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In-silico studies on selected Phytoconstituents of *Neolamarckia cadamba* (Roxb.) Stem Bark for Anticancer Activity

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Abstract

Aim*:* To study the anticancer potential of *Neolamarkia cadamba* (Roxb.) stem bark using molecular docking studies.

Materials&Methods: The dried bark extracted successively by soxhlet apparatus using n-Hexane, Dichloromethane and 80%Ethanol. Phytochemical screening and TLC analysis was done for all extracts. Dichloromethane extract (NCDME) evaluated for anticancer potential on MCF7, A549 and HepG2 cell lines using MTT assay and subjected to GC-MS analysis.5 phytocompounds are selected from GCMS report and studied against VEGFR2, HER2(erbB2) and EGFR tyrosine kinase through molecular docking studies.

Results: The IC_{50} values determined for the extract on all the cancer cell lines proves promising anticancer potential of the extract. The protein binding studies of the selected phytocompounds with reference to standard drugs reveals the same.

Conclusion: The study states that dichloromethane extract of *Neolamarkia cadama* (Roxb.) stem bark exhibited anticancer activity and some of the phytocompounds can be developed as natural anticancer agents.

Key Words: Cancer, GC-MS, In-silico analysis, *Neolamarkia cadamba*, Molecular docking, MTT Assay.

INTRODUCTION:

Plants are the best source of medicine from the start of human civilization on the earth. The knowledge preserved and transferred by the ancestors to the next generations reveals the medicinal applications of the plants and trees grown in their surroundings. The recorded data in ancient texts like Charaka samhitha, Susruta samhitha etcand folklore knowledge of medicinal plants inspire the researchers to investigate plants on modern scientific lines for their phytoconstituents and their use in treatment of different diseases. The plant derived drugs have been found to possess good effectiveness with less or no side effects compared to synthetic drugs [1]. Recent advancements in isolation and identification techniques like various column chromatography methods, LCMS, GCMS, *in-silico* screening techniques, pharmacokinetic modelling techniques facilitated and enhanced the research on individual plants yielding many new promising plant products [2, 6].

Cancer is a dreadful disease and became a major public health issue all over the world and it is the second leading cause of death in United States and the world over. Even though the death rate is declining in some cancers such as lung, colorectal, breast and prostrate cancers, number of cancer patients are increasing and treatment cost becoming a financial burden to the patients. The deaths due to lung cancer in 2017 was more than those of breast, prostrate, colorectal and brain cancers put together. There is a rapid increase in liver cancer mortality in women. According to WHO latsest statistics 9.6 bililion deaths in 2018 are because of different cancers. 70% of cancer patients are facing death are in low and middle income group. Dietray risk is one of the major causes for one third of deaths in cancer [3]. Hence, there is need to discover or invent new drug molecules with less side effects and cost effective strategies to treat cancer. Till now few drugs such as vincristine, vinblastine, vinorelbine, taxol, topotecan, irinotecan, etoposide, camptothecan etc. were discovered from natural sources and are very effective in treating cancer. Flavonoids like curcumin, quercetin, rutin etc are also showing promising anticancer potential [4,5,6]. Still many plants can be explored to get new lead molecules or specific phytoconstituents which can be used in prevention and treatment of specific cancer.

Neolamarkia cadamaba/Anthocephalus cadamba is a tropical evergreen tree belongs to Rubiaceae family.It is commonly known as “kadamaba”. In folklore medicine it was used to treat fever, uterine disorders, blood related diseases, leprosy, diarrhoea, skin infections etc. Different parts of the tree were screened for antimicrobial, analgesic, antipyretic,anti-inflammatory, antidiarrhoeal, diuretic and laxative, antihepatotoxic, hypolipidimic, antiplasmodial, analgesic, antidiabetic, antioxidant, antihelmintic, antifungal,antiparasitic,antifilarial,antimalarial,antiproliferative,antioxidant,antivenom activities[7,8,9]. However, Anticancer property was not evaluated exhaustively [10-16]

In-Silico evaluation of different plant extracts for many pharmacological activities including anticancer activity is the recent scientific trend all over the world [17-24]. Based on literature survey the present study aimed to find out phytocnstieunts with potential anticancer properties in stem bark of *Neolamarkia cadamba* (Roxb.) native to Telangana state, using advanced screening methods like GC-MS and molecular docking studies which had not been reported.

