International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 8 | Issue 1 | JAN-MAR| 2018 | 746-759
Research Article | Pharmaceutical Sciences | Open Access | MCI Approved| ज्ञान-विज्ञान विमुक्तये
|UGC Approved Journal |

# SYNTHESIS, ANTICANCER AND ANTIBACTERIAL ACTIVITY OF SOME NOVEL 1-PHENYL-4, 6-DISUBSTITUTED AMINO-1H-PYRAZOLO [3, 4-*d*] PYRIMIDINES

**P. Aravinda Reddy[1] and Ramya Sri. S[2]**

**[1] Department of Chemistry, Vaageswari College of Pharmacy, Karimnagar, Telangana-505527, India**

**[2]Department of Pharmacy, University College of Technology, Osmania University, Hyderabad, Telangana, 500007, India**

***Corresponding Author Email:** surapharmalabs1@gmail.com

**ABSTRACT**

*Each compound's Minimum Inhibitory Concentration (MIC) was also calculated after synthesis. A statistical analysis was performed on the antimicrobial data received. Using a sulforhodamine B test and statistical analysis, the chemicals in the title were looked at for their potential to kill human skin cancer cell lines. Antibacterial activity at 20 g/mL was comparable for all of the produced compounds (6a-6e), while compound 5 demonstrated the highest anticancer activity (3.32*10-5). A starting point for additional fine-tuning is provided by this.*

**KEY WORDS**

*Anticancer, Anti-Bacterial, Novel and Pyrimidines.*

## INTRODUCTION

On the account of the reported anticancer activity of pyrazolo[3,4-*d*]pyrimidines, a new series of pyrazolo[3,4-*d*]pyrimidine derivatives were synthesized and tested for in-vitro anticancer activity against Ehrlich Ascites Carcinoma (EAC) cell line. Moreover, one of the target products was evaluated for in-vivo radioprotective activity.[1]

Pyrazolo pyrimidine derivatives have attracted the attention of numerous researchers over many years due to their important biological activities. The structural similarity of pyrazolo[3,4-*d*] pyrimidines with purines[2] have made them a prime target for scientific research and in this context several reports dealing with the synthesis of these fused heterocyclic compounds have appeared in the literature[2].

An array of biological activities such as antibacterial, antifungal[3,4] antiphlogistic, antitumor[5], and herbicidal has been reported to be shown by various pyrazolopyrimidines. It has been proved that these heterocyclic compounds are effective as inhibitors of inflammatory mediators in intact cells[6], M. tuberculosis[7] and human enterovirus[8].

They also show inhibitory activity towards both tubulin polymerization, cyclin-dependent kinase[9] and enzymatic assays on Src and Abl tyrosine kinases[10]. Prompted by these claims and in continuing our synthetic studies on bioactive heterocycles [11], we have now synthesized a new series of some novel **1-phenyl-4,6-disubstitutedamino-1H-pyrazolo[3,4-*d*] pyrimidines to test their ability as anticancer & anti-bacterial agents.**

Cancer remains one of the most life-threatening diseases, taking nearly 7 million lives each year worldwide. It is realized that neither surgery nor radiation nor the two in combination can adequately control metastatic cancer; therefore, efforts to cure cancer have been focusing on conventional chemotherapy. However, this type of treatment usually does not discriminate between dividing normal cells and tumor cells, leading to severe side effects. In the last decade, the use of molecular targeted therapies (a new

generation of selective cancer drugs which interfere with specific receptors and signaling pathways that promote tumor cell growth) has made treatments more tumor specific. The chemistry of pyrazolo[3,4-d] pyrimidine derivatives has received great attention due to their structural similarity with purines and hence several pyrazolo[3,4-d] pyrimidine derivatives exhibit promising anticancer activity. Different mechanisms account for the cytotoxic effect of this class of compounds, where they had been reported to act as glycogen synthase kinase (GSK) inhibitors, cyclin dependent kinase (CDK) inhibitors, dual src/Ab1 kinase inhibitors and epidermal growth factor receptor (EGFR) inhibitors. Moreover, many 5-substituted-1-phenyl-1Hpyrazolo[3,4-d] pyrimidin-4-ones were reported to possess antiproliferative activity against breast carcinoma, MCF7. Examples of anticancer drugs currently used in anticancer therapy can be represented by erlotinib (TarcevaTM) and gefitinib (IressaTM) which have been approved for the chemotherapeutic treatment of patients with advanced non-small lung cancer. Also, lapatinib (TykerbTM) was approved for the treatment of breast cancer. In the view of the previous rationale and in continuation of an ongoing program on the synthesis of antitumor compounds, in the present study a new series of pyrazolo [3,4-d]pyrimidin-4-ones has been synthesized and screened in vitro for antitumor activity. The series comprises the derived 5,6-disubstituted pyrazolo[3,4-d]pyrimidin-4-one pharmacophore that is structurally related to erlotinib and lapatinib (Figure 1). In the present study, the substitution pattern at the 5,6-disubstituted pyrazolo[3,4-d] pyrimidin-4-one pharmacophore was manipulated so as to create different electronic environments that might affect the lipophilicity and hence the activity of target molecules. The rationale for the design of target compounds was based upon some structural modifications on the general features of anilino quinazoline-containing compounds (Figure 1). These modifications comprise a replacement of the benzene moiety in the quinazoline skeleton by a pyrazolo moiety as the pyrazolo moiety is naturally found in the body's purine bases and this is expected to enhance cytotoxic activity. Prompted by these claims, we present a new series of compounds containing 5,6-disubstituted pyrazolo[3,4-d]pyrimidin-4-ones core as anticancer agents. Our strategy is directed toward designing a variety of compounds with diverse chemical properties hypothesizing that the potency of these compounds might be increased by adding alternative binding groups such as a methyl group at position 6, and aroylhydrazone, phenylamino, amide, thioamide, thiosemicarbazide and substituted aryl at position 5 of the pyrazolo[3,4-d] pyrimidine ring.

## ANTIBACTERIAL ACTIVITY

### MATERIAL AND METHODS:

The synthesized compounds were screened for antibacterial activity. For determination of bacterial susceptibility test, both gram positive and gram-negative organisms are used. All the bacterial strains were obtained from the National Collection of Industrial Microorganisms A stock solution of amoxicillin was prepared and the dilutions are prepared. All the strains were maintained by weekly sub culturing on nutrient agar slant, stored at 4 $^0$C after previous 24 h incubation at 37 $^0$C. Before each experiment, the organism was activated by successive sub culturing and incubation. The activity is studied by using agar diffusion method.

**Gram Positive Bacteria:**

*Bacillus subtilis* : (NCIM-2545)
*Staphylococcus epidermidis* : (NCIM-2493)
*Staphylococcus aureus* : (NCIM-5021)

**Gram Negative bacteria:**

*Escherichia coli* : (NCIM-2803

**PROCEDURE:**

***Composition of nutrient agar medium:***

Peptone : 5.0g
Beef extract : 5.0g
NaCl : 5.0 mg
Agar : 2 %

Distilled water : up to 1000 mL

***Standardization of test microorganisms:***[40]

A 10 mL volume of sterile water was added to the agar slant containing a 24 h old culture of purified test microorganism and shaken carefully to harvest the organism. Subsequently, dilutions were carried out to get microbial population of $10^5$ cfu/mL by comparing with $BaSO_4$, equivalent to McFarland 0.5 standard.

