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Synthesis, Characterization and *In-Vitro*anti-Tumor Activity of Some Novel Pyrazole Derivatives

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Abstract: Pyrazole and its derivatives are considered a pharmacologically important active scaffold that possesses almost all types of pharmacological activities. The presence of this nucleus in pharmacological agents of diverse therapeutic categories such as a potent anti-inflammatory, antipsychotic , anti-obesity drug, an analgesic, and anti-depressant agents have proved the pharmacological potential of the pyrazole moiety. Owing to this diversity in the biological field, this nucleus has attracted the attention of many researchers to study its skeleton chemically and biologically. This heterocyclic can be traced in a number of well-established drugs belonging to different categories with diverse therapeutic activities. Aim of the present study is to synthesize and characterize various 4-[1-(2-hydroxy-4,4-dimethyl-6-oxocyclohex-1-en-1-yl)3,4-(phenyl]-5-methyl-2-phenyl-2,4-dihydro-3*H*-pyrazol-3-one derivatives using an efficient Scheme. The synthesized compounds (1-10) were evaluated for their *in vitro* anti-cancer activity.In order to develop potent anti-cancer agents, a novel series of pyrazole derivatives were synthesized and the structures of all compounds were confirmed by spectral studies. MTT assay has been employed to study anti-proliferative activity of these compounds with four human cancer cell lineDLD1 (Human Colorectal Adenocarcinoma) at various concentration (6.25-100µg/mL). Theresultsrevealedthat compounds VEPD and 4MEPD shows goodanti-tumoractivity and the other compounds shows moderate activity against theDLD1cell line.
Key Words: Pyrazole, antitumor, Pharmacological potential, Heterocycle, MTT assay, adenocarcinoma

1. Introduction

Pyrazoles (1*H*-pyrazoles, **3**) are constituted by an aromatic five-membered ring with three carbons and two nitrogen atoms, located at the 1- and 2-positions and are one of the most studied groups of compounds among the azole family. Drugs such as celecobix, rimonabant and sildenafil are currently used as therapeutic agents.[1]*N*-Unsubstituted pyrazoles may present three identical and non-separable tautomers, due to rapid inter-conversion in solution, and it is usually impossible to unequivocally assign the proton resonances of the pyrazole core in the proton-nuclear magnetic resonance spectra of these compounds.[2] Three partially reduced forms may also exist: 1-pyrazolines, 2-pyrazolines and 3-pyrazolines.The presence of the pyrazole nucleus in different structures leads to diversified applications in different areas such as technology, medicine and agriculture.[3] In particular, they are described as inhibitors of protein glycation, antibacterial[4], antifungal, anticancer, antidepressant, anti-inflammatory, anti-tuberculosis, antioxidant as well as antiviral agents.[5]

Cancer is a broad term. It describes the disease that results when cellular changes cause the uncontrolled growth and division of cells. Some types of cancer cause rapid cell growth, while others cause cells to grow and divide at a slower rate. Certain forms of cancer result in visible growths called tumors, while others, such as leukemia.[6]Most of the body's cells have specific functions and fixed lifespans. While it may sound like a bad thing, cell death is part of a natural and beneficial phenomenon called apoptosis. A cell receives instructions to die so that the body can replace it with a newer cell that functions better. Cancerous cells lack the components that instruct them to stop dividing and to die. As a result, they build up in the body, using oxygen and nutrients that would usually nourish other cells. Cancerous cells can form tumors, impair the immune system and cause other changes that prevent the body from functioning regularly. Cancerous cells may appear in one area, and then spread via the lymph nodes. These are clusters of immune cells located throughout the body.[6]

