ISSN:2249-1236
Annette Fernandez et al. IJRRPAS, (2017) Vol.7 Issue.1 Pg:-1426-1434
International Journal of Research and Reviews in Pharmacy and Applied sciences

Synthesis, characterization, *in silico* and *in vitro* studies of an amino derivative of embelin as potent anticancer agent against human breast cancer cell line MCF-7

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Abstract
The 4–aminoantipyrine derivative of embelin, 5–(1,5–Dimethyl–3–oxo–2–phenyl–2,3–dihydro–1H–pyrazol–4–ylamino)–2–hydroxy–3–undecyl–[1,4]benzoquinone (EAP) was synthesized and characterized by various physicochemical techniques including single crystal XRD. Embelin and its derivative EAP were subjected to *in silico* molecular docking studies against MCF–7 breast cancer cell lines. 5–flurouracil (5–FU) was taken as the standard. The docking results revealed EAP to possess greater affinity when compared to embelin and 5–flurouracil (5–FU). This was substantiated by *in vitro* analysis. The study reveals EAP to be a potential lead molecule to fight cancer.

Key words: Embelin, 4-aminoantipyrine, EAP, Docking, *in silico*, MCF–7, PI3K

1. Introduction

Cancer, a troublesome malignancy may be defined as a disease in which an assemblage of abnormal cells grow manically without paying attention to the customary cell division rules [1]. The malignant cells overtake the normal cell division by dividing faster, exploiting every kind of opportunity to do so. Breast cancer is the most widespread malignancy in females, characterized by the development of malignant cells in the tissues of the breast. The disease is quite universal and is on its rise. Practically one in four of all cancer cases reported in females belong to this category. The malignant cells invade and take possession of provinces in the body that are in general reserved for other cells. This demands immediate therapeutic attention to curb the situation. Some of the strategies practiced in the treatment of cancer are surgery, radiation therapy, hormonal therapy, chemotherapy, gene therapy, stem cell transplantation, targeted therapy and immuno therapy.

Chemotherapy (adjuvant or neo-adjuvant) is a popular method of treatment of breast cancer and involves intravenous or oral administration of drugs. Several drugs are now available in the market to serve the purpose. Cisplatin, Doxorubicin, Taxol, Tamoxifen, Epirubicin hydrochloride, 5–flurouracil, Cyclophosphamide, Zoladex, Vincristine etc. are a few to name. The type of drug to be administered in a patient is dependent on various factors like the stage of the disease, age etc. Chemotherapeutic compounds are in most cases associated with side effects like hair loss, nausea, vomiting, variation in apetite and Diarrhoea [2,3]. The absolute progress in survival after chemotherapy with the currently available drugs is also under question. Hence there is a strong incentive to develop anti cancer drugs with fewer side effects and enhanced survival rate.

Several studies have revealed the importance of phytochemicals in the treatment of various ailments. Medicinal plants have been employed to treat diseases in humans for many years due to their antioxidant and antimicrobial activities. This can be attributed to the presence of bioactive compounds present in them which act as free radical scavengers [4,5]. Medicinal plants are prospective in reducing the oxidative stress arising in cells and prevent the trauma due to cancer. It is amazing to note the multiple medicinal properties of phytochemicals and interestingly, a large number of modern drugs are being developed from them. Numerous plant species have anticancer properties and compounds derived from them and the derivatives of these compounds constitute over 50% of the anticancer drugs [6,7].

Embelin (2,5–dihydroxy–3–undecyl–1,4–benzoquinone) belong to the plant species *Embelia ribes* and its utility is cited in traditional medicines (Family: Myrsinaceae Genus: Embelia). Embelin is a bio-active constituent in the fruits of *Embelia ribes* possessing a broad spectrum of biological activities like anticancer, antitumor, antioxidant, anti-inflammatory, antihelmintic, and antimicrobial [8]. *Embelia ribes* have a long history of use in ayurvedic system of medicine and finds its use in several Ayurvedic preparations like *Sanjivani Vati, Pippalyasavam, Dhanwantara ghritham, Vidangarishta, Kaisoraguggulu vatica* [9]. Its ability to reduce the size of tumors as well as to hinder the activity of serum enzymes have been reported by many [10]. Embelin and some of its 2–hydroxy–5–substituted–3–undecylcyclohexa–2,5–diene–1, 4–diones were reported to be screened for anticancer activity against HBL-100 cell lines by MTT method [11]. Recent reports by Shah et al have shown that embelin inhibits cell proliferation, induces apoptosis and alter expression of breast cancer focused genes in MCF–7 breast cancer cell line [12]. The contribution of the mitochondrial pathway in embelin-induced apoptosis and the effect of embelin on the cell cycle were investigated by Li et al. It was concluded that embelin could induce apoptosis of MCF–7 breast cancer cells in a dose and time dependent fashion [13].

