Research Article

Synthesis, characterization, molecular docking and evaluation of anticancer activity of 2- pyrazoline derivatives

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Abstract

Objectives: 5-Substituted aryl-2-pyrazoline derivatives (5a-f) were synthesized by Claisen Schmit reaction method for their molecular docking and evaluation of their anticancer activity. **Materials and Methods:** 2-pyrazoline derivatives (5a-f) were characterized by spectroscopic (FTIR, ^{1}H NMR, ^{13}C NMR and Mass) analysis after synthesis. All compounds were screened for their *in vivo* anticancer activity using Ehrlich ascites carcinoma (EAC) cells and their *in vitro* anticancer activity also performed against Human breast adenocarcinoma cell line (MCF-7). Molecular docking study was performed using the CDOCKER protocol in Discovery Studio 3.5. **Results:** In the *in vivo* assay, 1-(2,4-dinitrophenyl)-5-(2-chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5b) and 1-(2,4-dinitrophenyl)-5-(3-nitrophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5f) exhibited significant anticancer activity as compared to the standard drug 5- Fluorouracil (5-FU). But *in vitro* assay, compound 1-(2, 4-dinitrophenyl)-5-(4-chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5c) and 1-(2,4-dinitrophenyl)-5-(4-N,N dimethylaminophenyl)-3-(4-methoxyphenyl)-2 pyrazoline (5e) were emerged as more potent inhibitor of MCF-7 with IC_{50} 2.42±0.26 mg/l and 2.75±0.33 mg/l respectively when compared with standard drug 5-Fluorouracil (5-FU) with IC_{50} was 9.84±2.57 mg/l).. Docking simulation of these compounds with epidermal growth factor receptor (EGFR) protein was used to determine the best pose binding mode and supportive mechanism of action. **Conclusions:** Hence compound 1-(2, 4-dinitrophenyl)-5-(4- chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5c) and compound 1-(2, 4-dinitrophenyl)-5-(4-N, N-dimetyl amino phenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5e) could be considered as bioactive molecules for future development and research and hope to get more target specific, less toxic and potent anticancer activity.

Keywords: 2-Pyrazoline, MCF-7, EAC cells, MTT assay, Molecular docking, EGFR, 5-Fluorouracil

Introduction

Cancer is the 2nd largest life-threatening disease in the world. Seven million peoples die in different type of cancer per year and it is expected that more than 16 million new cases will be found in every year by 2020 (Jemal et al., 2011). Although there has been progress in the prevalence of cancer in India, it is about 2.5 million with about 8, 00,000 new cases and 5, 50,000 deaths per annum (Ali et al., 2011). Synthetic organic chemistry has always been a fundamental part of the highly integrated and multidisciplinary process of anticancer drug development (Denny 2002). In current scenario, the therapeutic uses of synthetic drug has played important role in the control and prevention of cancer. Especially heterocyclic compounds are found to be very potent drug in different ailments. Among them pyrazoline is one of the most important class. 2-Pyrazoline derivatives have a wide range of biological activity like analgesic (Samshuddin et al., 2012; James and Bhat, 2012), anti-inflammatory (Sharma et al., 2012), antimicrobial (Siddiqui et al., 2011; Özdemir et al., 2007), antimalarial (Özdemir et al., 2007), antitubercular (Taj et al., 2011), antiameobic (Wani et al., 2012), anticonvulsant, antidepressant (Ozdemir et al., 2007; Palaska et al., 2001), antipatrkinsonism (Amr et al., 2008), antileishmanial (Rizvi

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et al., 2012), antihyperglyceamic (Ovais et al., 2013), antihepatotoxic (Khalilullah et al., 2011), antioxidant (Isloor et al., 2013), angiotensin converting enzyme inhibitory (Bonesi et al., 2010), momoamine oxidase inhibitors (Mathew et al., 2013) and anticancer activity (Bashir et al., 2011; Hayat et al., 2010; Wang et al., 2013; Havrylyuk et al., 2009; Johnson et al., 2007; Khalil et al., 2013; Havrylyuk et al., 2011; Shaharyar et al., 2010; Sharma et al., 2014).

We planned to synthesise a series of new 5-substituted aryl-2-pyrazoline derivatives and these compounds have been characterised by spectroscopic data. These compounds have been used to evaluate their *in vitro* anticancer activity against MCF-7 cell line by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay method and *in vivo* anticancer activity against EAC cell on mice model. These compounds have been optimised by Density functional theory (DFT) computation and these stimulates have been used for molecular docking evaluation to support the mechanism of anticancer action.

Materials and methods

All the chemicals used in this study were procured from Spectrochem Pvt. Ltd., Mumbai, Merck specialties Pvt. Ltd., Mumbai, and S.D. Fine Chem. Ltd., Mumbai, without further purification. The compounds (5a-f) were synthesized by reported method (Choudhary et al., 2011). The melting points were determined by open capillary method using Veego melting point apparatus (VMP-DS) and were uncorrected. The purity of compounds was checked by thin layer chromatography (TLC) on silica gel-G plate of 0.5 mm and developed plates were visualized by iodine vapour or UV light. Fourier transform Infrared (FT-IR) spectra were recorded on a Bruker FTIR ALPHA in the range 4000-400 cm^{-1}. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Ultra shield™300 MHz spectrometer using Deuterated chloroform ($CDCl_3$) as solvent and Tetramethylsylsilane (TMS) as internal standard (chemical shifts in δ ppm). Splitting patterns was designated as s (singlet), d (doublet), t (triplet), q (quart) and m (multiplet). Mass spectra were recorded on a G2 QTOF XEVO mass spectrometer. UV was measured by multimode plate reader Spectra Max M5; Molecular Devices. Elemental analysis was recorded on Perkin Elmer 2400 series II.

Procedure for the synthesis of 3- (substituted aryl)-1-(4-methoxyphenyl)-prop-2-en-1-one (3a-f)

A mixture of 4-methoxyacetopheone (0.01 mol) 1 and aromatic aldehyde (0.01 mole) 2 was dissolved in 10 ml ethanol in a 250 ml of round-bottom flask fitted with a magnetic stirrer. After 30 minutes 10 ml of sodium hydroxide solution (10%) was added drop wise into the reaction mixture. Then solution becomes turbid and the reaction mixture was maintained between 10-15^{0}C by using ice water batch. After vigorous stirring for 5-6 hr the mixture was neutralized by adding 0.1-0.2N HCl where the precipitation occurred. Then precipitate was collected by filtration, dried and recrystallized from ethanol/ethyl acetate to get corresponding 3- (substituted aryl)-1-(4-methoxyphenyl)-prop-2-en-1-one (3a-f) (Choudhary et al., 2011).