MATERIALS AND METHODS:

Plant material collection and identification

Neolamarkia cadamba stem bark was collected near Bapughat at Langar house, Mehdipatnam, Hyderabad, Telangana state. A vouc her specimen was prepared and the identification was authenticated by Botanical Survey of India, Attapur, Hyderabad. The collected bark dried under shade for two weeks and grounded to powder using cutting mill and stored in air tight containers.

Solvents and Reagents

n-Heaxane, Dichloromethane (LR) and TLC silica gel 60F254 (Merk) plates were purchased from Sri Venkateshwara scientific suppliers, Hyderabad. Ethanol (RS) procured from prohibition and excise department of Telangana state govt. DMEM (Dulbecco's modified Eagles medium), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], trypsin, EDTA Phosphate Buffered Saline (PBS) were purchased from Sigma Chemicals Co. (St. Louis, MO) and Fetal Bovine Serum (FBS) were purchased from Gibco. 25 cm^2 and 75 cm^2 flask and 96 well plated purchased from eppendroff India.

Cell lines

The Cancer cell lines were purchased from NCCS, Pune and the cells were maintained in MEM supplemented with 10 % FBS and the antibiotics penicillin (0.5 $mmolL^{-1}$), in atmosphere of 5% CO_2 /95% air at 37 ^{0} C.

Extraction

1.0 kg of stem bark powder was extracted with n-hexane, dichloromethane and 80% alcohol successively by hot percolation method using soxhlet apparatus. From the extracts, solvent was recovered using rotavapour, dried under vacuum and stored in a dessicator. The weight of the extracts was 6.7gm, 15.8 gm and 50.4 gm respectively. The colour and consistency of the extracts was noted.

Phytochemical Screening and TLC

The phytochemical screening of all the extracts was carried out with freshly prepared reagents. *Neolamarckia cadamaba* Dichloromethane Extract (NCDME) was subjected to TLC analysis on 8x5 cm plates using a solvent system of dichlorometehane and ethyl acetate in different ratios. Initially, the spots were observed in UV cabinet under short and longer UV light. Vanillin sulphuric acid sprayed on the plates followed by heating at 110^0C.

MTT Assay

The cytotoxic potential of NCDME was evaluated on HepG2 and A549 cell lines at Synteny Life Sciences Pvt.Ltd, Nallakunta , Hyderabad, Tealangana and on MCF -7 cell lines at IIT Hyderabad by MTT assay. It is a colorimetric assay that measures the reduction of yellow 3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase. The MTT Assay was performed with five concentrations of extract in triplicates on HepG2 and A549cells whereas 4 concentarations were used on MCF-7 cells. Cells were trypsinized and preformed the tryphan blue assay to know viable cells in cell suspension. Cells were counted by haemocytometer and seeded at density of 5.0 X 10 ^{3} cells / well in 100 µl media in 96 well plate culture medium and incubated overnight at 37 ^{0} C. After incubation, old media was removed and 100 µl of fresh media added with different concentrations of test compound in represented wells in 96 plates. After 48 hrs, the drug solution was discarded. Fresh medium and MTT solution (0.5 mg / $mL^{-1)}$ was added to each well and plates were incubated at 37 ^{0} C for 3 hrs. At the end of incubation time, precipitates were formed as a result of the reduction of the MTT salt to chromophore formazan crystals by the cells with metabolically active mitochondria. The optical density of solubilised crystals in DMSO was measured at 570 nm on a microplate reader. The percentage growth inhibition was calculated using the following formula.

$$\% \text{ Inhibition} = \frac{100\ (\text{Control} - \text{Treatment})}{\text{Control}}$$

The IC_{50} values were determined by using linear regression equation i.e. Y =mx+C, Here, Y = 50, m and C values are derived from the %viability graph [25].

