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Molecular Studies on Evaluation of Phytol as Cytoskeleton Targeting Element in Cancer

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Abstract:

The aims of the present in-vitro and in-silico studies were to isolate Phytol compound from that active crude extract of *Nymphaea pubescens* [Family Nymphaeaceae] leaf as well as to evaluate the anticancer effects of Phytol of *Nymphaea pubescen,* antioxidants activities, anti-inflammatory and lethality. The Hexane: Petroleum ether, Ethyl acetate and Methanol extracts of *Nymphaea pubescens* were used for DPPH, Super oxide scavenging Activity, Brine shrimp lethality method and 5-lipoxygenase inhibition. The structure of the isolated compounds were established by extensive spectroscopic studies (IR, 1HNMR, 13CNMR, and MASS) and identification of (3*E, 7R, 11R)-3, 7, 11, 15-tetramethylhexadec-2-en-1-ol* with a molecular mass of 296.54 Da. All the extracts showed potent inhibition of DPPH. Among them Methanol extract shows best biological activities and is a potential source of natural antioxidants. The novel phytol has been isolated from the water lily (*Nymphaea pubescens)* for the first time from *Nymphaea pubescens* plant. This compound might be having abundant natural antioxidant and that justified its uses in folkloric medicines. We observed that the phytol decreased the viability of cells in a dose dependent manner. The IC_{50} of phytol in MDA MB 231 breast cancer cell line was found to be 160µg. Molecular docking study revealed that the Actin of *Homo sapiens* having active site amino acids Serine-14, Methonine-16, Histidine-73, Arginine-183, Arginine-210, Lysin-336, and Serine-338 played a vital role in interacting with the phytol compound. The present study suggests that phytol of *N. pubescens* reduces the proliferation and self-renewal ability of actin-positive BCSCs and can be used as a drug for potential targeting of BCSCs.

Keywords:
Phytol of *Nymphaea pubescens*, solvent extracts, cytotoxic, Anti-inflammatory, MD simulation and Docking

Introduction:

Non-communicable diseases, like cancer, heart disease and cerebrovascular disease, remain the leading causes together accounting for 58% of death. Cancer is the leading cause of death all over the world, it accounted for 7.9 million cases of deaths (around 13% of all deaths) every year and projected to continue rising, with an estimated 17 million deaths by 2030 [1]. About 72% of all cancer deaths in 2007 occurred in low and middle-income countries. Cancer prevalence in India is estimated to be around 8, 00, 000 new cases and 5, 50,000 deaths occurring per year [2]. Functional analysis of the proteins discovered in fully sequenced genomes represent the next major challenge of life science research. Computational methods like comparative protein modeling play an increasingly important role in this activity or challenge. Our present work is an outcome of current research on actin protein which plays a role in regulation of cancer. Cancer is a multi-factorial disorder that has high population prevalence. Tumor genesis is a multistep process that can be activated by various environmental carcinogens (such as cigarette smoke, industrial emissions, gasoline vapors), inflammatory agents (such as tumor necrosis factor (TNF) and H_2O_2), and tumor promoters (such as phorbol esters and okadaic acid). These carcinogens are known to modulate the transcription factors (e.g. NF-kB, AP-1, STAT3), anti-apoptotic proteins (e.g., Akt, Bcl-2, Bcl-XL), proapoptotic proteins (e.g., caspases, PARP), protein kinases (e.g., IKK, EGFR, HER2, JNK, MAPK), cell cycle proteins (e.g., cyclins, cyclin-dependent kinases), cell adhesion molecules, COX-2, and growth factor signaling pathways. All cancer cells acquire similar sets of functional capacities such as: independence from mitogenic/proliferative regulatory

signals; loss of sensitivity to ‘‘anti-growth’’ signals, evasion of apoptosis, neo-angiogenic conversion, release from senescence, and acquisition of invasiveness, and metastasis. Cancer cells move within tissues during invasion and metastasis by their own, control of cancer cell migration is an important problem in tumor treatment. Cancer cell metastasis is a multi-stage process involving invasion into surrounding tissue, intravasation, transit in the blood or lymph, extravasation, and growth at a new site. Many of these steps require cell motility, which is driven by cycles of actin polymerization, cell adhesion and acto-myosin contraction. Cell migration has been well studied in non-neoplastic cells such as fibroblasts and epithelial cells, and the molecular mechanisms underlying cell migration are common to both non-neoplastic cells and cancer cells. Cell migration involves multiple processes that are regulated by various signaling molecules. The actin cytoskeleton and its regulatory proteins are crucial for cell migration in most of the cells. During cell migration, the actin cytoskeleton is dynamically remodeled and this reorganization produces the force necessary for cell migration, because inhibition of these processes decreases cell motility, elucidation of the molecular mechanisms of actin reorganization is important for cancer therapeutics. In 2010 Hanahan and Weinberg proposed four new hallmarks that are characterstics of a cancer cell [3]. 1). Deregulated metabolism: Most cancer cells use abnormal metabolic pathways to generate energy. 2). Evading the immune system: Cancer cells apperar to be invisible to the bodys immune system. 3). Unstable DNA: Cancer cells generally have severe chromosomal abnormalities which wrosen as the disease progresses. 4). Inflammation: cancer cells found to have a role in inducing local chronic inflammation.

Medicinal plants are the major source of drugs for the treatment of various health disorders especially in rural areas of India, Pakistan, China, Afghanistan, Iran and other countries of this region. The use of plant based medicines (local medicine) dates back to 4000-5000 B.C. Now-a-days huge number of allopathic medicines also contain plant based ingredients that are used for their preparation by different companies. There are about 400,000 species of higher plants in the world, as compared to animal species that are about 5-10 million. The plant materials contain thousands of chemicals which act against diseases and infections of humans and animals when properly used. Furthermore according to assessment of WHO about 80% of world population depend on medicinal plants for their health care needs, and more than 30% of the pharmaceutical preparations are also based on plants. Whereas some reports indicated that there are 90 popular medicinal plants and different Pharmaceutical companies are using extracts of these plants in various drugs. Scientists throughout the world are trying to explore the precious assets of medicinal plants to help the suffering humanity. However, the developed countries mostly import raw material from developing countries and after processing export it back as high priced prepared medicines to developing countries.