Procedure for the synthesis of 5-substituted aryl-2-pyrazoline derivatives (5a-f)

These 3- (substituted aryl)-1-(4-methoxyphenyl)-prop-2-en-1-one (0.01 mole) 3a-f and 2, 4-dinitrophenyl hydrazine (0.01 mole) 4 was dissolved in 20 ml of glacial acetic acid in a 250 ml of round bottom flask. Then reaction mixture was refluxed for 12-14 hrs. The completion of the reaction was monitored by TLC. After completion of the reaction the product was poured in crushed ice and allowed to stand overnight. The precipitate was collected by filtration, dried and recrystallized from water: methanol/ethanol, ethyl acetate to get desired 5-substituted aryl-2-pyrazoline derivatives (5a-f) (Samshuddin et al., 2012).

1-(2, 4-dinitrophenyl)-3, 5-bis-(4-methoxyphenyl)-2-pyrazoline (5a)

Light yellow; yield 64%: m.p 92-94^{0}C; R_f 0.67; Anal. calc for ($C_{23}H_{20}N_4O_6$), %: C=61.60, H=4.50, N=12.49, O=21.41; found: C=63.34, H=4.56, N=11.89, O=19.21. FT-IR ν_{max} Cm^{-1}: (aromatic C-H) 3011, (aliphatic C-H) 2841, (pyrazoline C=N) 1653, (aromatic NO_2)1418, 1506, (OCH_3)1166. ^{1}HNMR (300MHz, $CDCl_3$ δ, ppm): 1.95 [1H, d, H-4 (pyrazoline)]; 3.77 [3H, t, OCH_3-4]; 3.84 [3H, t, OCH_3-4']; 3.92 [1H, t, H-5 (pyrazoline)]; 6.86 [2H, d, H-3,5]; 6.94 [2H, d, H-3',5']; 6.94 [1H, d, H-6"]; 7.16 [1H, d, H-2,6]; 7.62 [2H, d, H-2'6']; 8.41 [1H, d, H-5"]; 8.42 [1H, s, H-3"]. ^{13}C NMR (300MHz, $CDCl_3$ δ, ppm): 55.08 (22-C), 55.16 (23-C), 76.57 (7-C), 77.00 (8-C), 114.11 (12-C, 14-C, 18-C, 20-C), 119.12 (3-C), 127.44 (6-C), 129.87 (10-C, 11-C, 15-C, 17-C, 21-C), 130.42 (2-C, 4-C), 130.98 (5-C), 143.49 (1-C), 161.25 (C-19), 163.02 (13-C). MS: m/z 449.0386 [M+1]. λmax:380 nm.

1-(2, 4-dinitrophenyl)-5-(2-chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5b)

Light yellow; yield 70%: m.p 91-93^{0}C; R_f 0.72; Anal. calc for ($C_{22}H_{17}ClN_4O_5$), %: C=58.35, H=3.78, Cl=7.83, N=12.37, O=17.67.; found C=69.48, H=4.40, N=1.33. FT-IR ν_{max} Cm^{-1} :(aromatic C-H) 3012, (aliphatic C-H) 2811, (pyrazoline C=N) 1650, (aromatic NO_2)1417, 1505, (OCH_3)1175, (C-Cl) 633, 752. ^{1}HNMR (300MHz, $CDCl_3$ δ, ppm): 1.95 [1H, d, H-4 (pyrazoline)]; 3.89 [3H, t, OCH_3-4']; 3.98 [1H, t, H-5 (pyrazoline)]; 6.99 [1H, d, H-6"]; 7.01 [1H,

d, H-6]; 7.05 [1H, t, H-5]; 7.13 [1H, t, H-4]; 7.18 [1H, d, H-3]; 7.33 [1H, d, H-3']; 7.34 [H, t, H-5']; 7.43 [1H, d, H-6']; 8.36 [1H, d, H-5"]; 9.06 [1H, s, H-3"]. ^{13}C NMR (300MHz, $CDCl_3$ δ, ppm): 55.29 (22-C), 76.58 (7-C), 77.00 (8-C), 113.70 (12-C, 14-C), 124.30 (3-C, 6-C), 126.90 (20-C), 127.55 (10-C, 11-C, 15-C, 18-C, 19-C, 21-C), 130.02 (5-C), 133.14 (17-C), 135.13 (2-C, 4-C), 139.40 (1-C), 163.36 (13-C).MS: m/z 453.1209 [M+1] λmax: 400 nm.

1-(2, 4-dinitrophenyl)-5-(4-chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5c)

Yellow; yield 65%: m.p 121-123^{0}C; R_f 0.65; Anal. calc for ($C_{22}H_{17}ClN_4O_5$), %: C=58.35, H=3.78, Cl= 7.83, N=12.37, O=17.67; found C=60.04, H=4.20, N=7.01. FT-IR ν_{max} Cm^{-1}: (aromatic C-H) 2961, (aliphatic C-H) 2839, (pyrazoline C=N) 1655, (aromatic NO_2)1406, 1509, (OCH_3)1175, (C-Cl) 631, 746. ^{1}HNMR (300MHz, $CDCl_3$ δ, ppm): 1.95 [1H, d, H-4 (pyrazoline)]; 3.89 [3H, t, OCH_3-4']; 3.93 [1H, t, H-5 (pyrazoline)]; 6.99 [1H, d, H-6"]; 7.05 [1H, d, H-2]; 7.10 [1H, d, H-3]; 7.15 [1H, d, H-5]; 7.33 [1H, d, H-3']; 7.34 [1H, d, H-5']; 7.43 [1H, d, H-6']; 8.36 [1H, d, H-5"]; 9.06 [1H, s, H-3"]. ^{13}C NMR (300MHz, $CDCl_3$ δ, ppm):55.39 (22-C), 76.57 (7-C), 77.00 (8-C), 113.79 (12-C, 14-C), 122.12 (3-C, 6-C), 129.40 (10-C, 11-C, 15-C, 17-C, 18-C, 20-C, 21-C), 130.72 (5-C), 133.43 (19-C), 136.05 (2-C, 4-C), 142.33 (1-C, 16-C), 163.44 (13-C). MS: m/z 475 [M+ Na]. λmax: 340 nm.