The MCF–7 (Michigan Cancer Foundation–7) human breast cancer cell line is the most studied breast cancer cell lines in the world since its isolation. It is found to preserve several characteristics of differentiated mammary epithelium, one of which is its ability to process estradiol by means of cytoplasmic estrogen receptors. These cell lines are estrogen receptive and are frequently used *in vitro* to study estrogen receptor positive breast cancers [14]. It is considered as one among the very few cell lines to express significant levels of estrogen receptor.

Numerous derivatives of embelin have been investigated for its utility as drugs for a wide range of clinical applications. 4-aminoantipyrine based heterocycles are rich in nature and found to possess wide range of pharmacological activities. It is also used as a deterrent against several diseases including cancer [15]. Embelin and its derivatives are examples of quinonoidal ligands. The therapeutic action of this class of ligands have been a theme of active research and their cytotoxic accomplishments have been attributed to a range of mechanisms like nduction of DNA strand breaks, free radical generation, alkylation via the formation of a quinone methide, redox cycling etc [16, 17].

The present study deals with the isolation of embelin from *Embelia ribes* and the synthesis of its 4–aminoantipyrine derivative. The synthesised derivate 5–(1,5–Dimethyl–3–oxo–2–phenyl–2,3–dihydro–1H–pyrazol–4–ylamino)–2–hydroxy–3–undecyl–[1,4] benzoquinone (EAP) was then characterized by X–ray diffraction studies, IR, ^{1}H–NMR and ^{13}C–NMR. Embelin and its derivative EAP were then subjected to *in silico* molecular docking studies against MCF–7. 5–flurouracil (5–FU) and doxorubicin (DOX) were taken as the standards owing to the presence of a quinone moiety and the target protein was assigned as phosphoinositide 3–kinase (PI3K). *In vitro* anticancer studies were conducted using MTT assay method. The results obtained were promising and complimented each other. It was found that the newly synthesized derivative of embelin demonstrated enhanced anticancer activity.

2. Materials and Methods

2.1 Extraction and isolation

4–aminoantipyrine was purchased from Alfa Aesar Company. All other chemicals used were of AR grade. Solvents were dried and purified according to standard procedures. Embelin was isolated by soxhlet extraction from the berries of *Embelia ribes*. Hexane was used as solvent and golden flakes of embelin were recrystallized from ethyl acetate. It was characterized by comparing its IR, NMR and mass spectral data with literature values [14].

The synthesis of 5–(1,5–dimethyl–3–oxo–2–phenyl–2,3–dihydro–1H–pyrazol–4–ylamino)–2–hydroxy–3–undecyl–[1, 4] benzoquinone (EAP) is as given in Scheme 1. Embelin (0.294g,1 mmol) and 4–aminoantipyrine (0.406, 2 mmol) were dissolved separately in glacial acetic acid, mixed together and heated under reflux on a water bath at 100 °C for 3 h. The cooled reaction mixture gave a deep red precipitate when poured into crushed ice. The product obtained was filtered, washed and dried. This was then subjected to column chromatographic separation (ethyl acetate: petroleum ether – 90:10) and

the obtained compound was recrystallized from methanol as blue crystals. Single crystal of the compound was grown and was employed for structural characterization by X–ray diffraction studies.

2.2 Insilico analysis

The X-ray structure of the protein PI3K (PDB ID: 4YKN) alpha lipid kinase with the active site inhibitor containing water molecules and heteroatoms was obtained from the RCSB Protein data bank (http://www.pdb.org/). The protein was pre-processed separately by deleting the existing ligand as well as the water molecules without hydrogen bond that were observed crystallographically followed by energy minimization. The chemistry of the protein was then corrected for missing hydrogen's.

Docking was performed using the graphical environment iGEMDOCK which is an automated docking program. It is software used for integrated structure based virtual screening and helps in recognizing pharmacological interactions that are useful for identifying lead molecules. After docking run was carried out using Gemdock scoring function, the individual binding pose of each ligand (Embelin, EAP and 5–FU) was observed and their binding affinity with the target protein was analyzed. The best binding pose and total energy of each ligand was analyzed in the post docking screening. The target protein after preparation was subjected to energy minimization using Universal Force Fields (UFF). All the potential active sites on PI3K were located and docking was performed on all these sites with the inhibitor ligands. After docking simulation, the best docked conformer of each ligand and receptor was merged and stepwise energy optimization was done. The various interactions of ligand with the receptor such as hydrogen bonding, hydrophobic bonding and van der Waal's interactions were then checked. The compound which gave the lowest binding energy i.e. highest negative docking score was chosen as the best inhibitor as it indicated the formation of energetically most stable drug receptor. The free version of Discovery Studio Visualizer 3.5 was used to visualize the docking images owing to its convenience and good quality images.