Research aimed for treatment of cancer had been the focus of many industries and academic groups since last two decades. Phytanic acid is a branched-chain fatty acid that accumulates in a variety of metabolic disorders. High levels of phytanic acid found in patients can exceed the millimolar range and lead to severe symptoms. Degradation of phytanic acid takes place by alpha-oxidation inside the peroxisome. A deficiency of its breakdown, leading to elevated levels, can result from either a general paroxysmal dysfunction or from a defect in one of the enzymes involved in alpha-oxidation. A chemical substance that comes from plants and is used to make vitamins E and K. Phytol is also found in soaps, beauty care products, and household products. *Nymphaea pubescens was* wild and widely distributed in tropical and temperate regions, inhabiting stagnant fresh water, ponds, lakes and swamps [4]. *Nymphaeaceae* is a perennial aquatic rhizomatous stoloniferous herb. It is commonly known as water lily, which includes about fifty species. The leaves, roots and flowers have a wide range of pharmacological activities and are used for diabetes, eruptive fevers and liver disorders in ayurvedic medicine [5]. In siddha system of medicine the whole plant was used in the treatment of eye disorders, diabetes and dyspepsia [6, 7, 8, 9; 10, 11; 12 & 13]. *Nymphaea nauchali* wild leaves extracts showed anti-hyperglycemic effect [14]. The leaves have been used in traditional Sudanese medicine as a remedy for dysentery, to treat tumors [15] and as an antibacterial [16]. With regard to chemical constituents, only six species have been previously investigated, and these studies describe the presence of two lignin’s from *N. odorata* [17], one hydrolysable tannin from *N. tetragona* [18]; several glycosyl flavonols from *N.* marliacea [19], *N. caerulea* [20], *N.lotus* [21], and *N. odorata* [17], and various acylated anthocyanins from *N. candida* [22], *N.* marliacea [19, 23] as well as two rare macrocyclic flavonoids from *N. lotus* [21]. In India, ten species of Nymphaea, both wild (*N. alba, N. candida, N. nouchali, N. pubescens, N. rubra and N.tetragona*) and cultivated (*N. caerulea, N.marliacea, N. micrantha and N. albavar. rubra*), are reported [24].

Actin is a cytoskeletal protein present within all eukaryotic cell types. The cell cytoskeleton is known to provide the basic infrastructure for maintaining cell morphology and functions such as adhesion, motility, exocytosis, endocytosis, and cell

division. The actin cytoskeleton and its regulatory proteins are crucial for cell migration and movement in most cells. The mechanism of cell movement involves actin remodeling which actually produces necessary force for cell migration. However, it has been observed that the alteration of actin polymerization or actin remodeling plays a pivotal role in regulating the morphology and phenotypic events of cancerous cells as a result of activation of oncogenic signaling pathways e.g., Ras and Src [25, 26, & 27]. Thus, elucidation of the molecular mechanisms of actin reorganization is important target for cancer therapeutics. There is however no scientific studies on antioxidant, anti-inflammatory and cytotoxic effect of *N. pubescens* leaves. Hence, the aim of this study is to investigate and evaluate their antioxidant, cytotoxic properties and to isolate pure compounds of active crude extract.

MATERIALS AND METHODS:

Preparation of leaf extracts:

Different analytical grade solvents viz., Hexane, Petroleum ether, Ethyl acetate and Methanol were used for extraction in increasing order of their polarity, following considerations of availability of reagents, cost and safety concerns [28]. 60 grams of dried leaf material was packed with Whattman no-1 filter paper and packed powder was placed into the extractor of a Soxhlet. The extraction was carried out by using solvents boiling point of respective solvents until the color change in leaf powder dark to pale. At the end of the extraction each extract was concentrated under reduced pressure in rotary-vacuum evaporator (Buchi, Switzerland) so dried extract material was packed under vacuum condition and stored until the further assay. At the time of antibacterial assay, 100 mg of the dried sample was dissolved first in 100µl of dimethyl sulphoxide (DMSO) and further diluted by adding 900µl of sterile distilled water to get the sample with a final concentration of 0.1 mg μl^{-1} [29].

Isolation and identification of bioactive compounds:

Extraction

Nymphaea pubescens leaf powder (2.459 kg) was extracted in a Soxhlet apparatus with hexane for 10 hrs for the removal of fatty matter. The defatted powder was extracted successively for 10 hrs. Each with ethyl acetate, acetone, MeOH, MeOH: water (80:20) at 60-70°C. The extracts were filtered and concentrated under vacuum (Buchi, Switzerland) separately to obtain viscous concentrate. This concentrate was subjected to freeze drying and the dried material was stored in -20°C until further use.

Column chromatography:

Admixture (crude sample + silica gel) was prepared by 45 gms of the material was dissolved in hexane and 900 gms of silica gel was added to it and make the material to get adsorbed in the surface of the silica gel.

Column was packed with silica gel (70-325mesh, 60g, and diameter 3cm×50h) using hexane as solvent, followed by adding admixture to the top of the column and column is continued with adding solvent in increasing order of polarity. The solvent is gradually increased from (0-100 hexane) and the various fractions collected is represented in the table

Identification & characterization of bioactive compounds:

Thin layer chromatography (TLC) analysis

The Extracted Nymphaea pubescens plant sample was eluted in thin layer chromatography for separation of compounds. The pre coated silica gel aluminum plates (Merck Germany) were cut in to 6cm×1cm size. A line lightly with a pencil was drawn about 1.5 cm from the bottom and 1.0 cm from the top. The capillary tubes (1mm) were washed with methanol for three times to avoid contamination on each time of spotting the samples were spotted using capillary tubes at the center of plate. Each sample was eluted on separate TLC plate. The chromatographic tank (10cm × 6cm) was filled the developing solvent system and equilibrated for about half an hours. The solvent system such as ethyl acetate: hexane (3:7) was used for hexane-extracted samples. Thin layer plate was placed gently in to the tank and allowed to stand till it reaches about 1cm from the top of the plate; the plate is removed and allowed to air dry. The compounds were detected using UV light (CAMAG TLC SCANNER).

Characterization of bioactive compounds:

The basic techniques for the structural elucidation of natural products have not changed much in the past few years. For identification, characterization and structural elucidation of the compounds

Substantial progress has also been made in the field of mass spectroscopy; particularly in terms of quadrate desk-top instruments which linked to gas chromatography systems serves as a powerful analytical tool.