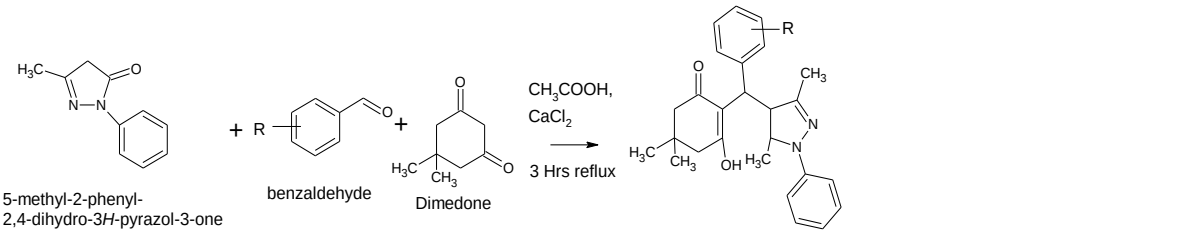
There are many causes of cancer, and some are preventable. For example, over 480,000 people die in the U.S. each year from smoking cigarettes, according to data reported in 2014.In addition to smoking, risk factors for cancer include: heavy alcohol consumption, excess body weight, physical inactivity and poor nutrition. Other causes of cancer are not preventable. Currently, the most significant unpreventable risk factor is age. According to the American Cancer Society, doctors in the U.S. diagnose 87 percent of cancer cases in people ages 50 years or older.[7]According to the National Cancer Institute, there are over 100 types of cancer. The most common type cancer in the U.S. is breast cancer, followed by lung and prostate cancers, which excluded non-melanoma skin cancers from these findings. Each year, more than 40,000 people in the country receive a diagnosis of one of the following types of cancer: bladder, colon and rectal, endometrial, kidney, leukemia, liver, melanoma, non-Hodgkin's lymphoma, pancreatic and thyroid.[8] other forms are less common. Innovative research has fueled the development of new medications and treatment technologies. Physicians usually prescribe treatments based on the type of cancer, its stage at diagnosis, and the person's overall health. The side effects of chemotherapy include hair loss. However, advances in treatment are improving the outlook for people with cancer.[9]

Recently, there is an urgent need to give much attention for design, synthesis and production of more potent and effective human therapeutic agents to treat cancer diseases, which is responsible for major deaths worldwide.[10] On the other hand, pyrazolo[1,5-a]pyrimidinesarepurineanalogue of particular significance inmedicinalchemistryduetotheirbroadscopeof remarkableantitumor andantibacterial activities.[11] A significant number of nitrogen heterocycles have been approved by theFDAas chemotherapeuticdrugs in recent years. Many of them incorporate a nitrogen-nitrogen (N-N) bond such as olaparibfor the treatment of advanced ovarian cancer, axitinib that has received approval for renal cellcarcinoma treatment, ponatinib for the treatment of chronic myeloid leukemia and Philadelphiachromosome-positive acute lymphoblastic leukemia, and ibrutinib as a first line treatment forchronic lymphocytic leukemia.[12]In the recent years, a number of review articles including pyrazoles have been published.They are concerned with the structure, with emphasis on the synthetic strategies with theirbiological activities in general or they even focus on their interesting photophysicalproperties.[13] In fact, a few of them include pyrazoline as a substructure of pyrazole.Furthermore it has been noticed that only one review exists on the therapeutic potential of pyrazoles and their anticancer activities.[14]

2. Material and Methods

2.1 Scheme

Mixture of 5-methyl-2-phenyl-2, 4-dihydro-3*H*-pyrazol-3-one (1 mmol), aldehyde (1 mmol) and Dimedone (1 mmol) in the presence of 10ml of acetic acid and pinch of $CaCl_2$was added. The mixture was refluxed for 3 hours. After completion of the reaction (TLC), the resulting solid was collected by filtration and recrystallized from ethanol.[15]



5-methyl-2-phenyl-2,4-dihydro-3*H*-pyrazol-3-one

4-[1-(2-hydroxy-4,4-dimethyl-6-oxocyclohex-1-en-1-yl)phenyl]-5-methyl-2-phenyl-2,4-dihydro-3*H*-pyrazol-3-one

R-Various substituted benzaldehydes: Veratraldehyde,Vanillin,Terophthane,3,4,5-trimethoxy benzaldehyde,4-nitro benzaldehyde,3-ethoxy benzaldehyde, 3-ethoxy4-hydroxy benzaldehyde,4-chlorobenzaldehyde,2-bromo benzaldehyde and4-dimethyl amino benzaldehyde.

2.2 Elemental analysis [16]

Elemental analysis (% C, H, N) was carried out by a Perkin- Elmer 2400 CHN analyzer. IR spectra of all compounds were recorded on a Perkin-Elmer FT-IR spectrophotometer in KBr, frequencies are reported in cm^{-1}. 1H NMR spectra were run on Varian Gemini 300 MHz and ^{13}C NMR spectra on Varian Mercury.

Compound1 Pyrazole (0.01 mol) and 4- Nitrobenaldehyde (0.01 mol) were dissolved in acetic acid (20 mL) and the reaction mixture was refluxed for 3 hr. After cooling, the crystals formed were filtered and purified by recrystallization from absolute alcohol.