2.3 In vitro antiproliferative affect determination by MTT assay

MCF–7 (Breast cancer) cell lines were cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37 ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of cells were then evaluated by direct observation of cells by inverted phase contrast microscope and followed by MTT assay method. Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension (5×10^4 cells/well) was seeded in 96 well tissue culture plate and incubated at 37 ºC in a humidified 5% CO_2 incubator. 1 mg of each compound was added to 1ml of DMEM and dissolved completely by cyclomixer. After that the extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility. After 24 hours, the growth medium was removed, freshly prepared samples in 5% DMEM were five times serially diluted by two fold dilution (100 µg, 50 µg, 25 µg, 12.5 µg, 6.25 µg in 100 µl of 5% MEM) and each concentration of 100 µl were added in triplicates to the respective wells and incubated at 37 ºC in a humidified 5% CO_2 incubator. Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

2.4 Cytotoxicity Assay by MTT Method:

Fifteen mg of MTT (Sigma, M–5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37 ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT solubilization solution DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 570 nm
The percentage of growth inhibition was calculated using the formula:

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$$\%\ \text{of viability} = \frac{\text{Mean Optical density of samples}}{\text{Mean Optical density of control group}} \times 100$$

3. Results and Discussion

3.1 Spectral analysis

The structure of the newly synthesized compound, EAP was established using various physicochemical studies and is depicted in Figure 1. The IR absorption band at 1672 cm^{-1} is attributed to the carbonyl group in the quinone ring. The band at 3275 cm^{-1} corresponds to the hydroxyl group. The bands corresponding to the long chain aliphatic C–H stretching as well as the aromatic C–H stretching are also evident from the spectra at 2851 cm^{-1} and 2922 cm^{-1} respectively. The C13 NMR spectrum did not show the ring carbon signals. Such a behaviour is normally observed in the case of embelin also particularly in the case of carbon attached to oxygen atoms. This is attributed to the fluxional effect caused by intramolecular hydrogen bonding. This results in long spin relaxation time, which leads to saturation of oxygen-carbon signals. However, in some cases, this result is observed to disappear when at least one of the hydroxyl group is removed [18].

Elemental analysis (%): calc. for $C_{28}H_{37}N_3O_4$ (479.28): C 70.12, H 7.78, N 8.76; found: C 70.38, H 7.54, N 8.11. ^{1}H NMR (δ): 7.4681–7.2943 (C2'–C6'), 5.1829 (s, C6, 1H), 3.1520 (s, N–CH_3, 3H), 2.1521(s, C–CH_3, 3H), 0.7814–2.3257 (aliphatic long chain). ^{13}C NMR (δ): 182.50, 180.72 (C=O of embelin–C1, C4), 161.11 (C1", C=O of 4–AAP), 150.62 (C2), 149.38 (C5, C–NH), 133.71–126.12 (C1'–C6'), 116.50 (C3), 105.08 (C2"), 95.32 (C6), 33.84 (C5", N–CH_3), 31.68–13.06 (11 C chain), 9.10 (C4"). MS (FAB) *m/z* 479.

3.2 Single crystal X-ray diffraction analysis

X–ray diffraction studies revealed that the crystal belongs to monoclinic crystal system with space group P $2_1/c$ and unit cell dimensions a = 22.352(3) Å, b = 8.2257(9) Å, c = 14.3776(13) Å; α= 90°, β= 95.307(5)°, γ= 90°. The number of particles per unit cell is four. The ORTEP of the crystal shown in Figure 2. The packing of EAP along c axis is given as Figure 3. The relevant experimental parameters of this compound are listed in Table 1. Selected bond lengths and bond angles are given in Table 2.

The carbon–oxygen bond lengths at C1 and C4 are 1.236 and 1.218 Å respectively. This corresponds to that of a carbonyl group and substantiates the presence of quinone moiety. The carbon–oxygen bond length at C1" is also in the keto range.

3.3 Insilico pharmacological studies

The Lipinski's rule of five was also verified for embelin and EAP. The same was done for the standard drug 5–FU as well. The results of the ADME test with their limiting values are given in Table 3. It is evident from the table that, the compounds under investigation obeyed the Lipinski's rule of five and have good pharmacokinetic parameters. EAP had just one violation which is admissible. Thus they are eligible to be selected as promising candidate for *in vitro* analysis.