The fast atom bombardment mass spectroscopy,GC-MS analysis and plasma desorption mass spectroscopy (PD-MS) are newer techniques that are developed in the field of mass spectroscopy. For flavonoids UV spectral data with shift reagents like NaOMe, $AlCl_3$/HCL, NaOAc and $AlCl_3/H_3Bo_3$ were used. UV spectrum: The UV

absorption spectrum of sterols are characteristic of the functional groups present and are in good agreement with those calculated from Woodward's and its main use is to detect conjugation. Simple acyclic dienes:217-222nm,Diene heteroannulat: 230-240nm, Diene homoannular: 256-260nm. These values changes with the number of substitutes.

***In vitro* Studies of Phytol on Cancer cell lines:**
Screening of Phytol of *Nymphea pubescenes* on Cancer Cell lines:
Maintenance of breast cancer cell line:

MDA-MB-231 human breast carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD). The cell line was maintained in tissue culture medium (Leibovitz-15 medium supplemented with 10% heat-inactivated fetal bovine serum,2mM L-glutamine, lx nonessential amino acids, 50 IU/mL penicillin, and 50 IU/mL streptomycin) at 37°C.

MTT Assay:

The cytotoxicity of phytol was determined by MTT test. MDA MB 231 cells present in their exponential growth phase were seeded at 1 × 10^4 cells per well in a 96-well plate (Corning). For a dose-response curve, DMSO stock solution of phytol was dissolved in culture medium to get final concentrations of 5– 320 µg. The cells were replenished with fresh media containing varying concentrations of drug on the next day and incubated for 48 hours at 37°C in 5% CO_2 saturated atmosphere. Wells containing only the cells and medium with no drug added, were taken as controls. After incubation the cells were fed with 200µl of fresh medium, 50µl of MTT (Sigma) solution (2 mg/ml PBS) was added and the plates were incubated for 3 hours. After incubation the media containing MTT solution was removed and the formazan crystals were dissolved by adding 200µl of DMSO to each well. The absorbance was read at 570nm for each well immediately using a multiwell spectrophotometer. The absorbance of control cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control. Each value is the mean of 8 wells with standard deviation.

In metabolically active cells the yellow, water soluble tetrazolium salt MTT (3, (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromid)is converted to purple, waterinsoluble formazan by dehydrogenase enzymes of the mitochondria. The resulting intracellular formazan is directly proportional to the number of metabolically active cells. This colorimetric reaction can be measured spectrophotometrically [30]. Measurements were made in triplicates. The relative viability of the treated cells as compared to the control cells was expressed as the % cytoviability, using the following formula:

% Cytoviability = [A540 of treated cells] x 100% / [A540 of control cells]

The IC50 (median inhibition concentration) was determined by nonlinear regression analysis of the corresponding dose response curve utilizing the analytical software package GraphPad Prism Version 4 (GraphPad Software, San Diego, CA). The results were presented as the mean ± standard error of the mean (S.E.M.) of four experiments and Student's *t*-test was used to assess the significance of the data.

Colony-formation Assay:

Human breast cancer cells (MDA MB 231) were grown in 6- well plates (10^4 cells per well). Each well contained 2mL of Leibovitz-15 medium supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 1% antibiotic-antimycotic solution in the presence or absence of 140 µg, 160 µg and 180 µg phytol. The cells were incubated at 37°C. After 48 hours, 5000 cells/plate were seeded in 35×10 mm petri plates and were allowed to grow for 7 days. The cells were then fixed in 4% formaldehyde and stained with methylene blue. The number of colonies was counted by two individuals separately who were blinded to the experiment. The experiment was done in triplicates.

Cytological observations:

Human breast cancer cells (MDA MB 231) were cultured both in the presence and absence of 140µg and 160µg phytol. Cells were fixed, stained with Mayer hematoxylin and eosin (H & E) and observed using inverted microscope. The experiment was done in triplicates.

Wound Healing Assay for cell migration:

MDA MB 231 cells were seeded in a 6 well plate. When 96-98% confluency was reached, a small scratch was made using melted pasteur pipette tip (bulb shaped). Floating and dead cells were washed out using media without FBS and respective media was added to wells. One well was kept untreated as control followed by addition of 140 µg phytol to second well. Migration of cells in the wound was assayed with a live-cell imaging setup. Images were taken after every 15 min for 72 hrs and the data was analyzed using ImagePro software. This assay was performed utilizing concentrations of drugs at which only 15-20% apoptosis or cell death was observed after 48 hrs treatment as determined by MTT assay.

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RESULTS AND DISCUSSION:

***Invitro* studies of Extraction, Isolation & Purification of Phytol:**

Various organic solvents were used for the extraction of bioactive compounds. The lea powder of *Nymphaea pubescens* was first extracted successfully with hexane, petroleum ether, ethyl acetate and methanol, by a Soxhlet apparatus. The extracts obtained were completely evaporated by using vacuum rotary evaporator. The concentrated crude extracts were used for biological studies. *Invitro* evaluation was carried out on different crude extracts by using polar to non-polar solvents (hexane: petroleum ether, ethyl acetate, and methanol) of *Nymphaea pubescens* for their bioactivity (Tab.1).

Table.1: Solvent crude extracts of *Nymphaea pubescens* dried leaf powder.

S. no	Solvent	Weight of extract
1	Hexane,petroleum Ether(50:50)-**NPH**	110gm
2	Ethyl acetate-**NPE**	60gm
3	Methanol- **NPM**	35gm

Methanol crude extract exhibited a significant antiradical effect, cytotoxicity and anti-inflammatory activities. So this NPM crude is used for further purification by column chromatography of different fractions are shown in Tab. 2.

Table. 2: The pure fractions were eluted at 100% $CHCl_3$/HEXANE

S. no	Fractions	Percentage of Solvents	Volume of Solvents
1	1-2	100% HEXANE	500ML
2	3-6	50% $CHCl_3$&HEX	1000ML
3	7-10	100% $CHCl_3$	l000ML
4	11 – 14	50%IPA-$CHCl_3$	l000ML
5	15 -18	100%IPA	l000ML
6	17-26	Hexane:Ethyl acetate	1000ML

Various organic solvents were used for the extraction of bioactive compounds. The leaf powder of *Nymphaea pubescens* was first extracted successfully with hexane: petroleum ether, ethyl acetate and methanol, by a Soxhlet apparatus. The extracts obtained were completely evaporated by using vacuum rotary evaporator. The concentrated crude extracts were used for biological studies.