2.3 In vitro Anticancer Activitydetermination by MTT assay[17]

DLD1 (Human Colorectal Adenocarcinoma) cells was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium, DMEM (Sigma aldrich, USA).The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany). The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

2.4 Cells seeding in 96 well plate[18]

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) wasseeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of compound stock:

1mg of sample was weighed and dissolved in 1mL DMEM using a cyclomixer. The sample solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

2.5 Anticancer Evaluation

After 24 hours the growth medium was removed, freshly prepared each compounds in 5% DMEM were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non treated control cells were also maintained.

2.6 Anticancer Assay by Direct Microscopic observation[19]

Entire plate was observed after 24 hours of treatment 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. 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 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide, DMSO, Sigma Aldrich, USA) 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 540 nm (Laura B. Talarico et al., 2004).

The percentage of growth inhibition was calculated using the formula:

$$\%\ of\ Viability = \frac{Mean\ OD\ Samples\ X\ \ 100}{Mean\ OD\ of\ control\ group}$$

3. Result and Discussion

3.1 Chemistry

The structure of synthesized compounds1-10 were confirmed and the structure was established by different spectral data(IR and 1H,C13 NMR)and elemental analysis. For example IR spectrum of **(**VEPD) 4-[1-(2-hydroxy-4,4-dimethyl-6-oxocyclohex-1-en-1-yl)3,4-(dimethoxy phenyl]-5-methyl-2-phenyl-2,4-dihydro-3*H*-pyrazol-3-one shows 3444(O-H),3315(Ar C-H), 2956(Alkyl C-H), 1621(C=O),1514(C=N),1467(C-N),1327(C-

O), 1226(C=C), 1194(C-C). 1H NMR (400 MHz, DMSO-*d*6) d: 7.64-6.52 (m, 7H) for benzene protons, 3.7-2.8 (m,7H) methane proton, 7.30-7.17 (m, 4H), 2.86-1.88 (s, 2H) methylene protons, 3.73 (s, 2H),1.11(s, 2H) methyl protons and the C13 NMR also shows in Fig.3 . Anal. calcd.(%) for $C_{27}H_{30}N_2O_5$: 462.5m/z.