The aptness of a molecule to perform as a drug is to be assessed by finding out its binding energy. This result articulates how well a drug binds to the target molecule. This is performed by docking the molecule with a protein. Molecular docking studies serve as a green technique which helps in screening small molecules by orienting them in the binding sites of proteins. In the present study, embelin, its EAP derivative and the standard anticancer drug 5–FU were docked with PI3K protein using iGEMDOCK software. The binding energies varied with the ligand revealing their different affinities with the target protein. These results are depicted in Table 4.

It is evident from the table that EAP possesses the highest negative dock score indicating the best activity among the three molecules under investigation. Also the docking score was significantly better (–111.63 kcal mol^{-1}) than that of the standard drug 5–FU (–67.74 kcal mol^{-1}). This indicates that embelin and particularly its derivative under study have the potential to be lead molecules in the treatment of cancer. The best docking poses of the ligand, EAP and its parent embelin are given in Figure 4 and Figure 5 respectively.

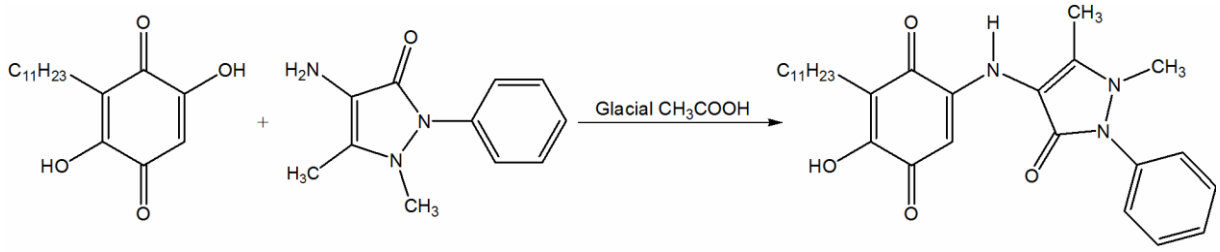
As the results of *in silico* analysis were promising, the compounds were subjected to *in vitro* antiproliferative effect determination by MTT assay and the cytotoxicity assay by MTT Method. EAP and embelin were tested on human breast cancer cell line MCF–7 at various concentrations ranging from 6.25 to 100 µg mL^{-1}. The results of the study are given in Table 5. The LD_{50} values of embelin and EAP were found to be 21.515 and 16.980 µg mL^{-1} respectively. The lower LD_{50} value of EAP shows that it is a better candidate as a lead molecule when compared with embelin. This value was comparable to that of the standard drug molecule, 5–FU (15.477 µg mL^{-1}). However it was slightly higher than that of doxorubicin (9.138 µg mL^{-1}). The variation of percentage viability with concentration can be better understood form the comparison given in Figure 6.

Conclusions:

Malignancy owing to cancer and its treatment by using phytochemicals was the impetus behind the present investigation. A new derivative of embelin using 4-aminoantipyrine, was prepared. The single crystals of this compound were grown and characterized using single crystal X-ray diffraction techniques. It was then subjected to docking analysis along with embelin to check its suitability as a drug molecule. The investigation revealed that EAP is a better candidate as a lead molecule in the treatment of cancer. The results were then subjected to experimental validation to verify the results obtained by computational studies. The experimental results substantiated the results obtained in *in silico* analysis. Clinical validation can be carried out to establish the efficacy of this compound as a potent anticancer drug.

Crystallographic data reported in this manuscript have been deposited with Cambridge Crystallographic Data Centre as supplementary publication No.CCDC-1485941. Copies of the data can be obtained free of charge from the Cambridge Crystallographic Data Centre, 12, Union Road, Cambridge, CB2 IEZ,UK, fax:441223336033;or (deposit @ccdc.cam.ac.uk)

1. Figures and tables



Scheme 1. Synthesis of EAP

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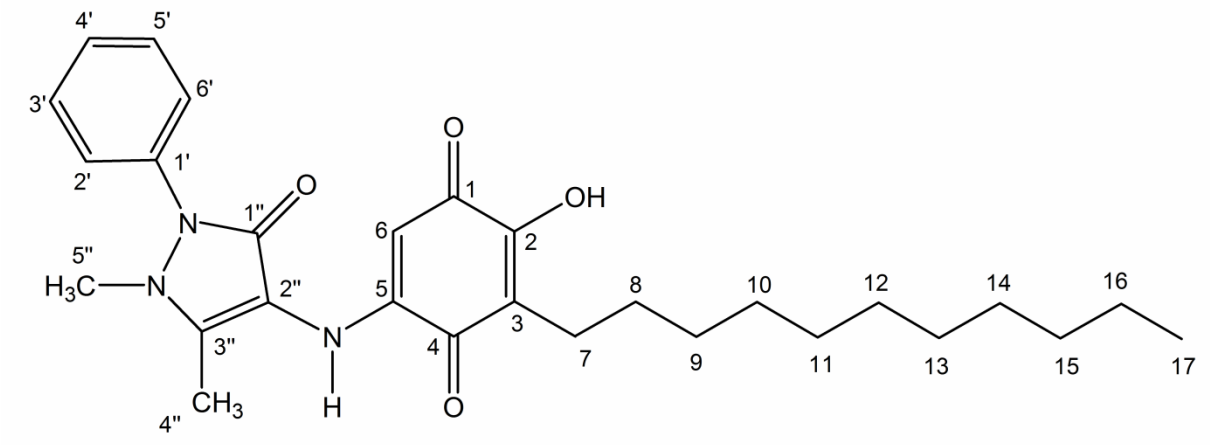