1-(2, 4-dinitrophenyl)-5-(2-furfuryl)-3-(4-methoxyphenyl)-2-pyrazoline (5d)

Light yellow; yield 55%: m.p 70-72^{0}C; R_f 0.52; Anal. calc for ($C_{20}H_{16}N_4O_6$), %: C=58.82, H=3.95, N=13.72, O=23.51; found C=57.35, H=4.49, N=9.82. FT-IR ν_{max} Cm^{-1}: (aromatic C-H)3072, (aliphatic C-H)2840, (pyrazoline C=N)1656, (aromatic NO_2)1414, 1503,(OCH_3)1168, (-O-)1009. ^{1}HNMR (300MHz, $CDCl_3$ δ, ppm): 1.61 [1H, d, H-4 (pyrazoline)]; 3.81 [3H, t, OCH_3-4']; 3.91 [1H, t, H-5 (pyrazoline)]; 6.50 [1H, d, H-3,5(furan)]; 6.51 [2H, t, H-4(furan)]; 6.99 [1H, d, H-6"]; 7.44 [1H, d, H-5']; 7.49 [1H, d, H-3']; 7.52 [1H, d, H-2']; 7.56 [1H, d, H-6']; 8.06 [1H, s, H-3"]; 8.09 [1H, d, H-5"]. ^{13}C NMR (300MHz, $CDCl_3$ δ, ppm): 55.51 (20-C), 76.65 (7-C), 77.07 (8-C), 112.64 (17-C, 18-C), 113.84 (12-C, 14-C), 119.16 (3-C), 130.76 (6-C, 10-C, 11-C, 15-C), 131.04 (5-C), 144.73 (1-C, 19-C), 151.80 (9-C, 16-C), 163.43 (13-C). MS: m/z 409 [M+1]. λmax:390 nm.

1-(2,4-dinitrophenyl)-5-(4-N,N-dimethylaminophenyl)-3-(4-methoxyphenyl)-pyrazoline (5e)

Brick red; yield 45%: m.p 78-80^{0}C; R_f 0.55; Anal. calc for ($C_{24}H_{23}N_5O_5$), %: C=62.46, H=5.02, N=15.18, O=17.34; found C=66.80, H=5.09, N=10.87. FT-IR ν_{max} Cm^{-1}: (aromatic C-H) 3005, (aliphatic C-H) 2839, (pyrazoline C=N) 1667, (aromatic NO_2)1420, 1509, (OCH_3)1171, (C-N<) 1253. ^{1}HNMR (300MHz, $CDCl_3$ δ, ppm): 1.68 [1H, d, H-4 (pyrazoline)]; 3.01 [3H, t, CH_3-4]; 3.11 [3H, t, CH_3-4]; 3.81 [3H, t, OCH_3-4']; 3.90 [1H, t, H-5 (pyrazoline)]; 6.97 [1H, d, H-6"]; 7.01 [1H, d, H-5]; 7.07 [2H, d, H-3,6]; 7.12 [1H, d, H-2]; 7.05 [1H, d, H-3']; 7.28 [1H, d, H-5']; 7.64 [2H, d, H-2'6']; 8.05 [1H, s, H-5"]; 8.40 [1H, d, H-3"]. ^{13}C NMR (300MHz, $CDCl_3$ δ, ppm): 43.63 (23-C, 24-C), 55.44 (22-C), 76.67 (7-C), 77.09 (8-C), 112.83 (18-C, 20-C), 114.31 (12-C, 14-C), 122.23 (3-C), 123.51 (6-C), 126.80 (17-C, 21-C), 128.40 (5-C, 10-C, 11-C, 15-C), 136.89 (16-C), 137.04 (2-C, 4-C), 140.22 (1-C), 150.43 (19-C), 153.93 (9-C), 161.55 (13-C). MS: m/z 461.2 [M+1]. λmax: 400 nm.

1-(2, 4-dinitrophenyl)-5-(3-nitrophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5f)

Brick red: yield 80%; m.p 150-151^{0}C; R_f 0.54; Anal. calc for ($C_{22}H_{17}O_7N_5$), %: C=57.02, H= 3.70, N= 15.11, O= 24.17; Found: C= 66.38; H=4.19, N=6.43. FT-IR ν_{max} Cm^{-1}: (aromatic C-H) 3070, (aliphatic C-H) 2844, (pyrazoline C=N) 1650, (aromatic NO_2)1420, 1503, (OCH_3)1174. ^{1}HNMR (300MHz, $CDCl_3$ δ, ppm): 1.65 [1H, d, H-4 (pyrazoline)]; 3.92 [3H, t, OCH_3-4']; 3.95 [1H, t, H-5 (pyrazoline)]; 7.01 [1H, d, H-6"]; 7.04 [2H, d, H-3',5']; 7.94 [2H, d, H-2'6']; 8.09 [2H, d, H-2,4]; 8.10 [1H, s, H-3"]; 8.53 [1H, d, H-5"]. ^{13}C NMR (300MHz, $CDCl_3$ δ, ppm): 55.52 (22-C), 76.62 (7-C), 77.04 (8-C), 113.98 (12-C, 14-C, 17-C, 19-C), 122.19 (3-C), 124.41 (6-C), 129.52 (10-C, 11-C, 15-C, 20-C, 21-C), 130.94 (5-C), 136.81 (2-C, 4-C), 140.75 (1-C), 148.65 (9-C, 16-C, 18-C), 163.78 (13-C). MS: m/z 463.11 [M+1]. λmax: 350 nm

***In vitro* MTT antiproliferative assay**

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method was used to determine the effects of 5-substituted aryl-2-pyrazoline derivatives (5a-f) on cell proliferation in the MCF-7 cell line. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) without phenol red and supplemented with 10% fetal bovine serum. The cell culture medium was maintained at 37°C in a humidified incubator containing 5% CO_2 atmosphere. To evaluate the cell cytotoxicity/viability, the cells were evenly distributed at a density of 5×10^3cells/well (optimal seeding density) in 96 well plates and it was incubated at 37°C in 5% CO_2 incubator (Model MCO-15AC; Sanyo Electric Biomedical Co. Ltd., Osaka, Japan) overnight. Then the medium in the wells was replaced by fresh medium with or without 5-substituted aryl-2-pyrazoline derivatives (5a-f) as well as standard drug 5-Fluorouracil (5-FU) with varying concentrations and incubated for 48 hours. Subsequently, the medium in each

well was replaced with 20 µl MTT (5mg/ml in PBS) and the incubation was continued for 4 hours. The medium in each well containing unbound MTT and death cells was removed by suction. The purple-blue formazan precipitate was dissolved in 100 µl dimethylsulfoxide and the absorbance of each well was measured at a wavelength of 570 nm on a Microplate reader (Multimode plate reader, Spectra Max M5; Molecular Devices, CA, USA). All the experiments were performed in triplicate. The IC_{50} was calculated as the concentration of compounds that achieved a 50% inhibition of cell viability. The percentage cell viability of the compound was calculated by:

$$\% \text{ Cell viability} = \left[\frac{(\text{Absorbance of test sample})}{(\text{Absorbance of the control sample})}\right] \times 100$$