***Preparation of $BaSO_4$, suspension equivalent to McFarland 0.5 Standard:***

To standardize the inoculums density for a susceptibility test, a $BaSO_4$, turbidity standard, equivalent to a 0.5 McFarland standard is used. The $BaSO_4$, McFarland 0.5 standard is prepared as follows. A 0.5 mL of 1.175 % w/v of $BaCl_2.2H_2O$ is added to 99.5 mL of 1% w/v of $H_2SO_4$

with constant stirring to maintain suspension. The correct density of the turbidity standard is verified by using a UV-spectrophotometer by determining the absorbance. The absorbance at 625 nm is 0.08-0.10 for this standard. This suspension is used to standardize the inoculums density.

***Preparation of stock solution and determination of zones of inhibition***

All the compounds to be tested were dissolved in DMSO to obtain a stock concentration of 1 mg/mL. The required final concentrations (10 µg/mL, 20 µg/mL, 40 µg/mL, and 50 µg/mL) were made from the stock solution, by using the same solvent. Amoxicillin was dissolved in water and a stock concentration of 1 mg/mL was prepared. The dilutions were prepared similar to the test compounds.

Inoculum of 100 µL solution from the standardized bacterial suspension was added to the molten agar (20 mL). The mixture was poured into sterile petri dishes, shaken slowly for uniform distribution and allowed to solidify. The plates were divided into three sections and cups were made in the agar plates, which were filled with the specific concentration of the prepared drug solution. The plates were incubated for 24 h at 35 $^0$C in an ambient air incubator. Solvents and growth controls were kept and the zones of inhibition were measured with millimeter ruler across the cup. The zone of inhibition obtained was compared to the interpretive standard (Amoxicillin). The petri dishes which were seeded with microorganism alone were regarded as negative controls and those dishes with bores containing reference drug solution were regarded as positive controls. The cups for each test compound were made in 3 petridishes so as to make (n=9), and the results were reported as mean ± SEM.

The same procedure was repeated for all the organisms with respect to all the test compounds.

## ANTICANCER ACTIVITY TESTING

### MATERIALS AND METHODS:

**Cell lines used:** Human Skin Cancer cell Line G361

**Cells/Well:** $5*10^3$

**Methods of testing:** Sulforhodamine B assay.

**Vehicle used:** Dimethyl sulfoxide (DMSO)

**Source of cell line:** NCCS, Pune.

**Positive control drug:** Doxorubicin (Adriamycin, ADR) Mfg: Pharmacia.

**SULFORHODAMINE B ASSAY:**

**Principle:**

The sulforhodamine B (SRB) assay was developed by Skehan and colleagues to measure drug-induced cytotoxicity and cell proliferation for large-scale drug-screening applications. Its principle is based on the ability of the protein dye sulforhodamine B to bind electrostatically. The activity is pH dependent on protein basic amino acid residues of trichloroacetic acid-fixed cells. Under mild acidic conditions it binds to and under mild basic conditions it can be extracted from cells and solubilized for measurement. The signal-to-noise ratio is favorable, and the resolution is 1000-2000 cells/well. Its performance is similar when compared to other cytotoxicity assays such as MTT or clonogenic assay. The SRB assay possesses a colorimetric end point and is nondestructive and indefinitely stable. These practical advances make the SRB assay an appropriate and sensitive assay to measure drug-induced cytotoxicity even at large-scale application. [41]

**Parameters reported:** $GI_{50}$, TGI, and $LC_{50}$

$GI_{50}$: Growth inhibition of 50 % ($GI_{50}$) calculated from drug concentration resulting in a 50 % reduction in the net protein increase.

TGI: Drug concentration resulting in total growth inhibition (TGI).

$LC_{50}$: Concentration of drug resulting in a 50 % reduction in the measured protein at the end of the drug treatment (concentration of drug causing lethality to 50 % of the cells as compared to that at the beginning) indicating a net loss of cells following treatment.

The *in vitro* testing for anticancer activity was carried out in Tata Memorial Centre [Advanced Centre for Treatment Research and Education in Cancer (ACTREC)], Mumbai.

## RESULTS AND DISCUSSION

**Antibacterial activity profile of 6-methyl-1-phenyl-4,6-disubstituted amino-1H-pyrazolo[3, 4-*d*] pyrimidines (6a-6e) for *Escherichia coli***

**Category:** **Gram Negative**

**Control:** **Amoxicillin**

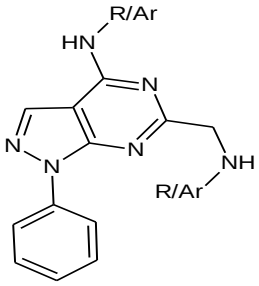
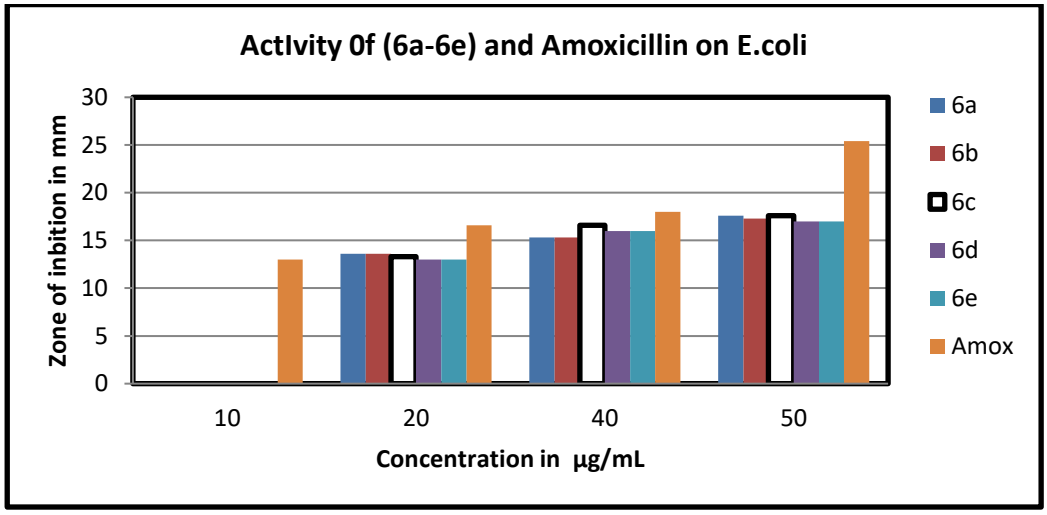



**Table: 12: Zone of inhibition in millimeters. (Average ± SEM) (n=9)**

| Compound No. | R | *Escherichia coli (µg/mL)* | | | |
|---|---|---|---|---|---|
| | | 10 | 20 | 40 | 50 |
| 6a | Aniline | -- | 13.3 ± 0.05 | 16.6 ± 0.05 | 17.3 ± 0.05 |
| 6b | Ethyl | -- | 13.3 ± 0.05 | 16.3 ± 0.05 | 17.3 ± 0.05 |
| 6c | n-Propyl | -- | 13.3 ± 0.05 | 16.0 ± 0.05 | 18.0 ± 0.08 |
| 6d | Iso Propyl | -- | 13.3 ± 0 | 16.0 ± 0 | 18.0 ± 0.08 |
| 6e | Morpholine | -- | 13.0 ± 0.05 | 16.3 ± 0.05 | 18.0 ± 0.10 |
| Amox | -- | 15.5 ± 0.07 | 18.7 ± 0.06 | 21.2 ± 0.09 | 24.8 ± 0.10 |




**Antibacterial activity profile of 6-methyl-1-phenyl-4,6-disubstituted amino-1H-pyrazolo [3, 4-*d*] pyrimidines (6a-6e) for *Bacillus subtilis***