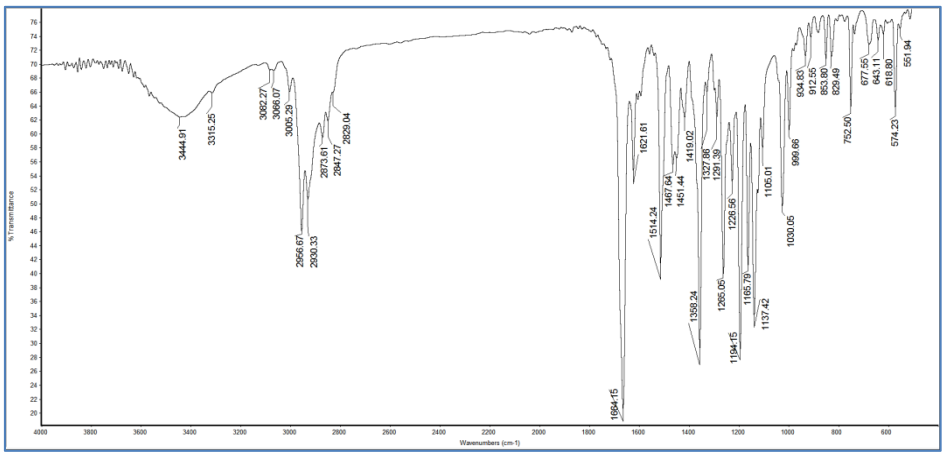


Fig 1: IR Spectrum of VEPD

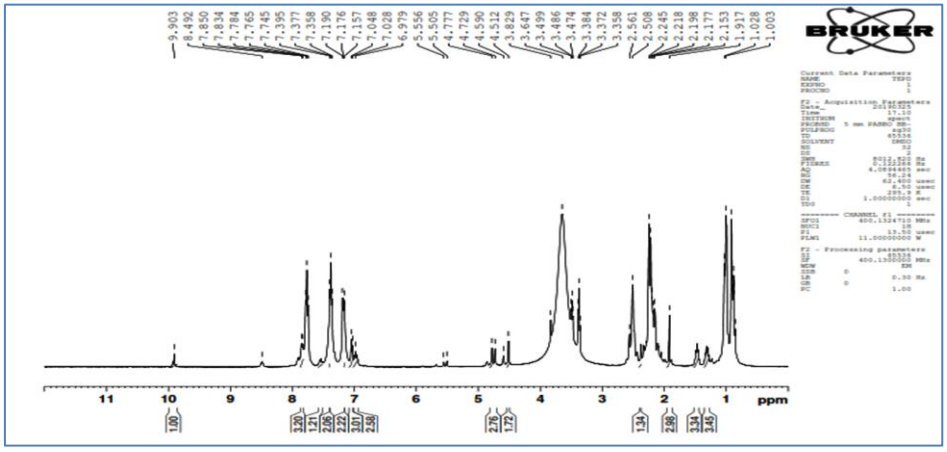


Fig 2: H1 NMR spectrum of VEPD

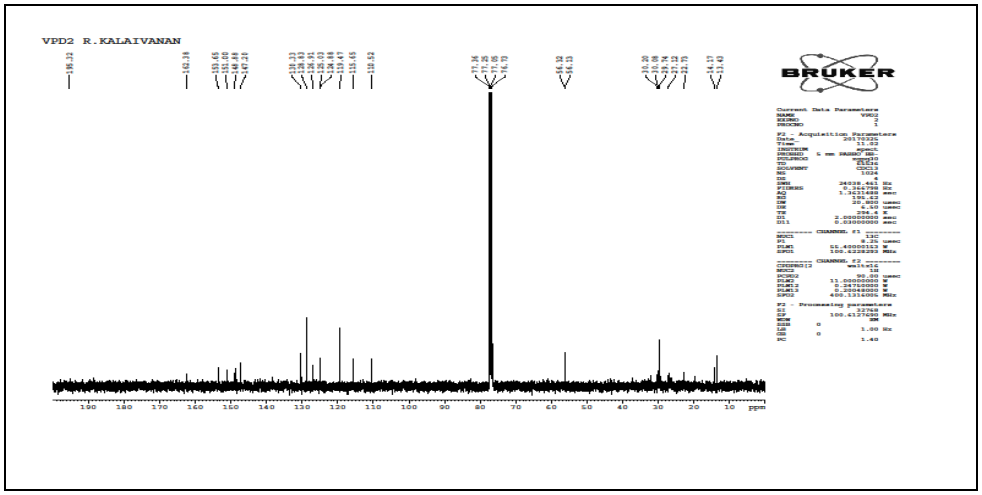
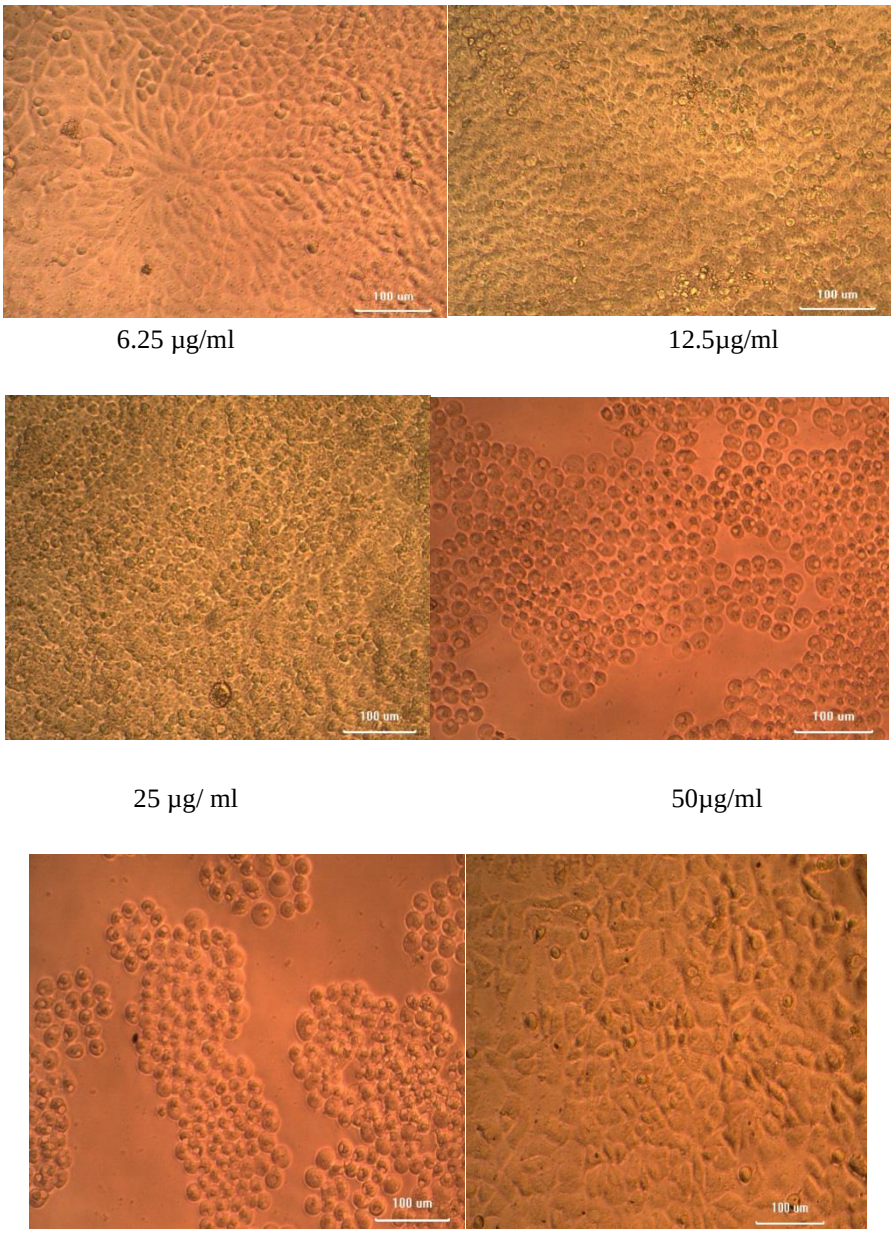