In-vitro evaluation was carried out on different crude extracts by using polar to non-polar solvents (hexane: petroleum ether, ethyl acetate, and methanol) of *Nymphaea pubescens* for their bioactivity. Among these three extractives NPH Showed good antioxidant activity (IC50 µg/ml=13.49) than NPE, NPM. NPM showed very good anti-inflammation activity (IC50 µg/ml=2.63) NNH also showed good anti inflammation activity (IC50 µg/ml=10.23) than NPE followed by NPE (IC50 µg/ml=14.26). All these three crudes showed moderate cytotoxic activity. NPM showed better cytotoxic activity (IC50 µg/ml=39.63) than NPH and NPE. All these biological activates were given in **table-2.**

Methanol crude extract exhibited a significant antiradical effect, cytotoxicity and anti-inflammatory activities. So this NPM crude is used for further purification by column chromatography of different fractions are shown in **table3.**

In the present study, the spectral data of Phytol (3E,7R,11R)-3,7,11,15-tetramethylhexadec-2-en-1-ol were presented for the first time from this plant of *Nymphea pubescens*.

Gas chromatography-mass spectrometry (GC-MS):

The combine's features of gas-liquid chromatography and mass spectrometry are combined in Gas chromatography-mass spectrometry (GC-MS) to identify different substances within a test sample. The Crude sample of *Nymphaea pubescens* were shown in Fig.01 & Tab.4, 5.

Tab.3: Total antioxidant activity of three crude extracts and soluble fractions are expressed as equivalents (mg/l of extract) was used as positive control

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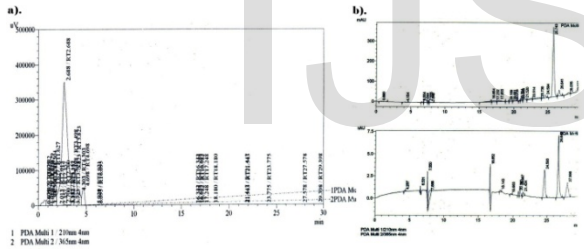
S. No	Plant extract	DPPH method IC50 µg/ml	Superoxide radical scavenging Activity IC50 µg/ml	5-lipoxygenase Inhibitoryactivity IC50 µg/ml	Brin shrimp lethality test ED50 µg/ml
1.	Control	Vitamin-c 3.59	Gallic acid 0.61	Indo Methacin 3.87	Podophyllotoxin 3.77
2.	Hexane : petroleum ether (50:50)-NPH	28.91	7.10	6.13	42023
3.	Ethyl acetate -NPE	13.32	15.54	7.26	47.6
4.	Methanol-NPM	-1.04	29.71	0.63	39.63

Fig.01: Analytical scale Gas chromatogram of phytol from *Nymphaea pubescens* of Crude *and (*25% H & 75% EA)



GC-MS have different application which includes drug detection, fire investigation, environmental studies, explosives detection, and determination of unknown samples. Airport security can also be used the GC/MS to identify substances in both luggage and human beings.

Tab. 04: Analytical scale Gas chromatogram of phytol from *Nymphaea pubescens of crude*

ID #	Name	Ret Time	Area	Height	Conc.
1	RT0.682	0.682	521717	22705	0.000
2	RT1.029	1.029	636063	33577	0.000
3	RT1.304	1.304	725756	45468	0.000
4	RT1.627	1.627	147275	72622	0.000
5	RT2.688	2.688	167652	37881	0.000
6	RT3.498	3.498	211880	12801	0.000
7	RT3.823	3.823	334791	14161	0.000
8	RT4.303	4.303	141064	84948	0.000
9	RT4.698	4.698	298425	10214	0.000
10	RT16.34	16.34	46194	5045	0.000
11	RT17.24	17.24	259819	4433	0.000
12	RT18.18	18.18	171495	2616	0.000
13	RT21.54	21.54	832533	10255	0.000
14	RT23.77	23.77	953159	29789	0.000
15	RT27.57	27.57	147953	18849	0.000
16	RT29.39	29.39	324119	6140	0.000
17	RT0.675	0.675	3536	309	0.000

GC-MS analyses of *N. pubescens* hexane & ethyl acetate fraction were performed to identify the volatile and semi volatile components. Analyses of the extract confirmed with the help of mass spectrometric characteristics and compared with the already reported compounds from the same species and other. Although the ion ratio of some compounds were the same but they were completely different compounds.

Tab.05: Analytical scale Gas chromatogram of phytol from *Nymphaea pubescens* 25% H & 75% EA

ID #	Name	Ret Time	Area	Height	Conc.
22	RT2.700	2.700	9183	4205	0.000
23	RT3.152	3.152	2686	2827	0.000
24	RT3.253	3.253	4994	3065	0.000
25	RT3.723	3.723	5076	2050	0.000
26	RT5.921	5.921	2662	382	0.000
27	RT6.095	6.095	6370	392	0.000
28	RT16.33	16.33	6939	1460	0.000
29	RT16.67	16.67	4906	457	0.000
30	RT16.78	16.78	4605	356	0.000
31	RT21.44	21.44	1547	80	0.000

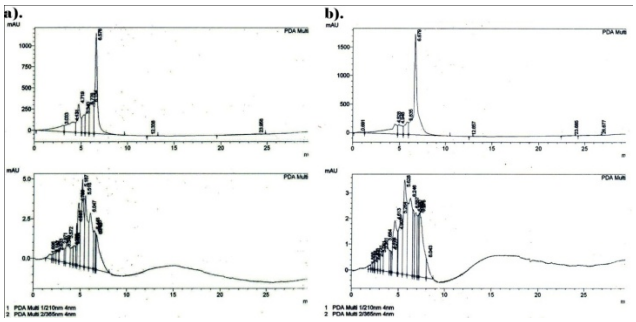


Fig.02: Analytical scale Gas chromatogram of phytol from *Nymphaea pubescens* 50% H & 50% EA & 100% EA

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GC/MS can also identify trace elements in materials that were far away of investigation previously and thought to have disintegrated beyond identification. The GC-MS is used to perform a specific test, it is therefore considered as a "gold standard" for forensic substance identification. It has also been used to identify a particular substance in a given sample. A non-specific test only shows that a substance falls into a category of substances. Although a non-specific test could statistically recommend the identity of the substance, this could lead to false positive identification.