Figure 1. Structure of EAP

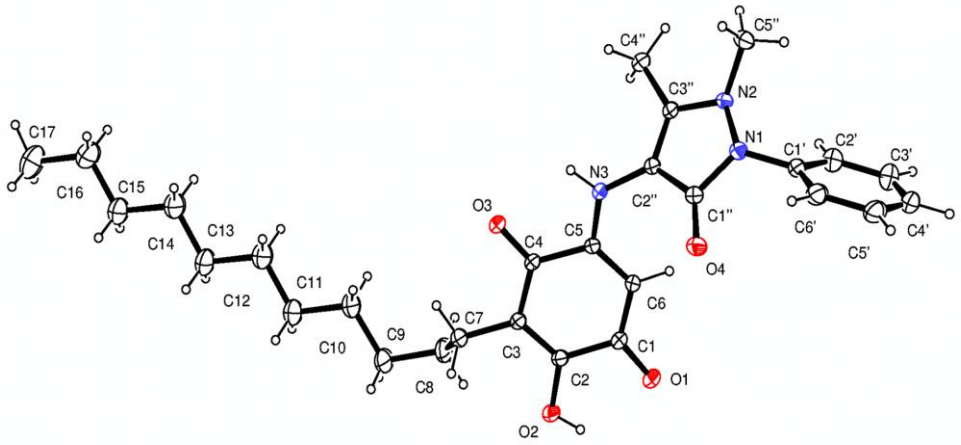

Figure2. ORTEP diagram of EAP

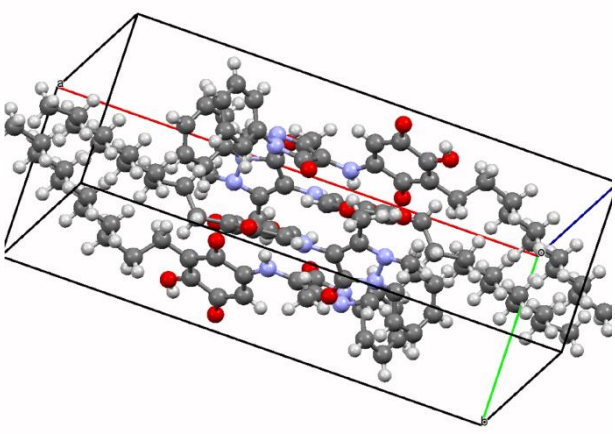

Figure 3. Packing of EAP along c axis

