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Original Article

SYNTHESIS, CHARACTERIZATION AND EVALUATION OF PYRIDOPYRIMIDINE CARBOXYLATE DERIVATIVES AS POTENTIAL ANTIMICROBIAL AND ANTICANCER AGENTS

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ABSTRACT

Objective: Some novel pyridopyrimidine carboxylate derivatives were synthesized through nucleophilic substitution reactions with the use of amidines, followed by 4-haloanilines and malonic acid and were evaluated for antimicrobial and anticancer activity.

Methods: The synthesized compounds A-F were characterized by UV, IR, ^{1}H NMR, mass and elemental analysis and were evaluated for their antimicrobial activity against *B. subtilis, S. aureus, E. coli, S. typhi, Candida albicans and Aspergillus niger* by disc diffusion method, anticancer activity against cancerous cells i.e. colon cancer (HT29), liver cancer (HepG2) and cervical cancer (Hela).

Results: All the compounds showed moderate to considerable antimicrobial and anticancer activity.

Conclusions: Out of the all synthesized compounds E and F showed excellent antimicrobial activity. The LC_{50} of the synthesized derivatives were found to be >100 µg/ml for all cancerous cell lines.

Keywords: Pyridopyrimidine, Amidines, 4-Haloanilines, Malonic Acid, Antimicrobial Activity, Anticancer Activity.

INTRODUCTION

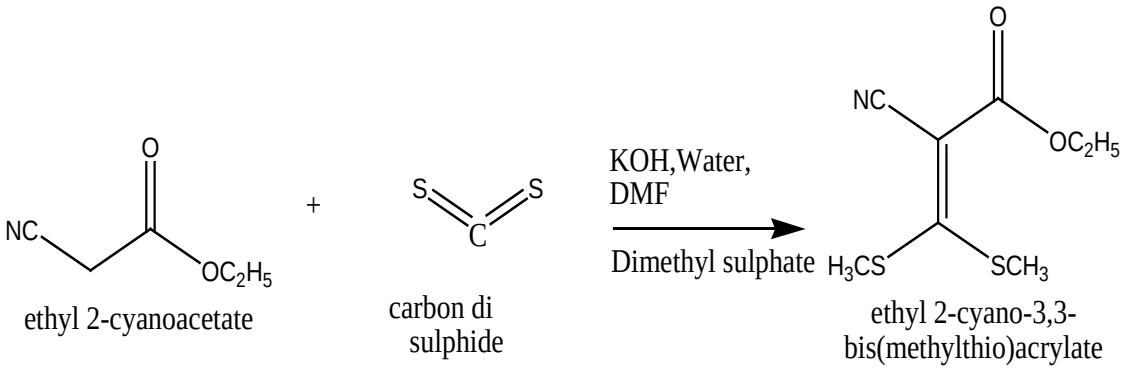
Compounds with pyridopyrimidine carboxylate structures are known to possess antibacterial [1-2], antifungal[3-4], analgesic [5], anti-inflammatory [6], anti HIV [7], antidiabetic [8], antiviral [9] and antitumor activity [10-12] etc. In the present study some novel pyridopyrimidine carboxylate derivatives (A to F) have been synthesized through nucleophilic substitution reactions with the use of amidines, followed by 4-haloanilines and malonic acid. The structures of the various synthesized compounds were assigned on the basis of elemental analysis, UV, IR and ^{1}H NMR spectral data. These compounds were also screened for their antimicrobial and anticancer activity.

MATERIALS AND METHODS

All the chemicals used were of synthetic grade procured from various chemical units like Loba Chemicals, SRL, Chem. Lab Mumbai. Melting points of all the synthesized compounds were determined in open capillary tubes and the values were uncorrected. The UV spectra were recorded by using double beam SHIMADZU 1700 UV spectrometer. The IR spectra were recorded on FT-IR 8101 (Shimadzu) spectrometer by KBr pellets technique. 1H-NMR spectra were recorded on JEOL JNM-α 400 spectrometer using DMSO-d6 as solvent and TMS as internal standard. Mass spectra were recorded on JEOL GC mate mass spectrometer. The purity of the compounds was checked by TLC.

