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Research Article

SYNTHESIS OF CERTAIN COUMARINYL PYRAZOLINE-5-ONE DERIVATIVES AND EVALUATION OF THEIR ANTIMICROBIAL, ANTIOXIDANT AND ANTI-TB ACTIVITIES

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ABSTRACT

The aim of the present work was to synthesize certain coumarinyl pyrazoline-5-one derivatives and to elucidate the potential role of these compounds as antimicrobial, antioxidant and anti-tubercular agents. The coumarinyl pyrazoline-5-one derivatives were prepared by condensing the unsaturated ester of diazotized Anilines with 6-bromo substituted Coumarin-3- acid hydrazides. All the synthesized compounds showed promising anti-tubercular activity against Mycobacterium tuberculosis and some of them showed significant *in vitro* antioxidant activity and antimicrobial activity

Keywords: Coumarinyl Pyrazoline-5- one derivatives, anti-tb, Antimicrobial, Antioxidant.

INTRODUCTION

Pyrazoline derivatives are reported to exhibit a wide range of biological activities such as analgesic, antipyretic, anti inflammatory, antibacterial, antifungal, antiviral, antitubercular, antioxidant, anticancer, antidiabetic and anticonvulsant activities.

Similarly Coumarins were also reported to exhibit antibacterial, antifungal, antitubercular, anticancer, C.N.S. depressant, stimulant and antipsychotic activities.

In view of considerable importance of the pyrazolins and coumarins, the present work is aimed to combine coumarins and Pyrazolin-5-one moieties together in a molecular framework and to find out the additive effects towards their biological activities such as antimicrobial, antioxidant and anti-tubercular activity.

MATERIALS AND METHODS

Melting points were determined in open capillaries and were uncorrected. IR spectra were recorded on a Perkin Elmer - Spectrum RX-IFTIR. ^{1}H NMR spectra were recorded on a FT NMR Spectrometer model Avance-II (Bruker 400 Mhz). Mass spectra were recorded on LCMS. The purity of the all compounds were established by single spot on TLC plates. Iodine vapour was used as developing agent. The solvent system used was cyclohexane:methanol(9:1).

Preparation of 6-bromo- 3-carbethoxy coumarins[1]

5.0gm of 6-bromo salicylaldehyde(0.041mol), 7.2gm of diethylmalonate(0.045mol), 25ml of absolute ethanol, 0.05ml of piperidine, 0.02(one drop) of glacial acetic acid and three boiling stones were placed in a dry round bottomed flask. The flask was equipped with a water cooled condenser protected from atmospheric moisture with calcium chloride drying tube. The solution was refluxed over the steam bath for 2 hours, and then the solution was transferred in to a 250ml flask. 35ml of cold water added and the solution was cooled in an icebath. After crystallization was completed, the crystals were filtered and washed twice with 3 ml portions of ice cold 50% aqueous ethanol and dried. Rection is given in the figure-1.

Preparation of 6-bromo coumarin 3- acid hydrazides[2]

A mixture of substituted 3-carbethoxy coumarin (0.01mol)and hydrazine hydrate (0.01mol) was placed in a round bottom flask fitted with a reflux condenser and the mixture was heated gently under reflux for 15 minutes. Then sufficient quantity of ethanol was added to give a clear solution. The mixture was refluxed for a further 2-3 hours. And then the mixture was concentrated to half its volume by distillation. The crystals of coumarin 3-acid hydrazide are filtered and recrystalized from an aqueous ethanolic solution. Rection is given in the figure-2.

Diazotization and Estrification[3]

Substituted aromatic amines(0.01mol) were dissolved in a mixture of 40 ml of hydrochloric acid (8ml) and water (6ml) then cooled to 0^{o}c in ice water. And a cold aqueous solution of sodium nitrite(0.03mol)was added. The diazotization salt solution was filtered directly in to the cold solution of ethylacetoacetate (0.01mol) and sodium acetate(0.122mol) in 50 ml of ethanol. The resultant solid was filterd, washed with water then recrystallised from ethanol. Rection is given in the figure-3.