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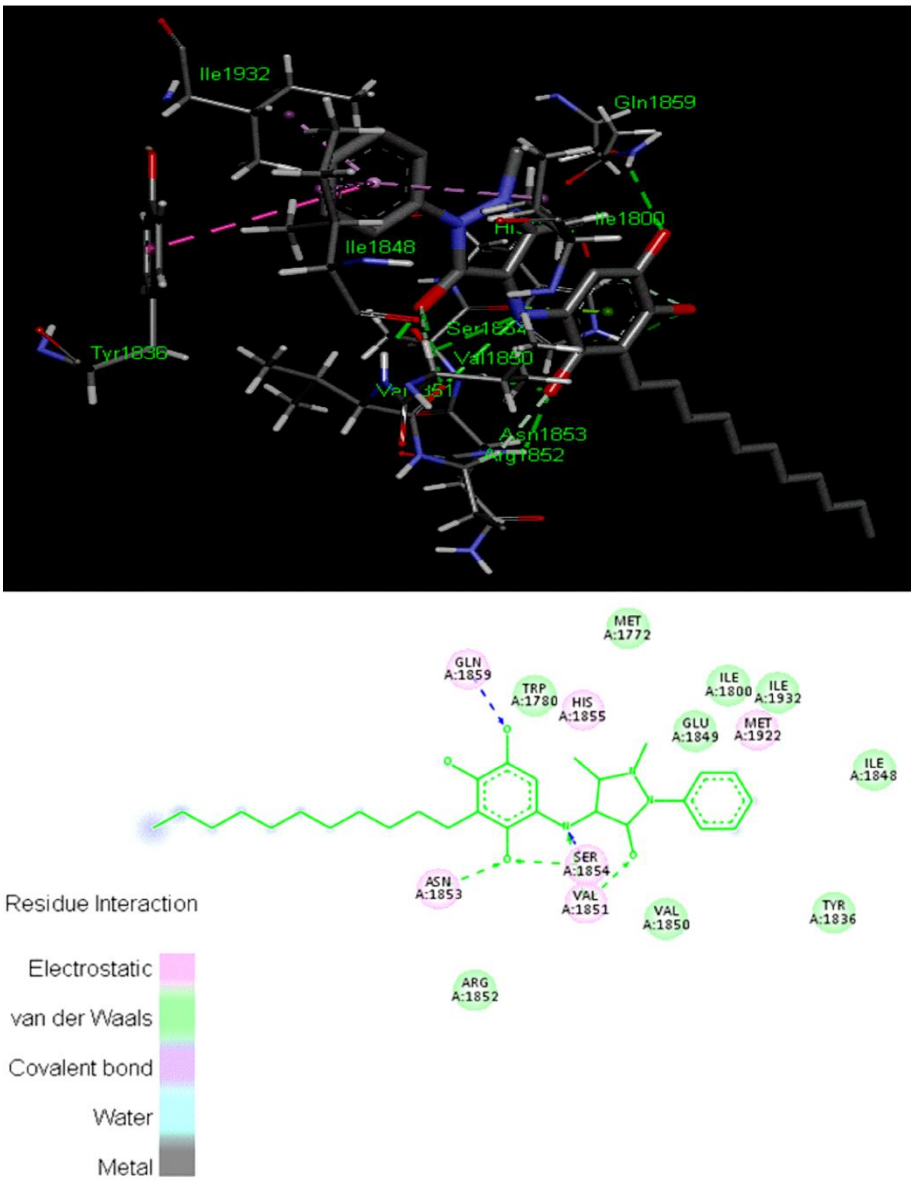

Figure 4. Docking pose of EAP

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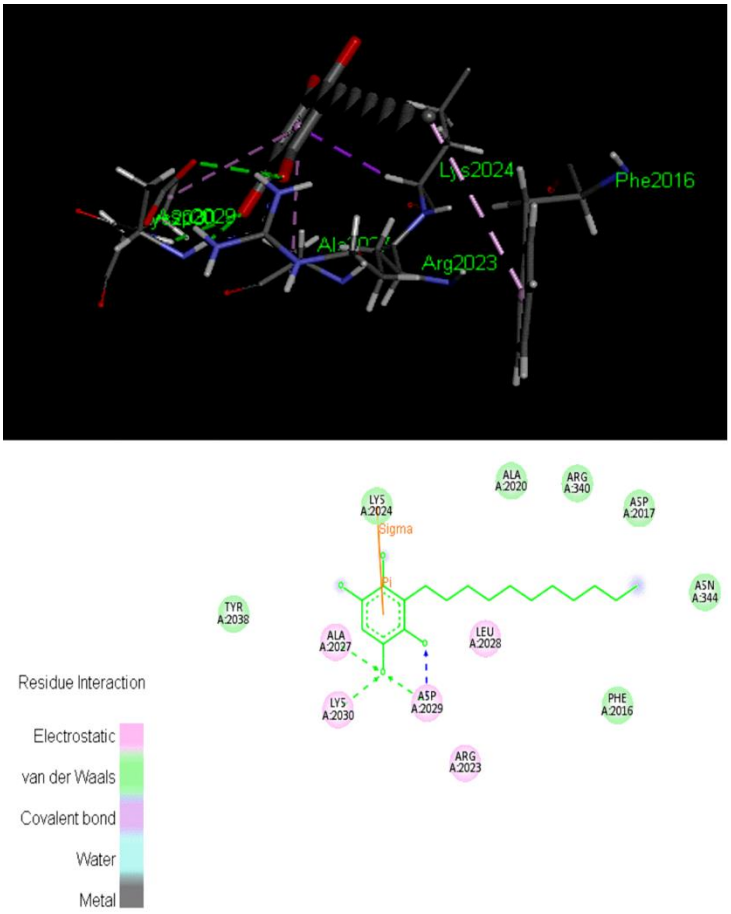