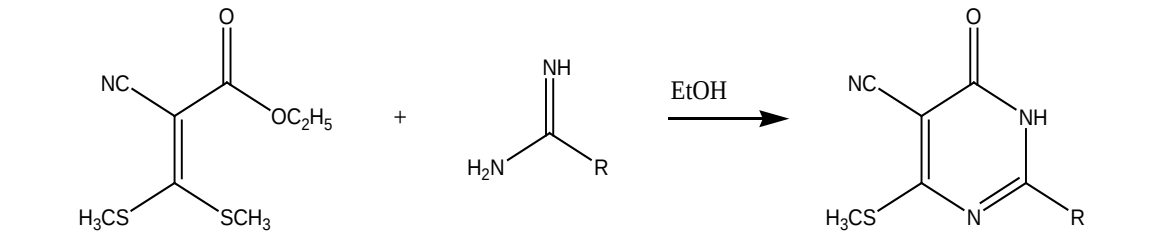
Step 1: **Preparation of Ethyl 3, 3 bis (methylthio) -2- cyanoacrylate:**

Dimethyl formamide (30 ml), ethylcyano acetate (0.1mol) and carbondisulfide (0.1 mol) were added in an ice cold solution of potassium hydroxide (0.2 mol) with continuous cooling and stirring. The mixture was stirred for one hour at room temperature, cooled and treated drop wise with dimethyl sulphate (0.2 mol) maintaining temperature at 20°C.The reaction mixture was allowed to stand at room temperature for 12 hours and poured into 500 ml of ice water mixture. The solid obtained was filtered, washed with cold water, recrystallized from n-hexane and dried.



Step 2: **Preparation of 2 – substituted - 4 - (methylthio) – 6 - oxo-1,6-dihydro pyrimidine -5-carbonitrile**

A mixture of ethyl 2- cyano-3,3-bis(methylthio) acrylates (0.02 mol) and freshly distilled aromatic amidines (0.02 mol) in 30 ml of ethanol were refluxed for one hour. The reaction mixture was allowed to stand at room temperature for 24 hours. The solid obtained was filtered, washed with cold ethanol, recrystallized from benzene-hexane mixture and dried.

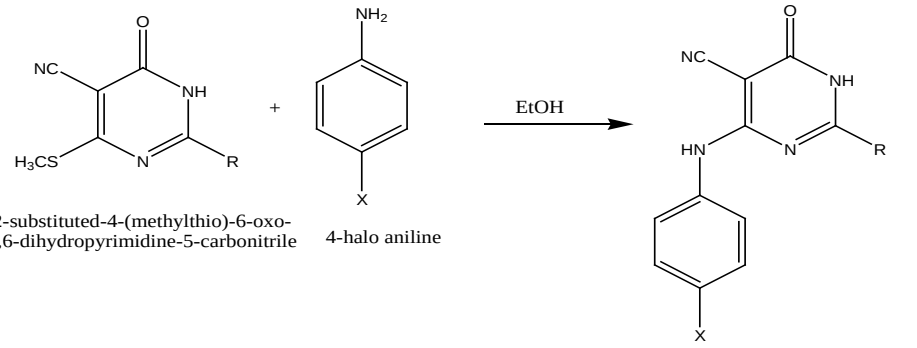


ethyl 2-cyano-3,3-bis(methylthio)acrylate — Amidine — 2-substituted-4-(methylthio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile

Where, R = C_6H_5 (Benzamidine), R = $COOCH_3$ (Formamidine acetate)

Step 3: Preparation of 4- (4-halo phenyl amino) -2-substituted-6-oxo-1,6-dihydro pyrimidine-5-carbonitrile