***In vivo* anticancer activity**

All tested compounds (5a-f) were administered at dose of 400 mg/kg, did not show any gross behavioural change or mortality up to 24 hr. Swiss albino mice of 10 weeks old with an average body weight 20 to 25 gm were used in this experiment. All mice were kept in good laboratory conditions supplied basal diet after dividing into nine groups (n=12). All methodology related to animal experiment were assessed and permitted by the University Animals Ethical Committee (Registration No: AEC/PHARMA/1501/08/2015), Jadavpur University, India. EAC cells were collected from Chittaranjan National Cancer Institute, Kolkata and suspended in sterile isotonic solution (0.9% w/v NaCl). The numbers of tumour cells per ml of this suspension were measured under microscope by using haemocytometer. All these groups of animals were injected with EAC cell (2 x 10^6 cell/mice) intraperitoneally except the normal control group (I). This was taken as zero days. Animals were allowed for 24 hr inoculation for the cancer to grow in the body before starting the drug administration. On 1st day 5 ml/kg, body weight of normal saline (0.9% w/v NaCl) was administered in normal control groups (I) as well as EAC control groups (II). After inoculation the synthesized compounds (5a-f) (400 mg/kg body weight/day) was given orally (III-VIII) and the standard drug 5-FU (20 mg/kg body weight/day) intraperitoneally were administered in groups (IX) for 7 days respectively. On the 9th day food and water were withheld 18 hr before start of dissection of animals. The weights of animals were recorded before and after sacrifice. Then blood was collected from heart; ascites fluids were collected from peritoneal cavity and liver was taken for histopathology study. Remaining animals were kept for observing mean survivable time (Singh et al., 2010).

The anticancer activities of the compounds were measured in EAC animals with respect to the following parameters such as: tumour weight, viable and non viable tumour cell counts were measured according to Ghosh et al. (2011) and Dash et al. (2010). The haematological parameter like haemoglobin (HB), red blood cells (RBC), white blood cells (WBC), Serum glutamic oxaloacetate transaminase (SGOT), Serum glutamic pyruvate transaminase (SGPT), bilirubin, total protein and creatinine were measured according to Ghosh et al. (2011).

The mean survival time (MST) of treated animals was calculated by using the formula:

$$\text{MST} = \frac{\text{Day of first death} + \text{day of last death of animal}}{2}$$

The percentage increases in the life span (% ILS) was calculated by using the formula:

$$\% \text{ ILS} = \left[\frac{(\text{Mean survival of treated group} - \text{mean survival of control group})}{(\text{Mean survival of control group})} - 1\right] \times 100$$

The MST and % ILS was calculated according to Bala et al. (2010).

Statistical analysis

All Statistical results were calculated by using Graph Pad Prism software. The values were recorded as mean ± standard error mean (SEM). The data were analyzed by using Analysis of variance (ANOVA); deference below the 0.001 level ($p<0.05$) were considered as statistically significant.

DFT and Docking studies

Geometry optimization of all the compounds was performed by DFT/B3LYP method using Gaussian 09 software (Becke 1993; Lee 1988). 6-311G+ (d,p) basis set were used for C, H, N, O. The vibrational frequency calculations were performed to ensure that the optimized geometries represent the local minima and there are only positive eigenvalues. Theoretical UV-Vis spectra were calculated by time-dependent-DFT/B3LYP method in methanol using conductor-like polarizable continuum model (CPCM) was used to calculate the fractional contributions of various groups to each molecular orbital (Barone et al., 1998; Cossi et al., 2001; Cossi et al., 2003; O'Boyle et al., 2008).

Molecular docking studies allow to predict the best interaction of small molecules (ligands) with proteins (enzyme)(Kirkpatrick 2004). In the present work, docking study was performed using the CDOCKER protocol in Discovery Studio 3.5 (Ojha and Roy, 2010). (Discovery Studio 3.5, Accelrys, Inc. San Diego, CA) in order to identify the interaction between compounds and EGFR or Human epidermal growth factor receptor-2 (HER-2) as well as to visualize the probable binding mode. Docking study has been conducted for all synthetic compounds into the active site of the receptor EGFR and HER-2. The crystal structures of EGFR (PDB code: 1M17) complex were retrieved from the RCSB Protein Data Bank

(http://www.rcsb.org/pdb). The site sphere was selected based on the ligand binding location after preparing the receptor and ligand. Ligand preparation was done by using Prepare Ligand module in Receptor-Ligand interactions tool of Discovery studio 3.5. Energy minimized ligand structure of the compounds were used for docking. Protein preparation was also done under Prepare Protein module of Receptor-Ligand interactions tool of Discovery Studio 3.5. The prepared protein was considered as receptor and active site was selected based on the ligand binding domain of the compounds, the pre-existing ligand was removed and prepared ligand was placed. Most favorable docked pose was selected based on the minimum free energy of protein-ligand complex and analyzed to investigate the interaction.

ADMET prediction

Adsorption, distribution, metabolism, excretion and toxicity (ADMET) is under descriptor module of small molecules protocol of Discovery studio client 3.5. Drug likeness of a compound could be checked following Lipniski's rule of five (Lipinski et al., 2001).

Results and discussion

In the present study a new series of 5-substituted aryl-2-pyrazoline derivatives (5a-f) have been synthesized by the synthetic route outlined in figure 1. The synthesized compounds were confirmed on the basis of melting point, IR, ^{1}H NMR, ^{13}C NMR, Mass, DFT and Elemental analysis.

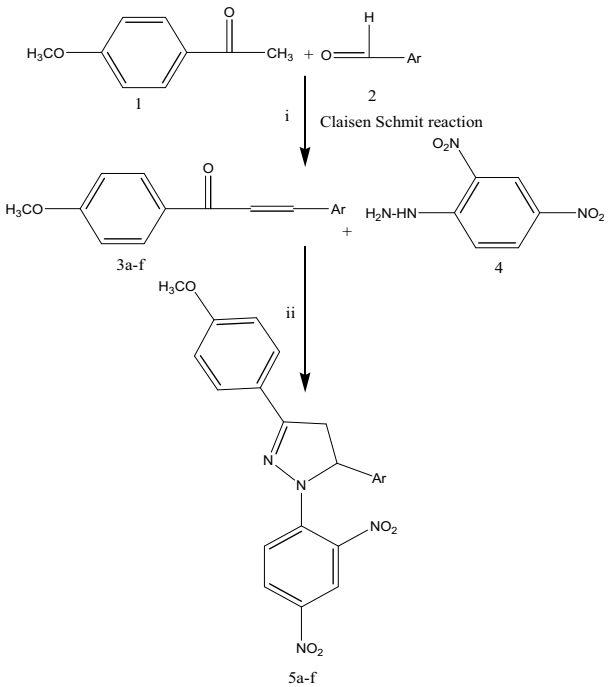

Figure 1. Scheme showed the synthesis of 5-substituted aryl-2-pyrazoline derivatives (5a-f), Reagent and conditions: (i) NaOH, C_2H_5OH, stirring, 6-8 h; (ii) Reagent and conditions: CH_3COOH, reflux, 12-14 h. Ar = -C_6H_4(*p*-OCH_3) (a), -C_6H_4(*o*-Cl) (b), -C_6H_4(*p*-Cl) (c), -C_4H_3O (d), -C_6H_4 (*p*-NMe_2) (e), -C_6H_4(*m*-NO_2) (f).