**Category:** **Gram Positive**

**Control:** **Amoxicillin**

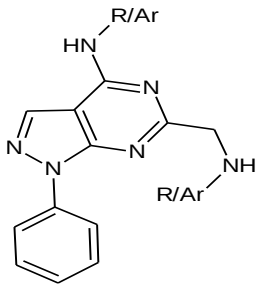
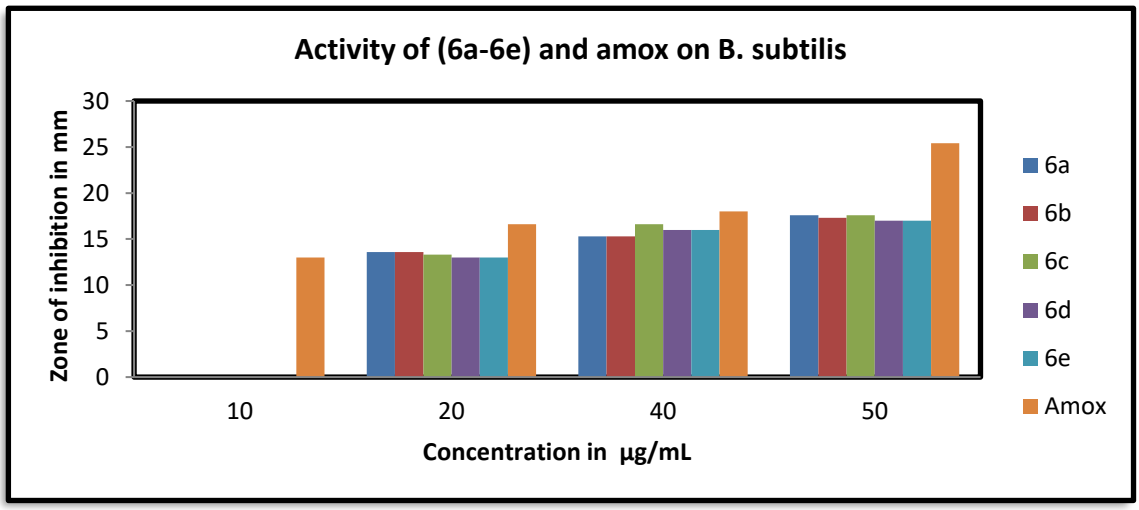


**Table: 13: Zone of inhibition in millimeters. (Average ± SEM) (n=9)**

| Compound No. | R | *Bacillus subtilis (µg/mL)* | | | |
|---|---|---|---|---|---|
| | | 10 | 20 | 40 | 50 |
| 6a | Aniline | -- | 12.7 ± 0.08 | 16.3 ± 0.05 | 18.3 ± 0.05 |
| 6b | Ethyl | -- | 13.6 ± 0.05 | 15.3 ± 0.05 | 18.0 ± 0.08 |
| 6c | n-Propyl | -- | 13.6 ± 0.05 | 15.3 ± 0.05 | 16.6 ± 0.13 |
| 6d | Iso Propyl | -- | 13.6 ± 0.05 | 15.3 ± 0.05 | 17.0 ± 0.05 |
| 6e | Morpholine | -- | 13.0 ± 0.05 | 15.4 ± 0.05 | 18.3 ± 0.05 |
| Amox | -- | 16.3 ± 0.05 | 19.0 ± 0.08 | 20.8 ± 0.09 | 25.2 ± 0.09 |




**Antibacterial activity profile of 6-methyl-1-phenyl-4,6-disubstituted amino-1H-pyrazolo [3, 4-*d*] pyrimidines (6a-6e) for *Staphylococcus aureus***

**Category:** **Gram Positive**

**Control:** **Amoxicillin**

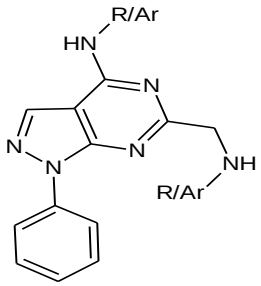
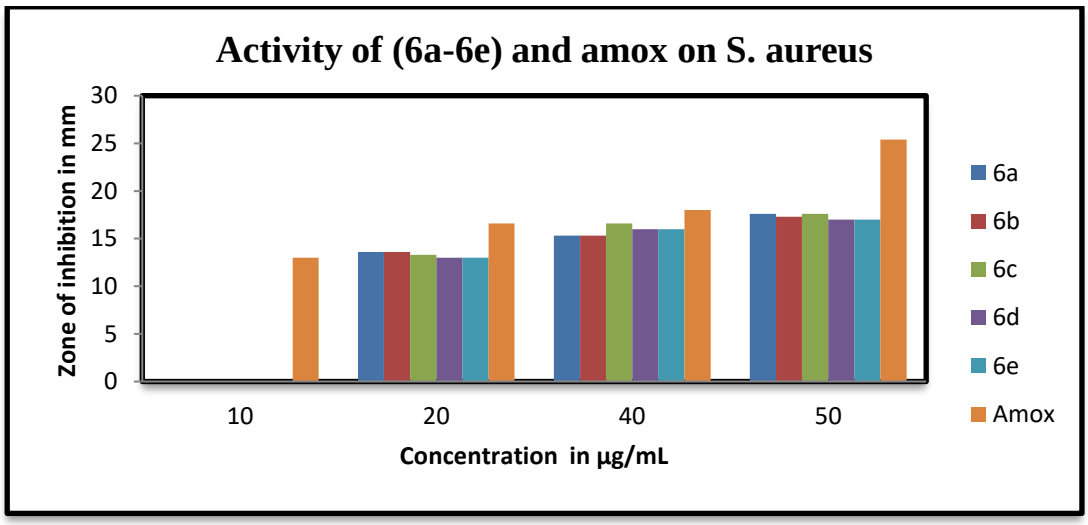



**Table: 14: Zone of inhibition in millimeters. (Average ± SEM) (n=9)**

| Compound No. | R | *Staphylococcus aureus (µg/mL)* | | | |
|---|---|---|---|---|---|
| | | 10 | 20 | 40 | 50 |
| 6a | Aniline | -- | 12.6 ± 0.05 | 15.3 ± 0.05 | 17.6 ± 0.05 |
| 6b | Ethyl | -- | 13.2 ± 0.04 | 15.3 ± 0.05 | 17.3 ± 0.05 |
| 6c | n-Propyl | -- | 13.3 ± 0.05 | 15.3 ± 0.05 | 17.6 ± 0.08 |
| 6d | Iso Propyl | -- | 13.0 ± 0.05 | 15.3 ± 0.05 | 17.6 ± 0.08 |
| 6e | Morpholine | -- | 13.0 ± 0.05 | 16.6 ± 0.04 | 18.2 ± 0.05 |
| Amox | -- | 16.6 ± 0.05 | 17.3 ± 0.13 | 21.7 ± 0.04 | 25.1 ± 0.09 |




**Antibacterial activity profile of 6-methyl-1-phenyl-4,6-disubstituted amino-1H-pyrazolo[3, 4-*d*] pyrimidines (6a-6e) for *Staphylococcus epidermidis***