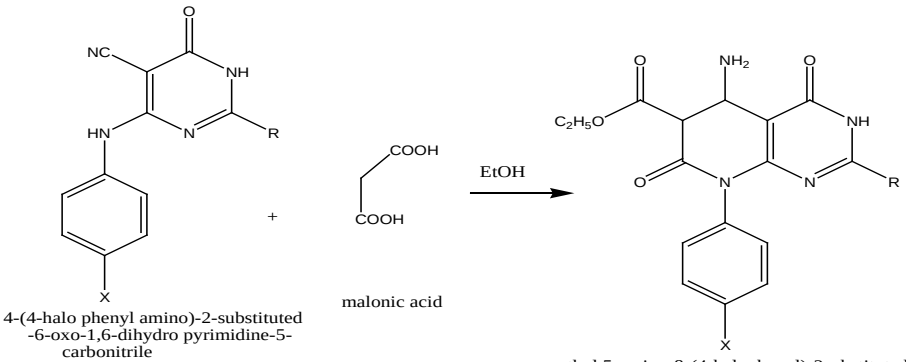
A mixture of 2-subsutitued-4 - (methylthio) - 6 - oxo-1,6 dihydropyrimidine-5-carbonitrile (0.02 mol) and freshly distilled aromatic halo anilines (0.01mol) in 30 ml of ethanol were refluxed for one hour. The reaction mixture was allowed to stand at room temperature for 24 hours. The solid obtained was filtered, washed with cold ethanol, recrystallized from n-hexane and dried. In this work different anilines like p-fluroaniline, p- chloroaniline, p-bromoaniline was used.



2-substituted-4-(methylthio)-6-oxo-1,6-dihydropyrimidine-5-carbonitrile — 4-halo aniline — 4-(4-halo phenyl amino)-2-substituted -6-oxo-1,6-dihydro pyrimidine-5-carbonitrile

Where X = F, Cl, Br etc

Step 4: Preparation of Ethyl-5-amino-8- (4-halo phenyl) -2-substituted-4, 7-dioxo- 3,4,5,6,7,8-hexa hydro pyrido (2,3-d) pyrimidine -6-carboxylate.



4-(4-halo phenyl amino)-2-substituted -6-oxo-1,6-dihydro pyrimidine-5-carbonitrile — malonic acid — ethyl 5-amino-8-(4-halophenyl)-2substituted-4,7-dioxo-3,4,5,6,7,8-hexahydropyrido [2,3-*d*]pyrimidine-6-carboxylate

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A mixture of step 3 product (0.01mol) and malonic acid (0.02mol) and 30 ml of ethanol were refluxed for one hour. The reaction mixture was allowed to stand at room temperature for 24 hours. The solid obtained was filtered, washed with cold ethanol, recrystallized from benzene-hexane mixture and dried

Antimicrobial activity [13]

Antimicrobial activity of the synthesized compounds was screened using the disc diffusion method against selected pathogens such as *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Salmonella typhi*, *Candida albicans* and *Aspergillus niger*. The compounds were dissolved in DMSO and sterilized by filtering through 0.45 µm millipore filter. Nutrient agar (anti bacterial activity) and sabouraud dextrose agar medium (antifungal activity) were prepared and sterilized by an autoclave (121ºC and 15 Ibs for 20 min) and transferred to previously sterilized petridishes (9 cm in diameter).

After solidification, petriplates were inoculated with bacterial organisms in sterile nutrient agar medium at 45 ^{0}C and fungal organism in sterile sabouraud's dextrose agar medium at 45 ^{0}C in aseptic condition. Sterile whatmann filter paper discs (previously sterilized in U.V. lamp) were impregnated with synthesized compounds at a concentration of 25,100 µg /disc was placed in the organism-impregnated petriplates under sterile condition. The plates were left for 30 min to allow the diffusion of compounds at room temperature. Antibiotic discs of ciprofloxacin (100 µg /disc) and ketoconazole (100 µg /disc) were used as positive control, while DMSO used as negative control. Then the plates were incubated for 24 hr at 37 ± 1 ^{0}C for antibacterial activity and 48 hr at 37±1 ^{0}C for antifungal activity. The zone of inhibition was calculated by measuring the minimum dimension of the zone of no microbial growth around the each disc.