The FTIR spectrum of all compounds (5a-f) showed bands ranging from 1176-1166 cm^{-1} for OCH_3. However, all compounds displayed bands ranges about 1538-1406 cm^{-1} for aromatic NO_2 and bands at 1667-1607 cm^{-1} for pyrazoline C=N group. In addition, all compounds displayed bands range 2850-2811 cm^{-1} for aliphatic C-H and bands range 3071-2961 cm^{-1} for aromatic ring. The ^{1}H NMR spectra of all compounds (5a-f) show 1H, d, H-4 (pyrazoline) as doublet signal at 1.65-1.95 ppm and 1H, d, H-5 (pyrazoline) as triplet at 3.90-3.95 ppm which proves 2-pyrazoline ring formation. The OCH_3 appears triplet at 3.87-3.91 pmm and aromatic hydrogen show triplet at 6.99-9.00 ppm was formed. ^{13}C NMR spectra of all compounds have shown that aliphatic-Cs resonate at 76-77 ppm while aromatic-Cs appear at 120-140 ppm. The composition of the compounds has been supported by mass spectroscopy and elemental analysis.

All the synthesized compounds (5a-f) have been evaluated for *in vitro* cytotoxic activity against MCF-7 cell line by MTT assay method. The results are summarized in table 1. Screening results of *in vitro* anticancer study (Table 1) revealed that the compound 1-(2,4-dinitrophenyl)-5-(4-chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5c) (IC_{50}=2.42 ±0.26 mg/l) and compound 1-(2,4-dinitrophenyl)-5-(4-N,N-dimethylaminophenyl)-3-(4-methoxyphenyl)-2 pyrazoline (5e) (IC_{50}=2.75±0.33 mg/l) exhibited most potent anticancer activities as compared to standard drug 5-FU (IC_{50}=9.84±2.57 mg/l). In addition, compounds 1-(2, 4-dinitrophenyl)-5-(2-furfurylphenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5d) (IC_{50}= 3.63±0.65 mg/l) and compound 1-(2, 4-dinitrophenyl)-3,5-bis-(4-methoxyphenyl)-2-pyrazoline (5a) (IC_{50}=3.93±0.51 mg/l) have displayed moderate activities. On the other hand, 1-(2, 4-dinitrophenyl)-5-(2-chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5b) (IC_{50}= 4.25±0.59 mg/l), 1-(2, 4-dinitrophenyl)-5-(3-nitrophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5f) (IC_{50}=4.82±0.72 mg/l) show low antiproliferative activities with respect to 5-FU.

Table 1. IC_{50} values of tested compound against MCF-7 Cell Line by MTT Assay

Compounds	MCF-7 IC_{50} (mg/L)
5a	3.93 ±0.51
5 b	4.25 ± 0.59
5 c	**2.42 ±0.26**
5 d	3.63 ±0.65
5 e	**2.75 ±0.33**
5 f	4.82 ±0.72
5-FU	9.84 ±2.57

Table 2. Anticancer activity of 2-pyrazoline derivatives (5a-f) against % of viable cell, % of non-viable cell, tumour weight, % TWI and tumour volume EAC bearing mice

Groups	Compounds	Dose of drug (mg/kg)	% of viable cell	% of non viable cell	Tumour weight (gm)	%TWI	Tumour volume (ml)
I	Normal control	#	-	-	-	-	-
II	EAC control	#	90.89±2.02	9.09±2.00	3.65±0.25	-	1.75±0.07
III	EAC+5a	400	87.11±1.22	12.88±1.22	2.31±0.11**	36.71	1.25±0.07*
IV	EAC+ 5b	400	54.73±1.90**	45.27±0.92**	1.82±0.07**	50.13	1.00±0.14**
V	EAC+5c	400	88.67±1.69	11.33±1.69	2.45±0.19**	32.87	1.31±0.16*
VI	EAC+5d	400	69.73±2.91**	18.36±2.84*	2.51±0.13**	31.23	1.33±0.05*
VII	EAC+5e	400	87.11±3.63	11.39±1.49	2.62±0.25**	28.21	1.53±0.10
VIII	EAC+5f	400	61.40±3.09**	30.50±2.90**	1.92±0.03**	47.39	0.95±0.08**
IX	EAC+5FU	20	18.93±1.77**	81.07±1.77**	0.91±0.10**	74.90	0.52±0.07**

= 5 ml/kg, normal saline; Values are mean ± SEM. n=6 animal in each group. Experimental groups were compared with EAC control; *p≤0.05, **p

Table 3. Anticancer activity of 2-pyrazoline derivatives against (5a-f) SGPT, SGOT, bilirubin, total protein and creatinine EAC bearing mice

Groups	Compounds	Dose of drug (mg/kg)	SGPT units/l	SGOT units/l	Bilirubin mg/dl	Total protein gm/dl	Creatinine mg/dl
I	Normal control	#	33.66±2.14	38.0±1.73	0.75±0.06	8.48±1.31	0.32±0.01
II	EAC control	#	129.2±2.66	104±2.55	2.10±0.17	4.44±0.19	0.51±0.06
III	EAC+5a	400	71.33±1.33**	49.67±2.33**	1.50±0.19	5.13±0,58	0.45±9.04
IV	EAC+ 5b	400	83.50±1.72**	47.66±2.72**	1.11±0.17**	6.30±0.28	0.37±0.07
V	EAC+5c	400	93.50±1.91**	82.33±2.24**	1.60±0.22**	5.18±0.28	0.46±0/04
VI	EAC+5d	400	43.50±3.99**	58.33±2.17**	1.75±0.25**	5.98±0.91	0.43±0.05
VII	EAC+5e	400	55.00±4.63**	50.33±2.92**	1.55±0.26**	5.71±0.18	0.48±0.03
VII	EAC+5f	400	38.50±3.86**	51.67±3.45**	1.00±0.08**	6.85±0.42*	0.42±0.09
IX	EAC+5 FU	20	41.67±1.60**	40.67±2.52**	0.90±0.03**	7.45±0.63**	0.35±0.01

= 5 ml/kg, normal saline; Values are mean ± SEM. n=6 animal in each group. Experimental groups were compared with EAC control; *p≤0.05, **p

From aforementioned, result (Table 1) it is recommended that the compounds 5c and 5e have shown > 50% inhibition at a concentration of 100 µl/ml which is preferred for their *in vitro* anticancer activity against MCF-7 cell line by using MTT assay method.