Fig 3: C13 NMR spectrum of VEPD

Anticancer Assay by MTT Method:



6.25 µg/ml 12.5µg/ml

25 µg/ ml 50µg/ml

100µg/ml Control

Fig 4: Anti-cancer activity of synthesized compounds using MTT assay against DLD1 (Human Colorectal Adenocarcinoma) cells in various concentration

In vitro anti-tumor activity thesynthesizedcompounds,4-[1-(2-hydroxy-4,4-dimethyl-6-oxocyclohex-1-en-1-yl)3,4-(phenyl]-5-methyl-2-phenyl-2,4-dihydro-3*H*-pyrazol-3-onederivatives 1–10, were examined in-vitroanti-tumor activities against DLD1 (Human Colorectal Adenocarcinoma) cells using MTT assay. The percentageofthe intact cells was measured in various concentrations (6.25- 100 µg/ mL)and compared to thecontrol(Fig1-6).The activities of these compounds against three carcinoma cells were comparedwiththatofdoxorubicin.The results indicatedthatalltestedcompoundsshowdose-dependent anti-cancer activitiesagainstDLD1cellline. Fromabove figureswecandeducethat at 100µgµg /mL, the IC_{50} value of synthesized compounds were 2BrPD- 158.754µg / mL ,3E4HPD- 214.598µg/ mL, 3STMPD-209.898 µg / mL,4CLPD-

128.726µg/mL ,4DMPD- 95.3382µg/mL, 4MEPD- 104.944µg/ mL, 4NPD- 161.955 µg / mL,TEPD- 160.467µg/mL , VAPD- 127.098µg/mL, VEPD- 73.6387µg/mL. Among them fourcompounds VEPD and4DMPD was showedgoodanti-canceractivitiesagainst DLD1 (Human Colorectal Adenocarcinoma) cells (100mmol/L). Rest of the compounds showedmoderateactivities againstthesamecellline. The IC_{50} (mg/mL) valueswere theconcentrationrequiredfor 50% inhibitionofcellgrowthandwerecompiledinTable1.