Anticancer activity [14]

Anticancer activity of synthesized pyridopyrimidine carboxilate derivatives were evaluated using three human cancer cell lines [colon cancer (HT29), liver cancer (HepG2), cervical cancer (Hela)] by MTT assay. 0.1ml of the cell suspension (containing 5x10^6 cells/100 µl) and 0.1 ml of the test solution (6.25 µgm to 100 µgm in 1% DMSO such that the final concentration of DMSO in media is less than 1%) were added to the 96 well plates and kept in 5% CO_2 incubator at 37^{0}C for 72 hrs. Blank contains only cell suspension and control wells contain 1% DMSO and cell suspension. After 72 hrs, 20 µl of MTT was added and kept in CO_2 incubator for 2 hrs. The plate was covered with aluminum foil to protect from light. Then the 96 well plates were kept in rotary shaker for 10 to 20 min.

After 10 to 20 min, the 96 well plates were processed on ELISA reader for absorption at 562 nm. The readings were averaged and viability of the test samples was compared with DMSO control. The percentage growth inhibition was calculated by using the following formula:

$$\% \text{ Growth of inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100$$

RESULTS AND DISCUSSION

The melting points of all synthesized compounds were found in open capillary tubes and readings were uncorrected. The structures of the synthesized compounds were supported by physical data (Table-1) and following spectral analysis.

Table 1: Physical data of the pyridopyrimidine carboxilate derivatives

Compound	Mol. formula	X	R	Mol. wt.	M.P.(^{o}C)	%Yield	R_f values
A	$C_{17}H_{17}N_4O_4F$	F	$COOCH_3$	360.34	350	78	0. 67
B	$C_{17}H_{17}N_4O_4Cl$	Cl	$COOCH_3$	376.79	358	80	0. 89
C	$C_{17}H_{17}N_4O_4Br$	Br	$COOCH_3$	421.25	360	79	0.77
D	$C_{22}H_{19}N_4O_4F$	F	C_6H_5	422.40	363	76	0. 78
E	$C_{22}H_{19}N_4O_4Cl$	Cl	C_6H_5	438.86	353	81	0.64
F	$C_{22}H_{19}N_4O_4Br$	Br	C_6H_5	483.31	359	82	0. 58

The lambda maxes of the compounds were measured by double beam SHIMADZU 1700 UV spectrometer. The results of the UV spectra were given in under the spectral detail heading. The IR spectra of the compounds were done in FT-IR 8101 (Shimadzu) spectrometer using KBr discs. The results of IR spectra were given in spectral detail heading which showed absorption bands for aromatic groups.

The ^{1}H NMR spectra of the synthesized compounds were recorded on JEOL JNM-α 400 spectrometer using TMS as internal standard (chemical shifts in δ, ppm) and DMSO as the solvent. The results of the ^{1}H NMR spectra were given under spectral detail heading showed that the numbers of hydrogen atoms present in all the synthesized compounds were exact when compared to the number of hydrogen atoms in the expected compounds.

The Mass spectra of the all the synthesized compounds were done on a JEOL GC mate spectrometer. The results presented in the spectral heading showed that the molecular mass of the synthesized compounds was nearer to the molecular mass of the expected compounds.

The spectral details of the synthesized compounds:

Ethyl-5-amino-8- (4-fluorophenyl) -2-acetyl-4,7-dioxo-3,4,5,6,7,8 hexahydro pyrido (2,3-d) pyrimidine-6-carboxylate (A)

UV λ_{max} (DMSO): 243 nm; IR (KBr) ν_{max} : 2915.05(C-H,Ar),3460.50(N-H),1322.33(C-N), 15 30.03(C=N),815.05(C-F),1610.06(C=O); ^{1}H NMR(DMSO- d_6) δ: 1.35 (T,3H),2.2 (S,1H),4.2 (M,1H),7.2 (T,3H),7.3 (M,1H),11.4 (S,1H); LC-MS: *m/z* 360.54 (M^+); $C_{17}H_{17}N_4O_4F$ (C:56.66,H:4.76,F:5.27,N:15.55,O:17.76)%.