Infrared Spectroscopy:

Infrared spectroscopy (IR spectroscopy) is also a part of spectroscopy that studies the infrared region of the electromagnetic spectrum. There are different techniques which are related with IR spectroscopy, the most common one is absorption spectroscopy. As with all other spectroscopic techniques, it can also be useful in identifying compounds or examination of sample composition. IR data shown in (Fig.03a),

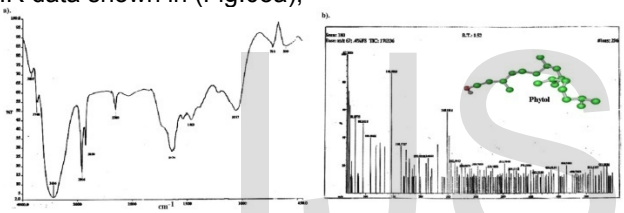

Fig. (03a): FT- IR spectrum & (03b) standard EI Mass spectrum of Phytol extracted from *Nymphaea pubescens*

The EI-mass spectrum of compound isolated from the petroleum ether fraction of methanol extract of *Nymphaea pubescens* revealed significant fragment ions at m/z 318[M]+ in figure-03b. This fragmentation ions indicated that this compound, which has the molecular formula $C_{20}H_{40}O$ $[M]^+$, must be a Phytol (3E, 7R, 11R)-3,7,11,15-tetramethylhexadec-2-en-1-ol.

Qualitative Phytochemical screening was performed as per the standard protocol to identify the different classes of compounds present in the crude extract because of their good antimicrobial activity. The results of phytochemical screening of ***Nymphaea pubescens*** were shown in tab.06

Tab. 06: Phytochemical analysis of Crude and Extacts-1, 2, 3 of *Nymphaea pubescens*

S. No	Name of the test	Crude	Extract-1	Extract-2	Extract-3
1	Steroid test (Conc. H_2SO_4	Presence	Presence	Absence	Absence
2	Alkaloid test (Dragon druff)	Presence	Presence	Absence	Absence
3	Flavonoid test ($AlCl_3$)	Presence	Presence	Presence	Presence
4	Tannin test ($FeCl_3$)	Presence	Absence	Absence	Presence
5	Terpinoid test	Presence	Presence	Presence	Presence
6	Carbohydrate test	Presence	Presence	Presence	Presence

Screening for the Antimicrobial Potential:

Preparation of plant sample:

10mg of plant crude sample was dissolved in the 1 ml of DMSO which is used for the screening. Test of micro organisms tested by the agar-diffusion assay against several bacteria shown in table 07. Plant extracts in organic solvent (hexane, petroleum ether, ethyl acetate and methanol) provided more consistent antimicrobial activity compared to those extracted in water. These observations can be rationalized in terms of the polarity of the compounds being extracted by each solvent and, in addition to their intrinsic bioactivity, by their ability to dissolve or diffuse in the different media used in the assay.

Tab. 07: Inhibition of micro organisms at different zones by extracts of *Nymphaea pubescens*

Micro organisms	Zone of inhibition(mm)		
	1St	2nd	3rd
Staphylococcus aureus (MTCC 3160)	3.14	7.07	5.31
Pseudomonas aeruginosa (ATCC9027)	8.04	3.80	7.07
Bacillus subtilis (ATCC 6633)	6.16	8.04	9.08
Escherichia coli (ATCC 35218)	5.31	7.07	5.31
Enterococcus faecalis (MTCC 439)	3.80	4.52	7.07
Xanthomonas campestris (MTCC 2286)	3.80	12.5	3.80
Streptococcus mutans (MTCC 497)	3.14	12.57	3.14
Lactobacillus casei(MTCC 1423)	3.80	5.31	3.80
Lactobacillus acidophilus (MTCC 495)	2.01	6.16	1.13

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Results for antibacterial activity illustrate that leaf extract of ***Nymphaea pubescens*** has shown highly significant antibacterial activity against all the bacterial strains tested so this extract can be fractionated in future to get active components responsible for antibacterial activity. The results of the present study support the folkloric usage of the studied ***Nymphaea pubescens*** plant and suggest that ***Nymphaea pubescens*** extracts possess compounds with antibacterial properties that can be used as antimicrobial agents in new drugs for the therapy of infectious diseases caused by pathogens.

Discussion of Extraction, Isolation and Purification of Phytol:

DPPH is the commonly used reagent to evaluate free radical scavenging activity of antioxidants [31]. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [32]. The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. Ascorbic acid was used as standard. The hexane: P.E extract of *N. pubescens* showed a significant dose dependent reduction in the DPPH radicals it may be due to the presence of active compounds like alkaloids, flavonoids, phenolic compounds and tannins [33]. Lipoxygenases (LOX's) are sensitive to antioxidants, and the most of their action may consist in inhibition of lipid hydroperoxide formation due to scavenging of lipidoxy or lipidperoxy-radicals formed in course of enzymic peroxidation. This can limit the availability of lipid hydroperoxide. 5-LOX inhibitors have been developed as drugs to treat inflammation in this study crude extracts exhibited significant inhibition of the lipoxygenase showing its strong potential to be developed as anti-inflammatory drug. The degree of lethality was found to be directly proportional to the concentration of the extract. Maximum mortalities took place at a concentration of 1000 µg/ml whereas least mortalities were at 10 µg/ml concentration. The LC50 values of the plant extracts were obtained by a plot of percentage of the shrimp nauplii killed against the concentrations of the extracts and the best-fit line was obtained from the data by means of regression analysis. This significant lethality of plant extracts to brine shrimp is an indicative of the presence of potent cytotoxic components which warrants further investigation.

Conclusion of Extraction, Isolation and Purification of Phytol:

Medicinal plants have been in use all over the world to treat various diseases including infections, cancer, inflammation, heart diseases etc. The use of natural products for treatment of all kind of diseases is due to their less harmful effects as compared to drugs synthesized in the laboratory. In all areas of the world, locally used herbal treatments are common and have been under investigation to get active principal of these remedies.