Figure 5. Docking pose of embelin

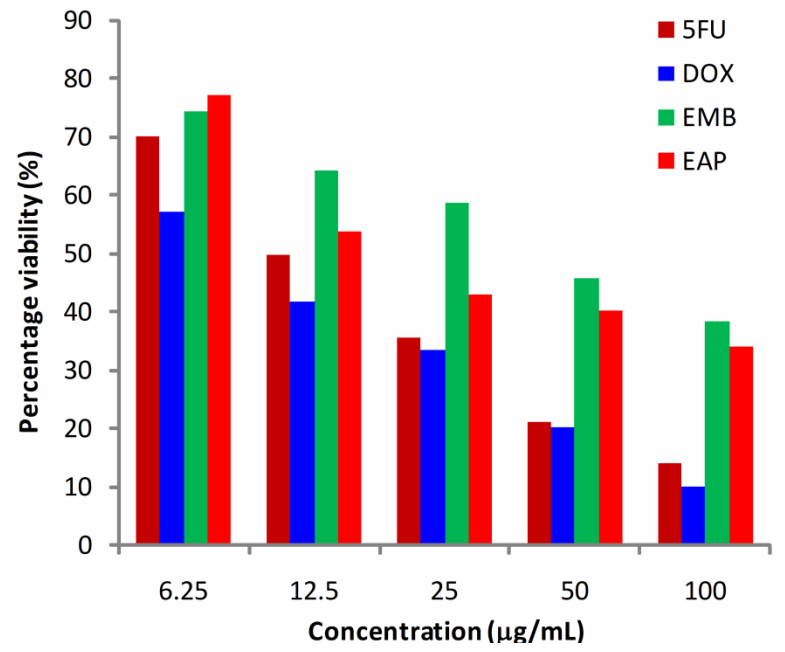

Figure 6. Variation of percentage viability with concentration

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Table 1. Crystallographic data for EAP

Empirical formula	$C_{28}H_{37}N_3O_4$
Formula weight	479.60
Temperature	296(2) K
Wavelength	0.71073 Å
Crystal system	Monoclinic
Space group	P $2_1/c$
Unit cell dimensions	a = 22.352(3) Å, b = 8.2257(9) Å, c = 14.3776(13) Å α = 90°, β = 95.307(5)°, γ = 90°.
Volume	2632.1(5) Å3
Z	4
Density (calculated)	1.210 mg/m3
Absorption coefficient	0.081 mm^{-1}
F(000)	1032
Crystal size	0.250 × 0.150 × 0.050 mm3
Theta range for data collection	2.640 to 25.997°
Index ranges	$-27 \leq h \leq 27$; $-10 \leq k \leq 8$; $-17 \leq l \leq 17$
Reflections collected	17825
Independent reflections	5157 [R(int) = 0.0527]
Completeness to theta = 25.242°	99.9 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.9960 and 0.9800
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	5157 / 0 / 317
Goodness-of-fit on F^2	1.003
Final R indices [I>2sigma(I)]	R1 = 0.0547, wR2 = 0.1294
R indices (all data)	R1 = 0.1341, wR2 = 0.1705
Extinction coefficient	n/a
Largest diff. peak and hole	0.262 and −0.244 e.$Å^{-3}$

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Table 2. Selected Bond lengths (Å) and bond angles (°) for EAP

