SYNTHESIS, CHARACTERIZATION OF COUMARIN DERIVATIEVES AND ITS PHARMACOLOGICAL EVALUATION

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ABSTRACT

Six novel coumarin derivatives (CM-1 to CM-6) were synthesized by simple synthesis of ,(z)-4-methyl-7-(1-phenylethylideneamino)-2H-chromen-2-one which involves reaction between 3-aminophenol with ethyl-3-oxobutanoate with to give 7-amino-4-methyl-2H-chromen-2-one. The compound undergoes reaction with benzaldehyde The newly synthesized derivatives were characterized by spectroscopical methods using IR, ^{1}H-NMR spectroscopy and Mass spectrometry.

The selected derivatives of the title compounds $CM_{1,}$ CM_5 are screened for the anti-tuberculosis by *in vitro* method using pyrazinamide & streptomycin as standard. The electron withdrawing groups such as chloro, nitro groups containing compounds (CM-2, CM-6) has shown the highest activity and the rest of compounds having electron donating groups (CM-1,CM-3,CM-4,&CM-5) shows mild to moderate activity.

The synthesized compounds were evaluated for their in vitro anthelmintic activity Pheretima posthuma .Albendazole was used as a standard drug at a dose of 20 mg/ml. The derivatives were screened for pharmacological studies that are anti-helminthic activity. The results are tabulated and among all the derivatives CM-2 has shown good anti-helminthic activity.

KEYWORDS: coumarin derivatives, of anti-tuberculosis & anthelementic activity,

INTRODUCTION

Coumarin is a simple molecule and many of its derivatives have been known for more than a century. Coumarin and coumarin-related compounds have been proved for many years to have significant therapeutic potential[1-3]. Normally Coumarin is synthesised by many of the named reactions with perkins reaction between salicylaldehyde and acetic anhydride as popular example. But in the present work 3-amino phenol and 3-oxo butanoate is used in presence of conc.H_2SO_4 as solvent and Zinc chloride used as base to obtain coumarin. Their physiological, anti-microbial, anti-cancer and anti-inflammatory activities make these compounds attractive for further backbone derivatisation and screening as novel therapeutic agents[19-22].

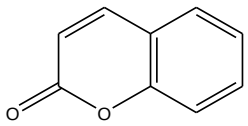


Fig :1 Coumarin

Materials and methods:

Compounds are weighed under weighing balance in precise amount. By using melting point apparatus were determining the melting point for newly synthesized compounds by using only one capillary tube. The reaction was monitored by using pre-coated with silica gel TLC plates.

After completion of the reaction recrystallized with a suitable solvent and the purity of the new compound were checked by using TLC plates are run in a solvent system like Ethanol Water and Ethyl acetate (3:1:3) the completion of reaction and purity of the product were evaluated. The spots are visualised under UV chamber.

Finally newly synthesised compounds are undergo for spectral characterization by IR, MASS AND NMR.

STEP- 1 **Synthesis of 7-amino-4-methyl Coumarin :**

Weigh the (22.0g,202mmol) of 3- amino phenol, and (18.0ml,234mmol) of Zinc chloride, followed by the addition of ethyl 3-oxo butanoate (25.0ml) and slowly add concentrated sulfuric acid with continuous stirring . the product was collected by filtration, washed twice with ethanol and then dried invaccum for characterization with very carefull operation to avoid the destruction. The resulted will be 7- Amino-4-methyl coumarin.

STEP-2 Preparation of (Z)-7-(benzylidene amino)-4- methyl-2H-chrome-2-one:

The above synthesized product was weighed about 0.1 M of 7-Amino- 4-methyl coumarin was taken into a round bottom flask. Then add 0.1M of substituted different aromatic aldehydes was dissolved in 35-40 ml of ethanol. Then the mixture was transferred into a round

bottom flask. The reaction mixture was stirred for 1-2 hours, and refluxed for 3 hours. Finally obtained product was collected and cooled at room temperature and poured into ice water. After cooling the product was filtered and dried at room temperature. Recrystallized with ethanol.