In the present study, all these synthesized compounds (5a-f) were evaluated for *in vivo* cytotoxic activity against EAC cell bearing mice model in Table-2 and Screening results of *in vivo* anticancer study suggest that, compound 5b and 5f showed tumour weight inhibition (50.13%) and (47.39%) with respect to control 5-FU (74.90%). However, the viable and non viable tumour cell count of compound 5c have shown most significant ($p<0.01$) as compared to EAC control group. Moreover, in figure 2 it is revealed that the count of red blood cells and haemoglobin of compound 5a and 5c have shown gradually increased ($p<0.05$) as compared to EAC control group. In addition, the WBC cells count of all drug treated groups have shown gradually decreased ($p<0.05$) with respect to EAC control group.

Furthermore, in (Table 3) enzymatic parameters like SGPT

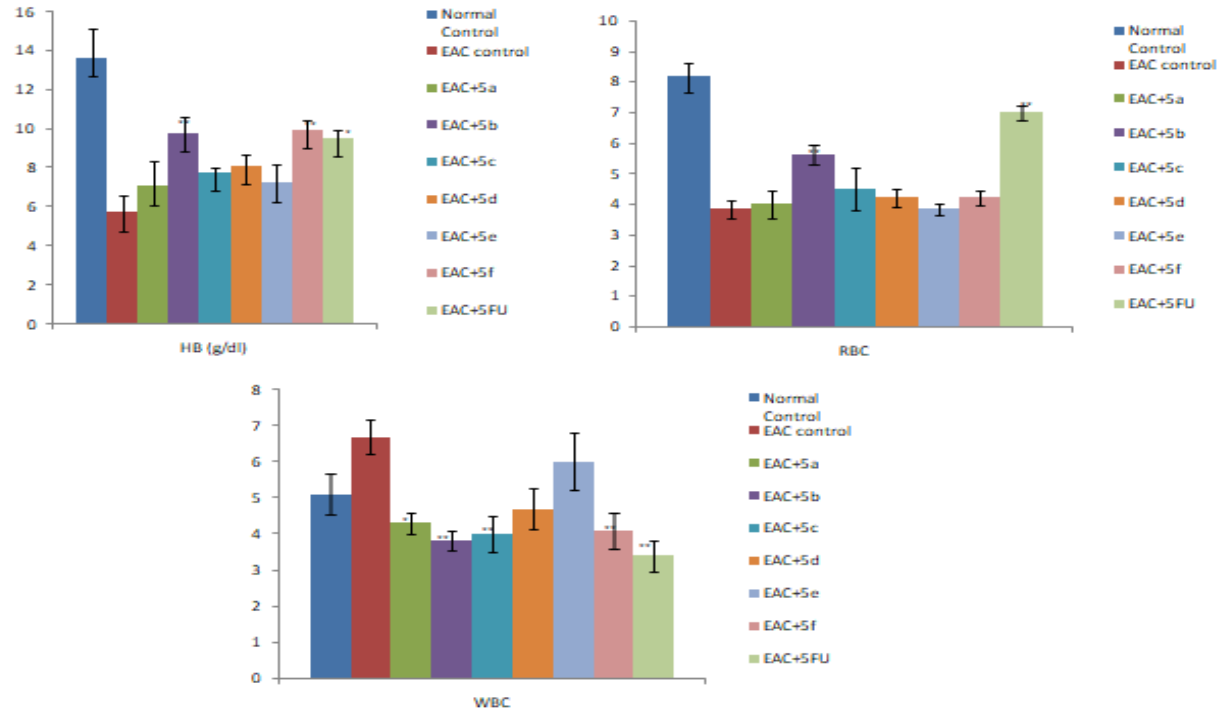

Figure 2. Effect of 2- pyrazoline derivatives (5a-f) on haematological parameters like HB, RBC, and WBC in EAC treated mice

Table 4. Anticancer activity of 2-pyrazoline derivatives (5a-f) against Mean Survival Time (MST) and increase in Life Spans (% ILS) EAC bearing mice

Groups	Compounds	MST (d)	%ILS
I	Normal control	-	-
II	EAC control	20	-
III	EAC+5a	28	40
IV	EAC+ 5b	34	70
V	EAC+5c	25	25
VI	EAC+5d	26	30
VII	EAC+5e	23	15
VIII	EAC+5f	30	50
IX	EAC+5FU	49	145

and SGOT level of all drug treated compounds exhibited significant difference ($p<0.01$) as compared to EAC control group. However, bilirubin levels of compound 5b and 5f have shown significant ($p<0.01$) decrease with respect to EAC control group. Whereas, the total protein level of drugs 5b and 5f treated groups have shown slight increase in level with respect to the EAC control group. In the table 4 reveals that compounds 5b and 5f shown mean survival time was 34 and 30 day respectably. In other hands the percentage of ILS estimation of compounds 5b and 5f shown to be 70% and 75% as compared to control 5- FU was 145%.

DFT computation and *in-silico* Docking study

The Density Functional Theory (DFT) computation has been used to generate stable optimised geometry (Figure 3) of the drugs and to calculate their population of molecular functions and energy. These data have been used to interpret the solution spectral properties and drug likeness feature as well as drug efficiency. The energy difference between HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital) functions refer to intramolecular charge transfer transitions that is supported by electronic spectral data. Crystallographic structure of EGFR is downloaded from RCSB protein data bank (PDB ID: 1M17) which was resolved at 2.60Å in X-Ray diffraction. Energy minimized structure of compounds (5a-f) are used for *in silico* protein ligand docking studies in the