**Category:** **Gram Positive**

**Control:** **Amoxicillin**

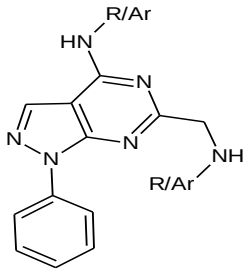



**Table: 15 Zone of inhibition in millimeters. (Average ± SEM) (n=9)**

| Compound No. | R | *Staphylococcus epidermidis (µg/mL)* | | | |
|---|---|---|---|---|---|
| | | 10 | 20 | 40 | 50 |
| 6a | Aniline | -- | 13.6 ± 0.05 | 15.4 ± 0.05 | 17.3 ± 0.1 |
| 6b | Ethyl | -- | 13.6 ± 0.05 | 15.6 ± 0.05 | 17.6 ± 0.05 |
| 6c | n-Propyl | -- | 13.3 ± 0.05 | 16.3 ± 0.05 | 17.0 ± 0.00 |
| 6d | Iso Propyl | -- | 13.3 ± 0.05 | 16.6 ± 0.05 | 17 ± 0.05 |
| 6e | Morpholine | -- | 13.3 ± 0.05 | 16 ± 0.08 | 18.3 ± 0.05 |
| Amox | -- | 13.0 ± 0.00 | 16.6 ± 0.05 | 18.0 ± 0.08 | 25.4 ± 0.08 |

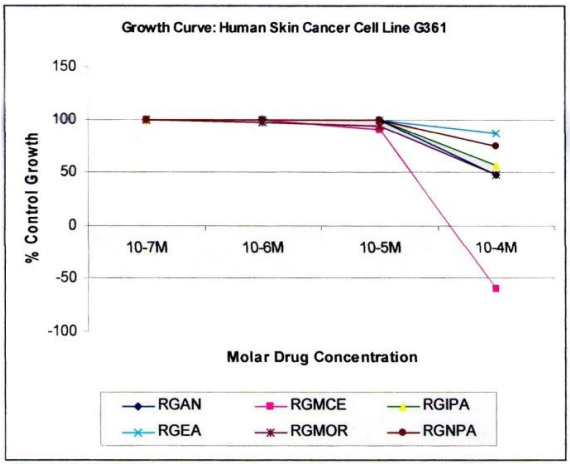
**RESULTS-ANTICANCER ACTIVITY**

**Table: 16 Reports of *in vitro* testing for anticancer activity**

| Compound No. | Human Skin Cancer Cell Line G361 | | | |
|---|---|---|---|---|
| | % Growth* | | | |
| | Molar Drug Concentration | | | |
| | $10^{-7}$M | $10^{-6}$M | $10^{-5}$M | $10^{-4}$M |
| 5 | 100.0 | 100.0 | **90.9** | **-59.3** |
| 6a | 100.0 | 100.0 | 99.7 | 48.5 |
| 6b | 100.0 | 100.0 | 100.0 | 87.7 |
| 6c | 100.0 | 99.6 | 100.0 | 75.1 |
| 6d | 100.0 | 100.0 | 99.7 | 56.5 |
| 6e | 99.7 | 97.8 | 94.3 | 47.6 |
| ADR | 46.9 | -76.1 | -73.6 | -76.4 |

*Average values of 3 experiments

| G 361 | Molar Drug Concentration | | |
|---|---|---|---|
| | LC 50† | TGI† | GI50† |
| 5 | $>10^{-4}$ | $3.32*10^{-5}$ | $2.08*10^{-6}$ |
| 6a | $>10^{-4}$ | $>10^{-4}$ | $>10^{-4}$ |
| 6b | $>10^{-4}$ | $>10^{-4}$ | $>10^{-4}$ |
| 6c | $>10^{-4}$ | $>10^{-4}$ | $>10^{-4}$ |
| 6d | $>10^{-4}$ | $>10^{-4}$ | $>10^{-4}$ |
| 6e | $>10^{-4}$ | $>10^{-4}$ | $>10^{-4}$ |
| | $2.03*10^{-6}$ | $1.27*10^{-7}$ | $<10^{-7}$ |

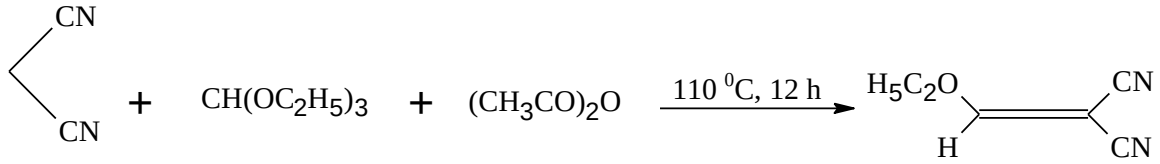



† Average of 3 experiments

**DISCUSSION-SYNTHETIC**

The synthesis of 1-phenyl-4,6-disubstituted amino-1H-pyrazolo[3,4-*d*]pyrimidines. were synthesized using the appropriate synthetic procedures.

**STEP:1 Synthesis of ethoxy methylene malanonitrile:**

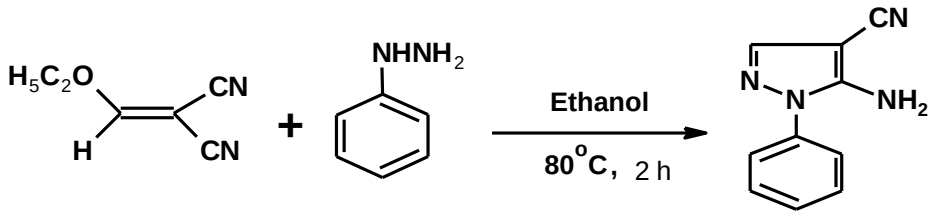



Malanonitrile Triethyl ortho formate Acetic anhydride

**(1)**

The reactants malanonitrile, triethylorthoformate and acetic anhydride were taken and heated at refluxing temperature for 12 h. The excess solvent was distilled off at negative pressure and finally, crushed ice was added to precipitate the product to give the yield of 64.75%. Acetic anhydride extracts proton from malanonitrile which in turn attacks triethyl orthoformate. This results in liberation of two molecules of ethylalcohol giving ethoxy methylene malanonitrile.

**STEP:2 Synthesis of 5-amino-4-cyano-1-phenyl pyrazole:**

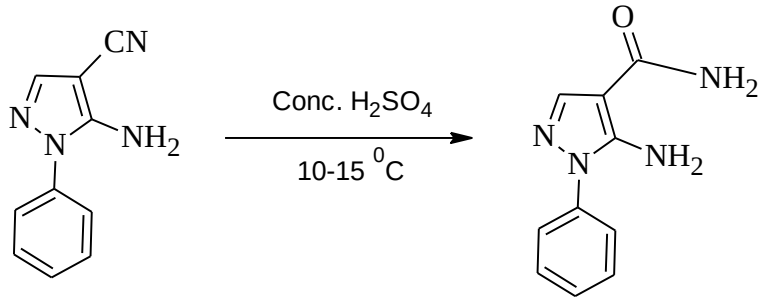



**(1)** **(2)**

The synthesis of the above mentioned compound is carried out as per the reported procedure. Ethoxy methylene malanonitrile was treated with phenyl hydrazine in solvent ethanol at refluxing temperature for 80 $^0$C to give a yield of 77.02%. The primary nitrogen of phenylhydrazine attacks ethoxy methylene malanonitrile liberating one molecule of ethylalcohol. Finally, 5-amino-4-cyano-1-phenylpyrazole was formed by internal cyclisation

.