Cyclization[4]

A mixture of diazotized and estrified aromatic substituted amines (0.002mol) were dissolved in glacial acetic acid (20ml) and the substituted coumarin 3-acid hydrazide (0.002mol) dissolved in 20mL glacial acetic acid was added. The mixture was refluxed for 5 hours, cooled and then allowed to stand over night. The resultant solid was filtered, dried and then crystallized from ethanol. The purity of all compounds was established by single spot on TLC plates as described above. Rections were given in figure 4, characterization data and spectral data's were given in the tables 1 and 2 respectively.

Antimicrobial Screening[5,6]

The antimicrobial activity of the synthesized compounds were tested by Cup plate method against gram positive organisms namely staphylococcus aureus and bacillus subtilis, gram negative organisms namely Escherichia coli and Pseudomonas aeruginosa using nutrient agar medium. The antifungal activity was tested against fungi Aspergillus Niger and candida albicans using Sabouraud Dextrose agar medium.

Sterile Agar plates were prepared and 0.1 ml of standardized test organism culture was spread uniformly. Wells were prepared by using a sterile borer of diameter 10mm and 100µl (To get the final concentration of 500 and 250 µg/ml) of the test substance, standard antibiotic were added in each

well separately. A standard antibiotic, ciprofloxacin (10µg/ml) was tested against bacteria and fluconazole(10µg/ml) against fungi. The plates were placed at 4^0C for 1 h to allow the diffusion of test solution into the medium and the plates were incubated at a temperature optimal for the test organism and for a period of time sufficient for the growth of at least 10 to15 generation, usually 24 hours at 37^0C for bacteria and 24- 48 hours at 25^0C for fungi. The zone of inhibition was measured in mm. Result swere tabulated in the tables 3,4 and 5.

Free-radical scavenging activity by DPPH assay method[7,8]

Free radical scavenging activity of the coumarino pyrazoline-5-one derivatives were determined by DPPH assay method and compared with (EC_{50}) of ascorbic acid as the standard.

Drugs stock solutions (1.0 mg / ml) were diluted to final concentrations of 2, 4,6,8 and 10 µg /ml in methanol. One ml of 0.3 mM (12 mg in 100 ml) DPPH solution in methanol was added to 2.5 ml of drug solution of different concentrations, and allowed to react at room temperature . After 30 minutes the absorbance values were measured at 518 nm and converted into the percentage antioxidant activity (AA). It is calculated.

Methanol (1.0 ml) and drug solution (2.5 ml) was used as a blank. DPPH solution (1.0ml; 0.3mM) and methanol (2.5ml) was used as a control. Methanol (1.0 ml),drug solution(2.5 ml) and DPPH solution (1.0ml;0.3mM) was used as sample. The positive controls were those using the standard solutions. The EC_{50} values which represents the concentration required for 50% antioxidant activity was calculated from the calibration curve (concentration of compounds µg / antioxidant activity). Results were given in the table-6

Anti-TB activity by Microplate Alamar blue assay (MABA)[9,10]

All the synthesized compounds were tested against M. tuberculosis using Microplate Alamar Blue assay (MABA). This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with propotional and BACTEC radiometric method. Briefly, 200µl of sterile deionzed water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. The 96 wells plate received 100 µl of the Middle brook 7H9 broth and serial dilution of compounds were made directly on plate. The final drug concentrations tested were 100 to 0.8 µg/ml. M.tb cultures (100µL innoculam of $2X10^5$cfu/mL)were added,yielding a final testing volume of 200µL. Plates were covered and sealed with Parafilm and incubated at 37ºC for five days. After this time, 25µl of freshly prepared 1:1 mixture of Almar Blue reagent and 10% tween 80 was added to the plate and incubated for 24 hrs. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink. MIC values were given in the table-7.