Characterization of NCDME by GC-MS Analysis

GC-MS analysis of the extract was performed at Skanda Life Sciences, Bangalore, Karnataka. 10 mg of NCDME was reconstituted in 1000µL methanol. 1 µl of this extract was injected into Agilent DB 5MS (30 meter X 0.25 mm) column fitted to thermo scientific GC trace 1310 instrument equipped with thermo scientific MS TSQ 8000.The temperature of ion source maintained at 230°C and that of transfer line at 300°C. EI mode of ionization method used to generate ions. Helium was used as carrier gas at flow rate of 1ml/min. An initial temperature of 60°C maintained for 5 min and then raised to 240°C at a rate of 10°C/min and held at the same temperature for 5min.Then, raised to 300°C at a rate of 10°C/min and held for 5min.The mass of the compounds and fragments recorded were matched with NIST database for identification of probable compounds present in the sample.

Extracted ion chromatograms were obtained from all the 28 peaks. Interpretation on mass spectrum of GC-MS was done using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The mass of compounds and fragments matching with NIST database and chemical +structures of major compounds matched with NIST Library.

In-Silico studies

Total five molecular structures have been selected from the GC MS data and their 3d structures were drawn using Marvin sketch 3d tool and the energies were minimized. These 3D structures were used for the *in silico* studies on three anticancer drug targets, VEGFR 2 kinase(lung cancer), ErbB2 Kinase (breast cancer) and EGFR kinase (liver cancer). Autodock was employed to dock the 3d molecules into the active site of three target proteins. The docking methodology followed according to the earlier published procedure. The protein structure of VEGFR-2 PDBID: 4ASD was downloaded from the protein data bank along with co-crystal ligand Sorafenib BAY 43-9006. The ligand and water molecules were removed and charges were added to the refined protein and its PDBQT file was created. The docking procedure was performed in the active site by defining the 3D grid coordinates in X, Y

and Z axes with grid dimensions -25.93, -1.63 and -10.66 respectively with gridpoints 50-50-50. The docking was performed for all the molecules using Lamarkian Genetic Algorithm for upto 10 successful runs and the final docking scores in terms of binding energies(kcal/mol)were noted . The binding energies of molecules were compared with the standard Sorafenib to identify potential anticancer molecules. Similarly the protein structures of Human HER2 (erbB2)-PDBID: 3PP0 and EGFR tyrosine kinase domain-PDBID: 4hjo and their co-crystal ligands of SYR and Erlotinib were downloaded from protein databank. The active site of the protein Human HER2 (erb2)-PDBID3PP0is defined by the X,Y and Z axes with grid dimensions -16.387,17.394 and 26.218 respectively with grid points 50-50-50. After docking, binding energies and binding poses of best docked confirmations and docking interactions were considered for test compounds and compared with that of standard. Molecular docking studies also performed on EGFR2 Kinase(4jho). Erlotinib has taken as standard and all the selected compounds docked into the active site of EGFR2 using same procedure described above. The grid dimensions selected were 25.53, 10.5, -0.75 for X, Y, Z axes respectively to define the active site on the target. [26]

RESULTS AND DISCUSSION

Phytochemical screening of the n-Hexane extract(NCNHE) shown positive test results for the presence of glycosides, carbohydrates, steroids and tannins. The dichloromethane extract (NCDME) and hydro alcoholic extract(NCHAE) revealed the presence of alkaloids ,glycosides, carbohydrates, steroids, flavonoids and tannins. TLC profile of NCDME with vanillin sulphuric acid reagent shown orange, yellow, pink and blue spots. 6spots observed in 90:10, 5 spots observed in 80:20, 50:50 and 20:80 whereas 2 spots found in 10:90 ratios of dichloromethane and ethyl acetate solvent system as shown in Figure 1 and 2.