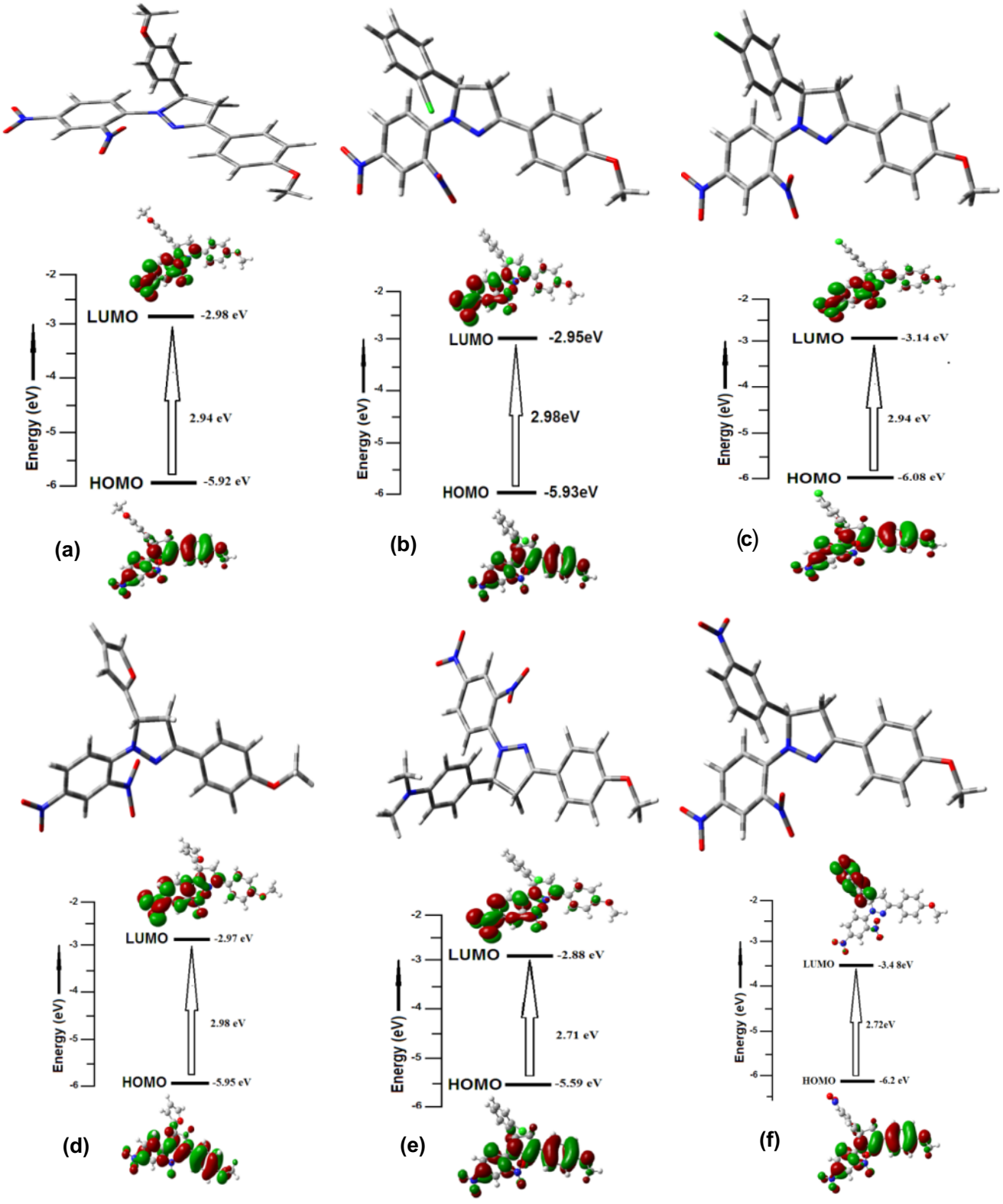

Figure 3. Optimised structures generated by DFT computation and HOMO-LUMO energy gap of (5a-f)

cavity of protein. There are total 12 amino acid residues (Leu694, val702, Ala719, Leu768, Met769, Gly772, Cys773, Asp776, Arg817, Asn818, Leu820, Asp831) surrounding the 5c in most favour binding position with the protein. On the other hand total 17 amino acids (Leu694, Phe699, Val702, Ala719, Lys721, Thr766, Leu768, Met769, Pro770, Phe771, Gly772, Cys773, Arg817, Asn818, Leu820, Thr830, Asp831) present in the binding sites those involve in the protein 5e

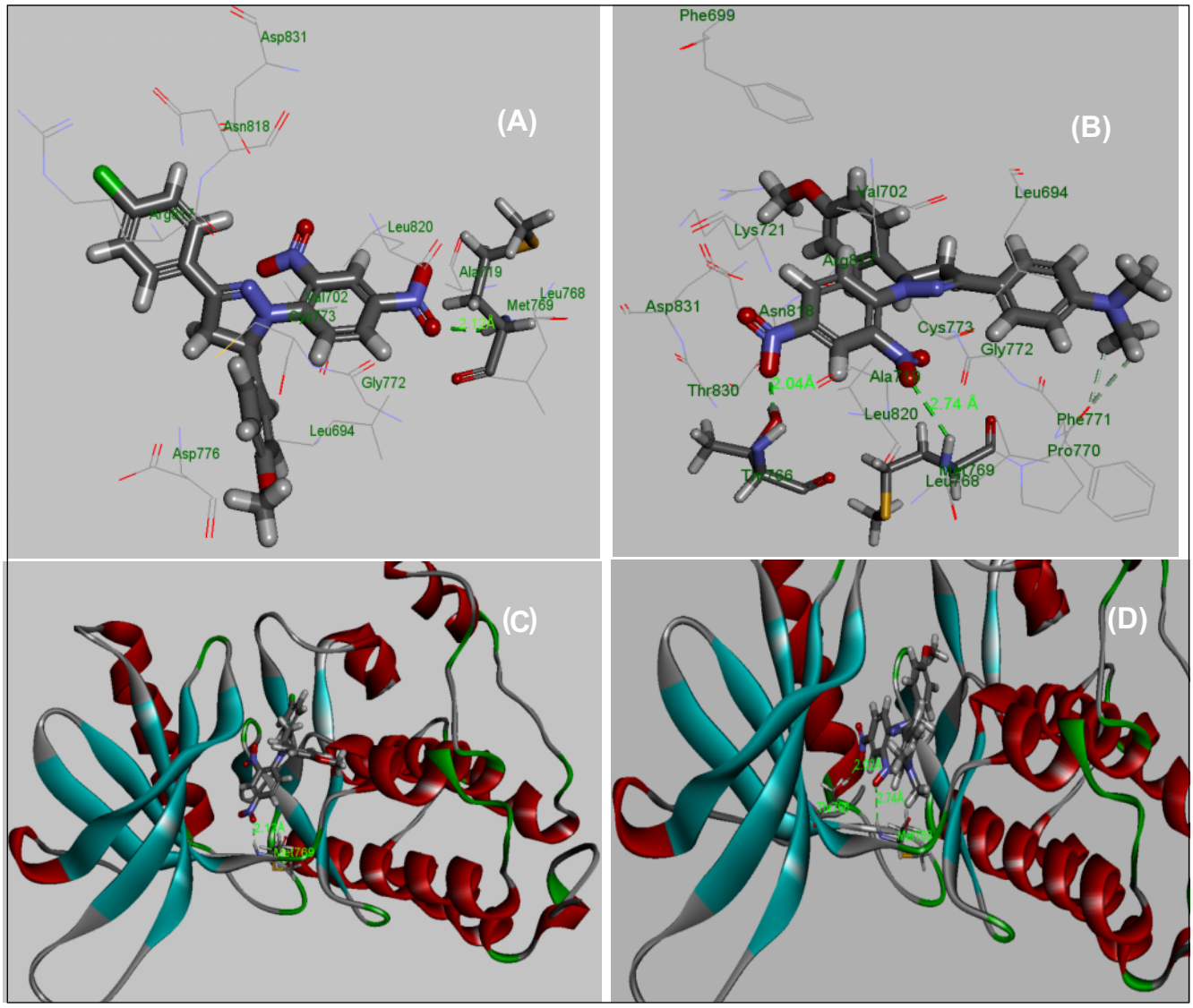

Figure 4. (A): 2 D molecular docking model of compound 5c with 1M17, (B). 2 D molecular docking model of compound 5e with 1M17, (C) 3D model of the interaction between compound 5c with1M17 binding site, (D)3D model of the interaction between compound 5e with 1M17 binding site