Table-1: Characterization data of synthesized compounds

Product code	Molecular formula	Molecular weight	Percentage yield	M.P ^{0}C	Rf value
I.CPZ-1	$C_{20}H_{12}BrClN_4O_4$	487.69	58%	324-326^0 C	0.642
I.CPZ-2	$C_{20}H_{12}BrClN_4O_4$	487.69	64%	114-116^0 C	0.651
I.CPZ-3	$C_{20}H_{12}BrClN_4O_4$	487.69	77%	290-292^0 C	0.671
I.CPZ-4	$C_{20}H_{12}Br2N_4O_4$	532.14	59%	264-266^0 C	0.591
I.CPZ-5	$C_{20}H_{12}BrN_5O_6$	498.24	69%	306-308^0 C	0.55

Table-2: spectral data of synthesized compounds

Product code	IR(v,cm^{-1})	^{1}H NMR(δ, ppm)(DMSO-d6)/ MS
I.CPZ-1	N-H 3304, C=O 1672, C=N 1610,C=C 1487, CN 1243.	2.20 (s,3H,CH_3 of Pyrazolone), 6.88-8.25 (m,8H, Ar-H), 8.86 (s,1H,NH).
I.CPZ-2	N-H 3153, C=O 1666, C=N 1623, C=C 1463, CN 1245.	2.20 (s,3H,CH_3 of Pyrazolone), 6.88-7.46 (m, 7H, Ar-H), 8.82 (s,1H,NH). m/z 486.6 $(M-1)^+$
I.CPZ-3	C=O 1704,C=N 1623,C=C 1463,CN 1250.	3.88(s,3H,CH_3 of Pyrazolone), 6.87-7.99 (m, 7H, Ar-H), 8.87 (s,1H,NH).
I.CPZ-4	C=O 1707, C=N 1624,C=C 1463, CN 1249.	3.91(s,3H, CH_3 of Pyrazolone), 6.90-7.12 (m, 7H, Ar-H), 8.81 (s,1H,NH). m/z 533.1 $(M+1)^+$
I.CPZ-5	NH 3567,C=N 1623,C=C 1464, CN 1248	3.91(s,3H,CH_3 of Pyrazolone), 6.90-7.79 (m, 7H, Ar-H), 8.83 (s,1H,NH).

Table-3 : quantitative screening of test compounds for antibacterial activit against gram positive organisms

Compound Code*	Diameter of Zone of Inhibition in mm(mean of three replicants)			
	Staphylococcus aureus		*Bacillus subtilis*	
	500µg/disc	250µg/disc	500µg/disc	250µg/disc
ICPZ -1	18.33	16.33	20	17.33
ICPZ-2	11	-	13.33	11
ICPZ-3	12	11	13	11
ICPZ-4	18.66	16.66	20.66	18.33
ICPZ-5	-	-	-	-
DMF (Blank)	-	-	-	-
Standard (Ciprofloxacin) 10 µg/disc	36		34	

(-) indicates no zone of inhibition

Table -4: quantitative screening of test compounds for antibacterial activity against gram negative organisms

Compound Code*	Diameter of Zone of Inhibition in mm (mean of three replicants)			
	Escherichia coli		*Pseudomonas aeroginosa*	
	500µg/disc	250µg/disc	500µg/disc	250µg/disc
ICPZ -1	16.00	13.33	-	-
ICPZ-2	14	12.33	-	-
ICPZ-3	15	14	12	11
ICPZ-4	15	12.66	-	-
ICPZ-5	14	13	-	-
DMF (Blank)	-	-	-	-
Standard (Ciprofloxacin) 10 µg/disc	25		21	

(-) indicates no zone of inhibition.

Table-5: quantitative screening of test compounds for antifungal activity

Compound Code*	Diameter of Zone of Inhibition in mm (mean of three replicants)			
	Candida albicans		*Aspergillus Niger*	
	500µg/disc	250µg/disc	500µg/disc	250µg/disc
ICPZ-1	17.33	12.33	-	-
ICPZ-2	12	-	-	-
ICPZ-3	-	-	-	-
ICPZ-4	14.66	12.33	16	13
ICPZ-5	-	-	-	-
DMF (Blank)	-	-	-	-
Standard (Fluconazole) 10 mcg/disc	25		19	