BIOLOGICAL ACTIVITIES:

IN VITRO ANTHELMINTHIC ACTIVITY:

For all of the newly synthesised derivatives, adult earthworms measuring 4-5 cm in length and 0.1-0.2 cm in width were utilised. All of the earthworms were gathered in the AP Indian district of Anantapur. Six worms were placed in each of the groups that were formed from the worms. To assemble the concentrations of 5, 10, and 20 mg/ml, all of the recently synthesised derivatives were dissolved in a minimum of 2% v/v Tween 80 and the volume was increased to 10 ml with normal saline. All of the derivatives and standard medication solutions were newly made prior to the start of the tests. Before being discharged into 10 ml of the appropriate formulation, each earthworm was thoroughly cleaned in normal saline solution. The formulations were as follows: vehicle (2% v/v Tween 80 in normal saline), albendazole (20 mg/ml), and derivatives (5,10,20 mg/ml). Six observations were made for each petri dish containing six worms of the same size in order to determine the anthelmintic activity. They were practical since they moved naturally and elicited reactions. Individual worms' paralysis and deaths were tracked in terms of timing. When the worms failed to awaken even in regular saline, paralysis was thought to have occurred. Worms were said to have died

when they stopped moving, which was followed by the fading of their body colour.

ANTI-TUBERCULAR ASSAY:

In this research work, firstly, all the derivatives were subjected to the susceptibility assay for Mycobacterium tuberculosis. In this point of view, micro plate alamar blue Assay method was selected based on the test, less time consumption, robust, and economical. Further to support the selection of MABA as the test assay for the synthesized derivatives, MABA possess the highest hits in PUBMED citations for estimation of anti- tubercular susceptibility of the derivatives.

MICRO PLATE ALAMAR BLUE ASSAY:

Microplate alamar blue assay method was employed for the antimicrobial activity of the synthesized derivatives against Mycobacterium tuberculosis. This method is nontoxic, employes a reagent having thermal stability and this method establishes good correlation with BACTEC radiometric and proportional method. Following is the flow of the method of assay

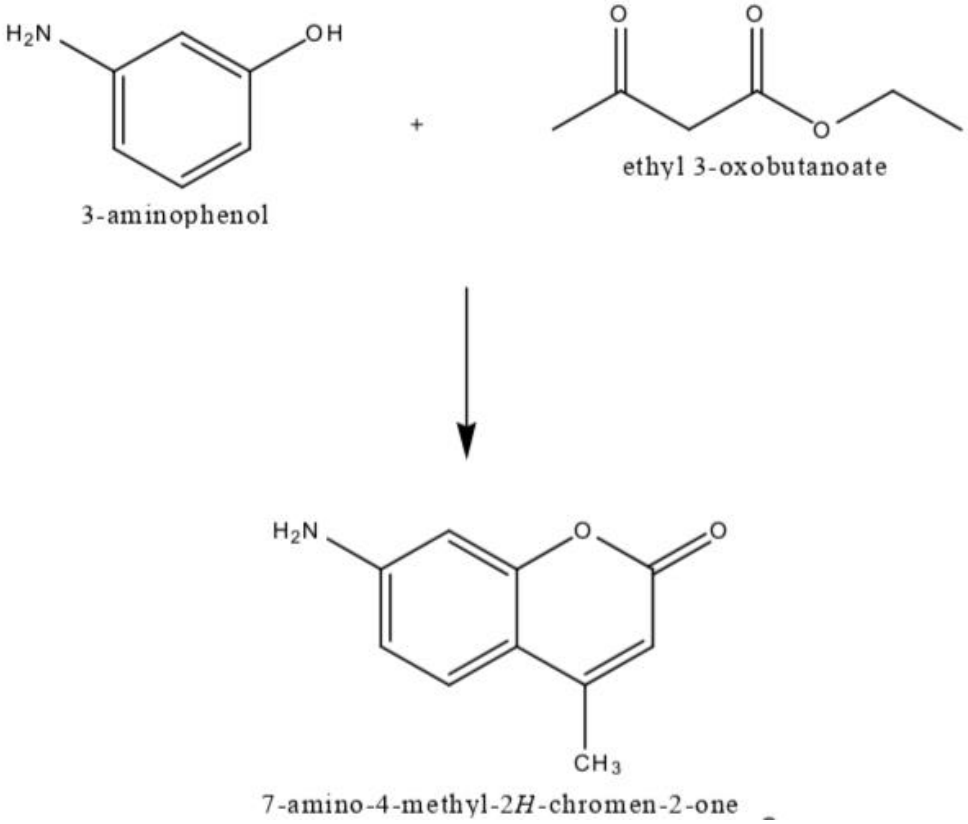
- A series of 96- well plate was used to run the microplate alamar blue assay method. In sll the peripheral wells, added 200µl of sterilised water to avoid desiccation during incubation.
- The microplate received with 100µl of Middlebrook 7H9 broth stock and then the serial dilutions of the derivatives and standards were made on the plate directly.
- The the 100µl of M. Tuberculosis suspension was added to all the wells containing compounds under to make the final volume up to 200µl.