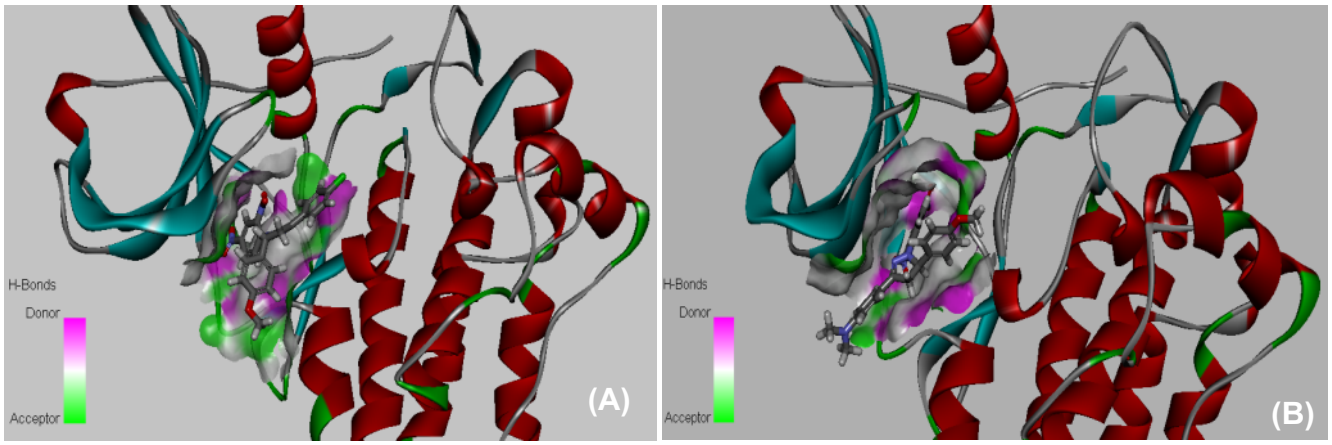

Figure 5 (A) The receptor surface model with 5c for EGFR and (B) the receptor surface model with 5e for EGFR

Table 5. ADME data of evaluated compounds (5a-f)

Compounds	5a	5b	5c	5d	5e	5f
Molecular Weight	448.42	452.84	452.84	408.36	461.47	463.40
ADMET solubility (aqueous)	-6.004	-6.769	-6.744	-5.649	-6.153	-6.051
ADMET solubility level (#)	1	1	1	2	1	1
ADMET absorption level(≠)	2(low)	2(low)	2(low)	2(low)	2(low)	3(very low)
ADMET_ AlogP98	4.811	5.492	5.492	4.223	4.990	4.722
No. Of H - bond acceptor	8	7	7	7	8	9
No. Of H - bond donor	0	0	0	0	0	0
Lipinski's filter	yes	yes	yes	yes	Yes	yes
Drug likeness inference	Very low but possible	Very low but possible	Very low but possible	low	Very low but possible	Very low but possible
Ames Prediction	Mutagen	Mutagen	Mutagen	Mutagen	Mutagen	Mutagen

0, Extremely low; 1, very low but possible; 2, yes low; 3, yes, good; 4, yes, optimal; 5, no, too soluble.

interaction. Most favoured binding mode of 5c and 5e are selected and are recorded in figure 4. In the best docked pose 5c forms one hydrogen bond with Met769 (2.03Å, Met (N-H)-(O) NO_2, 146º) of protein while 5e forms two H-bonds one with Thr766 (2.04 Å, Thr (O-H)-(O) NO_2, 156º). Surface model of binding of 5c and 5e with protein is noted in figure 5.

Drug likeness and ADMET prediction

Drug likeness of all the ligands were checked following Lipniski's rule of five. ADMET modules of discovery studio were used to check ADMET (absorption, distribution, metabolism, excretion and toxicity) property of the compounds. Toxicity prediction, Aqueous solubility, Blood Brain barrier penetration, Human Intestinal Absorption, Ames mutagenicity was predicted under the Calculate Molecular Property module of Small molecule tool of Discovery Studio client 3.5. Predicted data were summarized in table 5.

Conclusion

In summary, 5-substituted aryl-2-pyrazoline derivatives have been synthesized and structurally established by different spectral data. Their anticancer activities against MCF-7 cell lines and EAC cells have been evaluated. The results revealed that compound 1-(2, 4-dinitrophenyl)-5-(4-chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5c) with IC_{50} (2.42 ±0.26mg/l) and 1-(2,4-dinitrophenyl)-5-(4-N,N-dimetyl amino phenyl)-3-(4-methoxyphenyl) -2-pyrazoline (5e) with IC_{50} (2.75 ± 0.33 mg/l) exhibit significant anticancer activity against MCF-7 cell lines superior to 5-FU. However 1-(2, 4-dinitrophenyl)-5-(2-chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5b) and 1-(2,4-dinitrophenyl)-5-(3-nitrophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5f) exhibited the significant anticancer activity against EAC cells bearing mice model. Moreover we found compound 1-(2, 4-dinitrophenyl)-5-(4-chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5c) and 1-(2, 4-dinitrophenyl)-5-(4-N, N-dimetyl amino phenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5e) having the best interaction with the protein molecules and *in vitro* study also found that compound 1-(2, 4-dinitrophenyl) -5-(4-chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5c) and compound 1-(2,4-dinitrophenyl)-5-(4-N, N-dimetyl amino phenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5e) have potent cytotoxicity. Hence compound 1-(2, 4-dinitrophenyl)-5-(4- chlorophenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5c) and compound 1-(2, 4-dinitrophenyl)-5-(4-N, N-dimetyl amino phenyl)-3-(4-methoxyphenyl)-2-pyrazoline (5e) could be considered as bioactive molecules for future development and research and hope to get more target specific, less toxic and potent anticancer activity.

Conflict of Interest

The authors declare no conflict of interest of this work.

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