(-) indicates no zone of inhibition

Table -6: antioxidant studies

Compound code	% Reduction in absorbance (mean ± SD)					EC50
	2 µg/ml	4 µg/ml	6 µg/ml	8 µg/ml	10 µg/ml	
ICPZ-I	22.47 ± 0.19	31.30 ± 0.07	44.98 ± 0.71	51.48 ± 0.14	58.60 ± 0.14	7.79
ICPZ-2	18.76 ± 0.28	30.10 ± 0.19	52.89± 0.05	62.54 ±0.728	73.88 ± 0.07	6.33
ICPZ-3	16.24 ± 0.49	21.11 ± 0.20	27.93 ± 0.14	37.52 ± 0.12	51.36 ± 0.20	10.42
ICPZ-4	18.80 ± 0.04	26.74 ± 0.06	33.13 ± 0.11	40.62 ± 0.38	48.99 ± 0.40	10.40
ICPZ-5	17.75 ± 0.30	26.30 ± 0.22	38.49 ± 0.11	49.88 ± 0.13	55.70 ± 0.13	8.48
Ascorbic acid	26.76 ±0.12	39.51± 0.32	59.26± 0.09	66.26±0.19	73.58±0.16	5.48

Table -7 : Anti-Tb studies

compound code	100	50	25	12.5	6.25	3.125	1.6	0.8
cpz-i-1	s	s	s	s	s	s	s	s
cpz-i-2	s	s	s	s	s	s	s	s
cpz-i-3	s	s	s	s	s	s	s	s
cpz-i-4	s	s	s	s	s	s	s	s
cpz-i-5	s	s	s	s	s	s	s	r

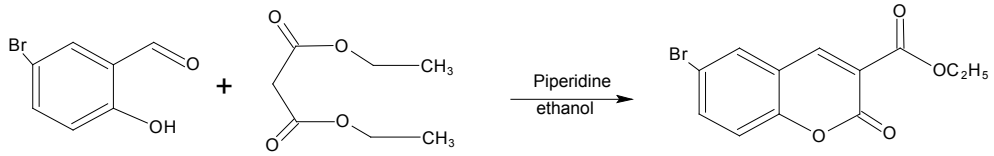


Figure-1: preparation of 6-bromo- 3-carbethoxy coumarins

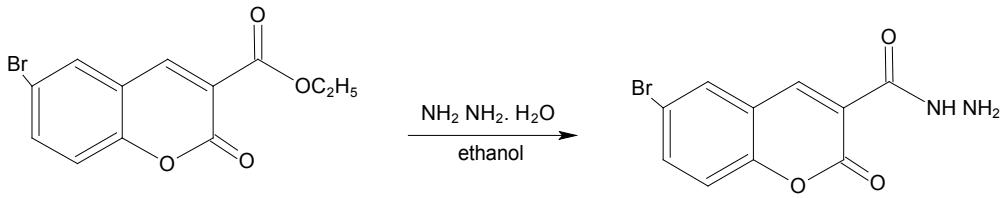
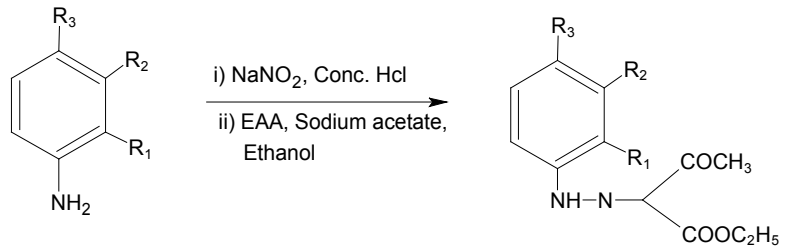