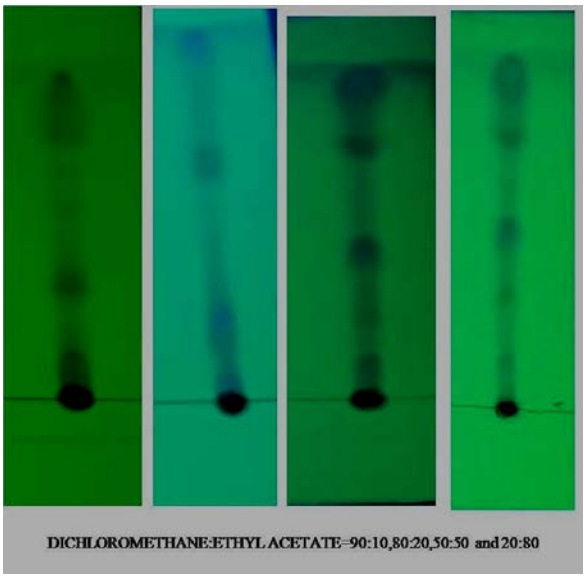
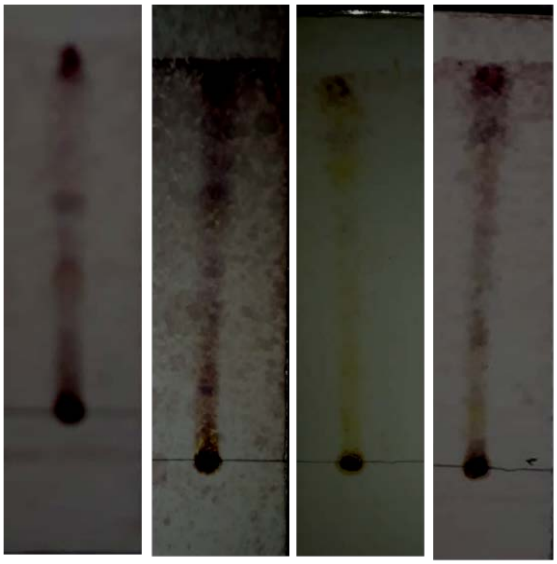


Fig.1: TLC profile under short UV light



After spraying with vanillin sulphuric acid

Fig 2: TLC profile after derivatization

TLC reveals the presence of considerable number of steroidal and terpenoidal compounds in the extract. Hence, it was assumed to possess good cytotoxic potential according to literature and hence MTT assay was carried out. The IC_{50} values of NCDME on MCF7, A549 and HepG2 cell lines are 91.22, 27.05 and 89.45 µg/ml respectively indicating excellent anticancer potential of the extract which can be attributed to the phytoconstituents present in it. The %inhibition of the cells is shown in tables 1, 2 and 3.

Table 1: MTT Assay on MCF-7 Cell line

NCDME Conc.(µg/ml)	Absorbance at 570nm*	% Inhibition	% Viability	IC_{50} Value (µg/ml)
50	1.795	40.95	59.05	91.22
100	1.483	51.22	48.78	
150	1.120	63.16	36.84	
200	0.674	77.88	22.12	
Untreated	3.040	0	100	
Blank	0	0	0	

*Average of 3 determinations.

Table 2: MTT Assay on A549 cell line

NCDME Conc.(µg/ml)	Absorbance at 570nm*	% Inhibition	% Viability	IC_{50} Value (µg/ml)
5	0.453	41.54	58.46	89.45
10	0.321	58.58	41.42	
25	0.253	67.35	32.65	
50	0.234	69.80	30.20	
100	0.343	55.74	44.26	
Untreated	0.775	0	100	
Blank	0	0	0	

