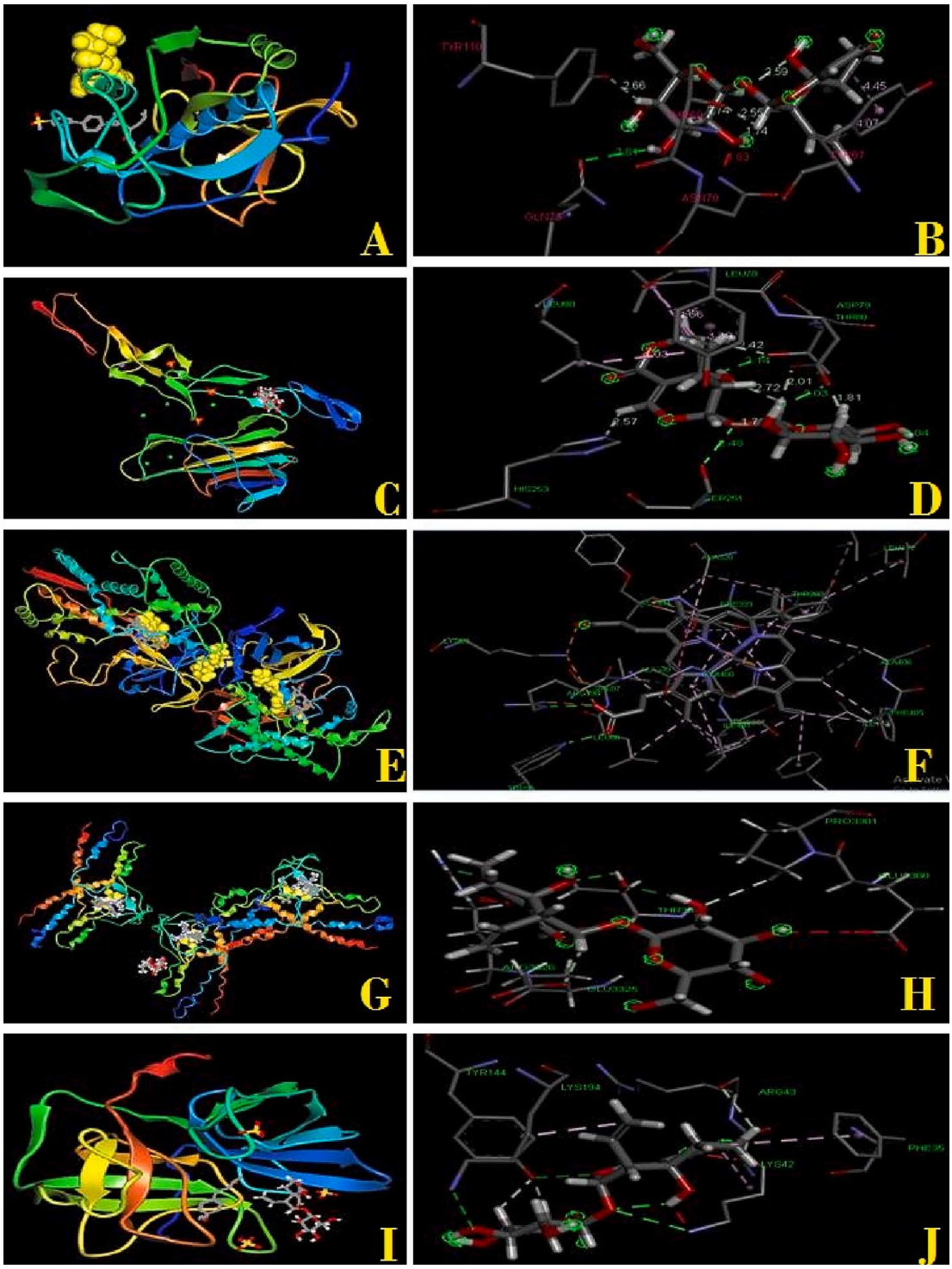


Fig. 7. Glide docking pose of swertiamarin molecule in the active binding site of studied proteins. A & B – 1VSN; C & D – 5BNQ; E & F – 6HN8; G & H – 7DN4; I & J – 3TJU.

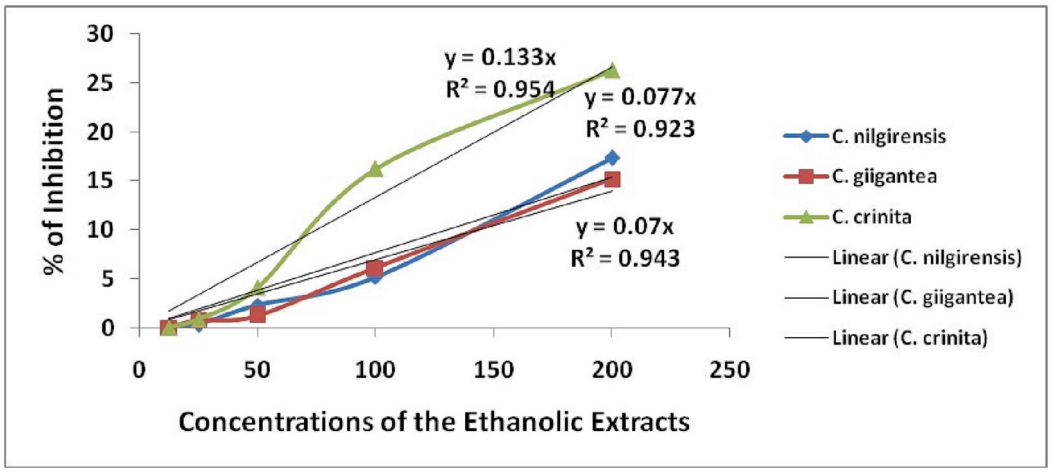


Fig. 8. Cytotoxicity of C. nilgirensis, C. crinita and C. gigantea ethanolic extracts.

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