Figure-2: preparation of 6-bromo coumarin 3- acid hydrazides



S.No.	R_1	R_2	R_3
1.	Cl	H	H
2.	H	Cl	H
3.	H	H	Cl
4.	H	H	Br
5.	NO_2	H	H

Figure-3: diazotization and estrification

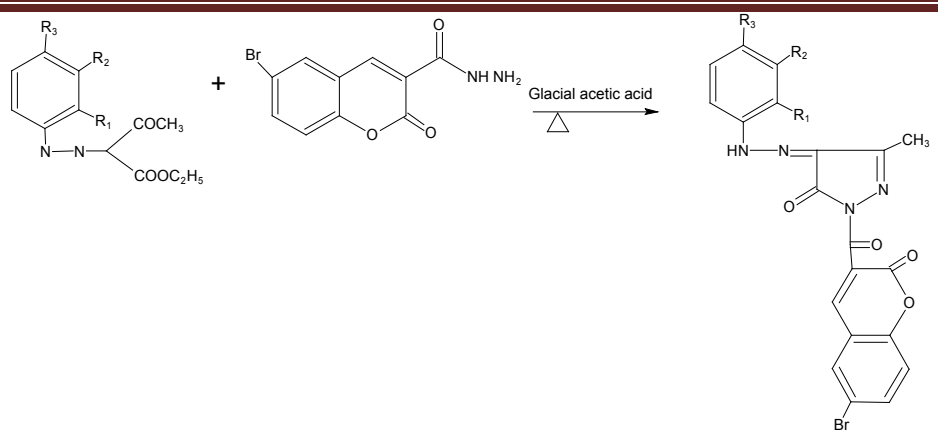


Figure-4: cyclization

RESULTS

Anti Microbial Screening

Antibacterial screening

Staphylococcus aureus was found to be sensitive to the synthesized compoundS I-CPZ-I and I-CPZ-4 at a concentration 500 mcg/well. Bacillus subtilis was found to be sensitive to the synthesized compounds I-CPZ-I and I-CPZ-4 even at a concentration 250mcg/well and moderatively sensitive to 500mcg/well concentration Of synthesized compound ICPZ-10. Gram negative organism Escherichia coli was moderately sensitive to ICPZ-1, ICPZ-2, ICPZ-3, ICPZ-4, ICPZ-5. Synthesized compounds ICPZ-1 was found to be moderately sensitive to Escherichia coli at 250mcg/well concentration. Gram negative micro organism Pseudomonas aeruginosa was found to be resistant to all the synthesized compounds. The MIC of test compounds ICPZ-1 and ICPZ-4 against staphylococcus aureus was found to be 31.25 mcg/ml. The MIC of test compounds ICPZ-1 and ICPZ-4 against Bacillus subtillis were found to be 15.62 mcg/ml.

Antifungal screening

Candida albicans was found to be sensitive to 500mcg/well concentration of the synthesized compounds ICPZ-1 and moderately sensitive to ICPZ-4, resistant to all other compounds. The test microorganism Aspergillus niger was found to be moderately sensitive to 500mcg/well concentration of the synthesized compounds ICPZ-4 and resistant to all other compounds.

Antioxidant Studies

ICPZ-2, was found to show good free radical scavenging activity and the compound ICPZ-1 showed moderate free radical scavenging activity, whereas ICPZ-5, showed the least antioxidant activity. But ICPZ-3, ICPZ-4, showed no antioxidant activity.

Anti-TB Screening

All the synthesized compounds showed promising Anti-TB activity against M.tuberculosis. When compared with standard drugs Pyrazinamide (MIC value -3.125µg/ml) and Streptomycin- (MIC value- 6.25µg/ml.)

Among the synthesized compounds ICPZ-1, ICPZ-2, ICPZ-3, ICPZ-4 were sensitive even in the least concentration (0.8 µg/ml).

The MIC values of the synthesized compounds ICPZ-5 was found to be 1.6µg/ml.

CONCLUSION

Results of antimicrobial screening shows that the gram positive organisms Staphylococcus aureus and Bacillus subtilis was found to be sensitive to the synthesized compounds ICPZ-I and ICPZ-4. Synthesized compounds ICPZ-I showed promising antifungal activity against Candida albicans at 500 µg/well concentration. The synthesized compounds ICPZ-2 was found to show good free radical scavenging activity. All the synthesized Pyrazoline-5-one derivatives of substituted coumarins showed promising Anti-TB activity against M.tuberculosis.

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