Ethyl-5-amino-8- (4-chlorophenyl) -2- acetyl -4,7-dioxo-3,4,5,6,7,8 hexahydro pyrido (2,3-d) pyrimidine-6-carboxylate (B)

UV λ_{max} (DMSO): 239 nm; IR (KBr) ν_{max} : 2918.05(C-H,Ar),3360.50(N-H),1312.33(C-N), 15 20.03(C=N),816.05(C-F),1611.06(C=O); ^{1}H NMR(DMSO- d_6) δ: 1.3 (M,1H),2.2 (S,1H),2.4 (S, 1H),3.4 (S,2H),3.5 (S,3H),4.2 (M,3H),6.5 (M,2H), 7.2 (M, 1H), 7.3 (M, 1H), 11.4 (S, 1H) ; LC-MS: *m/z* 376.79 (M^+); $C_{17}H_{17}N_4O_4Cl$ (C:54.18,H:4.56, Cl:9.42, N:14.55,O:16.78)%.

Ethyl-5-amino-8- (4-bromophenyl) -2- acetyl -4,7-dioxo-3,4,5,6,7,8 hexahydro pyrido (2,3-d) pyrimidine-6-carboxylate (C)

UV λ_{max} (DMSO): 231 nm; IR (KBr) ν_{max} : 2915.05(C-H,Ar),3367.50(N-H),1313.33(C-N), 15 22.03(C=N),818.05(C-Br),1612.06(C=O); ^{1}H NMR(DMSO- d_6) δ: 1.3 (T,1H), 2.2 (S,1H),4.2 (M,3H), 7.1 (M,1H),7.2 (M,1H),11.4 (S,1H) ; LC-MS: *m/z* 421.25 (M^+); $C_{17}H_{17}N_4O_4Br$ (C:48.47, H:4.07, Br:8.98,N:13.50,O:15.78)%.

Ethyl-2,5-diamino-8- (4-fluorophenyl) 2-benzyl- 4,7-dioxo-3,4,5,6,7,8 hexahydro pyrido (2,3-d) pyrimidine-6-carboxylate (D)

UV λ_{max} (DMSO): 279 nm; IR (KBr) ν_{max} : 2916.05(C-H,Ar),3460.50(N-H),1323.33(C-N), 16 30.03(C=N),816.05(C-F),1611.06(C=O); ^{1}H NMR(DMSO- d_6) δ: 0.07 (S,1H),1.2 (S,2H),2.08 (S,1H),2.9 (S,2H),3.4 (S,1H),3.7 (S,1H),6.6 (S,1H), 7.0 (T, 3H), 8.02 (S, 1H), 8.6 (S,1H); LC-MS: *m/z* 438.86 (M^+); $C_{22}H_{19}N_4O_4F$ (C:53.19,H:4.07,F:5.26,N:19.38,O:17.72)%.

Ethyl-2,5-diamino-8- (4-chlorophenyl) 2-benzyl -4,7-dioxo-3,4,5,6,7,8 hexahydro pyrido (2,3-d) pyrimidine-6-carboxylate (E)

UV λ_{max} (DMSO): 281 nm; IR (KBr) ν_{max} : 2922.05(C-H,Ar),3450.50(N-H),1310.33(C-N), 15 20.09(C=N),819.05(C-Cl),1618.06(C=O); ^{1}H NMR(DMSO- d_6) δ: 0.1 (M,1H),1.3 (M,3H),2.9 (S,1H),4.2 (M,1H),4.3 (M,3H),6.5 (D,1H) 6.8 (S,1H), 7.2 (M, 3H),7.4 (S,1H),10.8 (S,1H). ; LC-MS: *m/z* 376.79 (M^+); $C_{22}H_{19}N_4O_4Cl$(C:50.87,H:4.21,Cl:9.39,N:18.55,O:16.98)%.