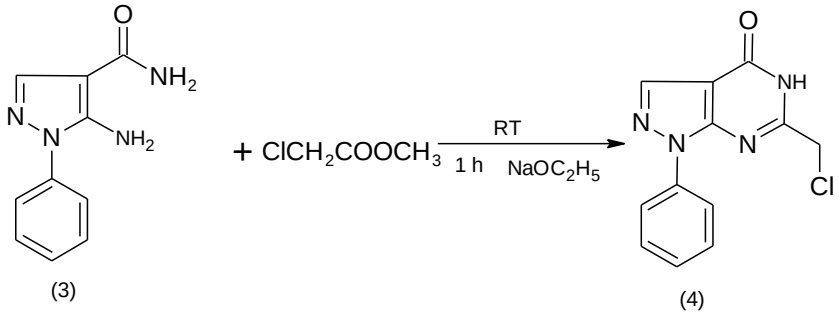
**STEP:3 Synthesis of 5-amino-1-phenyl pyrazole-4-carboxamide:**




**(2)** **(3)**

The preparation is carried out by acid hydrolysis of 5-amino-4-cyano-1-phenyl pyrazole **(2)**, with conc. sulphuric acid, as per the reported procedure. The temperature should be strictly maintained at 0-10 $^0$C, otherwise the compound gets destroyed, which is unresponsive to acid hydrolysis. The reactant must be added in small amounts and left with continuous stirring for 2 h after complete addition of the compound. The neutralization, which was carried out with dilute sodium hydroxide solution, must be carried out carefully, as the acidic solution gets bumped as soon as the base is added giving 99.64% yield.

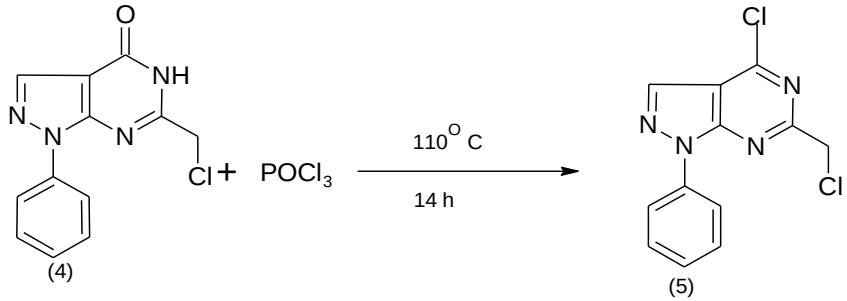
**STEP:4 Cyclisation of 5-amino-1-phenyl pyrazole-4-carboxamide:**




(3) (4)

The cyclisation process involves the reaction of 5-amino-1-phenyl pyrazole-4-carboxamide **(3)** with methylchloro acetate, in the presence of strong base such as sodium ethoxide. The sodium ethoxide must be highly concentrated i.e., the sodium metal which is added to ethanol in its preparation must be 3 % w/v. The sodium metal must be added in small pieces to ethanol until all the sodium metal dissolves in the solvent evolving hydrogen gas. This must be essentially carried out in inert gas, and anhydrous conditions. Here, in our work we used nitrogen ($N_2$) gas. Care must be taken regarding the pressure build up during the evolution of hydrogen gas. Finally, 5-amino-1-phenyl pyrazole-4-carboxamide **(3)**, ester (methylchloro acetate) is added in 1: 10 molar ratio and stirred at room temperature for 1 hour. The yeild obtained is 80.40%.

**STEP: 5 Chlorination of 6-(chloromethyl)-1-phenyl-1,5-dihydro-4*H*-pyrazolo [3,4 -*d*] pyrimidin-4-one:**

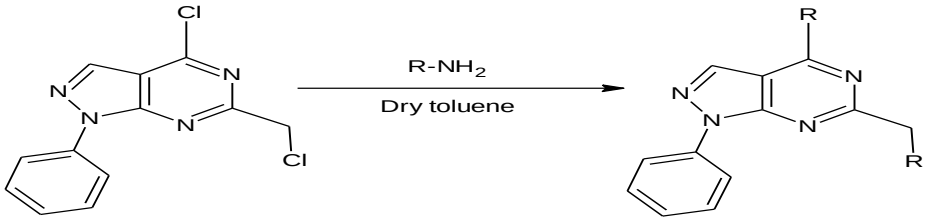



(4) (5)

The chlorination of pyrazolopyrimidines was done by using $POCl_3$ as it is the mostly used method for the chlorination[45]. In the conventional synthetic method, 6-(chloromethyl)-1-phenyl-1,5-dihydro-4*H*-pyrazolo[3,4-*d*] pyrimidin-4-one (5), and $POCl_3$ in approximately 1:15 ratio were used. Hence it is necessary to distill off the excess $POCl_3$ after completion of reaction, which makes the work up process tedious and it occasionally leads to

formation of sticky mass. In case of formation of sticky mass, the compound was extracted in ethyl acetate (50.0 mL) and the extract was dried by distillation and was directly used for the next step without any purification. The product is obtained at a yield of 88.22%.

**STEP:6 Amination of 4-chloro-6-(chloromethyl)-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidine:**




**(7)** **(8)**

The unstable chlorinated pyrazolo[3,4-*d*] pyrimidine **(5)** was stirred at room temperature with different amines for 24 hr. The excess toluene was distilled off from the reaction medium under reduced pressure. The mixture was added to crushed ice. It was neutralized with dilute hydrochloric acid. The resultant precipitate was crystallized by adding absolute ethanol to give the product. We have used 4-chloro-6-(chloromethyl)-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidine] and different amines in 1:10 molar ratio.

The spectral results of **(1), (2), (3), (4)** were already reported.[9,14,16] The carboxamide compound was cyclysed by the ester methyl chloro ester The $^1$H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 4.63, a singlet, integrating two protons indicating the hydrogens of –$CH_2$-Cl, at 6$^{th}$ position of pyrazolopyrimidine., The chlorinated compounds were treated with various amines, in dry toluene to produce the title compounds. The obtained compounds in IR showed prominent peaks at (3500-3200cm$^{-1}$) which are characteristic of primary and secondary amino groups indicated the formation of expected product.

The $^1$H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 6.58-6.62,triplet with one proton 4$^{111}$Ph , δ 6.76-6.62 doublet with two protons 2$^{111}$ & 6$^{111}$ Ph, δ 7.10-7.14 triplet with three protons 3$^{111}$,5$^{111}$ & 4$^{11}$ Ph indicated the formation anline substituent at 4$^{th}$ position, δ 7.50-7.54 triplet two protons 3$^{11}$ & 5$^{11}$ Ph, and δ 7.83-7.85 doublet with two protons 2$^1$ & 6$^1$ indicated the formation aniline substituent at 6$^{th}$ position, indicating the formation of disubstituted pyrazolopyrimidines with aniline.

The $^1$H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 0.69-0.73 triplet with three protons-$CH_2$-NH-$CH_2$-C<u>H</u>$_3$, δ 3.52-3.59 pentet with two protons-$CH_2$-NH-C<u>H</u>$_2$-$CH_3$ indicated ethylamine substituent at 6$^{th}$ postion, δ 1.22-1.25 triplet with three protons-NH-$CH_2$-C<u>H</u>$_3$, δ 3.47-3.52 pentet with two protons -NH-C<u>H</u>$_2$-$CH_3$ indicated the ethylamine substituent at 4 $^{th}$ postion indicating the formation of disubstitued pyrazolopyrimidines with ethylamine.

The $^1$H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 0.48-0.51 triplet with three protons-NH-$CH_2$-$CH_2$-C<u>H</u>$_3$, δ 1.60-1.69 sixlet with two protons -NH-$CH_2$-C<u>H</u>$_2$-$CH_3$, δ 3.47-3.52 quardlet with two protons -NH-C<u>H</u>$_2$-$CH_2$-$CH_3$ indicated the propyl amine substituent at 4$^{th}$ postion, δ 0.94-0.98 triplet with three protons -$CH_2$-NH-$CH_2$-$CH_2$-C<u>H</u>$_3$, δ 1.89-1.99 pentet with two protons -$CH_2$-NH-$CH_2$-C<u>H</u>$_2$-$CH_3$, δ 3.96-4.05 quardlet with two protons -$CH_2$-NH-C<u>H</u>$_2$-$CH_2$-$CH_3$ indicated the propyl amine substituent at 6$^{th}$ position indicating the formation of disubstituted pyrazolopyrimidines with propylamine.