Table 1: Anti-cancer activity (IC_{50} (mg/mL) values) ofsynthesized compounds using MTT assay against DLD1 (Human Colorectal Adenocarcinoma) cells in various concentration

Sample Concentration (µg/mL)	**OD value I**	**OD value II**	**OD value III**	**Average OD**	**Percentage Viability**
Control	1.0790	1.0725	0.9981	1.0499	100.00
Sample code: 2BrPD					
6.25	0.9848	0.9615	0.9691	0.9718	92.56
12.5	0.9597	0.9071	0.9218	0.9295	88.54
25	0.8628	0.8267	0.8460	0.8452	80.50
50	0.7918	0.7522	0.7825	0.7755	73.86
100	0.7044	0.6915	0.7266	0.7075	67.39
Sample code: 3E4HPD					
6.25	0.988	0.952	0.9722	0.9707	92.46
12.5	0.912	0.9064	0.9317	0.9167	87.31
25	0.8848	0.8712	0.8943	0.8834	84.14
50	0.7964	0.8342	0.8225	0.8177	77.88
100	0.7597	0.7826	0.7647	0.7690	73.25
Sample code: 3STMPD					
6.25	0.9935	0.9836	0.9822	0.9864	93.95
12.5	0.979	0.9711	0.957	0.9690	92.30
25	0.8692	0.9018	0.8922	0.8877	84.55
50	0.7732	0.8572	0.8466	0.8257	78.64
100	0.7511	0.8021	0.7976	0.7836	74.64
Sample code: 4CLPD					
6.25	0.9654	0.9826	0.9815	0.9765	93.01

12.5	0.8882	0.9074	0.9122	0.9026	85.97
25	0.7541	0.8231	0.8072	0.7948	75.70
50	0.6874	0.7411	0.726	0.7182	68.40
100	0.6475	0.6672	0.6417	0.6521	62.11
Sample code: 4DMPD					
6.25	0.9825	0.9436	0.9579	0.9613	91.56
12.5	0.927	0.8714	0.8344	0.8776	83.59
25	0.8026	0.7364	0.7528	0.7639	72.76
50	0.7015	0.6428	0.6244	0.6562	62.50
100	0.5826	0.5277	0.517	0.5424	51.67
Sample code: 4MEPD					
6.25	0.9354	0.9526	0.966	0.9513	90.61
12.5	0.8795	0.891	0.8992	0.8899	84.76
25	0.7725	0.7865	0.784	0.7810	74.39
50	0.6242	0.6514	0.6438	0.6398	60.94
100	0.5807	0.5922	0.6019	0.5916	56.35
Sample code: 4NPD					
6.25	0.9583	0.952	0.9617	0.9573	91.18
12.5	0.901	0.9136	0.9074	0.9073	86.42
25	0.8552	0.8815	0.8365	0.8577	81.70
50	0.7314	0.8076	0.7618	0.7669	73.05
100	0.6859	0.7322	0.7014	0.7065	67.29
Sample code: TEPD					
6.25	0.9443	0.9666	0.967	0.9593	91.37
12.5	0.8833	0.8917	0.8907	0.8886	84.63
25	0.7811	0.806	0.8235	0.8035	76.53
50	0.7521	0.7617	0.7825	0.7654	72.91
100	0.6825	0.6948	0.7148	0.6974	66.42

Sample code: VAPD					
6.25	0.9789	0.9624	0.9754	0.9722	92.60
12.5	0.9173	0.8915	0.9076	0.9055	86.24
25	0.8288	0.8036	0.8365	0.8230	78.39
50	0.7318	0.7015	0.7622	0.7318	69.71
100	0.6507	0.6036	0.6736	0.6426	61.21
Sample code: VEPD					
6.25	0.9657	0.9422	0.9467	0.9515	90.63
12.5	0.8239	0.8034	0.8188	0.8154	77.66
25	0.7514	0.6924	0.682	0.7086	67.49
50	0.6577	0.6019	0.5946	0.6181	58.87
100	0.4076	0.4326	0.4128	0.4177	39.78

4. Conclusion

In conclusion, the present study fosters a simple and easily scalable approachforthepreparationofanewseriesof4-[1-(2-hydroxy-4,4-dimethyl-6-oxocyclohex-1-en-1-yl)3,4-(phenyl]-5-methyl-2-phenyl-2,4-dihydro-3*H*-pyrazol-3-one.Allthenewlysynthesizedcompoundswerescreened for their in-vitro anti-tumor activityagainstDLD1 (Human Colorectal Adenocarcinoma) cell line using MTT cytotoxicityassayat various concentration (6.25-100µg/mL). The results revealed that compounds VEPD and 4MEPD shows good anti-tumor activity and the other compounds shows moderate activity againsttheDLD1cellline.Accordingly, this pyrazole derivative of compounds must be considered as excellent templates for future optimization or modification to obtain potent anti-tumor agents.

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