*Average of 3 determinations

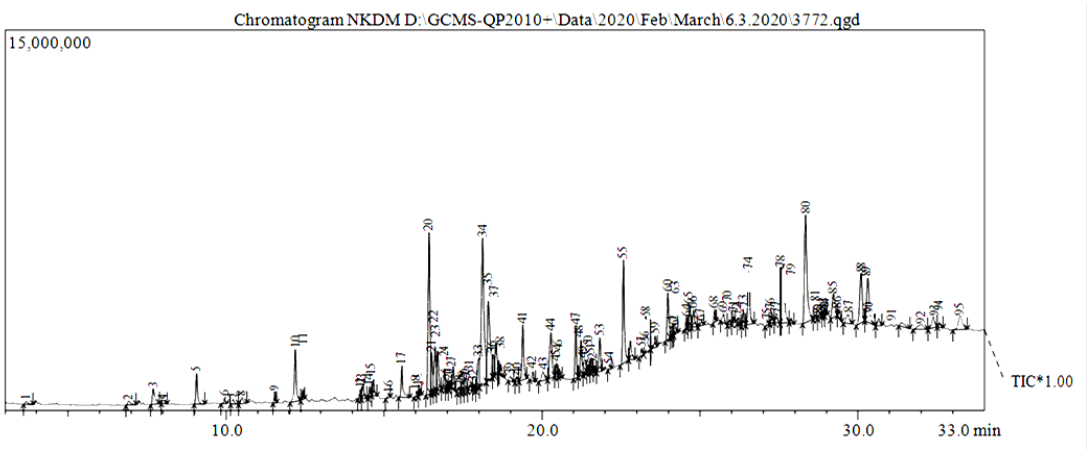


Fig 2: GC-MS analysis of Dichloromethane extract of *Neolamarkia cadamba*

Table 3: MTT Assay on HepG2 Cell line

NCDME Conc.(µg/ml)	Absorbance at 570nm*	% Inhibition	% Viability	IC_{50} Value (µg/ml)
5	0.512	31.55	68.45	27.05
10	0.326	56.41	43.59	
25	0.247	66.97	41.98	
50	0.314	58.02	33.02	
100	0.548	26.74	73.26	
Untreated	0.748	0	100	
Blank	0	0	0	

*Average of 3 determinations.

The GC-MS chromatogram has shown decenes, decanoic acids, carbaril, vanillin, tyramine, carboxylic acids, esters, acontanes, oleic acid, cholesterol and its derivatives, stigmasterol and its derivatives. Majority of the compounds are decanoic acids, decenes and acontanes. Hence, among these, few compounds were chosen for molecular docking studies against appropriate anticancer targets.

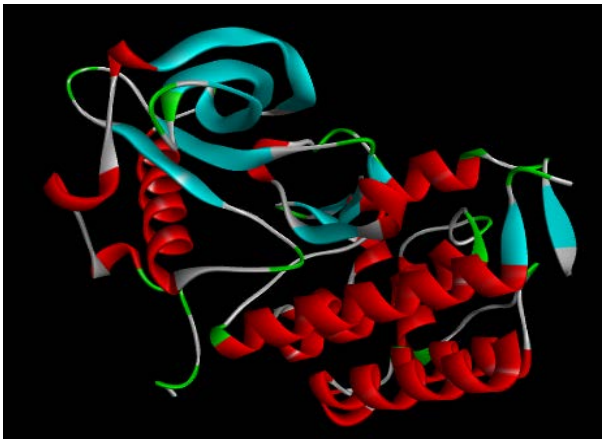


Fig 3: Crystal Structure of VEGFR2

The docking studies on protein 4ASD-VEGFR-2, the tested compound, Hexadecanoic acid has shown excellent binding energy (Table 4).The interactions of tested compounds with target were compared with those of Sorafenib. Sorafenib interacting with Glu885 with two Hydrogen bonds, one more hydrogen bond with Asp1046 and other important hydrophobic interactions were found with Val916 and Lys868. (Fig.4)The test compound Hexadecanoic acid also has shown similar interactions with Val916, Phe1047 and Asp1046 (Fig.6) Another compound Carbaril also shown good binding affinity with target (-7.3Kcal/mole) and also exhibited similar interactions. (Fig.5).

Table 4: Molecular docking studies on VEGFR2 kinase-PDBID: 4asd

Molecules (Ligand)	Binding Energies (Kcal/Mole)
Hexadecanoic acid	-8.4
Carbaril	-7.8
Tricosenoic acid	-7.3
Dotriacontane	-7.2
Tetrapentacontane	-6.9
Nonadec-1-ene	-6.6
Sorafenib	-12.5