The $^1$H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 1.26-1.28 doublet with six protons-NH-CH-(C<u>H</u>$_3$)$_2$, δ 4.43-4.47 quadlet with one proton –NH-C<u>H</u>-($CH_3$)$_2$ indicate isopropyl substituent at 4$^{th}$ position, δ 1.48-1.50 doublet with six protons-$CH_2$-NH-CH-(C<u>H</u>$_3$)$_2$, δ 3.04-3.11 quadlets with one proton –$CH_2$–NH-C<u>H</u>-($CH_3$)$_2$ indicate isopropyl substituent at 6$^{th}$ position indicating the formation of disubstituted pyrazolopyrimidines with isopropyl amine.

The $^1$H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 2.32-2.34 triplet with four protons 2$^{111}$ & 6$^{111}$ morph, δ 3.53-3.55 triplet with four protons 3$^{111}$ & 5$^{111}$ morph, indicate morpholine substituent at 4$^{th}$ position, δ 3.77-3.79 triplet with four protons 2$^{11}$ & 6$^{11}$ morph, δ 3.96-3.98 triplet with four protons 3$^{11}$ & 5$^{11}$ morph, indicate morpholine substituent at 6$^{th}$ position. indicating the formation of disubstitued pyrazolopyrimidines with morpholine.

The mass spectrum showed molecular ion peak [$M^+$] as the base peak and fragmentation pattern characteristic to its structure.

**ANTIMICROBIAL ACTIVITY**

The newly synthesized compounds were screened for antibacterial activity against Gram positive [Bacillus subtilis (NCIM-2545), Staphylococcus epidermidis (NCIM-2493), Staphylococcus aureus (NCIM-5021)] and Gram negative [(Escherichia coli (NCIM-2803)] bacteria by agar diffusion method. All the compounds were found to be active against all the four bacterial strains at 20 µg/mL concentration. And the activity of these compounds varied with the kind of organism. All compounds (**6a-6e)** showed similar zone of inhibitions. The compound 1-phenyl-*N*-propyl-6-[(propylamino)methyl]-1*H*-pyrazolo[3,4-*d*]pyrimidin-4-amine (6c) exhibited activity against *Escherichia coli, Staphylococcus aureus* and *Staphylococcus epidermidis,* at a concentration of 20 µg/mL. All the activity is done with Amoxicillin as reference.

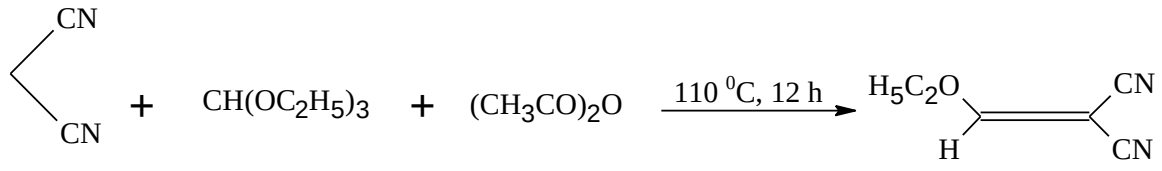
**ANTICANCER ACTIVITY**

The newly synthesized compounds were screened for their anticancer activity against Human Skin Cancer Cell Line G361 by Sulforhodamine B assay. Doxorubicin was used as a standard reference drug and the results obtained were shown in (Table:16,17). All compounds (**6a-6e)** showed low antiproliferative activity. The % Growth inhibition of the compound **(5)** was found to be considerable at a concentration of $10^{-4}$ M. $TGI_{50}$ (Growth inhibition of 50 % cells, calculated from drug concentration resulting in a 50 % reduction in the net protein increase) value of **(5)** is $3.32*10^{-5}$. As pyrimidinone derivative is the most active compound, it serves as a lead to further optimization in drug discovery process.

**DISCUSSION-SYNTHETIC**

The synthesis of 1-phenyl-4,6-disubstituted amino-1H-pyrazolo[3,4-*d*]pyrimidines. were synthesized using the appropriate synthetic procedures.

**STEP:1 Synthesis of ethoxy methylene malanonitrile:**

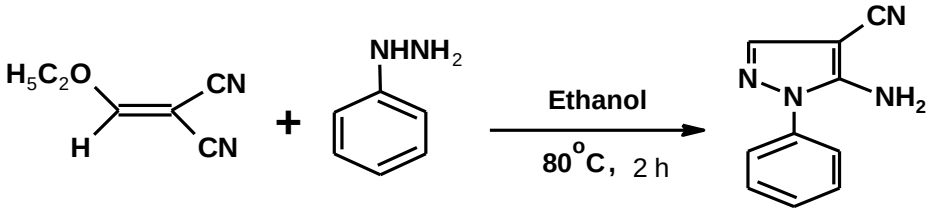



Malanonitrile Triethyl ortho formate Acetic anhydride

**(1)**

The reactants malanonitrile, triethylorthoformate and acetic anhydride were taken and heated at refluxing temperature for 12 h. The excess solvent was distilled off at negative pressure and finally, crushed ice was added to precipitate the product to give the yield of 64.75%. Acetic anhydride extracts proton from malanonitrile which in turn attacks triethyl orthoformate. This results in liberation of two molecules of ethylalcohol giving ethoxy methylene malanonitrile.

**STEP:2 Synthesis of 5-amino-4-cyano-1-phenyl pyrazole:**

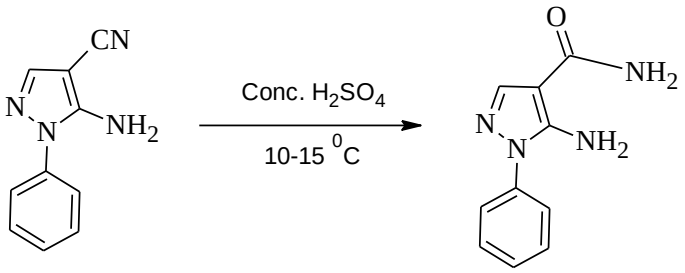



**(1)** **(2)**

The synthesis of the above-mentioned compound is carried out as per the reported procedure. Ethoxy methylene malanonitrile was treated with phenyl hydrazine in solvent ethanol at refluxing temperature for 80 $^0$C to give a yield of 77.02%. The primary nitrogen of phenylhydrazine attacks ethoxy methylene malanonitrile liberating one molecule of ethylalcohol.

Finally, 5-amino-4-cyano-1-phenylpyrazole was formed by internal cyclisation.

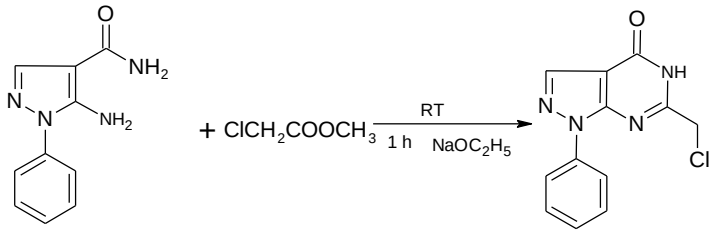
**STEP:3 Synthesis of 5-amino-1-phenyl pyrazole-4-carboxamide:**




**(2)** **(3)**

The preparation is carried out by acid hydrolysis of 5-amino-4-cyano-1-phenyl pyrazole **(2)**, with conc. sulphuric acid, as per the reported procedure. The temperature should be strictly maintained at 0-10 $^0$C, otherwise the compound gets destroyed, which is unresponsive to acid hydrolysis. The reactant must be added in small amounts and left with continuous stirring for 2 h after complete addition of the compound. The neutralization, which was carried out with dilute sodium hydroxide solution, must be carried out carefully, as the acidic solution gets bumped as soon as the base is added giving 99.64% yield.