Bond lengths			
C(1)–O(1)	1.236(3)	C(4)–C(5)	1.505(3)
C(1)–C(6)	1.403(3)	C(4')–C(5')	1.363(6)
C(1)–C(2)	1.502(3)	C(5)–N(3)	1.341(3)
C(1'')–O(4)	1.214(3)	C(5)–C(6)	1.352(3)
C(1'')–N(1)	1.407(3)	C(5'')–N(2)	1.464(3)
C(1'')–C(2'')	1.439(4)	C(5')–C(6')	1.373(4)
C(1')–C(2')	1.364(4)	C(7)–C(8)	1.501(4)
C(1')–C(6')	1.376(4)	C(8)–C(9)	1.532(4)
C(1')–N(1)	1.418(3)	C(9)–C(10)	1.508(4)
C(2)–O(2)	1.327(3)	C(10)–C(11)	1.515(4)
C(2)–C(3)	1.349(3)	C(11)–C(12)	1.502(5)
C(2'')–C(3'')	1.343(3)	C(12)–C(13)	1.516(4)
C(2'')–N(3)	1.405(3)	C(13)–C(14)	1.505(5)
C(2')–C(3')	1.376(4)	C(14)–C(15)	1.504(4)
C(3)–C(4)	1.445(3)	C(15)–C(16)	1.498(5)
C(3)–C(7)	1.500(3)	C(16)–C(17)	1.495(5)
C(3')–C(4')	1.354(6)	N(1)–N(2)	1.401(3)
C(3'')–N(2)	1.364(3)	N(3)–H(3)	0.8600
C(3'')–C(4'')	1.476(3)	O(2)–H(2)	0.8200
C(4)–O(3)	1.218(3)		
Bond angles			
O(1)–C(1)–C(6)	126.0(2)	C(3)–C(4)–C(5)	120.0(2)
O(1)–C(1)–C(2)	115.3(2)	C(3')–C(4')–C(5')	120.4(3)
C(6)–C(1)–C(2)	118.6(2)	N(3)–C(5)–C(6)	125.7(2)
O(4)–C(1'')–N(1)	123.3(2)	N(3)–C(5)–C(4)	113.4(2)
O(4)–C(1'')–C(2'')	132.7(2)	C(6)–C(5)–C(4)	120.9(2)
N(1)–C(1'')–C(2'')	104.0(2)	C(4')–C(5')–C(6')	120.6(4)
C(2')–C(1')–C(6')	120.2(3)	C(5)–C(6)–C(1)	119.8(2)
C(2')–C(1')–N(1)	121.0(3)	C(5')–C(6')–C(1')	118.9(3)
C(6')–C(1')–N(1)	118.7(3)	C(3)–C(7)–C(8)	114.7(3)
O(2)–C(2)–C(3)	121.2(2)	C(7)–C(8)–C(9)	113.1(3)
O(2)–C(2)–C(1)	115.0(2)	C(10)–C(9)–C(8)	112.9(3)
C(3)–C(2)–C(1)	123.8(2)	C(9)–C(10)–C(11)	114.6(3)
C(3'')–C(2'')–N(3)	126.3(2)	C(12)–C(11)–C(10)	113.3(3)
C(3'')–C(2'')–C(1'')	109.1(2)	C(11)–C(12)–C(13)	114.1(3)
N(3)–C(2'')–C(1'')	124.5(2)	C(14)–C(13)–C(12)	113.8(3)
C(1')–C(2')–C(3')	120.1(3)	C(15)–C(14)–C(13)	114.4(3)
C(2)–C(3)–C(4)	116.4(2)	C(16)–C(15)–C(14)	114.6(4)
C(2)–C(3)–C(7)	123.7(2)	C(17)–C(16)–C(15)	113.7(4)
C(4)–C(3)–C(7)	119.9(2)	N(2)–N(1)–C(1'')	109.21(19)
C(4')–C(3')–C(2')	119.7(4)	N(2)–N(1)–C(1')	118.6(2)
C(2'')–C(3'')–N(2)	110.1(2)	C(1'')–N(1)–C(1')	122.4(2)
C(2'')–C(3'')–C(4'')	129.3(2)	C(3'')–N(2)–N(1)	106.86(19)
N(2)–C(3'')–C(4'')	120.5(2)	C(3'')–N(2)–C(5'')	121.5(2)
O(3)–C(4)–C(3)	121.6(2)	N(1)–N(2)–C(5'')	114.6(2)
O(3)–C(4)–C(5)	118.4(2)	C(5)–N(3)–C(2'')	125.2(2)

Table 3. ADMET results of the ligands

	EMB	EAP	5–FU	Limiting value
S+log P	4.386	5.546	0.701	≤5
S+log D	2.517	3.725	0.918	<5
MlogP	1.906	3.511	0.005	<5
MWt	294.39	479.62	130.08	≤500
HBDH	2.000	2.000	2.000	≤5
M_NO	4.000	7.000	4.000	≤10
T_PSA	74.000	93.330	65.720	<140Å
Rule of 5	0	1	0	Rule of 5

Table 4. Binding energies of the ligands

Ligand	Total energy (kcal mol^{-1})	van der Waal's interaction	Hydrogen bonding	Electrostatic interaction
Embelin	−90.47	−71.1	−19.37	0
EAP	−111.63	−80.66	−30.97	0
5–FU	−67.74	−35.81	−31.93	0

Table 5. Antiproliferative effect of embelin and EAP

Concentration (µg mL^{-1})	Percentage Viability			
	5–FU	DOX	Embelin	EAP
Control	100	100	100	100
6.25	69.936±4.200	56.996±4.312	74.291±4.620	77.181±4.730
12.50	49.660±3.600	41.854±3.811	64.087±4.130	53.890±3.920
25.00	35.673±2.300	33.56±2.232	58.773±4.000	43.053±3.260
50.00	21.338±1.600	20.362±2.311	45.794±3.150	40.155±3.100
100.00	14.250±0.800	10.257±2.056	38.412±2.230	33.960±2.110
LD50 value	15.477	9.138	21.515	16.980

Acknowledgements:
The authors thank the Department of Computational Biology and Bioinformatics, Karyavattom, IISER Trivandrum, RGCB Trivandrum and Department of Chemistry, College of Engineering Trivandrum for providing the technical support and infrastructure.

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ISSN:2249-1236
Annette Fernandez et al. IJRRPAS, (2017) Vol.7 Issue.1 Pg:-1426-1434
International Journal of Research and Reviews in Pharmacy and Applied sciences