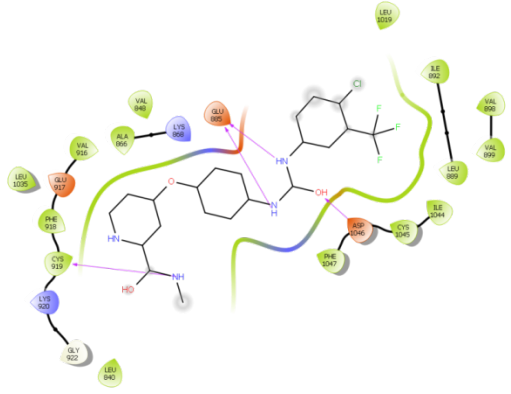


Fig 4: Interactions of Sorafenib

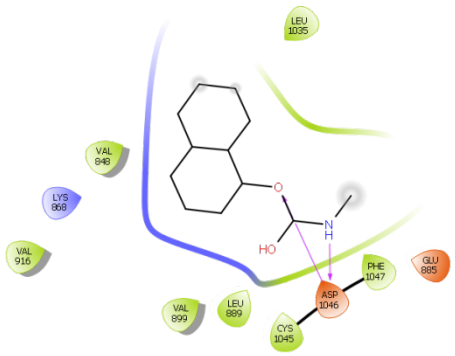


Fig.5: Interactions of Carbaril

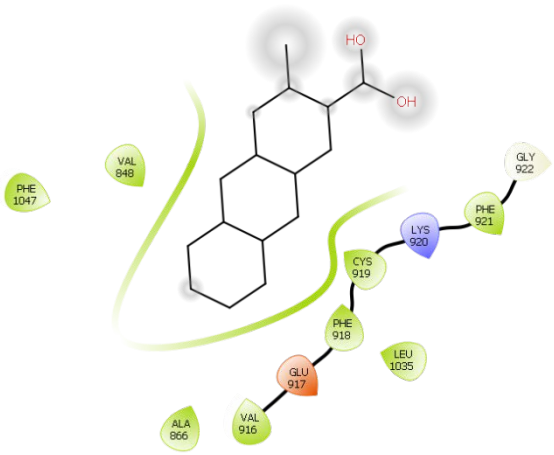


Fig 6: Intarctions of Hexadecanoicacid

In Docking on protein HER2, The selected compounds shown good binding affinity with target protein. However, tricosenic acid has shown highest binding affinity (-8.15Kcal/mole) (Table 5) and also exhibited similar interaction as those of standard. The interaction with Met801 and Leu852 were considered to be significant (Fig 8 and 9). Hence, the study revealed the anticancer ability of the selected compounds on human HER2 (erb2) to treat breast cancer.

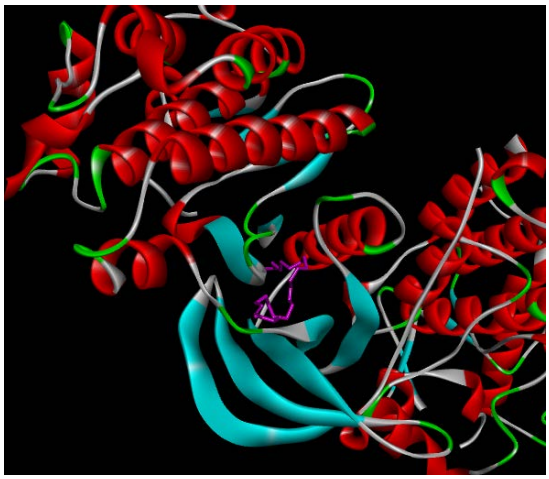


Fig 7: Docked conformation of Tricosenic acid in the active site of Human HER2 (erbB2)

Table 5: Molecular docking studies of selected molecules on Human HER2 (erbB2)-PDBID3PP0

Molecules (Ligand)	Binding Energies (Kcal/Mole)
SYR (Standard)	-10.74
Tricosenoic acid	-8.15
Hexadecanoic acid	-7.31
Carbaril	-7.16
Tricosenoic acid	-7.05
Nonadec-1-ene	-6.56
Tetrapentacontane	-6.54

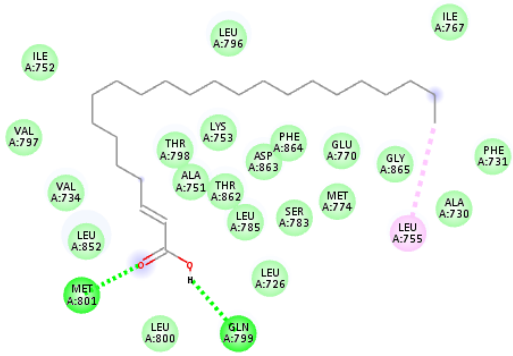


Fig 8: 2D interactions of of Tricosenoic acid with the active site of Human HER2 (erbB2).