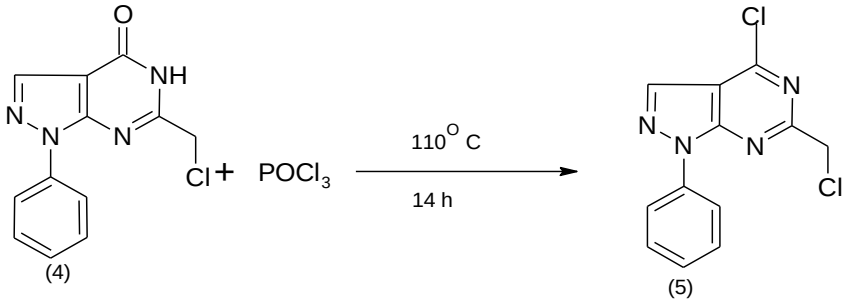
**STEP:4 Cyclisation of 5-amino-1-phenyl pyrazole-4-carboxamide:**




(3) (4)

The cyclisation process involves the reaction of 5-amino-1-phenyl pyrazole-4-carboxamide **(3)** with methylchloro acetate, in the presence of strong base such as sodium ethoxide. The sodium ethoxide must be highly concentrated i.e., the sodium metal which is added to ethanol in its preparation must be 3 % w/v. The sodium metal must be added in small pieces to ethanol until all the sodium metal dissolves in the solvent evolving hydrogen gas. This must be essentially carried out in inert gas, and anhydrous conditions. Here, in our work we used nitrogen ($N_2$) gas. Care must be taken regarding the pressure built up during the evolution of hydrogen gas. Finally, 5-amino-1-phenyl pyrazole-4-carboxamide **(3)**, ester (methylchloro acetate) is added in 1: 10 molar ratio and stirred at room temperature for 1 hour. The yeild obtained is 80.40%.

**STEP: 5 Chlorination of 6-(chloromethyl)-1-phenyl-1,5-dihydro-4*H*-pyrazolo [3,4 -*d*] pyrimidin-4-one:**

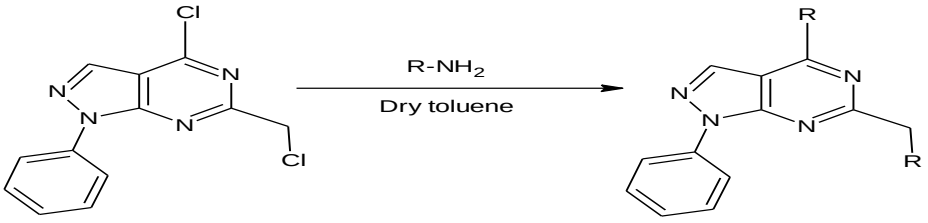



(4) (5)

The chlorination of pyrazolopyrimidines was done by using $POCl_3$ as it is the mostly used method for the chlorination[45]. In the conventional synthetic method, 6-(chloromethyl)-1-phenyl-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one (5), and $POCl_3$ in approximately 1:15 ratio were used. Hence it is necessary to distill off the excess $POCl_3$ after completion of reaction, which makes the work up process tedious and it occasionally leads to

formation of sticky mass. In case of formation of sticky mass, the compound was extracted in ethyl acetate (50.0 mL) and the extract was dried by distillation and was directly used for the next step without any purification. The product is obtained at a yield of 88.22%.

**STEP:6 Amination of 4-chloro-6-(chloromethyl)-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidine:**




**(7)** **(8)**

The unstable chlorinated pyrazolo[3,4-*d*] pyrimidine **(5)** was stirred at room temperature with different amines for 24 hr. The excess toluene was distilled off from the reaction medium under reduced pressure. The mixture was added to crushed ice. It was neutralized with dilute hydrochloric acid. The resultant precipitate was crystallized by adding absolute ethanol to give the product. We have used 4-chloro-6-(chloromethyl)-1-phenyl-1*H*-pyrazolo[3,4-*d*] pyrimidine] and different amines in 1:10 molar ratio.

The spectral results of **(1), (2), (3), (4)** were already reported.[9,14,16] The carboxamide compound was cyclysed by the ester methyl chloro ester The $^1$H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 4.63, a singlet, integrating two protons indicating the hydrogens of –$CH_2$-Cl, at 6$^{th}$ postion of pyrazolopyrimidine., The chlorinated compounds were treated with various amines, in dry toluene to produce the title compounds. The obtained compounds in IR showed prominent peaks at (3500-3200cm$^{-1}$) which are characteristic of primary and secondary amino groups indicated the formation of expected product.

The $^1$H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 6.58-6.62,triplet with one proton 4$^{111}$Ph , δ 6.76-6.62 doublet with two protons 2$^{111}$ & 6$^{111}$ Ph, δ 7.10-7.14 triplet with three protons 3$^{111}$,5$^{111}$ & 4$^{11}$ Ph indicated the formation anline substituent at 4$^{th}$ position, δ 7.50-7.54 triplet two protons 3$^{11}$ & 5$^{11}$ Ph, and δ 7.83-7.85 doublet with two protons 2$^{1}$ & 6$^{1}$ indicated the formation aniline substituent at 6$^{th}$ position, indicating the formation of disubstitued pyrazolopyrimidines with aniline.

The 1H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 0.69-0.73 triplet with three protons-$CH_2$-NH-$CH_2$-C<u>H</u>$_3$, δ 3.52-3.59 pentet with two protons-$CH_2$-NH-C<u>H</u>$_2$-$CH_3$ indicated ethylamine substituent at 6$^{th}$ postion, δ 1.22-1.25 triplet with three protons-NH-$CH_2$-C<u>H</u>$_3$, δ 3.47-3.52 pentet with two protons -NH-C<u>H</u>$_2$-$CH_3$ indicated the ethylamine substituent at 4 $^{th}$ postion indicating the formation of disubstitued pyrazolopyrimidines with ethylamine.

The $^1$H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 0.48-0.51 triplet with three protons-NH-$CH_2$-$CH_2$-C<u>H</u>$_3$, δ 1.60-1.69 sixlet with two protons -NH-$CH_2$-C<u>H</u>$_2$-$CH_3$, δ 3.47-3.52 quardlet with two protons -NH-C<u>H</u>$_2$-$CH_2$-$CH_3$ indicated the propyl amine substituent at 4$^{th}$ postion, δ 0.94-0.98 triplet with three protons -$CH_2$-NH-$CH_2$-$CH_2$-C<u>H</u>$_3$, δ 1.89-1.99 pentet with two protons -$CH_2$-NH-$CH_2$-C<u>H</u>$_2$-$CH_3$, δ 3.96-4.05 quardlet with two protons -$CH_2$-NH-C<u>H</u>$_2$-$CH_2$-$CH_3$ indicated the propyl amine substituent at 6$^{th}$ postion indicating the formation of disubstitued pyrazolopyrimidines with propylamine.

The $^1$H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 1.26-1.28 doublet with six protons-NH-CH-(C<u>H</u>$_3$)$_2$, δ 4.43-4.47 quardlet with one proton –NH-C<u>H</u>-$(CH_3)_2$ indicate isopropyl substituent at 4$^{th}$ position, δ 1.48-1.50 doublet with six protons-$CH_2$-NH-CH-(C<u>H</u>$_3$)$_2$, δ 3.04-3.11 quardlet with one proton –$CH_2$–NH-C<u>H</u>-$(CH_3)_2$ indicate isopropyl substituent at 6$^{th}$ position indicating the formation of disubstitued pyrazolopyrimidines with isopropylamine.