It can be concluded that medicinal value of this traditional plant has been supported by the results which show positive quenching activity against free radicals. Isolation of bioactive compounds in the extract would help to determine the potency and safety of *N. pubescens*. Further studies are needed for the bioactive compounds occurring in plant material consist of multi-component mixtures, their separation and determination still creates problems. A novel compound Phytol ($C_{20}H_{40}O$) has been isolated from the wild water lily *Nymphaea pubescens* for the first time from this plant. The structure of the isolated compounds was established by extensive spectroscopic studies (IR, GCMS, and EI MASS) and identified as PHYTOL (3E, 7R, 11R)-3, 7, 11, 15-tetramethylhexadec-2-en-1-ol with a molecular mass of 296.54 Da (Fig. 04). This is the first report of such a Phytol from this plant of *Nymphea pubescens*. The plant studied here can be seen as a potential source of useful drugs. The antimicrobial activities of these plants for the treatments of the diseases as claimed by traditional healers are also being investigated. The overall results show that the crude *Nymphaea pubescens* extracts evaluated in the present study can be fractionated in future and can lead to discovery of important chemotherapeutic agents. From the obtained results we could suggest that these compounds can be used for antioxidant and cytotoxic activity and suggest going for MD simulation and docking of *insilico* studies and cell culture work of *invitro* studies.

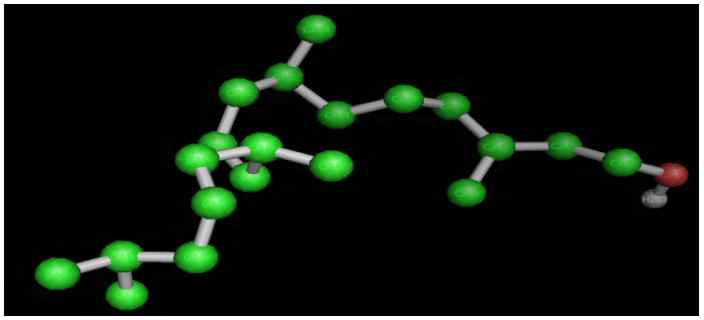

Fig. 04: The 3D structure of PHYTOL (3E, 7R, 11R)-3, 7, 11, 15-tetramethylhexadec-2-en-1-ol generated using Pymol software.

Results of *Invitro* study of phytol from *Nymphaea pubescens* screening with Cancer cell lines:

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Phytol inhibits proliferation of Breast Cancer Cells:

Phytol has been previously shown to inhibit proliferation and induce apoptosis in breast cancer cells. We evaluated the anti proliferative effects of phytol in the human breast cancer cell line MDA MB 231 by MTT assay. Cells in their log phase of growth were treated with increasing concentrations of ellipticine and paclitaxel for 48 hrs. The absorbance of control cells was taken as 100% viability and the values of treated cells were calculated as a percentage of control. We observed that phytol decreased the viability of cells in a dose dependent manner. The IC_{50} of phytol in MDA MB 231 breast cancer cell line was found to be 160µg.

Colony-formation Assay:

We assessed the effect of **phytol from *Nymphaea pubescens*** on the colony forming ability of human breast cancer cell line MDA.MD.231. The anticlonogenic effect of **phytol from *Nymphaea pubescens*** was much evident in the human breast cancer cell line MDA MB 231, as it significantly inhibited colony formation. In MDA MB 231 breast cancer cells, treated with different concentrations (140µg, 160µg and 180µg) of **phytol from *Nymphaea pubescens***, the number of colonies were inhibited by 20%, 58%, and 85% respectively compared with controls (Fig. 05a). (Tab.07). The peripheral cells of the control colonies displayed numerous cytoplasmic extensions, whereas in the **phytol from *Nymphaea pubescens*** treated colonies, the peripheral cell extensions were arrested (Figure 05b, 05c, 05d). These results suggest that **phytol from *Nymphaea pubescens*** treatment effects the morphology of the cells that may inhibit the cancer cell motility and thereby the metastatic capacity of the cancer cell.

Phytol abates migration of breast cancer cells:

Figure 05e, 05f displays the results after 0 h and 72 h of treatment. As expected the untreated sample showed migration of cells into the wound leading to wound closure. On the other hand doxorubicin treated cells also showed almost similar migration to untreated ones hence demonstrating induction of aggressiveness after treatment with doxorubicin. On the contrary, salinomycin treated cells showed no or negligible migration of cells leaving the wound open.

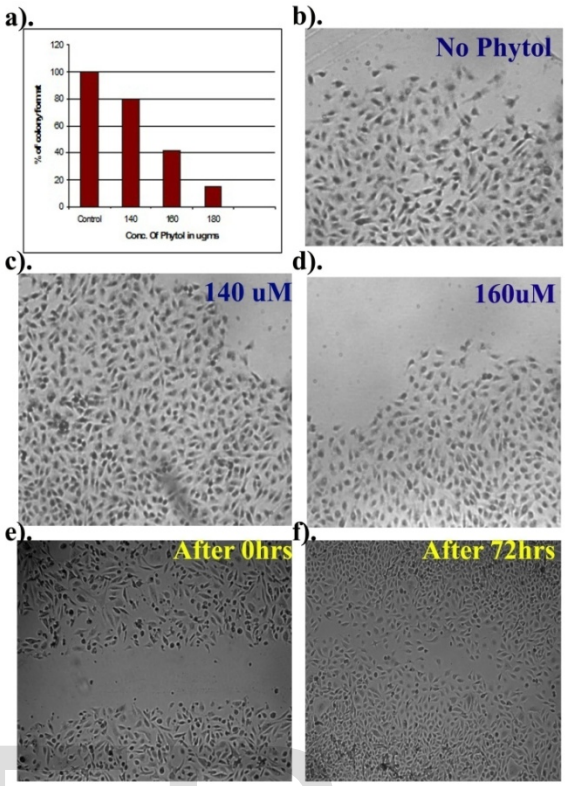

Figure 05: a).Colony Formation. 05 b, c, d). Cells showing cytoplasmic extensions after Phytol treatment (b) No Phytol, (c)140 µM & (d) 160µM. 05e & f).Wound healing assay: (e) At 0hrs of treatment (f) After 72hrs of treatment.

***Insilco* studies of phytol from *Nymphaea pubescens* with Actin of homo sapiens:**

Actin role in Homo sapiens:

Actin is a cytoskeletal protein present within all eukaryotic cell types. The cell cytoskeleton is known to provide the basic infrastructure for maintaining cell morphology and functions such as adhesion, motility, exocytosis, endocytosis, and cell division. The actin cytoskeleton and its regulatory proteins are crucial for cell migration and movement in most cells. The mechanism of cell movement involves actin remodeling which actually produces necessary force for cell migration. However, it has been observed that the alteration of actin polymerization or actin remodeling plays a pivotal role in regulating the morphology and phenotypic events of cancerous cells as a result of activation of oncogenic signaling pathways e.g., Ras and Src [26, 27 & 34]. Thus, elucidation of the molecular mechanisms of actin reorganization is important target for cancer therapeutics.