- The the final concentration of the compounds under test will range from 100 to 0.8µg/ml.
- Later the plates were marked and sealed with paraffin. They were then incubated for 5- days at 37℃.
- After the completion of incubation period, 25µl of 1:1 mixture of alamar blue reagent and 10% Tween 80 which is freshly prepared was added into the wells of the plate followed with 24hours of incubation.
- Finally the reading readings were noted based on the visual color change. pink color in the well indicates growth of the bacteria and blue color indicates no bacterial growth. In this method, the minimum inhibitory concentrations (MIC) of the compound can be determined as the concentration of the compound at which there is no color change from blue to pink

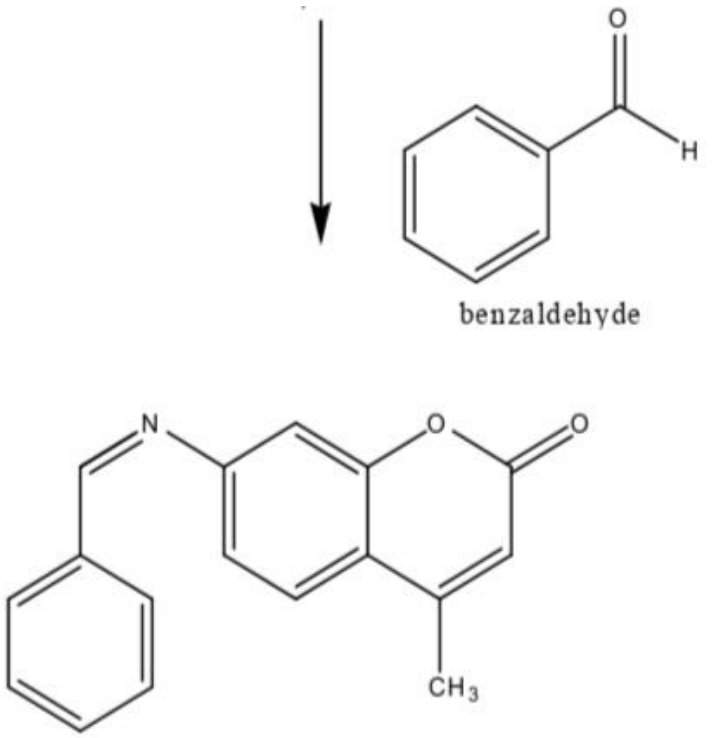
Synthetic scheme:

SCHEME

Step:1


H2N
OH
O
O
O
3-aminophenol
ethyl 3-oxobutanoate
H2N
O
O
CH3
7-amino-4-methyl-2H-chromen-2-one

STEP-2


O
H
benzaldehyde
N
O
O
CH3
(Z)-4-methyl-7-(1-phenylethylideneamino)-2H-chromen-2-one

Results and Discussion:

Characterization:

Compound (CM-1):

IR (KBr in cm^{-1}): C- C stretch 1610**,** N-H stretch 3346**,** O-H bending 834, Aromatic C=C1511

NMR Chemical Shift: δ=7.961 (S, Secondary amide R2-NH),δ=7−7.6 (S, 10 Ar H),

δ=4.172 (S, 1C, Ar NH2)

Mass: Base peak 241, Molecular ion peak 367.05

Compound (CM-2)

IR (KBr in cm^{-1}): C-N Stretch 1209, C=O stretch 1629, O-H stretch 3371, C-H bending 831

NMR Chemical Shift: δ=7.961 (S, Secondary amide R2-NH), δ=7−7.6 (S, 10 Ar H), δ=4.172 (S, 1H, Ar NH2)

Mass: Base Peak 184.0500, Molecular ion peak 402.5500

Compound (CM-3)

IR (KBr in cm^{-1}): O-H stretch 3372, C=C stretch 1632, C-O stretch 1265, Aromatic C=C 1514

NMR Chemical Shift: δ=3.112 (S, 1H, NH2), δ=7.2 −7.9 (S, 10 Ar H)

Mass: Base peak 241, Molecular ion peak 383.05

Compound (CM-4)

IR (KBr in cm^{-1}): C-N Stretch 1231, O-H stretch 3352, C-C stretch 1601, N-O stretch 1454

NMR Chemical Shift: δ=3.167 (S, 1C, =CH),δ=7.213–9.437 (S, (S, 10 Ar H)

Mass: Base Peak 122, Molecular ion peak 410.05

Compound (CM-5)

IR (KBr in cm^{-1}): C-H stretch 2920, C-C stretch 1594,C-H rocking 1359,C-N stretch 1165

Compound (CM-6)

IR (KBr in cm^{-1}):C=C stretch 1517,N-H stretch 3367,C-C stretch 1623,N O stretch 1349

Anti-tuberculosis Activity

The minimum inhibitory concentrations of the synthesized derivates against Mycobacterium tuberculosis obtained through Microplate Alamar Blue Assay method were represented below:

Table 5.14 MIC values of the synthesized compounds.

S. No	Compound code	MIC Values (µg/ml)
1	01	12.5
2	02	3.125
3	03	6.25
4	04	6.25
5	Pyrazinamide	3.125
6	Streptomycin	6.25
7	Ciprofloxacin	3.125

Anti-TB activity using Alamar Blue Dye:

1. The anti mycobacterial activity of compounds were assessed against M. tuberculosis using Microplate Alamar Blue Assay (MABA).
2. This methodology is non-toxic, uses a thermally stable reagent and shows good correlation with propotional and BACTEC radiometric method.
3. 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.
4. The 96 wells plate received 100 µl of the Middlebrook 7H9 broth and serial dilution of compounds were made directly on plate.
5. The final drug concentrations tested were 2 to 1024 µg/ml.
6. Plates were covered and sealed with parafilm and incubated at 37ºC for five days.
7. 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.
8. A blue color in the well was interpreted as no bacterial growth, and pink color was scored as growth.
9. The MIC was defined as lowest drug concentration which prevented the color change from blue to pink. The anti tubercular activity of synthesized compounds were performed by Microplate Alamar Blue Assay. The results of the assay were as follows:

Table 5.15 Anti-TB Activity results

S. NO	Samples	100µg|ml	50µg|ml	25µg|ml	12.5µg|ml	6.25µg|ml	3.12µm|ml	1.6µg|ml	0.8µg|ml
1	CM-1	S	S	S	S	R	R	R	R
2	CM-2	S	S	S	S	R	R	R	R
3	CM-3	S	S	S	S	S	R	R	R
4	CM-4	S	S	S	S	S	R	R	R
5	CM-5	S	S	S	S	S	R	R	R
6	CM-6	S	S	S	S	S	S	R	R

Note:

S- Sensitive

R- Resistant

Strain used: M.tuberculosis (H37RV strain): ATCCNo- 27294

Here are the **standard values** of the Anti-Tb test which was performed

Pyrazinamide – 3.125µg/ml

Streptomycin -6.25µg/ml

Ciprofloxacin -3.125µg/ml

5.3 IN-VITRO ANTHELMENTIC ACTIVITY

The synthesized compounds were evaluated for their in vitro anthelmintic activity Pheretima posthuma .Albendazole was used as a standard drug at a dose of 20 mg/ml.

COMPOUNDS	Time taken for Paralysis (min)			Time taken for death (min)		
	25mg/ml	50mg/ml	100mg/ml	25mg/ml	50mg/ml	100mg/ml
CM-1	43.09±1.25	32.47±1.04	18.43±1.5	59.21±2.74	45.13±1,17	24.41±1.10
CM-2	29±2.50	34.43±1.87	32.01±2,46	56.09±1.03	29.33±2.37	21..25±1,92
CM-3	22.19±2.34	29.58±2.03	18.46±2.37	39.18±2.47	21.06±1.94	32.53±2.07
CM-4	26.17±2.23	29.32±2.46	17.32±1.74	37.29±2.35	19.42±2.78	15.34±1.38
CM-5	51.52±1.08	43.56±1.98	43.33±1.09	76.45±1.35	34.28±1.76	28.36±2.78
CM-6	36 .02±1.87	27.35±2.37	18.42±2.05	17.38±0.98	7.54±2.98	9.36±1.87
Standard(Albendazole 20 mg|ml)	23.26±1.5			62±6.8		

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Table no 5.16 ; In vitro anthelminthic activity

Conclusion

Six novels substituted Coumarin derivatives were synthesized by two steps simple procedure, characterized and all derivatives screened for *in-vitro* anti-inflammatory and *in-vitro* anti-ulcer activities respectively. Structural Characyerization was performed by FT-IR,NMR,MASS Spectroscopy

The present research work, involves the synthesis of series of 6 novel substituted compounds of Coumarin. Here 3-Amino phenol taken as a starting material. Treatment of 3-oxo butanoate were dissolved in 18ml Zinc chloride, 2ml Sulphuric acid it forms 7-Amino-4-methyl Coumarin. To this compound various aromatic aldehydes were added along with 30-40ml of ethanol and kept for reflux for 3hrs and it forms a novel various colored coumarin derivative. Purification was done by recrystallization. Characterization of all derivatives were done by FT-IR, NMR, and Mass Spectroscopy.

All derivatives were screened for their *in-vitro* anthelimenthic and *in-vitro* anti-tuberculosis activities.

Anthelimentic activity:

All the derivatives (CM-1 to CM-6) were screened for *in-vitro* anti-helminthic activity by in vitro anthelminthic activity Pheretima posthuman method. The synthesized derivatives has shown the moderate inhibition.

The derivatives were screened for pharmacological studies that are anti-helminthic activity. The results are tabulated and among all the derivatives CM-2 has shown good anti-helminthic activity.

Anti-tuberculosis activity:

The selected derivatives were evaluated for *in-vitro* anti-tuberculosis activity by Micro plate Alamar blue assay method by using streptomycin as standard and in vitro anti-helminthic activity by using pheretima posthuman method. The electron withdrawing groups such as chloro, nitro groups containing compounds (CM-2, CM-6) has shown the highest activity and the rest of compounds having electron donating groups (CM-1,CM-3,CM-4,&CM-5) shows mild to moderate activity.

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