The $^1$H NMR (400 MHz, DMSO-$d_6$) showed peaks at δ 2.32-2.34 triplet with four protons 2$^{111}$ & 6$^{111}$ morph, δ 3.53-3.55 triplet with four protons 3$^{111}$ & 5$^{111}$ morph, indicate morpholine substituent at 4$^{th}$ position, δ 3.77-3.79 triplet with four protons 2$^{11}$ & 6$^{11}$ morph, δ 3.96-3.98 triplet with four protons 3$^{11}$ & 5$^{11}$ morph, indicate morpholine substituent at 6$^{th}$ position. indicating the formation of disubstitued pyrazolopyrimidines with morpholine.

The mass spectrum showed molecular ion peak [M$^+$] as the base peak and fragmentation pattern characteristic to its structure.

**ANTIMICROBIAL ACTIVITY**

The newly synthesized compounds were screened for antibacterial activity against Gram positive [Bacillus subtilis (NCIM-2545), Staphylococcus epidermidis (NCIM-2493), Staphylococcus aureus (NCIM-5021)] and Gram negative [(Escherichia coli (NCIM-2803)] bacteria by agar diffusion method. All the compounds were found to be active against all the four bacterial strains at 20 µg/mL concentration. And the activity of these compounds varied with the kind of organism. All compounds (**6a-6e)** showed similar zone of inhibitions. The compound 1-phenyl-*N*-propyl-6-[(propylamino) methyl]-1*H*-pyrazolo[3,4-*d*] pyrimidin-4-amine (6c) exhibited activity against *Escherichia coli, Staphylococcus aureus* and *Staphylococcus epidermidis,* at a concentration of 20 µg/mL. All the activity is done with Amoxicillin as reference.

**ANTICANCER ACTIVITY**

The newly synthesized compounds were screened for their anticancer activity against Human Skin Cancer Cell Line G361 by Sulforhodamine B assay. Doxorubicin was used as a standard reference drug and the results obtained were shown in (Table:16,17). All compounds (**6a-6e)** showed low antiproliferative activity. The % Growth inhibition of the compound **(5)** was found to be considerable at a concentration of $10^{-4}$ M. $TGI_{50}$ (Growth inhibition of 50 % cells, calculated from drug concentration resulting in a 50 % reduction in the net protein increase) value of **(5)** is $3.32*10^{-5}$. As pyrimidinone derivative is the most active compound, it serves as a lead to further optimization in drug discovery process.

**ACKNOWLEDGEMENT**

The Authors are thankful to the Management and Principal, Department of Chemistry, Vaageswari College of Pharmacy, Karimnagar, for extending support to carry out the research work. Finally, the authors express their gratitude to the Sura Labs, Dilsukhnagar, Hyderabad, for providing research equipment and facilities.

**REFERENCE**

1. M. Ghorab, A Fatma. I Ragab, I Saleh. M Alqasoumi, M Ahmed, A. Sarah A. Aboulmagd, *Europeon J. Med Chem*., **(2010)**; 45: 171–178.
2. A. Bendich, P.J. Russell, J.J. Fox, *J. Am. Chem. Soc*., **(1954)**; 76: 6073–6077.
3. B.S. Holla, M. Mahalinga, M.S. Karthikeyan, P.M. Akberali, N.S. Shetty. *Bioorg Med Chem.,* **(2006)**; 6: 2040-2047.
4. M. Bakavoli, G. Bagherzadeh, M. Vaseghifar, A. Shiri, M.Pordel, M,Mashreghi, P. Pordeli, M. Araghi. *Europeon J. Med Chem*., **(2010)**; 45: 647-650.
5. M. A El-Sherbeny, *Archiv der Pharmazie*., **(2000)**;333: 99-109.
6. H. Liu, H.-Q. Wang, Z.-j. Liu. *Bioorg Med Chem.,* **(2007)**; 17: 2203–2209.
7. J.M Quintela, C.Peinador, L.Gonzalez, I.Devesa, M.L. Ferrandiz, M.J.Alcaraz, R. Riguera. *Bioorg Med Chem.,* **(2003)**; 11: 863-868.
8. O. Moukha-Chafiq, M.L. Taha, H.B. Lazrek, C. Pannecouque, M. Witvrouw, E. De Clercq, *Nucleos. Nucleot. Nucleic Acids.,* **(2002)**;57:1797–1810.
9. J.H. Chern, K.S. Shia, T.A. Hsu, C.L. Tai, C.C. Lee, Y.C. Lee, C.S. Chang, S.N. Tseng,S.R. Shih, *Bioorg. Med. Chem.,* **(2004)** ;14:2519–2525.
10. V. Krystof, D. Moravcova, M. Paprskarova, P. Barbier, V. Peyrot,A. Hlobilkova, L. Havlicek, M. Strnad, *Europeon J. Med Chem*., **(2006)**;45: 1405–1411.
11. N.D. Adams, S.J. Schmidt, S.D. Knight, D. Dhanak, *Tetrahedron Lett*., **(2007)**; 48:3983–3986.
12. M. Bakavoli, G. Bagherzadeh, M. Vaseghifar, A. Shiri , M. Pordel , M.Mashreghi, P. Pordeli, M. Aragh. *Europeon J. Med Chem*., **(2010)**; 45: 647–650.
13. S.Schenone, O.Bruno, F.Bondavalli, A.Ranise, L.Mosti, G.Menozzi, P.Fossa, F. Manetti, L.Morbidelli, L.Trincavelli, C.Martini and A.Lucacchini *et al. Europeon J Med Chem*., **(2004)**; 39: 153–160.
14. P.J.Gilligan, C.Baldauf, A.Cocuzza, D.Chidester, R.Zaczek, L.W.Fitzgerald, J.McElroy, A.Smith, H-S.L. Shen, J.A.Saye, D.Christ, G.Trainor, D.W. Robertson and P.Hartig. *Bioorg Med Chem.,* **(2000)**; 8: 181-189.
15. H.Mukaiyama, T.Nishimura, S.Kobayashi, Y.Komatsu, S.Kikuchi, T.Ozawa, N.Kamada and H.Ohnot. *Bioorg Med Chem.,* **(2008)**; 16: 909–921.
16. B.A. Johns, K.S.Gudmundsson, E.M. Turner, S.H.Allen, D.K.Jung, C.J. Sexton, F. L. Boyd, Jr. and Mi.R. Peel. *Tetrahedron*., (**2003)**; 59: 9001–9011.
17. V.Krystof, D.Moravcová, M.Paprskárová, P.Barbier, V.Peyrot, A.Hlobilková, L.Havlícek, M.Strnad., . *Europeon J Med Chem*., **(2006)**; 41: 1405-1411.
18. J.He, F.Seela. *Tetrahedron.,* **(2002)**; 58: 4535-4542.
19. M.J. Quintela, C.Peinador, L.González, I.Devesa, M.L.Ferrándiz, J.Maria Alcaraz and R.Riguera. *Bioorg Med Chem.,* **(2003)**; 11: 863–868.
20. D.Robert. Hubbard, Y. Bamaung, F.Palazzo, Q.Zhang, P.Kovar, D.J.Osterling, X.Hu, J.L. Wilsbacher, E.F.Johnson, J.Bouska, J.Wang, R.L.Bell, S.K.Davidsen and G.S. Sheppard. *Bioorg Med Chem Lett.,* **(2007)**; 17: 5406–5409.

***Corresponding Author:**
***P. Aravinda Reddy and Ramya Sri.S***
*Email:* surapharmalabs1@gmail.com