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Tab. 07: Effect of phytol from *Nymphaea pubescens* MDA MB-231 cell colony- formation

Percentage inhibition	Values are represented as mean(±SD)					
	Control	Active enzyme concentration			Inactive enzyme concentration	
	100	1 µM	2µM	3µM	1µM	2µM	3µM
		63.3±0.57*	19.6±0.57*	3.3±3.0$	75.6±0.57$	25.3±0.57*	7.6±2.0$

Methodology -*insilico* studies:

In our previous work we had developed the 3-D structure human Actin have been constructed using the template crystalline profilin-beta-actin (PDB ID: 2BTF) [35]. The resulting FASTA sequence was used to build the 3-D structures. Further, the molecular model of human actin was constructed using Modeller9v11 package for homology modeling [27]. The model quality was assessed using PROCHECK [36], PROSA [38], ERRAT [41], VERIFY 3D [39, 40] and WHAT-IF [37]. The overall scores were used to choose the final model [27]. In the present work Protein-ligand docking was performed between the molecular model of human Actin with **phytol from *Nymphaea pubescens***. The detailed analyses of probable inhibition as well as interaction of the model were performed with high binding affinity. The studies presented our previous work useful to design molecules that may have anticancer activity. In the present *In-silico* study, we had previously developed 3D structure of Actin of *homo sapiens*[27], *now here* it was taken for *insilico* study of Protein- ligand docking.

Docking of phytol from *Nymphaea pubescens* with Actin of *homo sapiens*:

Design of Phytol of *Nymphaea pubescens* for Docking:

In the most basic sense, drug design involves design of small molecules that are complementary in shape and charge to the biomolecular target to which they interact and therefore will bind to it. Drug design frequently but not necessarily relies on computer modeling techniques. This type of modeling is often referred to as computer-aided drug design. Hence for the purified phytol of *Nymphaea pubescens* molecules which will interact with Actin of *homo sapiens* model, the phytol of *Nymphaea pubescens* has been selected as a parental molecule and PRODRG [42]softwares were used to pdb of phytol of *Nymphaea pubescens* ligand molecule was selected for docking on to Actin of *homo sapiens* model using AutoDock Tool [43].

Actin of *homo sapiens* model **docking with** Phytol of *Nymphaea pubescens* **lead molecules:**

The developed homology model for Actin of *homo sapiens* was submitted to Swiss-PDBViewer (SPDBV) [44] for energy minimization. It can repair distorted geometries by moving atoms to release internal constraints. Swiss-PdbViewer includes a version of the GROMOS 43B1 force field. This force field allows evaluating the energy of a structure as well as repairing distorted geometries through energy minimization. In this implementation, all computations are done in vacuous, without reaction field. The common force fields that is in use have been developed using high level quantum calculations and/or fitting to experimental data. The technique known as “energy minimization” is used to find positions of zero gradients for all atoms, in other words, a local energy minimum. Lower energy states are more stable and are commonly investigated because of their role in chemical and biological processes. Energy minimized target, PDB was further used for docking studies.

The isolated purified phytol ligand was tested for Lipinski’s Rule of Five using Molinspiration server [45] for their ability to follow ADME properties. The Auto Dock4 .2/ADT [43] program was used to investigate phytol ligand binding to structurally refined human actin model using a grid spacing of 0.375 Å and the grid points in X, Y and Z axis were set to 60×60×60. The search was based on the Lamarckian genetic algorithm [46] and the results were analyzed using binding energy. For each ligand, a docking experiment consisting of 100 stimulations was performed and the analysis was based on binding free energies and root mean square deviation (RMSD) values, and the ligand molecules were then ranked in the order of increasing docking energies. Each simulation consisted of 100 independent runs, with a population size of 200, 500 generations and a maximum of 25000000 energy evaluations. This creates a docking parameter file for each lead

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molecule and thus the AutoDock program for each lead molecule was run. Docking results were analyzed by using Pymol [47] (http://pymol.sourceforge.net) and VMD [48].

Docking of Phytol of *Nymphaea pubescens* molecule on to *Homo sapiens* Actin:

From the *Nymphaea pubescens* leaf extraction, isolation, and purification *of Phytol* lead molecule, is used for docking simulations on to Actin of *Homo sapiens* using Autodock 4.2. The lead molecule phytol was set with docking simulations of 100 for docking onto Actin of *Homo sapiens* and thus was used efficiently to study the lead molecule binding processes with the active sites, Number of distinct conformational clusters found = 34, out of 100 runs, Using an RMSD-tolerance of 2.0 A, molecule which were docked have shown to interact well with the active site of amino acids (Fig. 07).

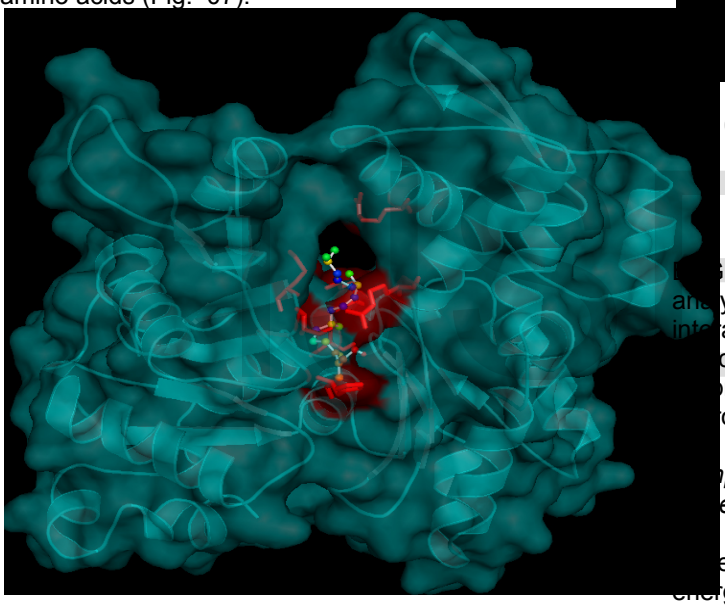


Fig. 07: Docking interaction of *Nymphaea pubescens* with Actin of *Homo sapiens*