Ethyl-2,5-diamino-8- (4-bromophenyl) 2-benzyl -4,7-dioxo-3,4,5,6,7,8 hexahydro pyrido (2,3-d) pyrimidine-6-carboxylate (F)

UV λ_{max} (DMSO): 278 nm; IR (KBr) ν_{max} : 2915.08(C-H,Ar),3367.40(N-H),1314.33(C-N), 15 24.03(C=N),819.05(C-Br),1611.06(C=O); ^{1}H NMR(DMSO- d_6) δ: 0.1 (D,1H),1.2 (S,1H),2.1 (D,1H),2.9 (D,2H),3.8 (S,1H),6.6 (M,1H),7.2 (M,3H), 7.4 (S,1H); LC-MS: *m/z* 421.25 (M^+); $C_{22}H_{19}N_4O_4Br$ (C:45.50,H:3.87, Br:18.98,N:16.50,O:15.18)%.

The antimicrobial activities of all synthesized compounds were screened by disc diffusion method. For all six compounds Minimum

Inhibitory Concentration was determined using standard Ciprofloxacin and Ketoconazole. All the compounds showed significant inhibitory activity against the microbes with the 100µg/ml which produces 100% inhibition against the microorganism. Out of the synthesized compounds E and F showed excellent antimicrobial activity. The results were tabulated in table 2 and 3 were given as zone of inhibition and MIC. Anticancer activity of synthesized pyridopyrimidine carboxilate derivatives were evaluated using three human cancer cell lines [colon cancer (HT29), liver cancer (HepG2), cervical cancer (Hela)] by MTT assay. All the synthesized compounds showed significant anticancer activity. The results were tabulated in table 4.

Table 2: *In vitro* antibacterial activity of pyridopyrimidine carboxilate derivatives

Compound	MIC (µg/ml)	Zone of inhibition (mm)			
		Gram positive		Gram negative	
		B. subtilis	*S.aureus*	*E. coli*	*S. typhi*
A	100	12	10	12	10
B	100	13	12	12	6
C	100	12	12	6	13
D	100	10	10	11	12
E	100	14	14	16	13
F	100	14	14	12	15
Standard (Ciprofloxacin)	100	24	22	21	22
Control(DMSO)	-	-	-	-	-

Table 3: *In vitro* antifungal activity of pyridopyrimidine carboxilate derivatives

Compound	MIC (µg/ml)	Zone of inhibition (mm)	
		Candida albicans	*Aspergillus niger*
A	100	12	11
B	100	10	13
C	100	13	14
D	100	12	11
E	100	15	15
F	100	16	14
Standard (Ketoconazole)	100	23	22
Standard(DMSO)	-	-	-

Table 4: anticancer activity of pyridopyrimidine carboxilate derivatives

Compound	GI_{50} (µg/ml)			TGI(µg/ml)			LC_{50} (µg/ml)		
	HT29	HepG2	Hela	HT29	HepG2	Hela	HT29	Hep2	Hela
A	25	23	24	58	48	47	>100	>100	>100
B	27	26	27	45	52	71	>100	>100	>100
C	24	28	30	57	55	67	>100	>100	>100
D	29	35	25	48	76	58	>100	>100	>100
E	22	18	28	38	41	62	>100	>100	>100
F	29	30	30	59	60	62	>100	>100	>100

CONCLUSION

The research work was oriented towards the finding of new pyridopyrimidine carboxylate derivatives with antimicrobial and anticancer activities. Compound E, F showed very good antimicrobial activity. The LC_{50} of the synthesized pyridopyrimidine carboxylate derivatives were found to be >100 µg/ml for all these cell lines. Based on cytotoxicity results the synthesized compounds possessed cytotoxic effect on these three human cancer cell lines.

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LIST OF ABBREVIATIONS

MTT - 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazoliumbromide

MTP - Micro Titer Plate

ELISA - Enzyme Linked Immuno Sorbant Assay

DMSO - Dimethylsulfoxide

HT29 - Colon cancer cells

HepG2 - Liver cancer cells

Hela - Cervical Carcinoma cells

GI_{50} - The concentration of drug to cause 50% reduction in growth of cancer cells

TGI - Total Growth Inhibition

LC_{50} - Lethal Concentration which kills 50% cancer cells

MIC _ Minimum Inhibitory Concentration

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