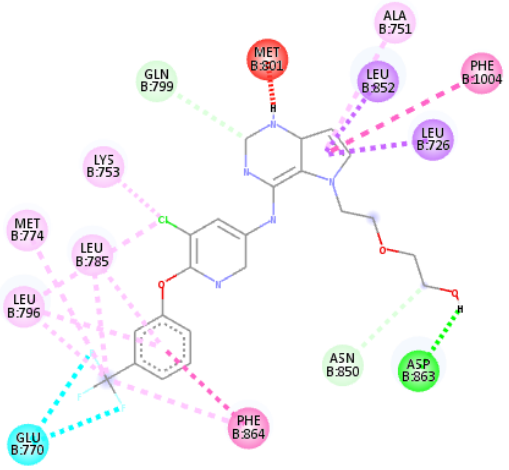


Fig 9: 2D interactions of of SYR Active co-crystal ligand with the active site of Human HER2 (erbB2)

Docking on EGFR tyrosine kinase,revealed that the docked compounds have shown significant binding affinity to the target and Hexadecanoic acid has shown maximum binding affinity with a binding energy -8.5Kcal/mole which is higher than Erlotinib as shown in the table 6. Binding energies and interactions are compared with those of standard, Erlotinib. Interactions of erlotinib have formed a hydrogen bonding with threonine 830(Fig.10).

Table 6: Molecular docking studies on EGFR tyrosine kinase domain-PDBID: 4hjo

Molecules (Ligand)	Binding Energies (Kcal/Mole)
Hexadecanoic acid	-8.5
Carbaril	-7.3
Tricosenoic acid	-6.3
Dotriacontane	-6.1
Nonadec-1-ene	-5.8
Tetrapentacontane	-5.8
Erlotinib	-8.2

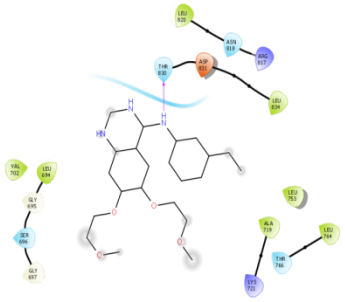


Fig 10: Interactions of Erlotinib

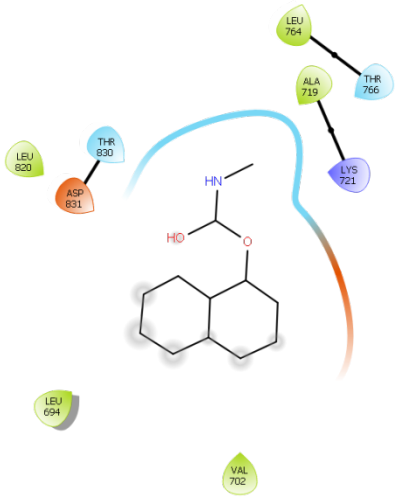


Fig 11: Interactions of Carbaril

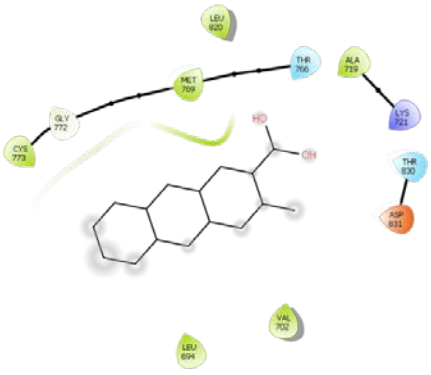


Fig 12: Interactions of Hexadecanoic acid

All the compounds have shown a binding energy above -5Kcal/mole. This indicates that all the compounds have potential for anticancer property and hence said to possess ability to become a drug for the treatment of cancer. The *in- silico* studies as well as *in- vitro* data revealed the therapeutic ability of the phytoconstituents of NCDME as anticancer agent.

CONCLUSION

MTT assay of NCDME performed and found to show good anticancer activity against MCF, A549 and HepG2 cell lines. Phytochemicals present in the extract were identified through GC-MS analysis and chemical nature of the majority of the compounds was noted. Based on this, few compounds were selected for molecular docking studies on proteins related to MCF, A549 and HepG2 cell lines. Binding energies and binding affinity to the target proteins reveals that almost all the compounds are having shown remarkable anticancer activity when compared to the respective standard substances.

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