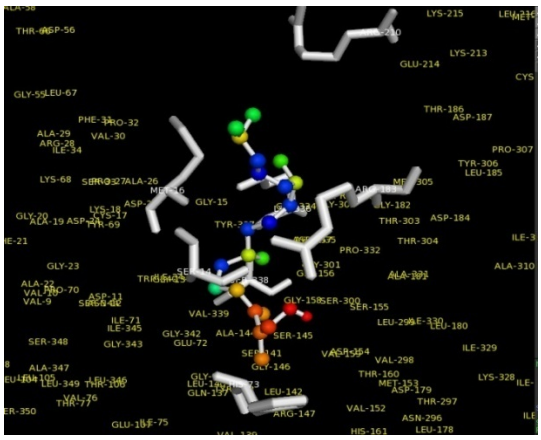
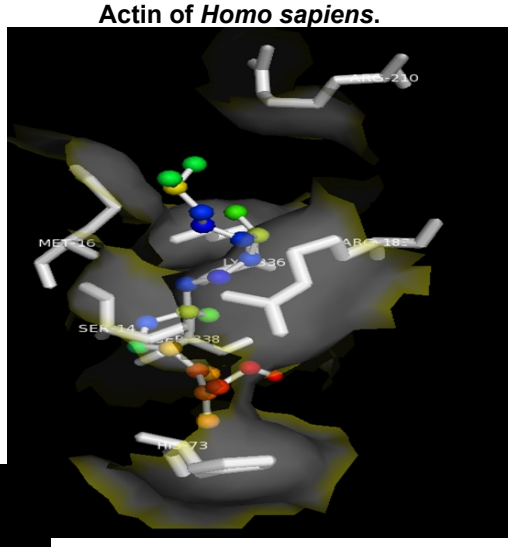


Fig.08. Overall binding interactions of Phytol with *Nymphaea pubescens* at active site amino acids of catalytic domain in Actin of *Homo sapiens*.



09: Binding interactions of Phytol of *Nymphaea pubescens* with active site amino acids of catalytic domain of Actin of *Homo sapiens*

The final result of docking has generated a file which helps during the due course of analysis, has shown that the lead molecule interacts with almost all the active site residues in catalytic pocket (fig. 07), the interactions may brought about by Vander Waals interactions or ogen bonding. The interaction of the Phytol molecule is shown in the Fig. 07 (Phytol of *phaea pubescens with* Actin of *Homo ens*).

The docking results of lead molecules are esented in the table 12 based on docking ergy and RMSD values. Phytol of *Nymphaea pubescens* molecule have shown interactions with the Actin of *Homo sapiens* active site amino acids Serine-14, Methonine-16, Histidine-73, Arginine-183, Arginine-210, Lysin-336, and Serine-338 (Fig.08, 09 & Tab.08).

These interactions may be due to the formation of Hydrogen bonds or by the establishment of Vander Waals forces. The interactions of lead molecules with the Actin of *Homo sapiens* have lead to the identification of the active site domain.

Conclusions of phytol from *Nymphaea pubescens*:

Based on the Protein ligand docking results, the interaction energy of docking between the **phytol from *Nymphaea pubescens*** and **human actin** was calculated and analyzed using

the HEX server. The efficient binding of **phytol from *Nymphaea pubescens*** and human actin revealed that the proteins could form hydrogen bond networks involving active amino acid residues. Several amino acid residues were identified to exclusively contributive to the binding of **phytol from *Nymphaea pubescens*** to Tyr166, His170, Ala173, Thr351, Gln354, Glu364, Ser365, 368, Arg372, and Phe375 of human actin. The generated homology model is expected to be useful for the structure-based drug design against cancer.

Our results show that the docking scores follow a distribution from which such predictions are possible and in good agreement with the experimentally determined agonist bound **phytol from *Nymphaea pubescens*** to human actin structures. Based on experimental data, molecular docking to explore the inhibition mechanism of **phytol from *Nymphaea pubescens*** toward human actin. Our results suggested that **phytol from *Nymphaea pubescens*** could exactly bond to the active pocket of human actin to display inhibition; the binding mode may alter with the change of some substituent's on certain positions. The **phytol from *Nymphaea pubescens*** compound orientation in the active site can greatly affect the stability of the protein ligand complex. Although our results are based on a rather detailed system setup, to some extent taking advantage of prior experimental knowledge, we believe that the fundamental ideas of this study can serve as inspiration when dealing with protein docking to flexible receptors.

Above findings signifies the potential of **phytol from *Nymphaea pubescens*** as an anticancer drug. The evidence leads us to propose that cell-surface actin may be a target for **phytol from *Nymphaea pubescens*** in cancer cells. Therefore, *phytol* competes with angiogenin for cell-surface actin and in this manner, blocks the formation of the actin-angiogenin complex required for cancer cell organization and angiogenesis in developing neoplastic tissue.

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post doctoral fellowship and the Molecular Biology Unit, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India for providing laboratory and technical support to our research work is gratefully acknowledged

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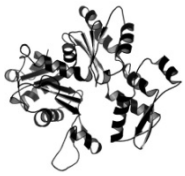
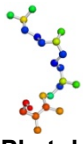
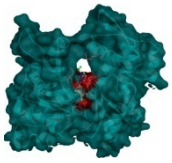
Table.08: Docking results of Phytol of *Nymphaea pubescens* molecule docked on to Actin of *Homo sapiens* model

S. No	Protein-Ligand Docking	RMSD from reference structure (A^0)	Estimated Free Energy of Binding (kcal/mol)	Docked energy (kcal/mol)	Estimated Inhib. Const, Ki uM or mM (micro/mill molar) [Temp= 298.15K]	Hydrogen Bonding Distance (A^0)	Active catalytic residues of Actin of Human
1.	Actin of Human  +  Phytol of *Nymphaea pubescens* = 	0.192	-5.63	-5.72	74.33 uM	1.962	Ser-14
2.		0. 185	-5.17	-5.21	162.65 uM	1.759	Met-16
3.		0.270	-5.16	-5.25	166.19 mM	1.632 1.956	His-73 Arg-183
4.		0.761	-5.02	-5.09	207.76 uM	1.923	Arg-210
5.		0.193	-4.75	-4.83	327.26 uM	1.852 1.632	Lys-336 Ser-338

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